# OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF ANTHOCYANINS FROM NOVA SCOTIAN LOWBUSH BLUEBERRIES *(Vaccinium Angustifolium* Aiton L.*)*

by

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# DEDICATION PAGE

*I dedicate this work to my parents and my brother, without none of this would have been possible. Thank you for your love, support and encouragement. This is for you.*

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# ABSTRACT

Ultrasound-assisted extraction of anthocyanin compounds from Nova Scotian lowbush blueberries was measured, modeled and optimized using response surface methodology (RSM). A Box-Behnken design was conducted to analyse the effect of extraction temperature, time, solvent concentration (acidified ethanol) and liquid to solid ratio on total anthocyanin content (TAC) and total phenolic content (TPC). The liquid to solid ratio had the most significant effect on TAC, followed by solvent concentration, while extraction temperature and time did not have a significant effect. Among the experimental range used for testing the variables, extraction with 60% solvent concentration, 50 mL/g liquid solid ratio, $65^0$C extraction temperature and 11.5 min extraction time resulted in the highest level of TAC (13.22 mg C3G/ g DW). The optimum point of TPC (47.05 mg GAE/ g DW) was obtained with solvent concentration 44.30%, liquid solid ratio of 50 mL/ g, extraction temperature $65^0$C and 19.99 min extraction time.

# LIST OF ABBREVIATIONS USED

| | |
|---|---|
| C3G | Cyanidin-3 glycoside |
| DW | Dry weight |
| FW | Fresh weight |
| g | Gram |
| GAE | Gallic acid equivalent |
| KCl | Potassium chloride |
| LSRatio | Liquid solid ratio |
| NaOH | Sodium hydroxide |
| mg | Milligram |
| RSM | Response surface methodology |
| SolvConc | Solvent concentration |
| Std. Dev. | Standard deviation |
| TAC | Total anthocyanin content |
| Temp | Temperature |
| TPC | Total phenolic content |
| USDA | United State Department of Agriculture |

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# 1 INTRODUCTION

Canada is the world's second largest producer of blueberries, after the United States. In 2010, the total marketed production of blueberries in Canada amounted to 83,507 metric tonnes, with a total blueberry farm gate value of $148.7 million compared to $110.5 million recorded in 2009. According to a report from Statistics Canada, British Columbia has the highest marketed volume of blueberries, followed by Nova Scotia. However, Nova Scotia has the highest marketed value for lowbush blueberries (Statistical Overview of the Canadian Blueberry Industry, 2010).

Blueberries are one of the richest natural sources of polyphenol compounds, which include anthocyanins. Complex anthocyanin patterns, depending on ripeness of the fruits, were observed for different blueberry cultivars. It is reported that blueberries exhibit one of the highest *in vitro* antioxidant capacities in comparison with a variety of fruits and vegetables such as strawberries and raspberries (Kalt et al., 1999; Kalt et al., 2003). It was reported that both highbush and lowbush varieties of blueberries have high antioxidant levels in comparison to other fruits, especially in regards to the total anthocyanin and phenolic content (Wu et al., 2004). In addition, many studies have been conducted on the health benefits of blueberry antioxidants. For example, consuming purified anthocyanin from blueberries rather than blueberry juice, was found to be effective in preventing obesity in mice (Prior et al., 2010). Other examples of health benefits associated with blueberry antioxidants include the inhibition of cancer cells, as well as improvements in subjects with atherosclerosis or diabetes (Adams et al., 2010; Grace et al., 2009; Seeram et al., 2006; Wu et al., 2004).

Recently, the interest in drugs and products derived from plants has made it important to search for effective extraction methods to maximize the yield of bioactive components such as vitamin C, anthocyanin and phenolic compounds. Work needs to be carried out to minimize the energy use, time, and cost associated with extraction. There are different chemical and mechanical procedures that can be used in the separation process of valuable compounds from fruits and vegetables, including solvent extraction and high pressure processing. Techniques to recover antioxidants from plants include high pressure processing, soxhlet extraction, room temperature extraction by shaking, extraction with intermittent cooling, pulsed electric fields, microwave and ultrasound-assisted extraction (Ghitescu et al., 2014; Kothari et al., 2012; Loginova et al., 2011; Jun, 2009). Ultrasound-assisted extraction is a promising technique that can be used to enhance conventional methods for the extraction of high-value molecules from natural sources.

The comparatively high level of polyphenols and anthocyanins in blueberries makes them a good material from which to extract antioxidants for the potential use as health supplements and functional food ingredients. The creation of such value-added products would be of great benefit to the lowbush blueberry industry and the Nova Scotia economy. In this study, ultrasound-assisted extraction was used to investigate the extraction of total anthocyanin and polyphenol content from lowbush blueberries followed by optimization of this process.

# 2 OBJECTIVES

This study investigates the ultrasound-assisted extraction of anthocyanins from Nova Scotian lowbush blueberries, using statistical screening and optimization methods. The specific objectives were as follows:

- To screen the factors with significant effects on total anthocyanin content involved in ultrasound-assisted extraction ;
- To establish a model to predict total anthocyanin content and to optimize extraction conditions;
- To establish a model for total phenolic content and to optimize extraction conditions;

# 3 LITERATURE REVIEW

## 3.1 Blueberry Varieties and Composition

Blueberries are categorized under the family *Ericaceae*, subfamily *Vacciniaceae*, genus *Vaccinium*, and subgenus *Cyanococcus* (Gough, 1994). There are different varieties of blueberries, including the wild-growing lowbush blueberries (*Vaccinium angustifolium*), cultivated highbush blueberries *(Vaccinium corymbosum)* and rabbiteye blueberries (*Vaccinium virgatum*). Blueberries are mostly cultivated in North America (Canada and USA), Europe, China and Australia (Lohachoompol et al., 2008; Wang et al., 2010). In addition to the previously-mentioned blueberry varieties, some types of blueberries have limited cultivars, which are popular in some specific countries. Bilberries (*Vaccinium myrtillus*) are an example of this category which belong to same genus as other blueberries. Bilberries are similar to the North American lowbush blueberries and are only grown in European countries (Gough, 1994).

Blueberries are considered by many to be a "super food" due to their high antioxidant and nutritional content. According to USDA National Nutrient Database for Standard Reference (2014), raw blueberries generally contain 9.96 g of sugar, 84.21 g water and 77 mg potassium per 100 g of fresh weight. The vitamin C content has been determined as 12.4–13.1 mg per 100 g of fresh fruit (Szajdek & Borowska, 2008). Blueberries have been found to contain between 20-27 anthocyanins, depending on the variety that can be categorized in highbush, rabbiteye and lowbush (Gao & Mazza, 1994). The health benefits from fruits and vegetables such as blueberries have been linked with their antioxidant activity against free radicals (Serafini et al., 2009). Antioxidant activity is dependent on factors such as the total anthocyanin content, total phenolic content, maturity

(Prior et al., 1998), genotypes, ripeness (Kalt et al., 2003), genotypic and environmental variation (Conner et al., 2002a), and postharvest conditions and cultivar type (Conner et al., 2002b). The total phenolic content and anthocyanin content are key parameters that are often reported as measurements from spectrophotometric assays (Singleton et al., 1999; Sondheimer & Kertesz, 1948). Further discussion of anthocyanin structure, types, and antioxidant activity are presented in later sections of this literature review.

### 3.2 Negative Health Effects Associated with Free Radicals

Normal physiological processes in living tissues can produce free radicals that can damage the structure of protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) molecules. These free radical species are reactive and short-lived derivatives of oxygen or nitrogen, also known as "reactive oxygen species (ROS)" and "reactive nitrogen species (NOS)", respectively. Superoxide, hydroxyl, peroxide and peroxynitrite are examples of reactive species (Fridovich, 1995; Galaris & Pantopoulos, 2008; Victor, 2014).

ROS at low concentrations may act as mediators in biochemical processes and are categorized as secondary messengers (Pham-Huy et al., 2008; Bae et al., 1997). Organisms at different pathophysiological/physiological states are associated with different levels of ROS, and there is a homeostatic balance between the production of reactive species and their elimination by specific enzymes (Galaris & Pantopoulos, 2008). Slight changes in this balance can change cell communication and activate signal transduction pathways which can cause problems for the human body (Roberts & Sindhu, 2009).

The superoxide anion ($O_2\cdot$) is the first ROS formed from the reduction of oxygen. Leakage of electrons from the electron transport chains of mitochondria, chloroplast and endoplasmic reticulum is believed to be main sources of forming this free radical. Although

($O_2^{\cdot}$) is not as reactive as other radicals, in biological systems it can be converted to other more reactive radicals such as peroxyl ($ROO^{\cdot}$) and a two-electron reduction product of ($O_2^{\cdot}$), hydrogen peroxide ($H_2O_2$). Nitric oxide ($NO^{\cdot}$) is an example of a NOS, which reacts slowly with non-radical molecules in the human body. Nitric oxide ($NO^{\cdot}$) reacts quickly with ROS including ($O_2^{\cdot}$), as well. The product of reacting nitric oxide and the superoxide anion is highly reactive peroxynitrite ($ONOO^-$), which can react with proteins and DNA (Roberts and Sindhu, 2009; Galaris and Pantopoulos, 2008; Dean et al., 1997; Huie and Padmaja, 1993).

In addition to the ROS and NOS produced from normal cellular processes, free radicals can also result from exogenous sources. Examples of exogenous sources include food, stress, tobacco smoke, the products from the burning of fossil fuels, certain pollutants such as ozone, ionizing radiation, and pesticides.

The body uses an antioxidant defense system, which is comprised of specific enzymes and other molecules, to balance the production and elimination of free radicals. Oxidative stress occurs when antioxidant capacity is insufficient to deal with the product of free radical species. Many pathological conditions are the result of this action (Neyestani, 2014). A summary of other human diseases attributed to oxidative stress from excess free radicals is presented in Figure 3.1.

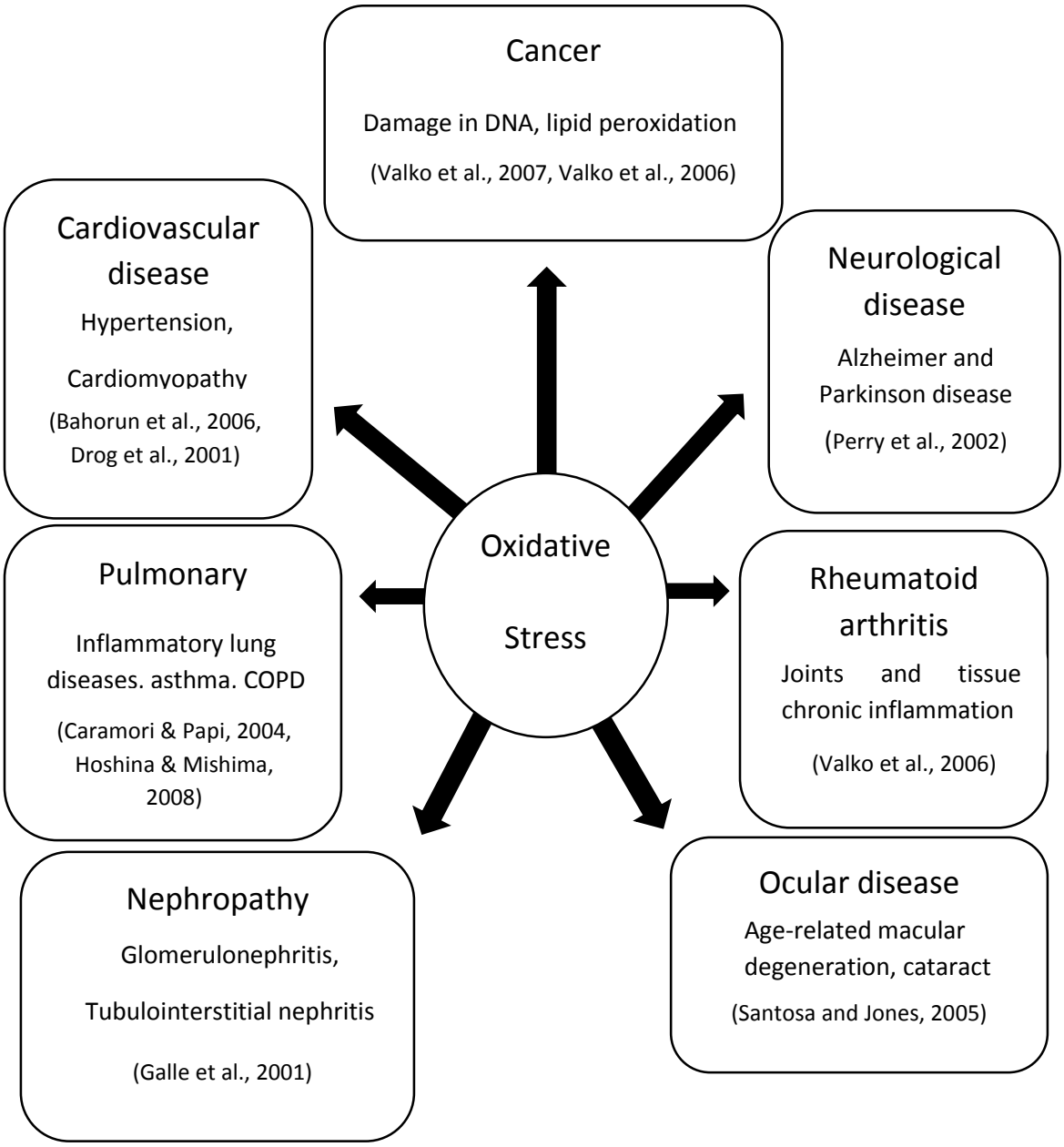



**Figure 3.1: Diseases attributed to oxidative stress from free radicals.**

### 3.3 Antioxidant Activity

The antioxidant defense system is the body's main defense mechanism against free radicals. "Antioxidants are defined as any substances that delay or inhibit oxidative damage to a target molecule." This action occurs when antioxidant molecules react with free radicals and neutralize them by donating their electrons (Sen et al., 2010) (Figure.3.2). Free

radicals are continuously removed by specific antioxidant compounds that can be categorized into two groups: endogenous and exogenous antioxidants, depending on whether they originate within the organism or outside of it. Endogenous antioxidants can then be classified in two groups: enzymatic and non-enzymatic compounds. The enzymatic compounds include enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Non-enzymatic antioxidants include vitamin C, vitamin E, and A (Sen et al., 2010). Nutrition antioxidants are exogenous antioxidants and need to be ingested in food, because they cannot be produced by the body. These compounds include trace metals (selenium, manganese, zinc), flavonoids and omega-3 fatty acids (Sen et al., 2010, Pham-Huy et al., 2008). Some antioxidant molecules such as vitamin C and E are synthesized by the body and also can be supplied by the diet (Tiwari, 2001; Pham-Huy et al., 2008).

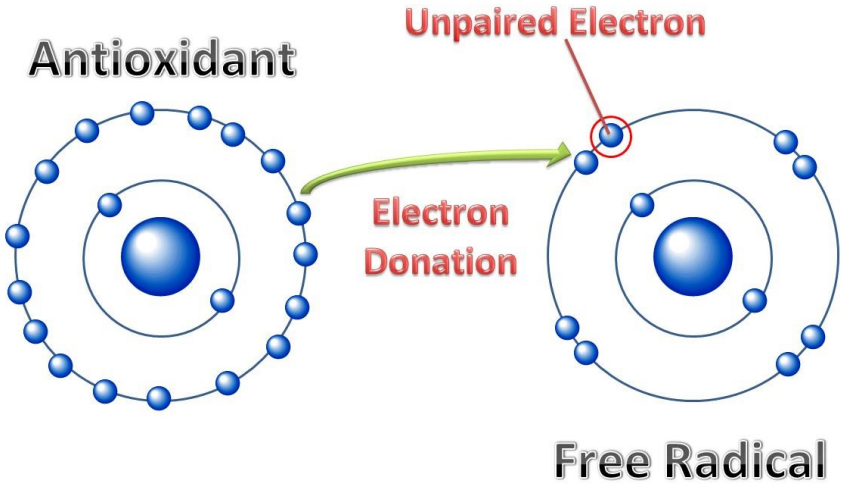



**Figure 3.2: Antioxidant molecule donating an electron to a free radical (Keeley, 2014)**

Fruits and vegetables contain antioxidants such as vitamins A, C, E, β-carotene, as well as important minerals, including selenium and zinc. Fruits, vegetables and medicinal herbs are the richest sources of antioxidant compounds (Yu, 1994). An inverse relationship between the consumption of natural antioxidants from plant products and the risk of serious health disorders caused by oxidative stress, such as degenerative diseases, have been reported (Wang et al., 1997). This effect can be attributed to their capacity for removing free radicals through the antioxidants they contain specifically phytochemicals (Ames et al., 1993). The studies indicate that plant polyphenols and anthocyanins are the agents responsible for the various protective effects (Wang et al., 1997; Ashor et al., 2014).

### 3.3.1 Polyphenols

Polyphenols are a class of antioxidant compounds that are present in many fruits, vegetables and plant-derived beverages, and products such as tea, coffee, chocolate, fruit juice and red wine. Phenolic compounds result from secondary metabolism in fruits and vegetables. They are produced as a defense barrier against seed dispersal, microorganisms and UV radiation. These compounds are natural pigments and can be used to add natural flavours to foods and vegetables (Pandey & Rizvi, 2009).

The diverse phenolic group contributes to the antioxidant activity observed in many plants products. As was explained previously, oxidative damage in DNA, proteins, lipids and other cell components is associated with degenerative diseases such as cancer and cardiovascular diseases. The phenolic groups within the polyphenol molecules disrupt the chain oxidation reactions in different cellular components by donating an electron or hydrogen to neutralize the free radicals, or by acting on protein or lipid kinase signaling (Marfella et al., 2014). Many polyphenols have been identified and classified into different

groups. Based on the number of phenol rings and the structural components that bind these rings to one another, these components can be classified (Manach et al., 2004). The sheer complexity and diversity of the structures associated with polyphenols is one of the main reasons that more research is needed on these compounds (Scalbert et al., 2005). A summary of the subclasses of polyphenol compounds is presented in Figure. 3.3 and 3.4.

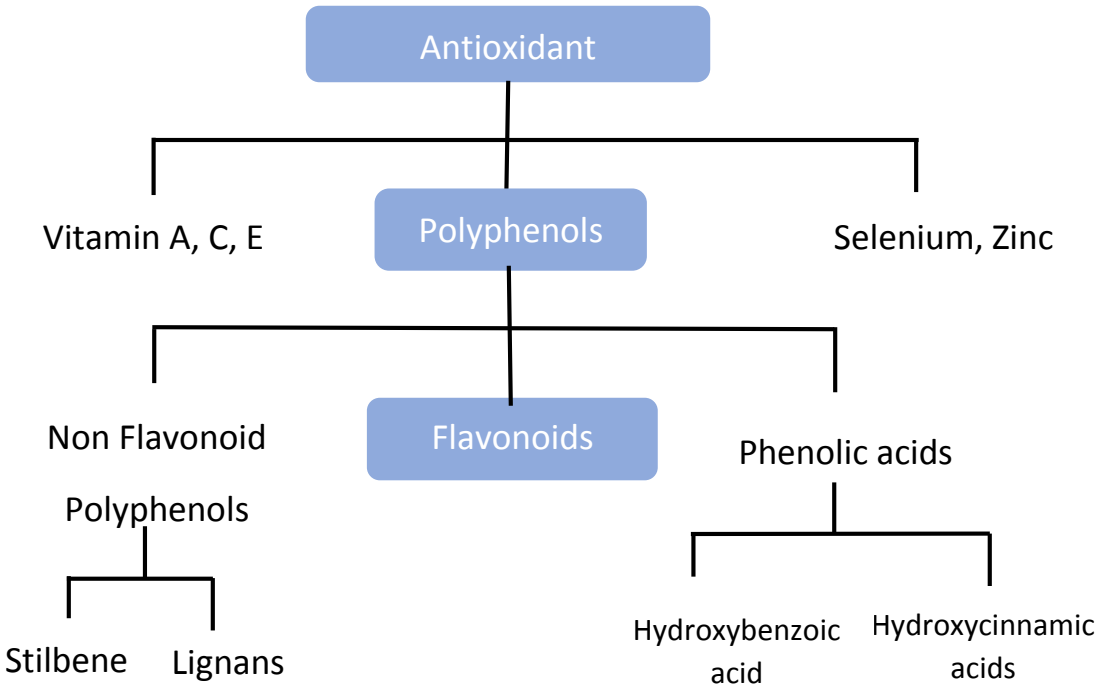



**Figure 3.3: Polyphenol classes and subclasses.**

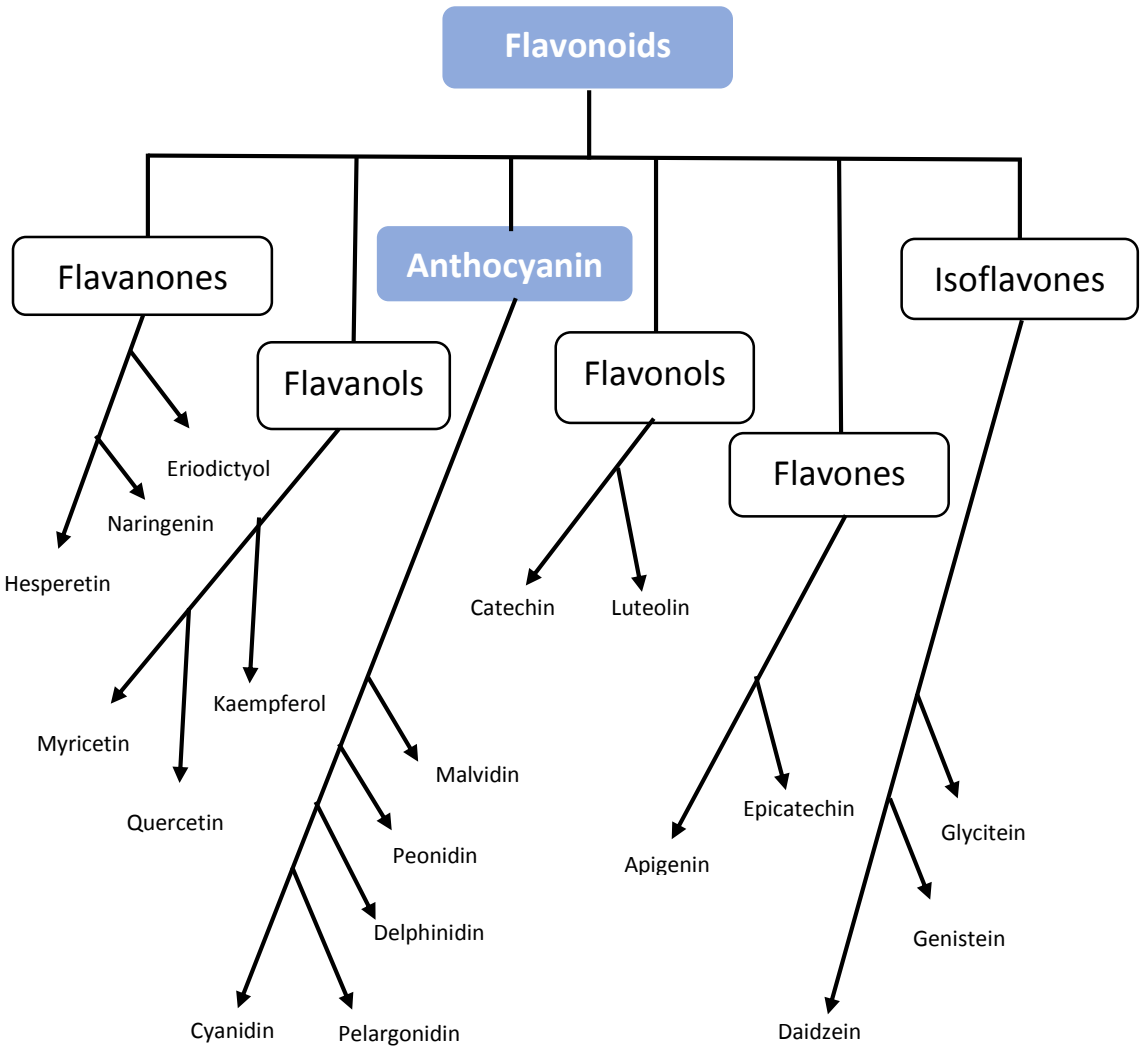



**Figure 3.4: Polyphenol classes and subclasses.**

Polyphenols can be categorized into two main groups: the flavonoids and phenolic acids (Scalbert et al., 2007), with non-flavonoid polyphenols being less prevalent. The phenolic acids can be further classified into two groups, derivatives of benzoic acid and derivatives of cinnamic acid. Hydroxybenzoic acids such as ellagitannins are found in a few fruit and vegetable sources, such as strawberries, raspberries and blackberries. Gallic acid, a trihydroxybenzoic acid is found in tea (Abdel-Hamid et al., 2013). The hydroxycinnamic acids are more common than the hydroxybenzoic acids. Fruits such as blueberries contain 0.5–2 g hydroxycinnamic acids/kg fresh weight (Manach et al., 2004). Examples of non-flavonoid polyphenols include lignans and stilbenes. Stilbenes have low quantities in the human diet. Resveratrol is one of the main compounds in the stilbenes category, and can be found in grapes (Rotches-Ribalta et al., 2012).

Flavonoids are the most prevalent polyphenols, and are further distributed amongst six subclasses. These include the flavonols, which are the most abundant flavonoids found in many fruits and vegetable such as grape berry. It is reported that flavonols play a key role in determining the quality of wine (Hilbert et al., 2015). The occurrence of flavones, another category of flavonoid, in fruits and vegetables is less common in comparison to the flavonols. The main sources of flavones are parsley and celery. Citrus fruits are the main source of a type of flavonoid called flavanones (Tomás-Barberán and Clifford, 2000). These compounds can also be found in other fruits and vegetables such as tomatoes (Justesen et al., 1998). A sub-category of flavonoids, isoflavones, are compounds mostly found in soybeans and its processed products (Lima et al., 2014). The flavanols can exist in both the monomer (catechins) and the polymer form (proanthocyanidins). Green tea and

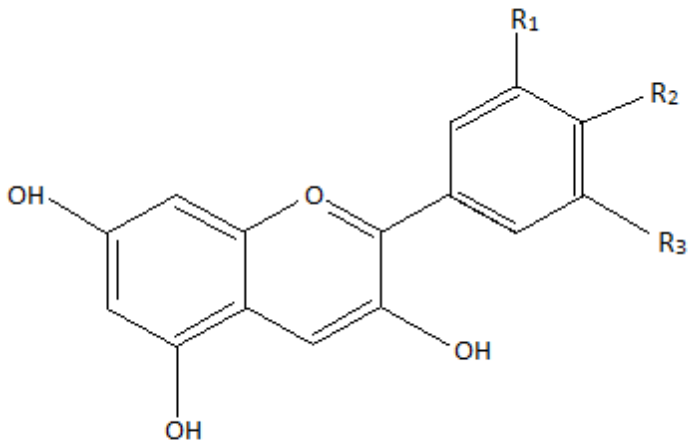
chocolate are the main sources of catechin (Sokolov et al., 2013). Red wine is another source of flavanols (Dias et al., 2010).

The polyphenols that are present in a fruit or vegetable are not limited to one type or class. Some natural sources such as green tea, grapes and blueberries contain a variety of polyphenol subclasses. One of the main sources of polyphenols are blueberries, which contain several phenolic compounds and their glycosides, including malvidin, delphidin, kaempherol, glucoside, galactoside and 3-O-rhamnoside of quercetin as well as gallic, syringic and vanillic acids (Kader et al., 1996). In addition to the polyphenol subclasses that have been discussed, anthocyanins are another sub-group of flavonoids that are extremely important and are associated with the pigments found in plant tissues. Anthocyanins are generally responsible for flowers and fruit appearing pink, red, blue, or purple. More details regarding the anthocyanins are presented in section 3.3.2.

### 3.3.2 Anthocyanins

The anthocyanins are one of the main classes of flavonoids. These compounds consist of an aglycone (anthocyanidin) as the main structure and sugar(s), and in many cases, acyl group(s). Figure .3.5 shows the structure of six common anthocyanidins. It is known that anthocyanins are water-soluble glycosides of 18 anthocyanidins, although new anthocyanidins have been identified (Anderson & Jordheim, 2010). More than 65% of the reported anthocyanins are acylated and the nature, number, and linkage positions of the acyl groups of the anthocyanins, result in the diversity of these compounds that are found in nature. According to a review by Anderson Anderson & Jordheim (2010), 539 different isolated natural anthocyanins and 23 anthocyanidins have been reported. Around 90% of anthocyanins are based on six main anthocyanidins: pelargonidin (Pg, 12%), cyanidin (Cy,

50%), peonidin (Pn, 12%), delphinidin (Dp, 12%), petunidin (Pt, 7%), and malvidin (Mv, 7%) (Kong et al., 2003; Andersen and Jordheim, 2010).




| *Anthocyanidin* | *R1* | *R2* | *R3* |
|---|---|---|---|
| *Cyanidin* | OH | OH | H |
| *Delphinidin* | OH | OH | OH |
| *Malvidin* | $OCH_3$ | OH | $OCH_3$ |
| *Pelargonidin* | H | OH | H |
| *Peonidin* | $OCH_3$ | OH | H |
| *Petunidin* | $OCH_3$ | OH | OH |

**Figure 3.5: Structure of the six most common anthocyanidins in nature.**

Anthocyanins are associated with the coloration of many fruits, flowers and vegetables (Kong et al., 2003). Berries (including blueberries, blackberries, chokeberries, elderberries, raspberries, and strawberries), grapes, cherries, purple corn, sweet potato and red onions are among plant products containing anthocyanin that can possibly affect the fruit color (Kahkonen et al., 2003; Fossen & Andersen, 2003). Various factors are

associated with different colours in fruits and vegetables, including the concentration and nature of the anthocyanidin, acylation, as well as external factors such as pH and the salts that are presented (Cabrita et al., 2000; Uddin et al., 2004).

Blueberries are one of the main sources of anthocyanin. Several blueberry cultivars have a wide range of anthocyanins that are present, including compounds that are specific for each cultivar (Vrhovsek et al., 2012). The most common anthocyanins in blueberries are the monoarabinosides, monoglucosides and monogalactosides of cyanidin (Cy), petunidin (Pt), peonidin (Pn), delphinidin (Dp) and malvidin (Mv) (Kader et al., 1996). Delphinidin and malvidin have been found to be the predominant anthocyanidins in blueberries. Acylated anthocyanins are another sub-class of anthocyanins found in blueberries, although total blueberry anthocyanins contain only a small portion of acylated anthocyanins (Rodriguez-Mateos et al., 2012; Yousef et al., 2013).

It is reported that anthocyanins can have therapeutic effects, due to their high antioxidant capacity, such as cardio- and neuro-protection as well as and anti-diabetic effects (Grace et al., 2009; Prior et al., 2008; Tarozzi et al., 2010). However, the mechanism of action of anthocyanin compounds in the body is complicated and that is due to the instability, low extraction content and low bioavailability of these compounds in the body (Castañeda-Ovando et al., 2008; Fernandes et al., 2014).

The degradation of anthocyanins during the processing and storage of fruits and vegetables can decrease their antioxidant properties (Skrede et al., 2000). There have been many studies on the degradation of anthocyanins, in which the effect of different factors on anthocyanin degradation have been investigated. For example, temperature has been shown to affect degradation; anthocyanins have demonstrated sensitivity to temperatures

(Wang and Xu, 2007; Sadilova et al., 2006). It is important to have enough accurate information about factors that can affect the anthocyanin content of fruits in order to predict the possible quality changes during storage and processing. An example would be the production of a nutraceutical or functional foods or beverages that contain a high level of anthocyanins after being enriched with an anthocyanin extract.

### 3.4 Ultrasound-Assisted Solvent Extraction

The recovery and extraction of bioactive compounds from vegetables and fruits is of interest to many industries that are involved in chemical, pharmaceutical and biological processes. Conventional solid-liquid extraction methods are often used to obtain enriched extracts of the bioactive compounds in plant materials (Belova et al., 2009). However, conventional solvent extraction techniques have some limitations. These include mass transfer resistances, the large amount of solvent used in classical extraction methods, long extraction time, high extraction temperature, health related risks and low efficiency and extraction yield (Japon-Lujan et al., 2006; Kimbaris et al., 2006; Barbero et al., 2008; Jadhav et al., 2009, Da Porto & Decorti, 2009; Khan et al., 2010; Adjé et al. 2010). Additional factors to consider are the properties of the bioactive compound and the raw material to be processed, such as its sensitivity to processing temperature.

Ultrasound-assisted extraction can be used as a tool to overcome the drawbacks of conventional solvent extraction methods and to improve some benefits of the solvent extraction process. The application of ultrasound generates cavitation, which is the generation of bubbles in the system. This cavitation helps to increase the rate of mass transfer between the solid plant material and the solvent medium by generating currents in the liquid (Da Porto & Decorti, 2009; Dolatowski et al., 2007). Cavitation on the surface

of the source material, results in cell disruption and particle breakdown (Paniwnyk et al., 2009; Riera et al., 2004), which also increases mass transfer from the sample to the solvent by increasing the surface area of the solid material. Scanning electron images of raw and ultrasonicated soybeans were compared and it was observed that the surface morphology of soybean flakes changed after the sonication process which found to be the reason resulting in high mass transfer (Li & Weiss., 2004).

The main physical parameter of the ultrasound bath is frequency (Hz). Ultrasound frequencies range above human hearing threshold, between 18 kHz and 100 MHz, which the upper limit has not been specifically defined. Two ranges are used in food industry: high or diagnostic ultrasound between 1 and 10 MHz, and power ultrasound between 20 and 100 kHz. An extended range of 20 kHz to 2 MHz ultrasound can result in improving the chemical reactions by physical and chemical effects in the system by cavitation causing particle disruption .There are two main types of ultrasonic systems that are used on the laboratory scale: ultrasound bath systems and ultrasound probe systems. For smaller volumes the probe system is considered to be more powerful. The ultrasonic cleaning bath is used for larger volumes and has low operation cost. Both probe and bath systems are both used in the industrial scale, depending on the application (Bendicho & Lavilla, 2000).

Ultrasound-assisted solvent extraction has been used on the laboratory scale for the extraction of many bioactive compounds. Some examples include: phenol-based natural compounds from olive leaves (Japón-Luján et al., 2006), essential oils from rose hip seeds (Szentmihalyi et al., 2002), antibiotics from animal feed (Morales-Munoz & de Castro, 2005) and antioxidants from morinda root (Hemwimol et al., 2006). There have also been studies comparing the added benefits of ultrasound-assisted extraction in comparison with

extractions without ultrasound and these advantages include better extraction yield, and a reduction in processing time, as well as in the amount of solvent and energy used. Some examples of these studies are discussed in the following paragraphs.

The effect of ultrasound on the extraction of phenolic compounds from grape mash was studied (Lieu & Li, 2010). The extraction was carried out after enzymatic treatment. The researchers reported an increase in the extraction yield and in the quality of the juice. The phenolic content, sugar content and total acid content were also improved resulting in a higher quality juice. The extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel using ethanol was investigated (Khan et al., 2009). In this study, the conventional solvent extraction method was compared with an optimum condition obtained from ultrasound-assisted extraction using 80% ethanol as solvent in both conditions. The flavanone concentrations and extraction yields obtained over a treatment time of 15 minutes were significantly higher than the results from the conventional extraction over a period of 60 minutes at 40 °C. Results showing similar benefits from ultrasound-assisted extraction of phenolic compounds from grape mash have also been observed in the literature (Lieu & Li, 2010). Traditionally during grape juice processing, grape mash is treated with enzymes to increase the juice volume and reduce the processing time. However, the cost of energy is high for this process due to the long enzymatic maceration time that is required.

The extraction of anthocyanins from berry fruits using ultrasound-assisted methods has also been reported (Galvan D'Alessandro et al., 2014). The extraction of anthocyanins from black chokeberry fruits was studied. They reported that ultrasound improved the extraction kinetics, mainly in the beginning of the extraction process and at low

temperatures. The effect of sonication on the retention of anthocyanins in blackberry juice was investigated in another study (Tiwari et al., 2009). Significant retention of anthocyanin content (>94%) was observed at maximum ultrasonic amplitude of 100% at a frequency of 20 kHz and maximum time of 10 minutes, indicating that sonication could be used in juice processing. Similar results have also been observed in the literature for the extraction of anthocyanins from red raspberries (Chen et al., 2007). In comparison with conventional solvent extraction, ultrasound-assisted extraction was more efficient and rapid in the extraction of anthocyanins, due to the strong disruption of fruit tissue structure under ultrasonic acoustic cavitation, which had been observed with the scanning electron microscopy (SEM). In addition, a comparison between the conventional extraction methods and ultrasound-assisted extraction of anthocyanins from blackberry and sweet cherry cultivars was studied (Oancea et al., 2013). Hydroethanolic solution and acidified ethanol were used to conventionally extract anthocyanins at 4 °C for two separate 24 hour periods. For the ultrasound-assisted extraction, the highest recovered anthocyanin content in blackberries (107.81 mg C3G/100 g FM) was obtained using a solvent consisting of 0.1% HCl in 80% ethanol with a 10:1 solvent: solid ratio (v/w) at 30 °C for five minutes. From the sweet cherries, the maximum yield (36.05 mg C3G /100 g FM) was obtained using a solvent containing 0.1% HCl in 60% ethanol, at an extraction temperature of 30 °C, with a 15:1 ratio of solid: solvent (w/v) and an extraction time of five minutes. These studies indicate ultrasound-assisted extraction can be more beneficial than conventional methods for the extraction of valuable compounds from fruits and vegetables.

# 4 EXPERIMENTAL MATERIALS AND METHODS

This chapter specifies the methods and materials used for the extraction of anthocyanin from lowbush Nova Scotian blueberries. Also discussed are the techniques used for determination of the total anthocyanin and phenolic content, as well as the experimental design used for the extraction of anthocyanin.

## 4.1 Plant Material and Reagents

Fresh wild blueberries were kindly donated by Glenmore Farms, Middle Musquodoboit, NS, Canada. Hydrochloric acid was purchased from EM Science (Darmstadt, Germany). Glacial acetic acid (Assay: ≥99.7% w/w), sodium acetate anhydrous (fused crystals certified ACS), Folin & Ciocalteu's phenol reagent 2.0 N (MP biomedical) was purchased from Fisher Scientific (Nepean, Ontario, CA) and reagent alcohol ACS reagent grade from Fisher Scientific (RICCA chemical, TX, USA). Potassium chloride ACS grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Gallic acid (97.5-102.5% (titration)) was purchased from Sigma Aldrich (Oakville, Ontario, CA). Anhydrous sodium carbonate grade ACS was purchased from VWR (BDH, Radnor, PA, USA). Distilled water was used for all the dilutions. Freeze dried blueberries were kept in a desiccator containing anhydrous calcium sulfate (Drierite, manufactured in USA by W. A. Hammomd Drierite company LTD.).

## 4.2 Equipment

A freeze dryer (Model 7750000, Labconco, Kansas City, MO, USA ) with an Ilmvac pump (model P12Z-301, chemvac, Ilmenau, Germany) was used to freeze dry the frozen blueberries. A domestic grinder (Everyday use essentials, E710) was used to grind the freeze dried blueberries. Ground samples were dried in a vacuum oven to determine the

moisture content. Extraction experiments were performed in an ultrasound bath (Branson B2510DTH Ultrasonic Cleaner, Danbury, CT, USA). Fifteen ml Falcon tubes (High clarify polypropylene conical tube) were purchased from Fisher Scientific and used as the extraction chamber. A refrigerated centrifuge (Thermo Fisher Scientific Centrifuge RT1, Germany) was used to separate solid and liquid phase. Disposable 0.45 μm Millex GS Filter Units were used to filter the extracts. Anthocyanin content was measured by using a UV/Vis spectrometer (Thermo Scientific Genesys 10S series, Madison, WI, USA) with a variable wavelength visible detector. The pH of the anthocyanin solutions were tested using Denver instrument pH meter (Ultrabasic, UB-10)

### 4.3 Sample Preparation

Fresh wild blueberries were stored one day after harvest in a domestic freezer at -16 °C for 48 hours one day after harvest, and then moved to cold storage facilities (located in the Dalhousie Food Science Laboratories) for 10 months, where they were kept at -35 °C before used in experiments. To prepare the blueberry extractions, frozen blueberries were cut into halves, lyophilized, ground and sieved.

To freeze-dry the frozen blueberries, they were first transferred to a -16C freezer overnight and then cut into halves. After this, they were transported to Dr. Gratzer's Biomaterials Laboratory located in the Dalhousie Dentistry Building. Here, they were kept at -80 ˚C for 10 minutes to ensure all samples were frozen before freeze drying. Freeze drying was conducted using a LABCONCO freeze dryer. Samples were processed in 350 g batches until a moisture content of less than 5% (w/w) was achieved. Each batch typically took five to six days and five batches were processed. These batches were combined into one batch and stored in the -16 °C freezer. Prior to moisture content analysis and

extractions, the frozen lyophilized blueberries were placed on the bench at 23 $^0$C (Relative humidity = 65%) for an hour to bring them to room temperature. The blueberries were then ground using the grinder to obtain a fine powder and then passed through a sieve (no. 35) to ensure that the particle diameters were less than 500 μm, because particle size has been identified as an important factor in the extraction yield of fruits and vegetables (Gião et al., 2009). The ground blueberries were then used immediately for moisture content determination or for extraction.

Freeze dried blueberries were tested for moisture content to confirm that the moisture content was less than 5% for determining the phenolic and anthocyanin content per gram of dry weight. The procedure is as follows. Empty aluminium dishes were placed in the oven at 70 $^0$C overnight. The dried dishes were placed in a desiccator containing anhydrous calcium sulfate for half an hour to cool down and then were weighed. Following this, 2 g of the ground freeze dried blueberries were transferred into the pre-weighed dish, spread using a spatula and placed into the vacuum oven overnight (15 hours) to dry at 70$^0$C. After drying, the dish was transferred into the desiccator to cool for half an hour and then reweighed. The procedure was repeated until a constant weight was observed (AOAC 934.06, 1996). The moisture content was calculated as follows:

$$\%\ \text{moisture} = \frac{(W_1 - W_2)}{W_1} \text{ x } 100 \qquad (1)$$

Where

$W_1$ = weight (g) of sample before drying

$W_2$ = weight (g) of sample after drying

After verifying that the lyophilized blueberry samples had a moisture content of less than 5%, the extraction was performed under various experimental conditions using an ultrasound bath, in accordance with the design of the experiment.

### 4.4 Extraction of Anthocyanin and Phenolic Compounds

Ultrasound-assisted extraction was used to extract the anthocyanin and phenolic compounds of lowbush Nova Scotian blueberries. The extraction procedure was carried out according to Zheng et al. (2013) with some modifications. The ground blueberry sample was mixed with the solvent for extraction (1% acidified ethanol) in 10 ml Falcon tubes. The ground blueberries were added to the solvent, and the mixture was vortexed for 30 s. The tubes were then placed into the ultrasound bath. Four factors were selected to test their effect on the extraction: the concentration of ethanol, the time and temperature of extraction, and the ratio of extraction solvent to the ground blueberries. Based on the literature and preliminary results, the range for each factor was determined, and then a factorial design was conducted to determine the most significant factors, followed by response surface methodology to optimize extraction of the total anthocyanin content. Total phenolic content was optimised as another response variable to indicate antioxidant activity.

As shown in Table 4.1, the selected four factors for this study were labelled as $X_1$, $X_2$, $X_3$, and $X_4$ and arranged into two levels, coded +1 and −1, for the high and low values, respectively. Further detail of the experimental design is described in Section 5. After extraction in the ultrasound bath, the samples were centrifuged for 20 minutes at 3000 rpm at 4 °C. The supernatant was removed and filtered through a 0.45 μm Millex GS Filter Unit. The filtered extract was stored in a freezer at -16 °C until required for TAC and TPC

tests. Just prior to analysis, extracts were thawed by leaving them at room temperature (Temperature = 21 °C, Relative humidity = 50% - 60%) for two hours.

**Table 4.1: Levels of four different variables.**

| Independent variables | Coded symbols | Levels | |
|---|---|---|---|
| | | -1 | 1 |
| Extraction Temp ($^0$C) | $X_1$ | 25 | 65 |
| Extraction Time (min) | $X_2$ | 3 | 20 |
| SolvConc (%) | $X_3$ | 20 | 100 |
| LSRatio (mL/g) | $X_4$ | 10 | 50 |

## 4.5 Total Anthocyanin Content

Total anthocyanin content (TAC) was determined according to the pH differential spectrophotometric method (Sondheimer & Kertész, 1948; Lee et al., 2005). Two buffer solutions were prepared prior to TAC analysis: potassium chloride (0.025 M, pH =1.0) and sodium acetate (0.4 M, pH = 4.5). In order to measure the pH of these solutions, the pH meter was calibrated prior to use. To prepare the 0.025 M potassium chloride buffer with pH =1.0, 1.86 g KCl was mixed with 200 mL distilled water in a beaker and left on stirring plate for 10 minutes. The pH of the system was adjusted to 1.0 by adding HCl to the solution while stirring. The solution was transferred to a one liter volumetric flask and diluted to volume with distilled water.

To prepare the sodium acetate buffer with a concentration of 0.4 M, 54.43 g sodium acetate ($CH_3CO_2Na.3H_2O$) and approximately 200 ml distilled water were mixed in a beaker and left on a stirring plate for 30 minutes to dissolve the salt. The pH of the system was adjusted to 4.5 using HCl. The solution was transferred to a 1- liter volumetric flask and diluted to volume with distilled water. The prepared buffers were mixed thoroughly.

Then, the solutions were transferred into two 500 mL glassware containers, sealed and kept under the fume hood and away from light before further analysis.

Prior to analysis, the appropriate dilution factor was determined by diluting the thawed blueberry extracts with potassium chloride buffer (pH 1.0) in a glass sample tube. The dilution factor was calculated by dividing the final volume of the sample by the initial volume, where the final volume was 5 ml. Care was taken so that the sample did not exceed 20% of the total volume (i.e. the buffer capacity). The wavelength at which the diluted sample exhibited maximum absorbance ($\lambda_{max}$) was determined to be 520 nm using a UV visible spectrophotometer.

Distilled water was used as blank to zero the spectrophotometer at all wavelengths. Blueberry extracts were diluted in both buffers (pH 1.0 and 4.5) according to the dilution factor previously determined. The samples were then left in the dark at room temperature for between 15 minutes and one hour, and then the absorbance of each mixture was measured at 520 and 700 nm (The reason for measuring the absorbance at 700 nm is to correct for haze). The samples were tested in duplicate.

The absorbance of the diluted samples was calculated as follows:

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (2)$$

$A_{520}$ = Absorbance of diluted samples at 520 nm wavelength

$A_{700}$ = Absorbance of diluted samples at 700 nm wavelength

From this equation, the total anthocyanin content (TAC) was determined:

$$\text{TAC (mg/L)} = \frac{A.MW.DF.1000}{\varepsilon(1)} \quad (3)$$

Where:

MW = molecular weight of the standard (cyanidin 3-glucoside = 449.2 g/mol)

DF = dilution factor (e.g.: 0.2 mL sample is diluted to 3 mL, DF = 15)

1000 = Factor to convert g to mg

$\varepsilon$ = Molar absorptivity (Cyandin 3-glucoside = 26,900 L.mol-1.cm-1)

1 = Cuvette path length (cm)

Results of equation 3 were converted and expressed as mg of cyanidin 3-glucoside equivalents per gram of fruit (on a dry weight basis).

### 4.6 Total Phenolic Content

The total phenolic content (TPC) of blueberry extracts was determined using the Folin & Ciocalteu colorimetric method described by Singleton et al., (1999) with some modification. A standard gallic acid solution of 2.00 mg/ ml was prepared and then diluted to 1.00, 0.5, 0.25, and 0.125 mg/mL to determine the standard curve. Samples of blueberry extracts were brought to room temperature prior to analysis as it was described in section 4.3. Fifty µl of extract, or standard solution of Gallic acid, or water (for the blank) were diluted with 2.7 ml dH2O in glass sample tubes and mixed thoroughly. Then 0.2 ml of Folin & Ciocalteu phenol reagent was added to each solution and mixed thoroughly. Samples were left at room temperature for five minutes. Then 2 ml of 7.5% $Na_2CO_3$ solution was added to the mixture. The samples were incubated for 90 to 120 minutes at room temperature in the dark. Concentrated samples were diluted with water to fall within the standard curve range. The absorbance of the samples was measured at 750 nm using a

spectrophotometer. Based on the standard curve and moisture content, the total phenolic content of blueberries was expressed as gallic acid equivalents per 100 g of freeze dried blueberries.

# 5 EXPERIMENTAL DESIGN

## 5.1 Factorial Design

One of the main purposes for screening the extraction procedure is to identify the effective variables. This requires selecting the appropriate values for the different variables that have an effect on the anthocyanin extraction and could significantly improve the quality of extracts. In a single factor design, the effect of each variable is studied individually, and this can be time consuming. The factorial design is a well-known technique that can minimize the throwbacks of single factor design by combining a series of independent studies into one and by testing all the effective variables at a same time. As a result fewer experiments are required (Barka et al., 2014; Gottipati & Mishra, 2010).

Factorial design is a technique that is used to estimate the overall main factors and the interaction effects among them. For factorial design, a combination of two or more factors can be tested. The levels of factors are given by (–) and (+). The negative sign shows the lowest level of each variable while the positive sign shows the highest level of that variable. If there are k factors each at two-levels, a factorial design has $2^k$ runs. For experiments with two variables the experiments can be described by the corners in a quadrant (see Figure 5.1) and for a design with three variables, these are represented by the corners in a cube (see Figure 5.2) (Lundstedt et al., 1998; Montgomery, 2008). Two level factorial design is the simplest format of a factorial design consisting of k variables, for which each variable has two levels.

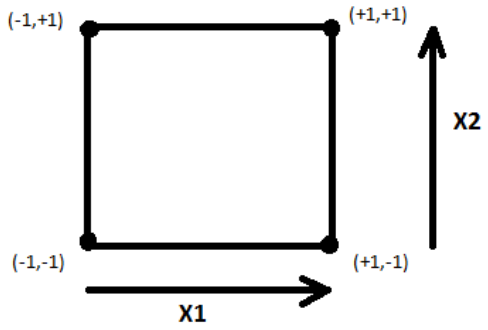



**Figure 5.1: The experiments in a factorial design with two variables.**

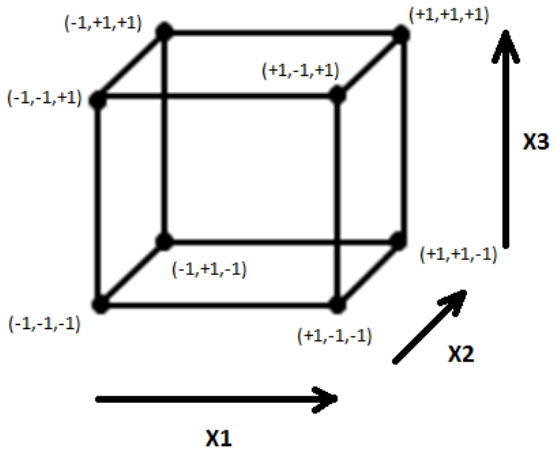



**Figure 5.2: The experiment in a factorial design with three variables.**

To screen the effect of selected variables and levels of the system a $2^4$ factorial design was used, which resulted in 16 experimental studies in duplicate, total of 32 experiments (Table 5.1) created by Minitab 17 software. Linear and interaction coefficients were determined by least squares regression followed by analysis of variance (ANOVA).

**Table 5.1: Two level factorial design of the experiment in duplicate to screen the effect of selected variables on TAC.**

| Run numbers | Temp($X_1$) | Time($X_2$) | SolvConc($X_3$) | LSRatio($X_4$) |
|---|---|---|---|---|
| 1 | -1(25) | -1(3) | -1(20) | -1(10) |
| 2 | -1(25) | -1(3) | -1(20) | 1(50) |
| 3 | -1(25) | -1(3) | 1(100) | -1(10) |
| 4 | -1(25) | -1(3) | 1(100) | 1(50) |
| 5 | -1(25) | 1(20) | -1(20) | -1(10) |
| 6 | -1(25) | 1(20) | -1(20) | 1(50) |
| 7 | -1(25) | 1(20) | 1(100) | -1(10) |
| 8 | -1(25) | 1(20) | 1(100) | 1(50) |
| 9 | 1(65) | -1(3) | -1(20) | -1(10) |
| 10 | 1(65) | -1(3) | -1(20) | 1(50) |
| 11 | 1(65) | -1(3) | 1(100) | -1(10) |
| 12 | 1(65) | -1(3) | 1(100) | 1(50) |
| 13 | 1(65) | 1(20) | -1(20) | -1(10) |
| 14 | 1(65) | 1(20) | -1(20) | 1(50) |
| 15 | 1(65) | 1(20) | 1(100) | -1(10) |
| 16 | 1(65) | 1(20) | 1(100) | 1(50) |
| 17 | -1(25) | -1(3) | -1(20) | -1(10) |
| 18 | -1(25) | -1(3) | -1(20) | 1(50) |
| 19 | -1(25) | -1(3) | 1(100) | -1(10) |
| 20 | -1(25) | -1(3) | 1(100) | 1(50) |
| 21 | -1(25) | 1(20) | -1(20) | -1(10) |
| 22 | -1(25) | 1(20) | -1(20) | 1(50) |
| 23 | -1(25) | 1(20) | 1(100) | -1(10) |
| 24 | -1(25) | 1(20) | 1(100) | 1(50) |
| 25 | 1(65) | -1(3) | -1(20) | -1(10) |
| 26 | 1(65) | -1(3) | -1(20) | 1(50) |
| 27 | 1(65) | -1(3) | 1(100) | -1(10) |
| 28 | 1(65) | -1(3) | 1(100) | 1(50) |
| 29 | 1(65) | 1(20) | -1(20) | -1(10) |
| 30 | 1(65) | 1(20) | -1(20) | 1(50) |
| 31 | 1(65) | 1(20) | 1(100) | -1(10) |
| 32 | 1(65) | 1(20) | 1(100) | 1(50) |

## 5.2 Response Surface Methodolog

Response surface methodology is a powerful technique that allows optimization and determination of the best conditions to maximize the desired responses. There are different multilevel designs that have been used for the optimization of variables in many studies such as central composite design (CCD) and Box–Behnken design (BBD) (Grosso et al., 2014; Ngan et al., 2014).

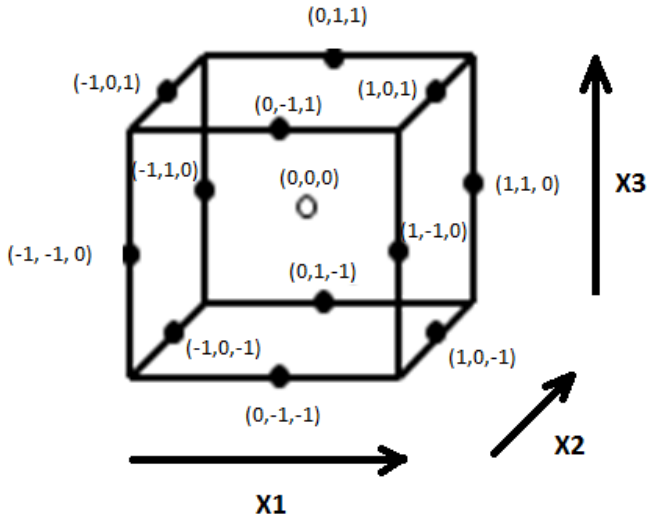



**Figure 5.3. The experiment in a Box-Behnken design with three variables.**

The Box Behnken design consists of the center point and middle points of the edges from a cube (Figure 5.3). This design, which is based on three-level incomplete factorial design is a spherical, rotatable, or nearly rotatable second-order design. The Box-Behnken design was selected in this study as it has a higher efficiency compared with CCD and is more efficient than a factorial design (Ferreira et al., 2007).

The extraction was carried out as described in the preliminary extraction assay (see Section 4.4.). The independent variables were analysed at three levels ($X_1$: 25 $^0$C (−1),

$45^0$C (0), 65 $^0$C (+1); $X_2$: 3 min (−1), 10 min (0), 20 min (+1); $X_3$: 20% (−1), 60% (0), 100% (+1); $X_4$: 10 (−1), 30 (0), 50 (+1)) comprising 27 runs that were conducted in triplicate (Table 5.2). The measurement of total anthocyanin content (TAC) and total phenolic content (TPC) were expressed as mg/ g of dry plant material. In addition, three replicates at the center point were performed to estimate the pure error.

Experimental data were fitted by the following second-order polynomial model according to the following equation (Montgomery 1997):

$$\hat{y} = \beta_{0+} \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \beta_{ii} x_i^2 + \beta_{jj} x_j^2 + e \quad (4)$$

Where Y was the response variable; $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ represent the regression coefficients of constant, linear, quadratic, and interactions terms, respectively, while $X_i$ represents the independent variable. $X_j$ and $X_i^2$ represent the interaction and quadratic terms, respectively.

**Table 5.2: Response surface methodology variables of the experiment to optimise TAC and TPC.**

| Run number | Temp($X_1$) | Time($X_2$) | SolvConc($X_3$) | LSRatio($X_4$) |
|---|---|---|---|---|
| 1 | -1(25) | -1(3) | 0(60) | 0(30) |
| 2 | 1(25) | -1(3) | 0(60) | 0(30) |
| 3 | -1(25) | 1(20) | 0(60) | 0(30) |
| 4 | 1(65) | 1(20) | 0(60) | 0(30) |
| 5 | 0(45) | 0(11.5) | -1(20) | -1(10) |
| 6 | 0(45) | 0(11.5) | 1(100) | -1(10) |
| 7 | 0(45) | 0(11.5) | -1(20) | 1(50) |
| 8 | 0(45) | 0(11.5) | 1(100) | 1(50) |
| 9 | -1(25) | 0(11.5) | 0(60) | -1(10) |
| 10 | 1(65) | 0(11.5) | 0(60) | -1(10) |
| 11 | -1(25) | 0(11.5) | 0(60) | 1(50) |
| 12 | 1(65) | 0(11.5) | 0(60) | 1(50) |
| 13 | 0(45) | -1(3) | -1(20) | 0(30) |
| 14 | 0(45) | 1(20) | -1(20) | 0(30) |
| 15 | 0(45) | -1(3) | 1(100) | 0(30) |
| 16 | 0(45) | 1(20) | 1(100) | 0(30) |
| 17 | -1(25) | 0(11.5) | -1(20) | 0(30) |
| 18 | 1(65) | 0(11.5) | -1(20) | 0(30) |
| 19 | -1(25) | 0(11.5) | 1(100) | 0(30) |
| 20 | 1(65) | 0(11.5) | 1(100) | 0(30) |
| 21 | 0(45) | -1(3) | 0(60) | -1(10) |
| 22 | 0(45) | 1(20) | 0(60) | -1(10) |
| 23 | 0(45) | -1(3) | 0(60) | 1(50) |
| 24 | 0(45) | 1(20) | 0(60) | 1(50) |
| 25 | 0(45) | 0(11.5) | 0(60) | 0(30) |
| 26 | 0(45) | 0(11.5) | 0(60) | 0(30) |
| 27 | 0(45) | 0(11.5) | 0(60) | 0(30) |

The optimum conditions were found by analyzing the response surface plots aiming for the highest reachable response variable for each independent parameter. Fischer's F-test determined the second-order model equation at a probability (P) level, which for this study was selected as 0.01. The adequacy of the model was determined by assessing the lack of fit, the coefficient of determination ($R^2$) and the F test value obtained from the analysis of variance (ANOVA).

# 6 RESULTS AND DISCUSSION

## 6.1 Extraction Screening

The screening of the experimental variables was carried out using a two level factorial design. In this study, the main effects of the four selected factors on total anthocyanin content (TAC) were considered, in addition to the interactions amongst the variables. Table 6.1 presents the average of total anthocyanin content obtained through extraction, using a two level factorial design to generate the experimental procedure. As was described in Section 4.4., four variables, temperature ($X_1$), time ($X_2$), solvent concentration ($X_3$) and liquid solid ratio ($X_4$) were selected with two levels (-1, +1) for each variable to monitor the total anthocyanin content. The total anthocyanin content ranged from 2.86 to 12.55 mg C3G/ g DW.

**Table 6.1: Two level factorial design and results.**

| Run number | Temp ($X_1$) | Time ($X_2$) | SolvConc($X_3$) | LSRatio ($X_4$) | TAC* (mg C3G/g DW) |
|---|---|---|---|---|---|
| 1 | -1 | -1 | -1 | -1 | 7.10 |
| 2 | -1 | -1 | -1 | 1 | 10.29 |
| 3 | -1 | -1 | 1 | -1 | 2.86 |
| 4 | -1 | -1 | 1 | 1 | 7.34 |
| 5 | -1 | 1 | -1 | -1 | 7.73 |
| 6 | -1 | 1 | -1 | 1 | 10.73 |
| 7 | -1 | 1 | 1 | -1 | 4.10 |
| 8 | -1 | 1 | 1 | 1 | 9.69 |
| 9 | 1 | -1 | -1 | -1 | 9.36 |
| 10 | 1 | -1 | -1 | 1 | 11.66 |
| 11 | 1 | -1 | 1 | -1 | 5.51 |
| 12 | 1 | -1 | 1 | 1 | 11.52 |
| 13 | 1 | 1 | -1 | -1 | 10.50 |
| 14 | 1 | 1 | -1 | 1 | 11.68 |
| 15 | 1 | 1 | 1 | -1 | 10.85 |
| 16 | 1 | 1 | 1 | 1 | 12.55 |
| 17 | -1 | -1 | -1 | -1 | 7.82 |
| 18 | -1 | -1 | -1 | 1 | 10.90 |
| 19 | -1 | -1 | 1 | -1 | 3.47 |
| 20 | -1 | -1 | 1 | 1 | 7.02 |
| 21 | -1 | 1 | -1 | -1 | 7.95 |
| 22 | -1 | 1 | -1 | 1 | 11.16 |
| 23 | -1 | 1 | 1 | -1 | 3.91 |
| 24 | -1 | 1 | 1 | 1 | 10.54 |
| 25 | 1 | -1 | -1 | -1 | 10.42 |
| 26 | 1 | -1 | -1 | 1 | 12.37 |
| 27 | 1 | -1 | 1 | -1 | 4.51 |
| 28 | 1 | -1 | 1 | 1 | 7.40 |
| 29 | 1 | 1 | -1 | -1 | 9.89 |
| 30 | 1 | 1 | -1 | 1 | 11.83 |
| 31 | 1 | 1 | 1 | -1 | 8.91 |
| 32 | 1 | 1 | 1 | 1 | 12.45 |

* TAC is reported as mg cyanidin-3-glycoside per g dry weight of blueberries.

Analysis of variance (ANOVA) was used to check the significance of the variables. The significance of each independent variable and their interaction effects are presented in

Table 6.2, where the P-value, F-value and adjusted sum of squares for each factor in addition to the interaction between factors up to two level is included. The significant level was chosen as $\alpha = 0.01$, and variables with p-value less than 0.01 were considered significant. According to the results, the temperature ($X_1$), time ($X_2$), solvent concentration ($X_3$), and liquid solid ratio ($X_4$), all had significant effects on the response factor (TAC). According to the Pareto chart (Figure 6.1), the liquid solid ratio was found to have the most impact on TAC, followed by solvent concentration and temperature. The conditions that provided the highest extraction of total anthocyanin content extraction were: T = 65$^0$C, t = 20 min, SolvConc = 20%, and LSRatio= 50 mL/g.

**Table 6.2: The results of 2-level factorial design analysis.**

| Parameters | Adjusted sum of square | F-value | p-value |
|---|---|---|---|
| Model | 235.769 | 24.7 | <0.005 |
| Linear | 205.289 | 53.78 | <0.005 |
| $X_1$-Extraction temperature($^0$C) | 47.093 | 49.35 | <0.005 |
| $X_2$-Extraction Time (min) | 19.419 | 20.35 | <0.005 |
| $X_3$-Solvent Concentration (%) | 46.912 | 49.16 | <0.005 |
| $X_4$-Liquid to solid ratio (mL/g) | 91.865 | 96.26 | <0.005 |
| 2-way Interaction | 30.48 | 5.32 | 0.002 |
| $X_1X_2$-Temp*Time | 1.489 | 1.56 | 0.225 |
| $X_1X_3$-Temp*SolvConc | 3.622 | 3.8 | 0.065 |
| $X_1X_4$-Temp*LSRatio | 3.917 | 4.1 | 0.056 |
| $X_2X_3$-Time*SolvConc | 14.845 | 15.56 | 0.001 |
| $X_2X_4$-Time*LSRatio | 0.013 | 0.01 | 0.906 |
| $X_3X_4$-SolvConc*LSRatio | 6.593 | 6.91 | 0.016 |
| Error | 20.041 | | |
| Lack-of-Fit | 6.932 | 1.69 | 0.194 |
| Pure Error | 13.109 | | |
| Total | 255.81 | | |

$R^2 = 94.88\%$

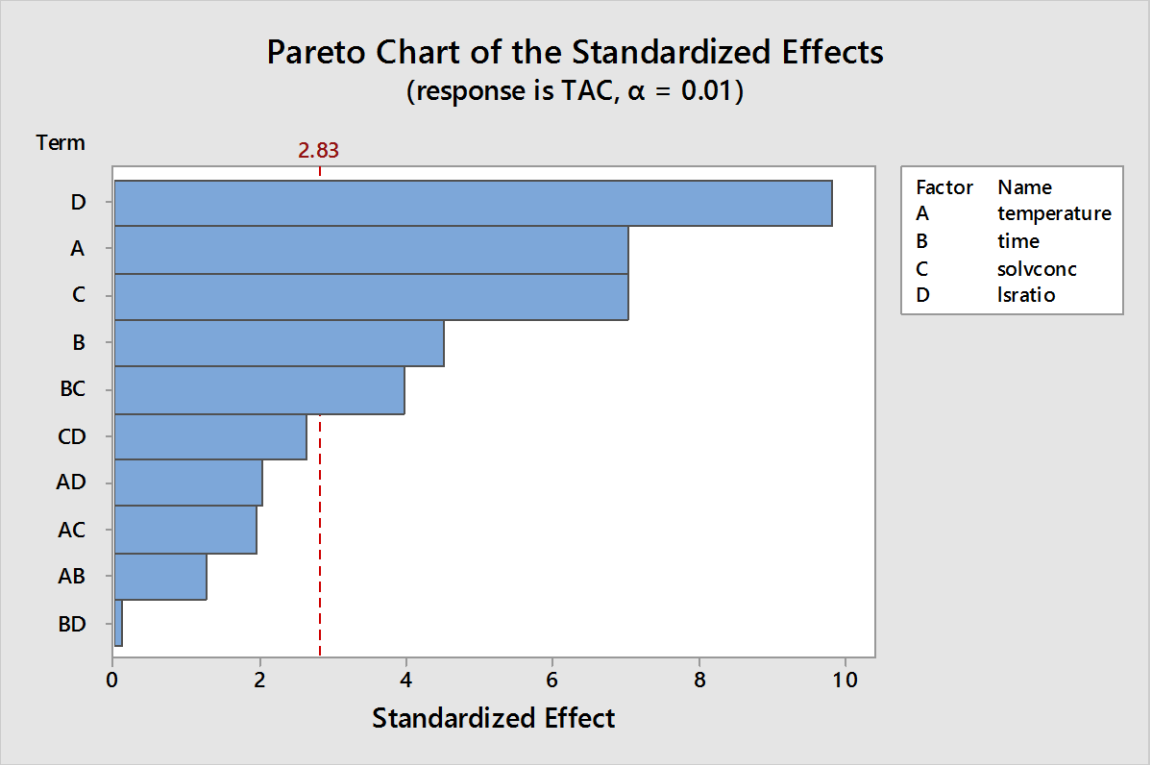



**Figure 6.1: Pareto chart of two level factorial design on TAC of lowbush blueberries, representing the liquid solid ratio as the most significant variable.**

The main effect plot for each factor is shown in Figure 6.2. Temperature, time and liquid solid ratio all had a positive effect on total anthocyanin content. The higher the temperature of the ultrasound bath, the faster solvent molecules move and penetrate in the plant material. Increase in extraction time and solvent (acidified ethanol) helped with the anthocyanin extraction as well. Solvent concentration had a negative effect and by increasing the concentration of ethanol in the system the anthocyanin content decreased significantly.

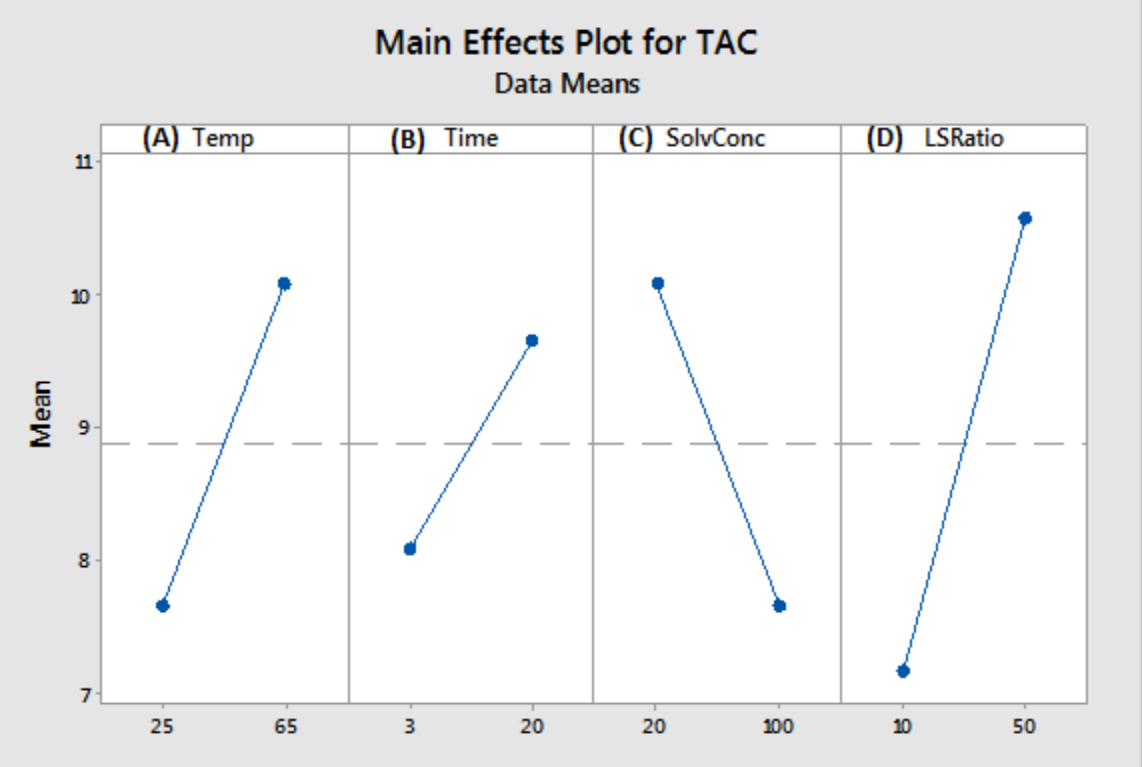



**Figure 6.2: Positive effect of (A) temperature, (B) time, (C) liquid solid ratio and negative effect of (C) solvent concentration on the TAC (mg C3G/g DW) extracted from lowbush blueberries.**

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An interaction effect plot is presented in Figure 6.3, where the non-parallel lines for time ($X_2$) and solvent concentration ($X_3$) indicate that there is an interaction between the factors time and solvent concentration, this interaction is also supported by the p-value of $X_1X_3$ (0.001) presented in Table 6.2. This interaction means that the effect of one variable depends on the effect of the other. To clarify the effects, further analysis is required. Response surface methodology, using Box-Behnken design was used to obtain more information on the effects of each variable, as well as to optimize each significant factor.

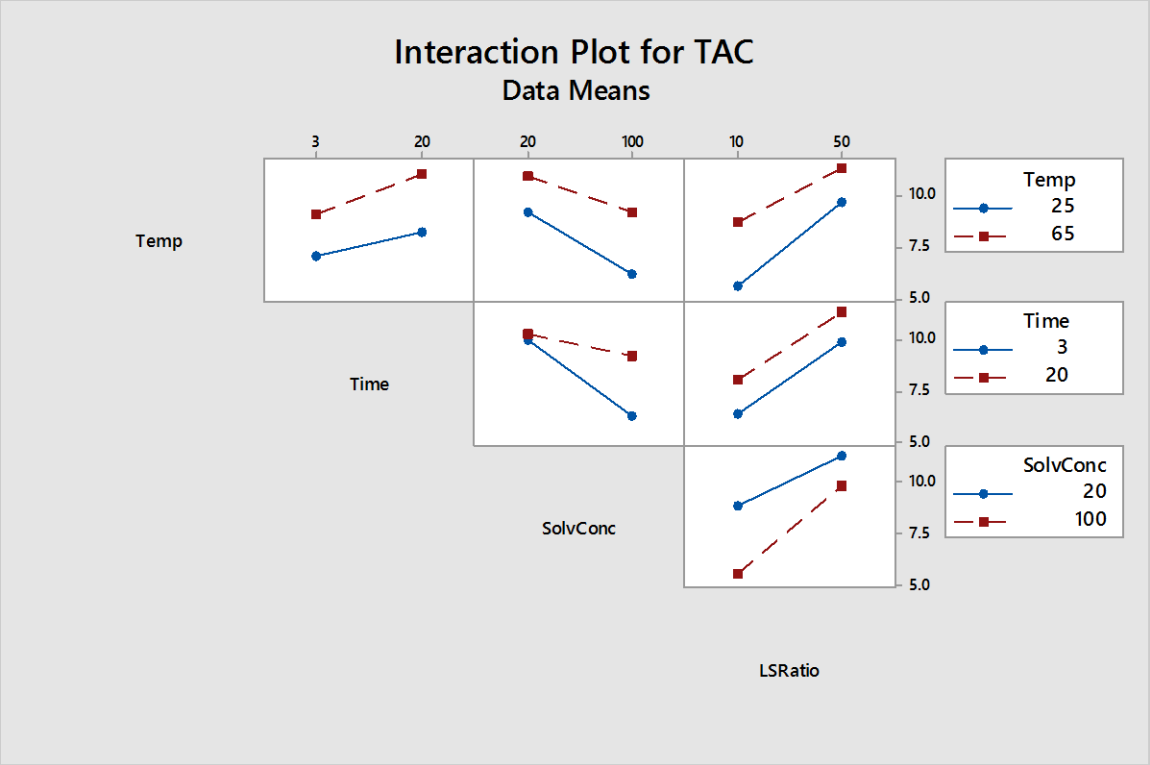


**Figure 6.3: Two-way interaction plot of variables on TAC (mg C3G/g DW).**

## 6.2 Extraction Optimization

Response surface methodology was used to establish a statistical model to predict total anthocyanin content and to determine the optimal extraction conditions. The effect and interactions of the variables on total anthocyanin content were shown in individual response surface plots and discussed. A reduced model to predict total anthocyanin content was then proposed, followed by verification of both the full and reduced models with experimental data. As a comparison, a statistical model was also developed for total phenolic content, using response surface methodology, and individual response surface plots for the variables were analyzed and discussed.

### 6.2.1 Total Anthocyanin Content

Design Expert 9.0 was used to generate the experimental conditions based on Box-Behnken design for analysis by response surface methodology. This resulted in 27 experiments in which the samples were analyzed for total anthocyanin content and stored for later total phenolic analysis.

Table 6.3 shows the experimental conditions and resultant total anthocyanin content according to the design of the experiment. Also, raw data and detailed TAC content of each replicate is presented in Appendix B. The total anthocyanin content ranged from 5.68 to 13.22 mg C3G/g DW (assuming water content of blueberry 85%: 0.85 to 1.98 mg C3G/g FW). Other researchers who have reported the anthocyanin extracts from lowbush blueberries have obtained anthocyanin contents of 4.35 μmol malvidin 3-glucoside/g FW at harvest (Kalt et al., 1999) and 96.60 μmol Torox equivalent/ g FW (Wu et al., 2004).

**Table 6.3: Response surface methodology results of TAC.**

| Run number | Temp ($X_1$) | Time ($X_2$) | SolvConc ($X_3$) | LSRatio ($X_4$) | TAC*(mg C3G/g DW) |
|---|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 0 | 11.95 |
| 2 | 1 | -1 | 0 | 0 | 12.89 |
| 3 | -1 | 1 | 0 | 0 | 11.50 |
| 4 | 1 | 1 | 0 | 0 | 12.12 |
| 5 | 0 | 0 | -1 | -1 | 9.04 |
| 6 | 0 | 0 | 1 | -1 | 5.68 |
| 7 | 0 | 0 | -1 | 1 | 10.56 |
| 8 | 0 | 0 | 1 | 1 | 10.84 |
| 9 | -1 | 0 | 0 | -1 | 10.25 |
| 10 | 1 | 0 | 0 | -1 | 9.32 |
| 11 | -1 | 0 | 0 | 1 | 12.43 |
| 12 | 1 | 0 | 0 | 1 | 13.22 |
| 13 | 0 | -1 | -1 | 0 | 10.34 |
| 14 | 0 | 1 | -1 | 0 | 10.95 |
| 15 | 0 | -1 | 1 | 0 | 7.77 |
| 16 | 0 | 1 | 1 | 0 | 8.50 |
| 17 | -1 | 0 | -1 | 0 | 10.40 |
| 18 | 1 | 0 | -1 | 0 | 10.94 |
| 19 | -1 | 0 | 1 | 0 | 6.01 |
| 20 | 1 | 0 | 1 | 0 | 11.97 |
| 21 | 0 | -1 | 0 | -1 | 10.40 |
| 22 | 0 | 1 | 0 | -1 | 9.02 |
| 23 | 0 | -1 | 0 | 1 | 12.70 |
| 24 | 0 | 1 | 0 | 1 | 12.10 |
| 25 | 0 | 0 | 0 | 0 | 12.12 |
| 26 | 0 | 0 | 0 | 0 | 11.57 |
| 27 | 0 | 0 | 0 | 0 | 11.39 |

*TAC is expressed as milligram of cyanidin-3-glycoside per gram of dry weight.

#### 6.2.1.1 Full Model for the Optimization of TAC

By performing multiple regression analysis on the experimental data, the response variable (TAC) and the test variables were related by the following second-order polynomial equation considering all the interactions between variables:

$$TAC = 11.69192 + 0.659669\ X_1 - 0.15587\ X_2 - 0.95482\ X_3 + 1.511415\ X_4 + -0.07694\ X_1X_2 + 1.356352\ X_1X_3 + 0.430086\ X_1X_4 + 0.028967\ X_2X_3 + 0.195819\ X_2X_4 + 0.908675\ X_3X_4 + 0.326412\ X_1^2 - 0.02118\ X_2^2 - 2.17516\ X_3^2 - 0.60451\ X_4^2 \quad (2)$$

Where $X_1$ (temperature), $X_2$ (time), $X_3$ (solvent concentration) and $X_4$ (ratio of liquid to solids) were the coded variables, and the minimum, midpoint and maximum values were -1, 0 and +1, respectively.

The model from Equation 2 can also be presented as Equation 3 below, where instead of the coded values, the experimental ranges for each variable are applied.

$$TAC = 13.2387712 - 0.169236979\ X_1 - 0.030894379\ X_2 + 0.027916753\ X_3 + 0.036466018\ X_4 - 0.00045\ X_1X_2 + 0.00169544\ X_1X_3 + 0.001075214\ X_1X_4 + 8.5196E\text{-}05\ X_2X_3 + 0.001151878\ X_2X_4 + 0.001135843\ X_3X_4 + 0.00081603\ X_1^2 - 0.000293206\ X_2^2 - 0.001359475\ X_3^2 - 0.001511285\ X_4^2 \quad (3)$$

The regression coefficient values of Eq. (2) and (3) are listed in Table 6.4.

**Table 6.4: Coded and uncoded Regression coefficients of the predicted second-order model for TAC.**

| Term | Coded Coefficients | Uncoded Coefficients |
|---|---|---|
| Constant | 11.69192477 | 13.23877 |
| $X_1$ | 0.65966922 | -0.16924 |
| $X_2$ | -0.155870385 | -0.03089 |
| $X_3$ | -0.954815985 | 0.027917 |
| $X_4$ | 1.511415018 | 0.036466 |
| $X_1X_2$ | -0.076944586 | -0.00045 |
| $X_1X_3$ | 1.356351684 | 0.001695 |
| $X_1X_4$ | 0.430085551 | 0.001075 |
| $X_2X_3$ | 0.028966652 | 8.52E-05 |
| $X_2X_4$ | 0.195819285 | 0.001152 |
| $X_3X_4$ | 0.908674713 | 0.001136 |
| $X_1^2$ | 0.326412099 | 0.000816 |
| $X_2^2$ | -0.021184126 | -0.00029 |
| $X_3^2$ | -2.175159881 | -0.00136 |
| $X_4^2$ | -0.604513956 | -0.00151 |

To determine if the quadratic model was significant, the statistical significance of the regression equation was checked by analysis of variance (ANOVA), as summarized in Table 6.5. The significance of each coefficient was determined by the F-test and p-value. The variables are considered more significant when the corresponding p-values are below 0.01. Fisher's F-value (8.74) of the quadratic model with a very low p-value (0.000291436) implies that the model is significant and suitable to be used in this experiment. The lack of fit is non-significant (p-value> 0.01), indicating the model could adequately fit the data. Temperature and time showed no significant effect on total anthocyanin content (TAC). However, the liquid to solid ratio ($X_4$) was the most significant parameter influencing the total anthocyanin content, followed by solvent concentration

($X_3$). Also, quadratic term coefficients ($X_3^2$) were significant with very small p-value, indicating an optimum point for solvent concentration. The p-value of the interaction factor between temperature and solvent concentration (0.0078) indicated that there is a significant interaction between these two factors. The rest of the coefficients were not significant.

**Table 6.5: Analysis of variance for the full quadratic model of TAC.**

| Source | Sum of Squares | df | Mean Square | F-Value | p-value |
|---|---|---|---|---|---|
| Model | 88.91 | 14 | 6.35 | 8.74 | 0.000291436 |
| $X_1$-Temp | 5.22 | 1 | 5.22 | 7.19 | 0.020009913 |
| $X_2$-Time | 0.29 | 1 | 0.29 | 0.40 | 0.538346025 |
| $X_3$-SolvConc | 10.94 | 1 | 10.94 | 15.06 | 0.002187355 |
| $X_4$-LSRatio | 27.41 | 1 | 27.41 | 37.72 | 5.00649E-05 |
| $X_1X_2$ | 0.02 | 1 | 0.02 | 0.03 | 0.86 |
| $X_1X_3$ | 7.36 | 1 | 7.36 | 10.13 | 0.0078 |
| $X_1X_4$ | 0.74 | 1 | 0.74 | 1.02 | 0.33 |
| $X_2X_3$ | 0.00 | 1 | 0.00 | 0.00 | 0.95 |
| $X_2X_4$ | 0.15 | 1 | 0.15 | 0.21 | 0.65 |
| $X_3X_4$ | 3.30 | 1 | 3.30 | 4.55 | 0.054 |
| $X_1^2$ | 0.57 | 1 | 0.57 | 0.78 | 0.39 |
| $X_2^2$ | 0.00 | 1 | 0.00 | 0.00 | 0.96 |
| $X_3^2$ | 25.23 | 1 | 25.23 | 34.73 | 0.00007 |
| $X_4^2$ | 1.95 | 1 | 1.95 | 2.68 | 0.13 |
| Residual | 8.72 | 12 | 0.73 | | |
| Lack of Fit | 8.43 | 10 | 0.84 | 5.85 | 0.15 |
| Pure Error | 0.29 | 2 | 0.14 | | |
| Total | 97.63 | 26 | | | |

The adequacy of the model can be assessed by a number of parameters to determine whether the model fits the data – these are summarized in Table 6.6. The coefficient of determination indicates the correlation between the actual values and predicted ones, which in this study was $R^2$ = 91.07%. This represents a satisfactory correlation between the experimental data and predicted values. However, adjusted $R^2$ and predicted $R^2$ are not

close which is not satisfactory (Design expert software suggests maximum of 0.2 difference). The adequate precision value measures the signal to noise ratio, for which a ratio above 4 is desirable. In this study, the ratio is 13.3430 indicating an adequate signal. Another factor to analyze the sufficiency of the model is the coefficient of variance (C.V. %) which is defined by Equation (4) :

$$\mathrm{CV} = \frac{\text{Standard Deviation}}{\text{Mean}} \mathrm{X}\ 100 \qquad (4)$$

The coefficient of variance in this study was found to be 8.05%, indicating a high degree of precision and reliability of the experimental values (Design expert software suggests CVs of 10% or higher means weak method performance). From this analysis, it can be concluded that the model is adequate for predicting total anthocyanin content within the experimental ranges specified for the variables. However, the low predicted $R^2$ can be improved by model reduction.

**Table 6.6: Fit statistics for the full model predicting Y (TAC).**

| | |
|---|---|
| $R^2$ | 91.07% |
| Adjusted $R^2$ | 80.56% |
| Predicted $R^2$ | 49.59% |
| Adequate Precision | 13.3430 |
| Std. Dev. | 0.85 |
| C.V. % | 8.05 |

#### 6.2.1.2 Effect of Extraction Factors on TAC

Optimum values of the variables were obtained according to the regression equation (Eq. (2)) using Design Expert 9.0 software. These are presented in Table 6.7, with the predicted maximum total anthocyanin content of 13.22 mg C3G/g DW.

**Table 6.7: Variables optimum values of the TAC full model.**

| Temperature ($^0$C) | Time (min) | SolvConc (%) | LSRatio (mL/g) | TAC (mg C3G/g DW) |
|---|---|---|---|---|
| 65 | 11.5 | 60 | 50 | 13.22 |

The effects of each variable and interactions of the variables on total anthocyanin content were analysed using graphical representations of Eq. (2) in the form of three dimensional response surface plots and two dimensional contour plots. In these plots the effect of two factors is considered, while the other two factors were kept constant at their respective level zero (0), representing the center value of the testing ranges. The response is the total anthocyanin content (TAC, mg C3G/g DW).

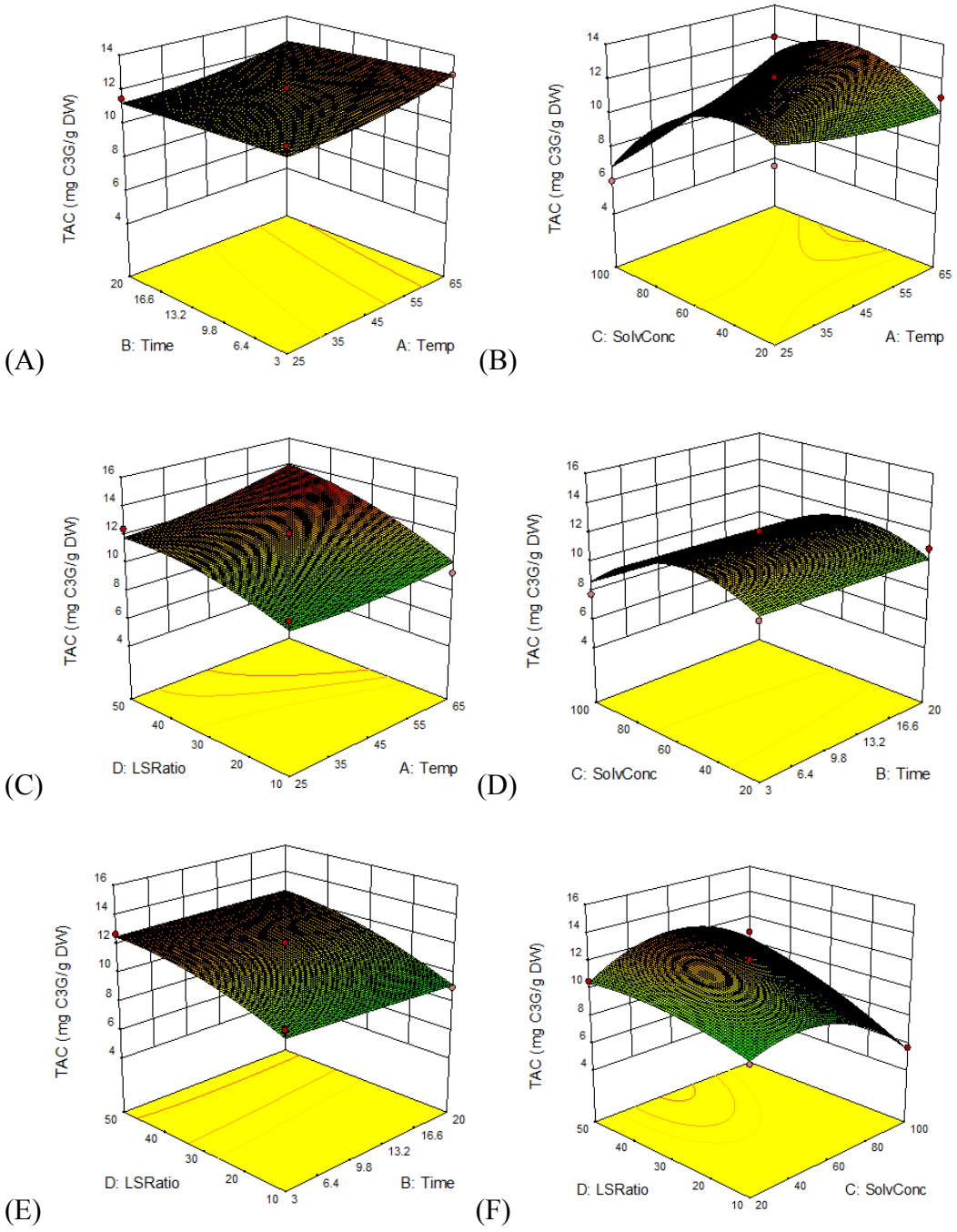


**Figure 6.4: Response surface plots (3-D) showing the effects of variables (X1: extraction temp; X2: extraction time; X3: solvent concentration; X4: liquid solid ratio) on the response Y (TAC).**

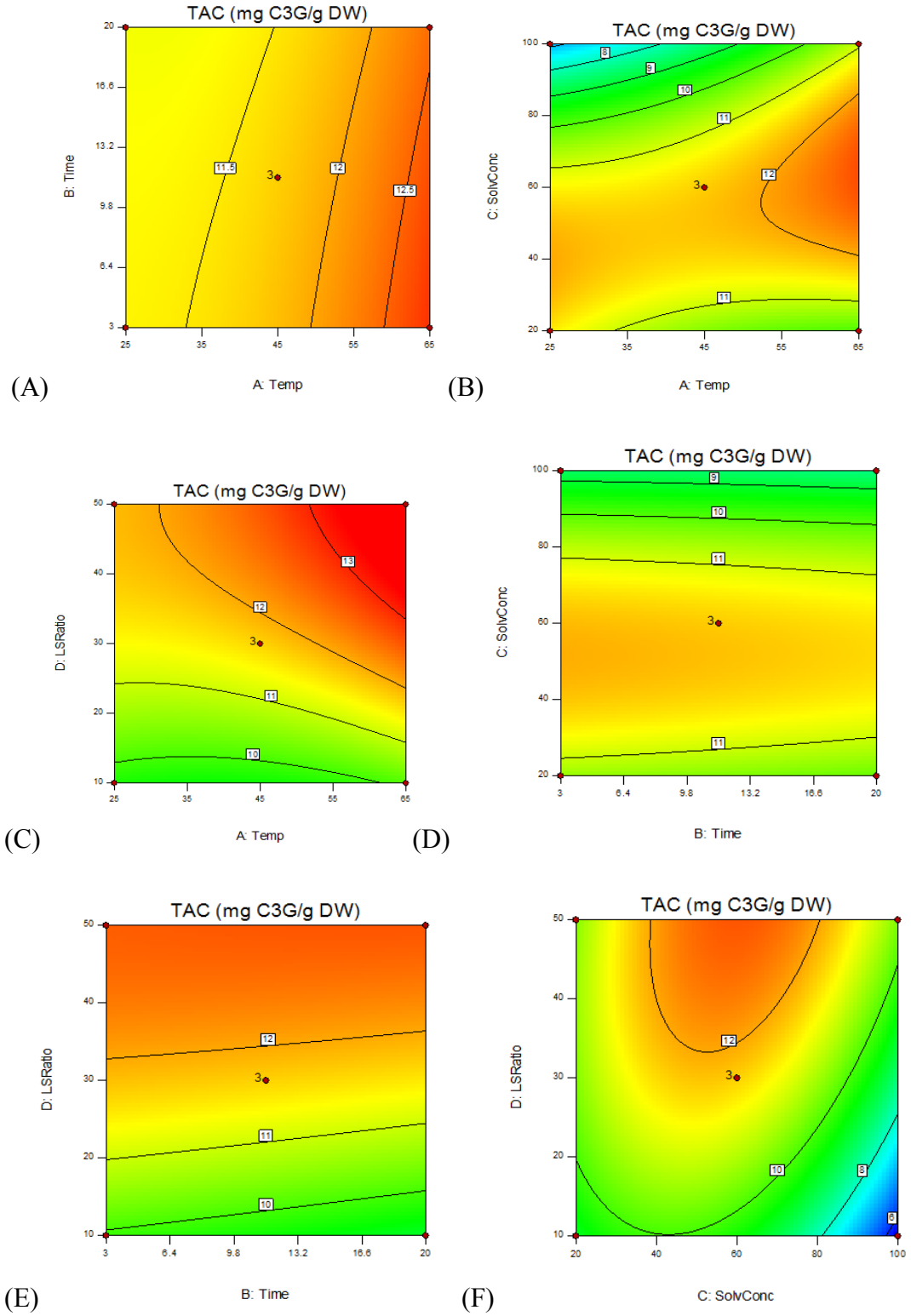


**Figure 6.5: Contour plots (2-D) showing the effects of variables (X1: extraction temp; X2: extraction time; X3: solvent concentration; X4: liquid solid ratio) on the response Y (TAC).**

Temperature, time and the interaction between these two variables were analysed according to the RSM diagrams. It can be seen from Figure 6.4A and 6.5A that the effects of temperature and time are not significant and no optimum point is found among the range of the variables. This may be due to the selected range of temperature and times for this study (25 to 65 °C, 3 to 20 minutes) being too limited and not including values in the optimal range. In another study, it was reported an effective range of extraction time and temperature was 10 min to 50 minutes and 30 °C to 60 °C, respectively, for optimizing the extraction of TPC and TAC of wine lees (Tao et al., 2015). The difference between these results and the present study may be attributed to the difference in the composition of phenolic and anthocyanin compounds within the fruit samples used for extraction and the associated differences in extraction time and temperature effects; however, further analysis such as HPLC would need to confirm this. Also, high temperatures would speed up the softening and swelling of the wine lees, could result in increasing the solubility of extracted compounds. It is possible that by increasing the extraction temperature range, a significant effect would be observed, especially as degradation of anthocyanin compounds would be expected at very high temperatures. In addition, the physical characteristics of the fruit sample would be important. For example, most of the phenolics from wine lees were assumed to be located on the external surface of the particles and within the particles with broken walls. As a result, a rapid extraction rate was observed during the first 10 minutes of extraction; after 10 minutes the extraction rate increased slowly. In the present study of anthocyanin extracts, the freezedried blueberries were ground to 500 microns prior to extraction, and it is possible that a rapid extraction rate may have occurred before the three

minute extraction time, so that in the chosen time range for this study (3 to 20 mins), no further significant difference in TAC was observed.

Another variable examined in this research was solvent concentration. Figure 6.4B and 6.5B indicate that there is a significant interaction between temperature and solvent concentration. It can be observed from the graph that at the lower temperature (25 $^{o}$C), the maximum total anthocyanin content is achieved with solvent concentration of around 20% to 60% while at the highest temperature (65 $^{o}$C) with solvent concentration of 40% to 80%, maximum TAC is achieved.

The ratio of liquid to the solid ground blueberry was a variable in determining the total anthocyanin content. According to Figure 6.4C and 6.5C, liquid to solid ratio has a significant effect on the total anthocyanin content while temperature does not. When the ratio of liquid to solid was increased, the TAC increased. If the liquid to solid ratio is too small there will not be enough liquid to fully extract the solute. If the ratio is too big, the cost of the process is high. As a result, a suitable ratio of liquid to solid material should be selected to extract the anthocyanins from plant material due to the cost of having high liquid to solid ratio. The anthocyanin extraction was increased by increasing the liquid solid ratio up to 50 mL/g DW. In another study, optimum extraction of anthocyanins from wine lees was also achieved by using 50 ml/g of liquid to solid ratio (Tao et al., 2014). The solubility of different molecules depends on the intermolecular or interionic forces between solute and solvent. In general, a larger solvent volume can dissolve the elements more effectively due to the high concentration gradient between the solid raw materials and the bulk solvent, which is the driving force during mass transfer and leads to the improvement of the extraction (Li et al., 2005, Tao & Sun, 2013).

The effects of solvent concentration and time are also shown in Figure 6.4D and 6.5D. The extracted TAC is at its maximum with solvent concentration of 40% to 60%, while no effect of variable time and no interaction between time and solvent concentration were found. Also, Figure 6.4E and 6.5E showed that time had no significant effect while the liquid solid ratio had a significant effect.

According to Figure 6.4F and 6.5F, the shape of the contour plot is elliptical, which indicates that the two variables (liquid solid ratio and solvent concentration) have significant effects on TAC. The amount of anthocyanin extraction was less in pure ethanol than in the ethanol-water mixture, which agrees with other studies in the literature (Spigno et al., 2007; Dong et al., 2010). For the range of solvent concentrations tested (20%, 60%, 100%), the highest anthocyanin content (13.22 mg C3G/ g DW) was achieved at a solvent concentration of 60%. The maximum TAC was reached at a liquid solid ratio of about 30 to 50 mL/g with 60% solvent concentration. The TAC decreased as the solvent concentration was increased from 80% to 100%. Samples with a liquid solid ratio less than about 30 mL/g resulted in low values of TAC. The effect of solvent concentration on anthocyanin extraction can be attributed to changes in the solvent polarity as the water-ethanol concentration in the solvent mixture is modified, thus affecting the solubility of anthocyanin compounds. Using ethanol–water mixtures with a lower ethanol content would have advantages when scaling up extraction processes to industrial-scale operations. By reducing the cost of the solvent and by producing less ethanol as waste, which would benefit the environment.

No information has been found on ultrasound-assisted extraction of lowbush blueberries using acidified ethanol as a solvent. Thus, there is no proper source with which

to compare the results obtained from this study with literature. However, there are some studies on optimizing the extraction of phenolic compounds from a variety of fruits using Box-Behnken design to develop the experimental plan. The extraction of antioxidant phenolic compounds from quinoa seeds was investigated and it was reported that a solvent with 50% ethanol had a three-fold higher extraction yield than pure water (Galvan D'Alessandro et al., 2014). The optimization of phenolic compounds from apples using Box-Behnken design was also studied (Alberti et al., 2014). Extraction with 84.5% methanol for 15 min at 28 $^{o}$C and extraction with 65% acetone for 20 min at 10 $^{o}$C were found to provide the best results. These results indicate that factors such as different solvents and fruit can lead to different optimal conditions.

#### 6.2.1.3 Model Reduction

From the ANOVA analysis of the full model and the examination of the p-values, it was found that not all four variables are significant in their effect on anthocyanin extraction (Table 6.5). The ethanol concentration and the solvent-to-solid ratio had significant effects on the total anthocyanin extraction; however, temperature and time were not statistically significant factors. Although the full model for predicting total anthocyanin content (represented by Eq. (2) and (3)), resulted in adequate fit parameters (presented in Table 6.8), it can be improved and presented in a more convenient form by removing non-significant terms. Thus, the non-significant interactions and quadratic terms were removed from the full model and a reduced model was obtained using Design Expert 9.0 software. The reduced model, written in terms of coded variables, is given by:

$$TAC = 11.53230558 + 0.65966922\ X_1 - 0.155870385\ X_2 - 0.954815985\ X_3 + 1.511415018\ X_4 + 1.356351684\ X_1X_3 - 2.115302685\ X_3^2 \quad (5)$$

**Table 6.8: Analysis of variance for the reduced quadratic model of TAC.**

| Source | Sum of Squares | df | Mean Square | F-Value | p-value |
|---|---|---|---|---|---|
| Model | 81.05 | 6 | 13.51 | 16.3 | 9.41E-07 |
| $X_1$-Temp | 5.22 | 1 | 5.22 | 6.3 | 0.02079 |
| $X_2$-Time | 0.29 | 1 | 0.29 | 0.35 | 0.5598 |
| $X_3$-SolvConc | 10.94 | 1 | 10.94 | 13.2 | 0.00166 |
| $X_4$-LSRatio | 27.41 | 1 | 27.41 | 33.07 | 1.26E-05 |
| $X_1X_3$ | 7.36 | 1 | 7.36 | 8.88 | 0.00741 |
| $X_3^2$ | 29.83 | 1 | 29.83 | 35.99 | 7.26E-06 |
| Residual | 16.58 | 20 | 0.83 | | |
| Lack of Fit | 16.29 | 18 | 0.91 | 6.28 | 0.14606 |
| Pure Error | 0.29 | 2 | 0.14 | | |
| Total | 97.63 | 26 | | | |

It can be concluded from Table 6.8 that the reduced model has a higher F-value and lower p-value than the full model (16.3> 8.74 and 9.41E-07<0.000291436, respectively. The R- squared, adjusted $R^2$, predicted $R^2$, adequate precision and C.V% values indicate that the reduced model is significant (Table 6.9) and that the model can be used to navigate the design space.

**Table 6.9: Fit statistics of reduced model for Y (TAC).**

| | |
|---|---|
| $R^2$ | 83.02 |
| Adjusted $R^2$ | 77.92 |
| Predicted $R^2$ | 60.34 |
| Adequate Precision | 15.65 |
| Std. Dev. | 0.91 |
| C.V. % | 8.60 |

#### 6.2.1.4 Verification of the Full and Reduced Models

To verify the full and reduced models for predicting the optimum response values, five different experimental conditions were used to conduct the experimental extraction and then compared to the predicted model results. Mean values from experimental runs

conducted in duplicate are shown in Table 6.10 and 6.11. The full model tended to over-predict the TAC response by 36.99% and under-predict by 6.82%, whereas the reduced model under-predicted TAC response by 17.86% and over-predicted TAC response by 20.96%. By comparing the errors of running number four which is close to the optimum point and sample five which is an optimum point, with running samples 1-3, it can be concluded that the presented model (both full and reduced models) can predict the optimum points well enough and better than non-optimum points (6.90% and 4.53% error compare to 36.99% and 17.86%). This demonstrated the validation of both the RSM models, indicating that the models are adequate for predicting optimum points of total anthocyanin extraction (Table 6.10 and 6.11).

**Table 6.10: Comparison between experimental a predicted value of TAC.**

| Temp (°C) | Time (min) | SolvConc (%) | LSRatio(mL/g) | Full model prediction | Experimental value | Error% |
|---|---|---|---|---|---|---|
| 25 | 3 | 20 | 10 | 11 | 8.03 | 36.99 |
| 45 | 3 | 50 | 50 | 11.42 | 16.2 | -29.51 |
| 65 | 3.33 | 80 | 42 | 13.63 | 14.63 | -6.84 |
| 45 | 20 | 60 | 50 | 12.62 | 11.58 | 8.98 |
| 65 | 11.5 | 60 | 50 | 14.01 | 13.11 | 6.86 |

**Table 6.11: Comparison between experimental a predicted value of TAC.**

| Temp (°C) | Time (min) | SolvConc (%) | LSRatio(mL/g) | Reduced model prediction | Experimental value | Error% |
|---|---|---|---|---|---|---|
| 25 | 3 | 20 | 10 | 9.71 | 8.03 | 20.92 |
| 45 | 3 | 50 | 50 | 13.31 | 16.2 | -17.84 |
| 65 | 3.33 | 80 | 42 | 12.92 | 14.63 | -11.69 |
| 45 | 20 | 60 | 50 | 12.89 | 11.58 | 11.31 |
| 65 | 11.5 | 60 | 50 | 13.7 | 13.11 | 4.50 |

### 6.2.2 Total Phenolic Content

As was described in Section 3.3.1 anthocyanin compounds are a subclasses of polyphenol compounds. Response surface methodology was used to determine a model for predicting and optimizing the extraction of total phenolic content from lowbush blueberries, in order to make a comparison with the model for predicting total anthocyanin content. The Box-Behnken design based on the optimization method described in Section 4.7.2 was used with the same extracts described earlier; however, the extracts were analyzed for total phenolic content using the Folin & Ciocalteu assay. The analysis was done on the same day that samples were extracted, after three hours of storage time in the freezer (-16 °C). Table 6.12 summarizes the experimental design and the average TPC results of triplicate runs. The range of values for the total phenolic content of the samples is from 6.41 to 47.05 mg GAE/g DW.

**Table 6.12: Total phenolic content of lowbush Nova Scotian blueberries.**

| Number of runs | Temp ($X_1$) | Time ($X_2$) | SolvConc ($X_3$) | LSRatio ($X_4$) | TPC* (mg GAE/g DW) |
|---|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 0 | 26.75 |
| 2 | 1 | -1 | 0 | 0 | 30.14 |
| 3 | -1 | 1 | 0 | 0 | 27.51 |
| 4 | 1 | 1 | 0 | 0 | 29.29 |
| 5 | 0 | 0 | -1 | -1 | 7.57 |
| 6 | 0 | 0 | 1 | -1 | 6.41 |
| 7 | 0 | 0 | -1 | 1 | 27.18 |
| 8 | 0 | 0 | 1 | 1 | 19.13 |
| 9 | -1 | 0 | 0 | -1 | 13.14 |
| 10 | 1 | 0 | 0 | -1 | 18.46 |
| 11 | -1 | 0 | 0 | 1 | 32.63 |
| 12 | 1 | 0 | 0 | 1 | 47.05 |
| 13 | 0 | -1 | -1 | 0 | 24.65 |
| 14 | 0 | 1 | -1 | 0 | 23.86 |
| 15 | 0 | -1 | 1 | 0 | 13.85 |
| 16 | 0 | 1 | 1 | 0 | 15.53 |
| 17 | -1 | 0 | -1 | 0 | 22.39 |
| 18 | 1 | 0 | -1 | 0 | 26.47 |
| 19 | -1 | 0 | 1 | 0 | 11.85 |
| 20 | 1 | 0 | 1 | 0 | 18.91 |
| 21 | 0 | -1 | 0 | -1 | 12.92 |
| 22 | 0 | 1 | 0 | -1 | 14.67 |
| 23 | 0 | -1 | 0 | 1 | 14.65 |
| 24 | 0 | 1 | 0 | 1 | 30.50 |
| 25 | 0 | 0 | 0 | 0 | 16.16 |
| 26 | 0 | 0 | 0 | 0 | 15.98 |
| 27 | 0 | 0 | 0 | 0 | 28.24 |

*Total phenolic content is expressed as mg gallic acid equivalent per gram of DW.

#### 6.2.2.1 Full Model for the Optimization of TPC

The results of the experiment planned using the Box-Behnken design were analyzed by applying regression analysis on the response variable (TPC) using Design Expert 9.0 software. A second- order polynomial equation was suggested as the model equation,

where $X_1$, $X_2$, $X_3$ and $X_4$ are the coded values of extraction describing temperature ($X_1$), time ($X_2$), ethanol concentration ($X_3$), and the ratio of liquid to material ($X_4$), respectively.

$$TPC = 20.12 + 3.01\ X_1 + 1.53\ X_2 - 3.87\ X_3 + 8.16\ X_4 - 0.40\ X_1X_2 + 0.75\ X_1X_3 + 2.27\ X_1X_4 + 0.62\ X_2X_3 + 3.53\ X_2X_4 - 1.72\ X_3X_4 + 6.53\ X_1^2 + 1.50\ X_2^2 - 4.32\ X_3^2 - 1.00\ X_4^2 \quad (6)$$

The final equation in terms of non-coded factors is presented in equation (5):

$$TPC = 42.17 - 1.52\ X_1 - 0.92\ X_2 + 0.23\ X_3 + 0.19\ X_4 - 0.0024\ X_1X_2 + 0.00093\ X_1X_3 - 0.00568\ X_1X_4 + 0.00181\ X_2X_3 + 0.02074\ X_2X_4 - 0.00215\ X_3X_4 + 0.01633\ X_1^2 + 0.02074\ X_2^2 - 0.00270\ X_3^2 - 0.00251\ X_4^2 \quad (7)$$

The regression coefficient values of Eq. (2) and (3) are listed in Table 6.13.

**Table 6.13: Coded and uncoded regression coefficients of the predicted second-order model for TPC.**

| Term | Coded Coefficients | Uncoded Coefficients |
|---|---|---|
| Constant | 20.12 | 42.17 |
| $X_1$ | 3.01 | -1.52 |
| $X_2$ | 1.53 | -0.92 |
| $X_3$ | -3.87 | 0.23 |
| $X_4$ | 8.16 | 0.19 |
| $X_1X_2$ | -0.40 | -0.0024 |
| $X_1X_3$ | 0.75 | 0.00093 |
| $X_1X_4$ | 2.27 | 0.00568 |
| $X_2X_3$ | 0.62 | 0.00181 |
| $X_2X_4$ | 3.53 | 0.02074 |
| $X_3X_4$ | -1.72 | -0.00215 |
| $X_1^2$ | 6.53 | 0.01633 |
| $X_2^2$ | 1.50 | 0.02074 |
| $X_3^2$ | -4.32 | -0.00270 |
| $X_4^2$ | -1.00 | -0.00251 |

ANOVA was used to determine the variables that were significant (Table 6.14). In this case, variables with p-value less than 0.05 were considered to have a significant effect on the total phenolic content of the extracts.

**Table 6.14: Analysis of variance for the full quadratic model of TPC.**

| Source | Sum of Squares | df | Mean Square | F-Value | p-value |
|---|---|---|---|---|---|
| Model | 1709.27 | 14 | 122.09 | 3.74 | 0.013813 |
| $X_1$-Temp | 108.40 | 1 | 108.40 | 3.32 | 0.093409 |
| $X_2$-Time | 28.23 | 1 | 28.23 | 0.86 | 0.370728 |
| $X_3$-SolvConc | 179.59 | 1 | 179.59 | 5.50 | 0.037006 |
| $X_4$-LSRatio | 799.77 | 1 | 799.77 | 24.50 | 0.000336 |
| $X_1X_2$ | 0.65 | 1 | 0.65 | 0.02 | 0.889938 |
| $X_1X_3$ | 2.23 | 1 | 2.23 | 0.07 | 0.798119 |
| $X_1X_4$ | 20.67 | 1 | 20.67 | 0.63 | 0.441658 |
| $X_2X_3$ | 1.52 | 1 | 1.52 | 0.05 | 0.833007 |
| $X_2X_4$ | 49.74 | 1 | 49.74 | 1.52 | 0.240656 |
| $X_3X_4$ | 11.87 | 1 | 11.87 | 0.36 | 0.557759 |
| $X_1^2$ | 227.50 | 1 | 227.50 | 6.97 | 0.021573 |
| $X_2^2$ | 11.98 | 1 | 11.98 | 0.37 | 0.555927 |
| $X_3^2$ | 99.41 | 1 | 99.41 | 3.05 | 0.106487 |
| $X_4^2$ | 5.37 | 1 | 5.37 | 0.16 | 0.692133 |
| Residual | 391.70 | 12 | 32.64 | | |
| Lack of Fit | 293.00 | 10 | 29.30 | 0.59 | 0.765822 |
| Pure Error | 98.70 | 2 | 49.35 | | |
| Total | 2100.97 | 26 | | | |

The p-value and Fisher's F-value indicate that the model is significant. The non-significant lack of fit (p-value of 0.765822) confirms that the model is significant for predicting total phenolic content. Among the four selected factors, solvent concentration and liquid to solid ratio were found to have significant effects on total phenolic content while temperature and time had no significant effect on the total phenolic content of extracts. According to the p-value of the parameters, the liquid solid ratio had the most significant effect on the total phenolic content, followed by solvent concentration. An

increase of the liquid solid ratio increased the concentration gradient, and as a result the diffusion rate of the compounds to the solvent increased as well (Cacace & Mazza, 2003). There is no significant interaction between variables, however the quadratic term ($X_1^2$) has a significant effect on TPC (p-value of 0.021573).

To confirm the adequacy of the model, $R^2$, adjusted and predicted $R^2$, as well as adequate precision and coefficient of variation were checked (Table 6.15). The adequacy precision was greater 4 and the R-squared value was 81.36%, indicating that the model was adequate. However, in comparison with both the full and reduced models for predicting total anthocyanin content, the C.V. % of 26.79% is much higher and the adjusted and predicted $R^2$ value is lower, indicating that both TAC models fit the data better than the model for TPC.

**Table 6.15: Fit statistics of full model for Y (TPC).**

| | |
|---|---|
| $R^2$ | 81.35% |
| Adjusted $R^2$ | 59.6% |
| Predicted $R^2$ | 9.1% |
| Adequate Precision | 8.125256 |
| Std. Dev. | 5.713283 |
| C.V. % | 26.78691 |

#### 6.2.2.2 Optimization of Extraction Conditions of TPC

Three dimensional surface plots and two dimensional contour plots (graphical representations of Eq. (6) present the effect of interactions between two variables while the rest of the variables are set at the center point. These are shown in Figures 6.6 and 6.7.

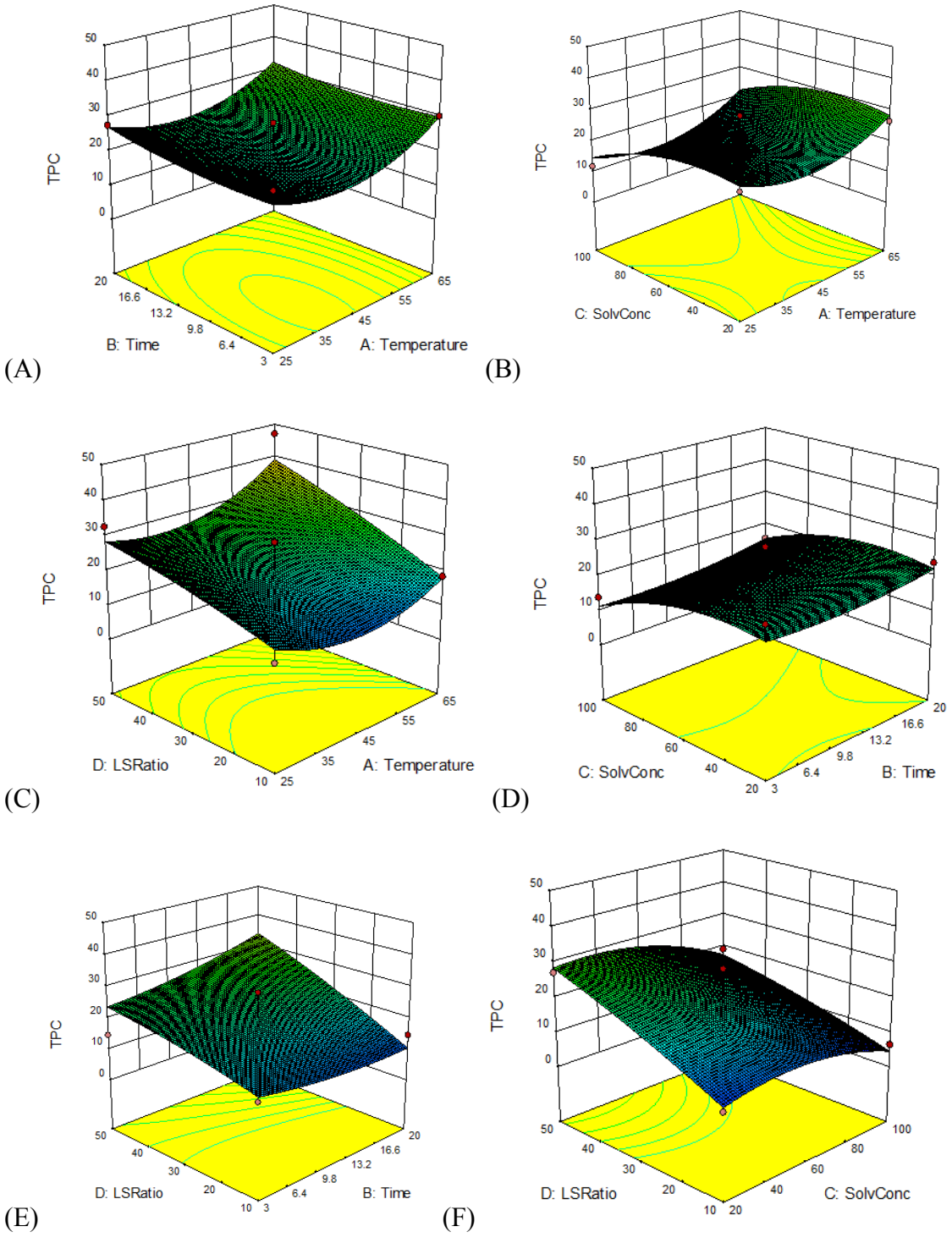


**Figure 6.6: Response surface plots (3-D) showing the effects of variables (X1: extraction temperature; X2: extraction time; X3: solvent concentration; X4: liquid solid ratio) on the response Y (TPC).**

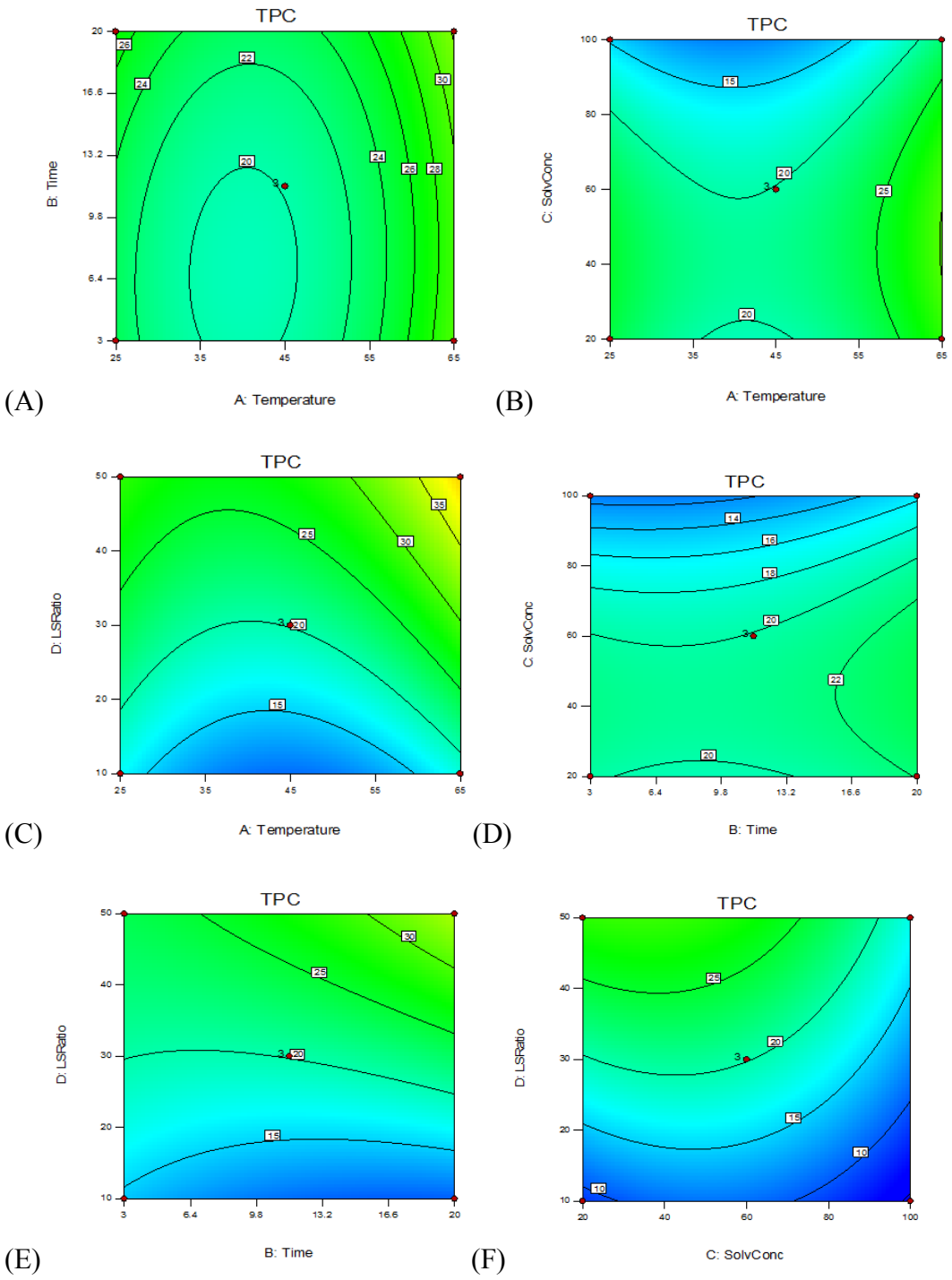


**Figure 6.7: Contour plots (2-D) showing the effects of variables (X1: extraction temp; X2: extraction time; X3: solvent concentration; X4: liquid solid ratio) on the response Y (TPC).**

As shown in Figure 6.6A and 6.7A, temperature and time are not significant factors however temperature has a minimum at 35 °C to 45 °C which confirms the significant p-

value of quadratic term $X_1^2$. Solvent concentration was one of the main factors affecting the total phenolic content significantly. Figure 6.6B and 6.7B showed the significant effect of solvent concentration, including the minimum total anthocyanin content at a solvent concentration of 100% and temperature at 25 °C to 45 °C, while two other factors are held at center points (time = 11.5 min, liquid solid ratio = 30 mL/g). The reason can be attributed to the possibility of changing the solubilities of compounds by modifying the ethanol concentration, and this may influence the extraction of phenolics. Water has polar hydrogen bonds that might be more difficult to break through for dissolving the active compounds compared to ethanol with covalent bonds. So, adding ethanol to the system up to 60% can help dissolving anthocyanin compounds in the solution. However, by adding ethanol more than 60%, the decrease in polarity of the solution leads in lower TAC. The effects of the solvent concentration and of the liquid solid ratio on the extraction of total phenolic content from blackcurrants were tested (Cacace & Mazza, 2003). The effect of solvent concentration was similar to that found for blueberries. It was reported that the total phenolic content increased with ethanol concentration up to a maximum at approximately 60% and then decreased with further increase in ethanol concentration, regardless of any change in other factors, including the liquid solid ratio.

According to Figure 6.6C and 6.7C, the effect of liquid solid ratio is significant. Samples with liquid to solid ratios of 50 mL/g and under 65°C showed the maximum TPC while the other two parameters were kept at center point values. The extraction of grape seed polyphenols under 50% aqueous ethanol for 200 min, at different liquid solid ratio, 10, 20, 30, and 40 mL/g and temperatures was studied, resulting in total polyphenol

concentrations in the range from 14.72 mg GAE/gdb to 66.81 mg GAE/gdb. The highest extraction yield was obtained at a solid liquid ratio of 40 mL/g (Bucic- Kujic et al., 2007)

It can be observed from Figure 6.6D and 6.7D that time does not have any effect on TPC and samples treated for different extraction times showed the same TPC level. According to Figure 6.6E, 6.7E, 6.6F and 6.7F, the liquid solid ratio has a significant effect on TPC and as the liquid (diluted ethanol) increased, the total phenolic content also increased. Samples with a liquid solid ratio of 50 mL/g and a solvent concentration of 20% to 60% found to have the maximum TPC while the other two parameters were kept at the center point (time: 11.5 min and temperature: 45 °C).

Liquid solid ratio had the most significant effect on both total phenolic content and total anthocyanin content, followed by solvent concentration. Significant effect of Temp, the interaction between solvent concentration and Temp, as well as the quadratic term for solvent concentration that were observed for total anthocyanin content, were not significant in the model for TPC. However, the coefficient of the quadratic term for temperature ($X_1^2$) was significant in optimizing for TPC. One of the possible reasons for this phenomenon was that phenolic compounds such as phenolic acids may act differently in presence of ultrasound waves since they are thermally unstable (De Paepe et al., 2014).

In order to compare the results from this study with values in the literature, TPC and TAC values for this study were calculated per 100 g fresh weight of lowbush blueberries, (considering 85% initial moisture content (Bastin & Henken, 1997). The TPC ranged from 96.202 to 705.68 mg GAE/100 g FW and the TAC ranged from 85.22 to 198.34 mg C3G/ 100 g FW, which were consistent with the reported range of similar studies on total anthocyanin and phenolic content of other varieties of blueberries (Moyer

et al., 2002; Sellappan et al., 2002; Grace et al 2009; You et al., 2011) and higher than some of the other reported fruits and vegetables (Balasundram et al., 2006). Anthocyanin extracts from the blackberry cultivar *Cacanska Bestrna*, in which an ultrasound bath was used for the extraction have been studied (Ivanovic et al., 2013). The highest total anthocyanin content that was reported was 1.38 g/100 g DW (expressed as cyanidin 3-glucoside equivalent) while the highest TPC was 2.658 g GAE/100 g DW. On a dry basis, according to the results of our study, the highest TAC that was achieved was 1.32 (g C3G/ 100 g DW) and the highest TPC was 4.70 (g GAE/ 100 g DW). Comparing these results and similar data from other vegetables and fruits, indicates that lowbush blueberry extracts produced by ultrasound-assisted extraction, are high in total anthocyanin and phenolic content.

# 7 FUTURE WORK

It is recommended that future work be focused on improving the model for blueberry anthocyanin extracts. Increasing the experimental range of the variables would be beneficial for obtaining a more adequate model. In addition, the selection of ultrasound bath or probe should be investigated, as each has advantages and disadvantages. Selecting an ultrasound bath with higher temperature range or/and testing and comparison between probe and bath sonication methods may be useful in studying the effect of temperature on the extraction of TAC and TPC (Ozcan, 2006). Kinetic studies also can be helpful in studying the diffusion rate of active compounds into the solvent, and would be useful in determining the point during the extraction where the maximum response is achieved. This additional step would help to determine the minimum time needed for the extraction (D'Alessandro et al., 2013).

In this study, it was expected that the changes in each variable would follow the same trend for TAC and TPC extraction, however this was not the case for the factor "extraction time", which may have affected the adequacy of the model for TPC. The phenolic group contains diverse compounds such as flavonoids and phenolic acids that may act differently in response to temperature and ultrasound, in comparison to anthocyanin compounds (Fischer et al., 2013; De Paepe et al., 2014). For these reasons, it may have been important to have conducted a separate screening of variables prior to optimization of TPC, as was done for TAC extraction, and so this should be considered for future work. After screening the effect of variables on TPC, optimization analysis would need to be repeated to find the optimum point of each parameter for TPC.

Finally, it would be interesting to use HPLC to study the extraction of individual anthocyanin and phenolic compounds from lowbush blueberries, and the effects of extraction conditions. By doing so, more information on the anthocyanin and phenolic profiles would be obtained, which may be useful in nutraceutical applications using specific molecules associated with particular health benefits.

# 8 CONCLUSION

An ultrasound-assisted extraction method was utilized to extract anthocyanin and phenolic compounds from lowbush Nova Scotian blueberries. The optimal conditions for total anthocyanins and total phenolic were obtained through Box-Behnken design within the current experimental range. Using the surface and contour plots in RSM was effective for estimating the effect of four independent variables (extraction temperature, extraction time, solvent concentration and ratio of liquid to raw material). Optimum condition of TAC was found to be Temperature: $65^0$C, Time: 11.5 min, solvent concentration: 60%, liquid solid ratio: 50mL/g resulting in total anthocyanin of 13.22 mg C3G/ g DW. We were able to reduce the full model for total anthocyanin content to have fewer factors and achieve higher adequacy for predicting TAC, although the adequacy of full model and reduced model have both been confirmed. The reduced model was able to predict the response for TAC within an error of -11% to 20%. The TPC model was determined and the adequacy of the model was tested. It was found that both the full and reduced TAC models were able to fit the data better than the TPC model. Liquid solid ratio had the most significant effect on TAC as well as TPC, followed by solvent concentration. TAC ranged from 0.57 g C3G/100 g DW to 1.32 g C3G/100 g DW, and TPC from 0.64 g GAE/100 g DW to 4.7 g GAE/100 g DW, which are comparable or higher than values found in the literature. Thus, lowbush Nova Scotian blueberries can be considered as a potential source for antioxidants and pigments, and ultrasound-assisted extraction is an effective extraction method.

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# APPENDIX A: FOLIN & CIOCALTEU ASSAY STANDARD CURVE

Standard curve was prepared for Folin-Ciocalteu assay analysis. 50.2 mg gallic acid was dissolved in 25 mL distilled water to have solution with 2 mg/ mL concentration. This solution was kept at -16 C in the freezer. Before testing TPC the original solution was diluted to 1, 0.5, 0.25 and 0.125 mg/ mL. Three standard curves were prepared for triplicate samples.

**Table A.1: Gallic acid absorbency in different concentration (First replicate).**

| Concentration(mg/mL) | ABS |
|---|---|
| 2 | 1.886 |
| 1 | 0.668 |
| 0.5 | 0.415 |
| 0.25 | 0.231 |
| 0.125 | 0.094 |

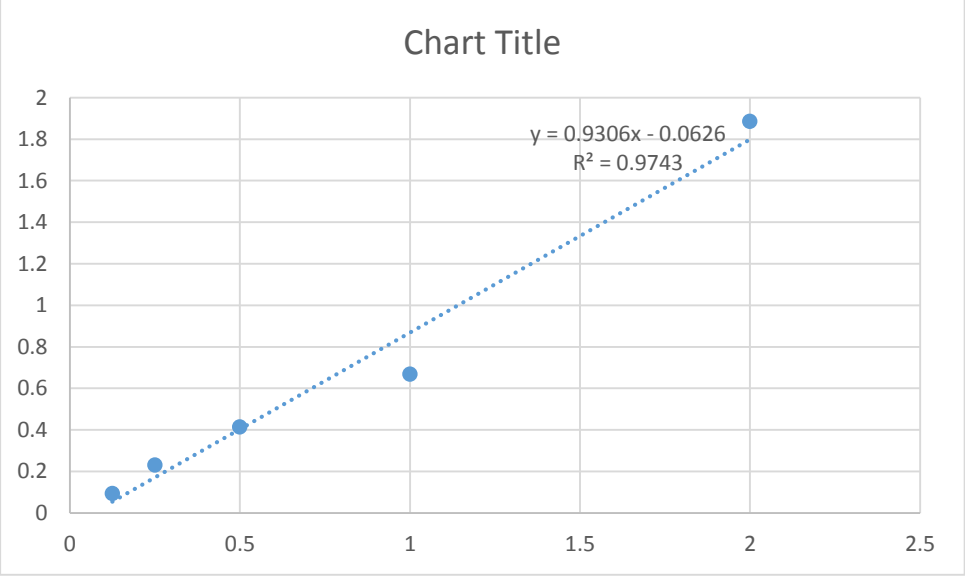


**Figure A.1: Gallic Acid Standard Curve for First replicate.**

**Table A.2: Gallic acid absorbency in different concentration (Second replicate).**

| Concentration(mg/L) | ABS |
|---|---|
| 2 | 2.019 |
| 1 | 1.08 |
| 0.5 | 0.552 |
| 0.25 | 0.271 |
| 0.125 | 0.128 |

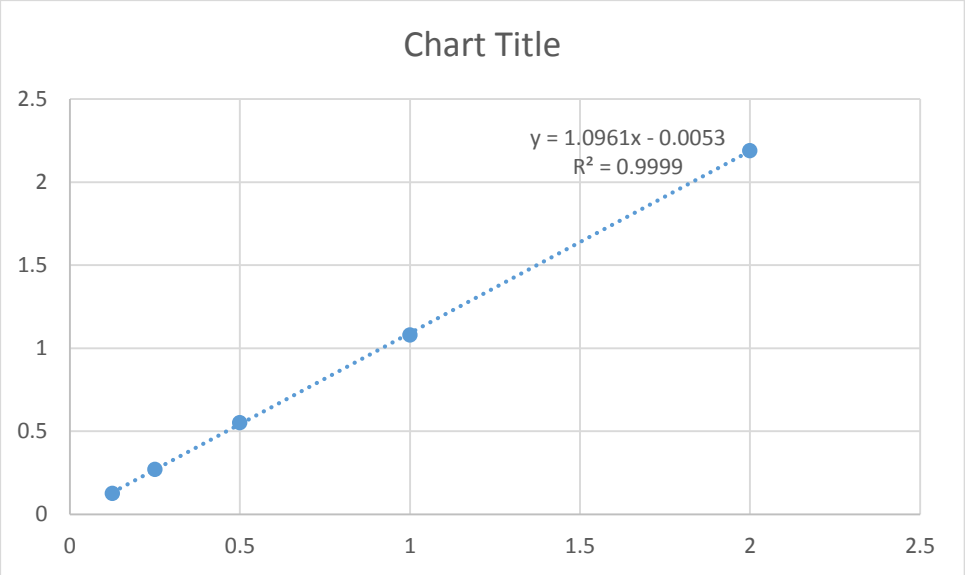


**Figure A.2: Gallic Acid Standard Curve for second replicate.**

**Table A.3: Gallic acid absorbency in different concentration (Third replicate).**

| Concentration(mg/L) | ABS |
|---|---|
| 2 | 2.17 |
| 1 | 1.135 |
| 0.5 | 0.527 |
| 0.25 | 0.226 |
| 0.125 | 0.157 |

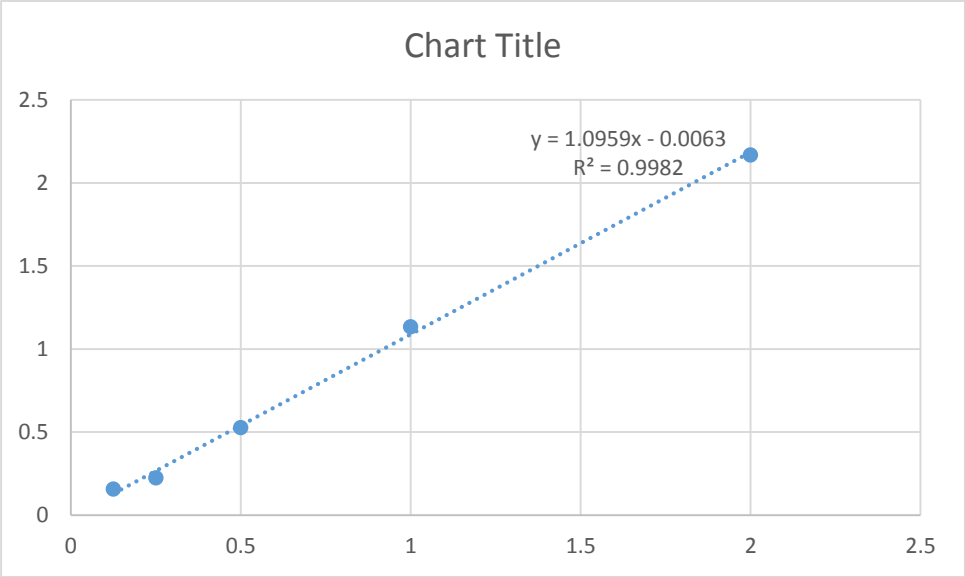


**Figure A.3: Gallic acid Standard Curve for third replicate.**

# AEENDIX B: RAW DATA

**Table B.1: Raw data for TAC factorial design conditions for first replicate.**

| No. of runs | Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight | DF |
|---|---|---|---|---|---|---|
| 1 | 65 | 20 | 20 | 10 | 1.0399 | 25 |
| 2 | 65 | 20 | 20 | 50 | 0.2003 | 10 |
| 3 | 65 | 20 | 100 | 10 | 1.0805 | 25 |
| 4 | 65 | 20 | 100 | 50 | 0.1985 | 10 |
| 5 | 65 | 3 | 20 | 10 | 1.0172 | 25 |
| 6 | 65 | 3 | 20 | 50 | 0.2048 | 10 |
| 7 | 65 | 3 | 100 | 10 | 0.9924 | 25 |
| 8 | 65 | 3 | 100 | 50 | 0.2034 | 10 |
| 9 | 25 | 20 | 20 | 10 | 1.0079 | 25 |
| 10 | 25 | 20 | 20 | 50 | 0.2132 | 10 |
| 11 | 25 | 20 | 100 | 10 | 1.0486 | 10 |
| 12 | 25 | 20 | 100 | 50 | 0.2163 | 10 |
| 13 | 25 | 3 | 20 | 10 | 1.0893 | 25 |
| 14 | 25 | 3 | 20 | 50 | 0.216 | 10 |
| 15 | 25 | 3 | 100 | 10 | 1.0267 | 10 |
| 16 | 25 | 3 | 100 | 50 | 0.2131 | 10 |

**Table B.2: Raw data for TAC values of factorial design for first replicate (I).**

| ABS at pH=1 | | ABS at pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/L) | TAC(mg/g of DW) |
| 2.50 | 0.01 | 0.30 | 0.12 | 2.32 | 968.95 | 9.64 |
| 1.49 | 0.00 | 0.13 | 0.00 | 1.36 | 227.44 | 11.74 |
| 2.86 | 0.23 | 0.49 | 0.19 | 2.33 | 972.29 | 9.31 |
| 1.71 | 0.12 | 0.29 | 0.11 | 1.40 | 234.12 | 12.20 |
| 2.72 | 0.01 | 0.26 | 0.01 | 2.45 | 1024.06 | 10.41 |
| 1.60 | 0.00 | 0.14 | 0.00 | 1.47 | 244.64 | 12.35 |
| 1.10 | 0.15 | 0.34 | 0.15 | 0.76 | 316.44 | 3.30 |
| 1.07 | 0.08 | 0.21 | 0.09 | 0.86 | 143.28 | 7.28 |
| 1.99 | 0.00 | 0.20 | 0.00 | 1.79 | 746.44 | 7.66 |
| 1.52 | 0.00 | 0.14 | 0.00 | 1.39 | 231.28 | 11.22 |
| 3.36 | 0.57 | 0.98 | 0.53 | 2.34 | 389.92 | 3.85 |
| 1.59 | 0.11 | 0.30 | 0.12 | 1.30 | 217.25 | 10.39 |
| 2.11 | 0.00 | 0.23 | 0.00 | 1.88 | 784.01 | 7.44 |
| 1.47 | 0.00 | 0.12 | 0.00 | 1.35 | 225.94 | 10.82 |
| 2.80 | 0.40 | 0.84 | 0.41 | 1.98 | 331.14 | 3.34 |
| 1.08 | 0.10 | 0.23 | 0.10 | 0.85 | 142.11 | 6.90 |

**Table B.3: Raw data for TAC values of factorial design for first replicate (II).**

| ABS at pH=1 | | ABS at pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC (mg/L) | TAC(mg/g of DW) |
| 2.63 | 0.01 | 0.30 | 0.12 | 2.45 | 1020.72 | 10.15 |
| 1.51 | 0.00 | 0.13 | 0.00 | 1.38 | 230.95 | 11.92 |
| 2.65 | 0.21 | 0.52 | 0.20 | 2.13 | 889.22 | 8.51 |
| 1.78 | 0.12 | 0.33 | 0.13 | 1.46 | 243.97 | 12.71 |
| 2.72 | 0.01 | 0.27 | 0.01 | 2.46 | 1025.73 | 10.43 |
| 1.61 | 0.00 | 0.14 | 0.00 | 1.47 | 245.14 | 12.38 |
| 1.67 | 0.15 | 0.36 | 0.15 | 1.32 | 549.81 | 5.73 |
| 1.10 | 0.08 | 0.22 | 0.09 | 0.89 | 147.79 | 7.51 |
| 2.13 | 0.00 | 0.20 | 0.00 | 1.93 | 804.05 | 8.25 |
| 1.50 | 0.00 | 0.13 | 0.00 | 1.37 | 228.94 | 11.10 |
| 3.46 | 0.59 | 0.99 | 0.53 | 2.41 | 402.61 | 3.97 |
| 1.64 | 0.12 | 0.30 | 0.12 | 1.34 | 223.60 | 10.69 |
| 2.30 | 0.01 | 0.23 | 0.00 | 2.07 | 863.75 | 8.20 |
| 1.51 | 0.00 | 0.13 | 0.00 | 1.37 | 229.44 | 10.98 |
| 2.95 | 0.41 | 0.77 | 0.38 | 2.14 | 358.02 | 3.61 |
| 1.11 | 0.10 | 0.24 | 0.10 | 0.88 | 147.12 | 7.14 |

**Table B.4: Raw data for TAC factorial design conditions for second replicate**

| No. of runs | Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|---|
| 1 | 65 | 20 | 20 | 10 | 0.9949 | 25 |
| 2 | 65 | 20 | 20 | 50 | 0.2077 | 10 |
| 3 | 65 | 20 | 100 | 10 | 1.0075 | 25 |
| 4 | 65 | 20 | 100 | 50 | 0.2087 | 10 |
| 5 | 65 | 3 | 20 | 10 | 1.0353 | 25 |
| 6 | 65 | 3 | 20 | 50 | 0.216 | 10 |
| 7 | 65 | 3 | 100 | 10 | 1.0115 | 25 |
| 8 | 65 | 3 | 100 | 50 | 0.2103 | 10 |
| 9 | 25 | 20 | 20 | 10 | 1.003 | 25 |
| 10 | 25 | 20 | 20 | 50 | 0.2043 | 10 |
| 11 | 25 | 20 | 100 | 10 | 1.0354 | 10 |
| 12 | 25 | 20 | 100 | 50 | 0.2046 | 10 |
| 13 | 25 | 3 | 20 | 10 | 1.0292 | 25 |
| 14 | 25 | 3 | 20 | 50 | 0.2149 | 10 |
| 15 | 25 | 3 | 100 | 10 | 1.0612 | 10 |
| 16 | 25 | 3 | 100 | 50 | 0.2025 | 10 |

**Table B.5: Raw data for TAC values of factorial design for second replicate (I).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC (mg/L) | TAC(mg/g of DW) |
| 2.86 | 0.01 | 0.26 | 0.00 | 2.60 | 1083.34 | 11.26 |
| 1.50 | 0.00 | 0.13 | 0.00 | 1.36 | 227.77 | 11.34 |
| 2.98 | 0.22 | 0.56 | 0.20 | 2.41 | 1006.11 | 10.33 |
| 1.80 | 0.13 | 0.34 | 0.14 | 1.47 | 245.14 | 12.15 |
| 2.45 | 0.01 | 0.25 | 0.01 | 2.20 | 918.86 | 9.18 |
| 1.63 | 0.00 | 0.14 | 0.00 | 1.50 | 249.65 | 11.95 |
| 1.63 | 0.18 | 0.45 | 0.20 | 1.21 | 504.31 | 5.16 |
| 1.66 | 0.12 | 0.33 | 0.13 | 1.35 | 224.60 | 11.04 |
| 1.84 | 0.00 | - | - | - | - | - |
| 1.35 | 0.00 | 0.12 | 0.00 | 1.24 | 206.57 | 10.46 |
| 3.60 | 0.65 | 1.06 | 0.57 | 2.46 | 410.63 | 4.10 |
| 1.46 | 0.14 | 0.35 | 0.16 | 1.13 | 188.86 | 9.55 |
| 1.93 | 0.01 | 0.19 | 0.01 | 1.75 | 728.49 | 7.32 |
| 1.43 | 0.00 | 0.12 | 0.00 | 1.31 | 218.09 | 10.49 |
| 2.64 | 0.57 | 0.99 | 0.64 | 1.73 | 288.56 | 2.81 |
| 0.92 | 0.02 | 0.09 | 0.02 | 0.82 | 137.60 | 7.03 |

**Table B.6: Raw data for TAC values of factorial design for second replicate (II).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC (mg/L) | TAC(mg/g of DW) |
| 2.51 | 0.01 | 0.27 | 0.00 | 2.24 | 936.39 | 9.73 |
| 1.58 | 0.00 | 0.14 | 0.00 | 1.45 | 241.47 | 12.02 |
| 3.24 | 0.23 | 0.56 | 0.20 | 2.66 | 1108.81 | 11.38 |
| 1.85 | 0.14 | 0.34 | 0.19 | 1.57 | 261.34 | 12.95 |
| 2.56 | 0.01 | 0.27 | 0.01 | 2.29 | 954.34 | 9.53 |
| 1.56 | 0.00 | 0.14 | 0.00 | 1.42 | 237.46 | 11.37 |
| 1.83 | 0.19 | 0.47 | 0.21 | 1.38 | 574.44 | 5.87 |
| 1.80 | 0.13 | 0.36 | 0.14 | 1.46 | 244.14 | 12.01 |
| 1.96 | 0.00 | 0.21 | 0.01 | 1.76 | 733.08 | 7.56 |
| 1.42 | 0.00 | 0.12 | 0.00 | 1.30 | 217.25 | 11.00 |
| 3.61 | 0.64 | 1.10 | 0.59 | 2.46 | 411.29 | 4.11 |
| 1.53 | 0.14 | 0.39 | 0.17 | 1.16 | 194.38 | 9.82 |
| 1.84 | 0.00 | 0.20 | 0.01 | 1.64 | 684.65 | 6.88 |
| 1.38 | 0.00 | 0.13 | 0.00 | 1.25 | 209.40 | 10.08 |
| 2.75 | 0.60 | 1.05 | 0.69 | 1.79 | 298.41 | 2.91 |
| 0.99 | 0.02 | 0.09 | 0.02 | 0.90 | 149.79 | 7.65 |

**Table B.7: Raw data for TAC RSM conditions for first replicate.**

| Temp($^{O}$C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight | DF |
|---|---|---|---|---|---|
| 65 | 3 | 60 | 30 | 0.3374 | 25 |
| 65 | 20 | 60 | 30 | 0.3384 | 25 |
| 65 | 11.5 | 60 | 10 | 1.0762 | 25 |
| 65 | 11.5 | 60 | 50 | 0.2016 | 25 |
| 65 | 11.5 | 20 | 30 | 0.3326 | 25 |
| 65 | 11.5 | 100 | 30 | 0.3318 | 25 |
| 45 | 20 | 20 | 30 | 0.3331 | 25 |
| 45 | 20 | 100 | 30 | 0.3444 | 25 |
| 45 | 20 | 60 | 10 | 1.0129 | 25 |
| 45 | 20 | 60 | 50 | 0.2002 | 25 |
| 45 | 11.5 | 20 | 10 | 1.0181 | 25 |
| 45 | 11.5 | 100 | 10 | 1.0262 | 25 |
| 45 | 11.5 | 20 | 50 | 0.2023 | 25 |
| 45 | 11.5 | 100 | 50 | 0.2099 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3445 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3315 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3442 | 25 |
| 45 | 3 | 20 | 30 | 0.3429 | 25 |
| 45 | 3 | 100 | 30 | 0.3363 | 25 |
| 45 | 3 | 60 | 10 | 1.0492 | 25 |
| 45 | 3 | 60 | 50 | 0.3493 | 25 |
| 25 | 3 | 60 | 30 | 0.3413 | 25 |
| 25 | 20 | 60 | 30 | 0.3357 | 25 |
| 25 | 11.5 | 60 | 10 | 1.0313 | 25 |
| 25 | 11.5 | 60 | 50 | 0.21 | 25 |
| 25 | 11.5 | 20 | 30 | 0.3407 | 25 |
| 25 | 11.5 | 100 | 30 | 0.3369 | 25 |

**Table B.8: Raw data for TAC values of RSM design for first replicate (I).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/l) | TAC(mg/g of DW) |
| 1.07 | 0.01 | 0.10 | 0.01 | 0.97 | 404.11 | 12.39 |
| 1.05 | 0.01 | 0.09 | 0.01 | 0.95 | 398.27 | 12.17 |
| 2.76 | 0.01 | 0.26 | 0.01 | 2.50 | 1045.35 | 10.04 |
| 0.67 | 0.01 | 0.06 | 0.01 | 0.61 | 254.66 | 13.06 |
| 0.93 | 0.00 | 0.08 | 0.00 | 0.85 | 356.52 | 11.09 |
| 1.01 | 0.07 | 0.15 | 0.05 | 0.84 | 351.93 | 10.97 |
| 0.96 | 0.00 | 0.08 | 0.00 | 0.88 | 367.38 | 11.41 |
| 0.85 | 0.06 | 0.01 | 0.05 | 0.83 | 346.79 | 10.41 |
| 2.69 | 0.02 | 0.24 | 0.02 | 2.44 | 1019.05 | 10.40 |
| 0.67 | 0.01 | 0.06 | 0.00 | 0.62 | 257.58 | 13.31 |
| 2.46 | 0.01 | 0.23 | 0.01 | 2.23 | 928.88 | 9.43 |
| 1.84 | 0.20 | 0.44 | 0.21 | 1.41 | 586.55 | 5.91 |
| 0.59 | 0.00 | 0.06 | 0.00 | 0.53 | 222.93 | 11.40 |
| 0.65 | 0.04 | 0.10 | 0.04 | 0.54 | 224.60 | 11.07 |
| 1.07 | 0.01 | 0.09 | 0.01 | 0.98 | 409.12 | 12.28 |
| 1.04 | 0.01 | 0.09 | 0.01 | 0.96 | 398.69 | 12.44 |
| 1.06 | 0.01 | 0.09 | 0.01 | 0.97 | 404.53 | 12.15 |
| 0.92 | 0.00 | 0.08 | 0.00 | 0.83 | 348.17 | 10.50 |
| 0.94 | 0.06 | 0.15 | 0.05 | 0.79 | 328.55 | 10.10 |
| 2.73 | 0.01 | 0.30 | 0.01 | 2.43 | 1014.87 | 10.00 |
| 1.22 | 0.01 | 0.09 | 0.01 | 1.12 | 466.32 | 13.81 |
| 1.13 | 0.01 | 0.09 | 0.00 | 1.04 | 434.59 | 13.17 |
| 1.08 | 0.01 | 0.08 | 0.01 | 1.00 | 416.22 | 12.82 |
| 2.81 | 0.01 | 0.02 | 0.01 | 2.78 | 1161.49 | 11.65 |
| 0.66 | 0.00 | 0.05 | 0.00 | 0.61 | 254.24 | 12.52 |
| 0.81 | 0.00 | 0.07 | 0.00 | 0.74 | 310.18 | 9.41 |
| 0.63 | 0.06 | 0.14 | 0.04 | 0.48 | 199.97 | 6.14 |

**Table B.9: Raw data for TAC RSM conditions for first replicate (II).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC (mg/L) | TAC(mg/g of DW) |
| 0.87 | 0.00 | 0.09 | 0.01 | 0.78 | 326.88 | 10.02 |
| 0.85 | 0.00 | 0.08 | 0.00 | 0.76 | 318.11 | 9.72 |
| 2.14 | 0.01 | 0.26 | 0.01 | 1.88 | 784.43 | 7.54 |
| 0.54 | 0.00 | 0.05 | 0.00 | 0.48 | 200.80 | 10.30 |
| 0.87 | 0.00 | 0.07 | 0.00 | 0.80 | 333.14 | 10.36 |
| 1.07 | 0.08 | 0.14 | 0.04 | 0.90 | 374.89 | 11.68 |
| 0.91 | 0.00 | 0.08 | 0.00 | 0.83 | 347.75 | 10.80 |
| 0.80 | 0.60 | 0.12 | 0.04 | 0.12 | 51.35 | 1.54 |
| 2.18 | 0.02 | 0.28 | 0.02 | 1.90 | 794.45 | 8.11 |
| 0.57 | 0.01 | 0.05 | 0.00 | 0.51 | 214.58 | 11.08 |
| 2.34 | 0.01 | 0.23 | 0.01 | 2.10 | 877.53 | 8.91 |
| 1.60 | 0.17 | 0.44 | 0.19 | 1.18 | 493.03 | 4.97 |
| 0.62 | 0.00 | 0.05 | 0.00 | 0.57 | 237.96 | 12.16 |
| 0.61 | 0.04 | 0.08 | 0.02 | 0.50 | 209.99 | 10.35 |
| 1.06 | 0.01 | 0.09 | 0.01 | 0.97 | 404.53 | 12.14 |
| 0.87 | 0.01 | 0.09 | 0.00 | 0.78 | 326.46 | 10.18 |
| 0.95 | 0.01 | 0.08 | 0.00 | 0.87 | 361.95 | 10.87 |
| 0.96 | 0.00 | 0.09 | 0.00 | 0.87 | 363.62 | 10.97 |
| 0.88 | 0.07 | 0.12 | 0.04 | 0.73 | 306.01 | 9.41 |
| 2.57 | 0.01 | 0.29 | 0.10 | 2.37 | 989.83 | 9.76 |
| 0.95 | 0.01 | 0.09 | 0.00 | 0.86 | 358.61 | 10.62 |
| 0.89 | 0.00 | 0.09 | 0.00 | 0.81 | 337.32 | 10.22 |
| 0.92 | 0.02 | 0.08 | 0.00 | 0.82 | 341.91 | 10.53 |
| 2.38 | 0.01 | 0.26 | 0.01 | 2.12 | 883.79 | 8.86 |
| 0.59 | 0.00 | 0.05 | 0.00 | 0.53 | 222.93 | 10.98 |
| 0.80 | 0.00 | 0.06 | 0.00 | 0.74 | 307.26 | 9.33 |
| 0.52 | 0.04 | 0.08 | 0.02 | 0.42 | 175.34 | 5.38 |

**Table B.10: Raw data for TAC RSM conditions for second replicate.`**

| Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|
| 65 | 3 | 60 | 30 | 0.3364 | 25 |
| 65 | 20 | 60 | 30 | 0.3394 | 25 |
| 65 | 11.5 | 60 | 10 | 1.0728 | 25 |
| 65 | 11.5 | 60 | 50 | 0.2078 | 25 |
| 65 | 11.5 | 20 | 30 | 0.3485 | 25 |
| 65 | 11.5 | 100 | 30 | 0.341 | 25 |
| 45 | 20 | 20 | 30 | 0.3496 | 25 |
| 45 | 20 | 100 | 30 | 0.337 | 25 |
| 45 | 20 | 60 | 10 | 1.0241 | 25 |
| 45 | 20 | 60 | 50 | 0.2011 | 25 |
| 45 | 11.5 | 20 | 10 | 1.025 | 25 |
| 45 | 11.5 | 100 | 10 | 1.0651 | 25 |
| 45 | 11.5 | 20 | 50 | 0.2047 | 25 |
| 45 | 11.5 | 100 | 50 | 0.2037 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3437 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3447 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3474 | 25 |
| 45 | 3 | 20 | 30 | 0.3417 | 25 |
| 45 | 3 | 100 | 30 | 0.332 | 25 |
| 45 | 3 | 60 | 10 | 1.0821 | 25 |
| 45 | 3 | 60 | 50 | 0.2033 | 25 |
| 25 | 3 | 60 | 30 | 0.3322 | 25 |
| 25 | 20 | 60 | 30 | 0.3413 | 25 |
| 25 | 11.5 | 60 | 10 | 0.9908 | 25 |
| 25 | 11.5 | 60 | 50 | 0.2004 | 25 |
| 25 | 11.5 | 20 | 30 | 0.344 | 25 |
| 25 | 11.5 | 100 | 30 | 0.335 | 25 |

**Table B.11: Raw data for TAC RSM conditions for second replicate (I).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/l) | TAC(mg/g of DW) |
| 1.16 | 0.01 | 0.12 | 0.01 | 1.04 | 433.34 | 13.32 |
| 1.10 | 0.01 | 0.08 | 0.00 | 1.02 | 427.07 | 13.01 |
| 2.44 | 0.01 | 0.24 | 0.01 | 2.20 | 916.77 | 8.84 |
| 0.76 | 0.00 | 0.06 | 0.00 | 0.71 | 294.32 | 14.65 |
| 0.66 | 0.00 | 0.08 | 0.00 | 0.58 | 240.88 | 7.15 |
| 1.03 | 0.02 | 0.14 | 0.28 | 1.14 | 476.75 | 14.46 |
| 0.87 | 0.00 | 0.07 | 0.00 | 0.80 | 333.98 | 9.88 |
| 0.89 | 0.09 | 0.19 | 0.08 | 0.69 | 288.06 | 8.84 |
| 2.19 | 0.01 | 0.18 | 0.00 | 2.00 | 836.61 | 8.45 |
| 0.51 | 0.00 | 0.05 | 0.00 | 0.46 | 190.78 | 9.81 |
| 2.26 | 0.01 | 0.21 | 0.01 | 2.05 | 854.98 | 8.63 |
| 1.71 | 0.23 | 0.49 | 0.24 | 1.22 | 510.99 | 4.96 |
| 0.58 | 0.00 | 0.05 | 0.00 | 0.54 | 224.60 | 11.35 |
| 0.43 | 0.04 | 0.11 | 0.04 | 0.32 | 134.43 | 6.82 |
| 0.96 | 0.01 | 0.07 | 0.00 | 0.89 | 370.72 | 11.15 |
| 0.94 | 0.00 | 0.12 | 0.00 | 0.82 | 343.16 | 10.30 |
| 0.84 | 0.01 | 0.10 | 0.01 | 0.75 | 311.85 | 9.28 |
| 0.64 | 0.00 | 0.08 | 0.00 | 0.57 | 236.71 | 7.16 |
| 0.58 | 0.04 | 0.11 | 0.04 | 0.47 | 196.63 | 6.12 |
| 2.40 | 0.01 | 0.26 | 0.01 | 2.15 | 896.31 | 8.57 |
| 0.71 | 0.01 | 0.05 | 0.01 | 0.65 | 272.19 | 13.85 |
| 1.03 | 0.00 | 0.08 | 0.00 | 0.95 | 397.02 | 12.36 |
| 0.95 | 0.00 | 0.08 | 0.00 | 0.87 | 364.45 | 11.04 |
| 2.21 | 0.01 | 0.18 | 0.01 | 2.04 | 849.97 | 8.87 |
| 0.54 | 0.00 | 0.05 | 0.00 | 0.49 | 204.14 | 10.53 |
| 0.87 | 0.00 | 0.07 | 0.00 | 0.80 | 334.40 | 10.05 |
| 0.33 | 0.04 | 0.07 | 0.02 | 0.24 | 101.03 | 3.12 |

**Table B.12: Raw data for TAC RSM conditions for second replicate (II).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/L) | TAC(mg/g of DW) |
| 1.13 | 0.01 | 0.11 | 0.01 | 1.02 | 426.24 | 13.10 |
| 1.10 | 0.01 | 0.10 | 0.01 | 1.00 | 417.47 | 12.72 |
| 2.82 | 0.01 | 0.28 | 0.01 | 2.53 | 1057.87 | 10.20 |
| 0.71 | 0.00 | 0.07 | 0.01 | 0.64 | 266.35 | 13.25 |
| 1.08 | 0.00 | 0.09 | 0.00 | 0.99 | 413.30 | 12.26 |
| 1.08 | 0.02 | 0.10 | 0.02 | 0.98 | 407.45 | 12.36 |
| 0.88 | 0.00 | 0.07 | 0.00 | 0.82 | 340.24 | 10.06 |
| 0.90 | 0.09 | 0.21 | 0.09 | 0.69 | 289.31 | 8.88 |
| 2.54 | 0.01 | 0.23 | 0.01 | 2.31 | 964.78 | 9.74 |
| 0.62 | 0.00 | 0.06 | 0.00 | 0.56 | 232.95 | 11.98 |
| 2.41 | 0.09 | 0.23 | 0.01 | 2.10 | 875.44 | 8.83 |
| 1.90 | 0.25 | 0.51 | 0.25 | 1.38 | 577.36 | 5.61 |
| 0.55 | 0.00 | 0.06 | 0.01 | 0.50 | 209.99 | 10.61 |
| 0.59 | 0.06 | 0.10 | 0.04 | 0.47 | 195.38 | 9.92 |
| 0.97 | 0.01 | 0.08 | 0.00 | 0.89 | 370.30 | 11.14 |
| 1.03 | 0.01 | 0.09 | 0.01 | 0.94 | 393.26 | 11.80 |
| 1.06 | 0.01 | 0.09 | 0.01 | 0.97 | 403.70 | 12.02 |
| 1.04 | 0.00 | 0.08 | 0.00 | 0.96 | 400.77 | 12.13 |
| 0.61 | 0.05 | 0.12 | 0.04 | 0.49 | 205.81 | 6.41 |
| 3.03 | 0.01 | 0.31 | 0.01 | 2.72 | 1133.85 | 10.84 |
| 0.63 | 0.01 | 0.06 | 0.01 | 0.57 | 238.38 | 12.13 |
| 0.99 | 0.01 | 0.09 | 0.01 | 0.90 | 375.31 | 11.68 |
| 0.97 | 0.00 | 0.09 | 0.01 | 0.88 | 368.63 | 11.17 |
| 2.46 | 0.01 | 0.22 | 0.01 | 2.24 | 934.30 | 9.75 |
| 0.61 | 0.00 | 0.05 | 0.00 | 0.55 | 231.28 | 11.93 |
| 0.89 | 0.00 | 0.07 | 0.00 | 0.82 | 341.91 | 10.28 |
| 0.47 | 0.06 | 0.07 | 0.00 | 0.35 | 144.03 | 4.45 |

**Table B.13: Raw data for TAC RSM conditions for third replicate.**

| Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|
| 65 | 3 | 60 | 30 | 0.3325 | 25 |
| 65 | 20 | 60 | 30 | 0.3477 | 25 |
| 65 | 11.5 | 60 | 10 | 1.016 | 25 |
| 65 | 11.5 | 60 | 50 | 0.2067 | 25 |
| 65 | 11.5 | 20 | 30 | 0.3256 | 25 |
| 65 | 11.5 | 100 | 30 | 0.3444 | 25 |
| 45 | 20 | 20 | 30 | 0.3395 | 25 |
| 45 | 20 | 100 | 30 | 0.3483 | 25 |
| 45 | 20 | 60 | 10 | 0.9987 | 25 |
| 45 | 20 | 60 | 50 | 0.2173 | 25 |
| 45 | 11.5 | 20 | 10 | 0.9952 | 25 |
| 45 | 11.5 | 100 | 10 | 1.072 | 25 |
| 45 | 11.5 | 20 | 50 | 0.2006 | 25 |
| 45 | 11.5 | 100 | 50 | 0.2068 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3345 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3321 | 25 |
| 45 | 11.5 | 60 | 30 | 0.3472 | 25 |
| 45 | 3 | 20 | 30 | 0.3402 | 25 |
| 45 | 3 | 100 | 30 | 0.3356 | 25 |
| 45 | 3 | 60 | 10 | 1.006 | 25 |
| 45 | 3 | 60 | 50 | 0.208 | 25 |
| 25 | 3 | 60 | 30 | 0.336 | 25 |
| 25 | 20 | 60 | 30 | 0.3353 | 25 |
| 25 | 11.5 | 60 | 10 | 0.9958 | 25 |
| 25 | 11.5 | 60 | 50 | 0.2059 | 25 |
| 25 | 11.5 | 20 | 30 | 0.342 | 25 |
| 25 | 11.5 | 100 | 30 | 0.3333 | 25 |

**Table B.14: Raw data for TAC RSM conditions for third replicate (I).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/L) | TAC(mg/g of DW) |
| 1.23 | 0.01 | 0.09 | 0.01 | 1.13 | 473.41 | 14.72 |
| 1.07 | 0.01 | 0.10 | 0.01 | 0.97 | 403.28 | 11.99 |
| 2.33 | 0.01 | 0.27 | 0.01 | 2.06 | 859.16 | 8.74 |
| 0.71 | 0.00 | 0.06 | 0.00 | 0.65 | 271.77 | 13.60 |
| 1.02 | 0.00 | 0.09 | 0.00 | 0.93 | 388.25 | 12.33 |
| 1.03 | 0.04 | 0.13 | 0.04 | 0.89 | 373.22 | 11.21 |
| 0.96 | 0.00 | 0.09 | 0.01 | 0.87 | 364.87 | 11.11 |
| 1.02 | 0.06 | 0.15 | 0.05 | 0.86 | 359.03 | 10.66 |
| 1.97 | 0.01 | 0.22 | 0.01 | 1.76 | 732.66 | 7.59 |
| 0.73 | 0.00 | 0.06 | 0.00 | 0.67 | 278.45 | 13.25 |
| 2.33 | 0.00 | 0.22 | 0.01 | 2.11 | 880.45 | 9.15 |
| 2.01 | 0.13 | 0.34 | 0.12 | 1.66 | 692.59 | 6.68 |
| 0.68 | 0.00 | 0.06 | 0.00 | 0.62 | 260.50 | 13.43 |
| 0.77 | 0.03 | 0.09 | 0.03 | 0.67 | 279.71 | 13.99 |
| 1.09 | 0.01 | 0.09 | 0.01 | 1.00 | 416.64 | 12.88 |
| 0.95 | 0.01 | 0.10 | 0.01 | 0.85 | 355.27 | 11.06 |
| 1.08 | 0.00 | 0.09 | 0.00 | 0.99 | 413.71 | 12.32 |
| 0.92 | 0.00 | 0.09 | 0.00 | 0.84 | 350.68 | 10.66 |
| 0.74 | 0.03 | 0.25 | 0.01 | 0.47 | 196.63 | 6.06 |
| 2.72 | 0.01 | 0.11 | 0.03 | 2.64 | 1100.46 | 11.31 |
| 0.63 | 0.00 | 0.04 | 0.00 | 0.58 | 240.05 | 11.93 |
| 1.03 | 0.00 | 0.08 | 0.00 | 0.95 | 394.68 | 12.15 |
| 0.87 | 0.00 | 0.08 | 0.01 | 0.79 | 328.55 | 10.13 |
| 2.89 | 0.01 | 0.28 | 0.01 | 2.61 | 1088.77 | 11.31 |
| 0.70 | 0.00 | 0.05 | 0.00 | 0.65 | 272.19 | 13.67 |
| 1.02 | 0.00 | 0.09 | 0.00 | 0.94 | 391.59 | 11.84 |
| 0.73 | 0.03 | 0.09 | 0.03 | 0.64 | 265.93 | 8.25 |

**Table B.15: Raw data for TAC RSM conditions for third replicate (II).**

| pH=1 | | pH=4.5 | | | | |
|---|---|---|---|---|---|---|
| 550 | 700 | 550 | 700 | A | TAC(mg/L) | TAC(mg/g of DW) |
| 1.16 | 0.01 | 0.11 | 0.01 | 1.06 | 442.52 | 13.76 |
| 1.16 | 0.01 | 0.10 | 0.01 | 1.06 | 440.43 | 13.10 |
| 2.77 | 0.01 | 0.28 | 0.01 | 2.49 | 1037.42 | 10.56 |
| 0.76 | 0.01 | 0.06 | 0.01 | 0.69 | 289.31 | 14.47 |
| 1.03 | 0.00 | 0.09 | 0.00 | 0.94 | 392.84 | 12.48 |
| 1.03 | 0.04 | 0.13 | 0.04 | 0.89 | 371.97 | 11.17 |
| 1.06 | 0.00 | 0.09 | 0.00 | 0.98 | 407.87 | 12.42 |
| 1.06 | 0.09 | 0.16 | 0.06 | 0.86 | 359.03 | 10.66 |
| 2.52 | 0.01 | 0.24 | 0.01 | 2.28 | 949.75 | 9.83 |
| 0.73 | 0.00 | 0.07 | 0.01 | 0.66 | 276.78 | 13.17 |
| 2.37 | 0.01 | 0.23 | 0.01 | 2.14 | 892.56 | 9.27 |
| 1.83 | 0.12 | 0.36 | 0.13 | 1.48 | 617.86 | 5.96 |
| 0.71 | 0.00 | 0.50 | 0.00 | 0.21 | 85.58 | 4.41 |
| 0.73 | 0.03 | 0.11 | 0.03 | 0.62 | 257.58 | 12.88 |
| 1.15 | 0.01 | 0.13 | 0.01 | 1.02 | 423.73 | 13.10 |
| 1.16 | 0.01 | 0.11 | 0.01 | 1.05 | 438.76 | 13.66 |
| 1.04 | 0.00 | 0.11 | 0.01 | 0.94 | 391.59 | 11.66 |
| 0.92 | 0.00 | 0.09 | 0.00 | 0.84 | 349.01 | 10.61 |
| 0.78 | 0.03 | 0.12 | 0.04 | 0.66 | 276.95 | 8.53 |
| 3.08 | 0.01 | 0.29 | 0.01 | 2.78 | 1161.82 | 11.94 |
| 0.73 | 0.01 | 0.07 | 0.01 | 0.67 | 278.87 | 13.86 |
| 1.03 | 0.00 | 0.08 | 0.00 | 0.95 | 394.68 | 12.15 |
| 1.14 | 0.01 | 0.11 | 0.01 | 1.03 | 430.41 | 13.27 |
| 2.90 | 0.01 | 0.35 | 0.01 | 2.56 | 1066.64 | 11.08 |
| 0.78 | 0.00 | 0.07 | 0.01 | 0.71 | 298.08 | 14.97 |
| 1.00 | 0.00 | 0.09 | 0.00 | 0.91 | 380.73 | 11.51 |
| 0.78 | 0.03 | 0.10 | 0.03 | 0.67 | 280.96 | 8.72 |

**Table B.16: Raw data for TPC conditions for first replicate.**

| No. of rums | Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|---|
| 1 | 65.00 | 3.00 | 60.00 | 30.00 | 0.34 | 1.00 |
| 2 | 65.00 | 20.00 | 60.00 | 30.00 | 0.34 | 1.00 |
| 3 | 65.00 | 11.50 | 60.00 | 10.00 | 1.08 | 2.00 |
| 4 | 65.00 | 11.50 | 60.00 | 50.00 | 0.20 | 1.00 |
| 5 | 65.00 | 11.50 | 20.00 | 30.00 | 0.33 | 1.00 |
| 6 | 65.00 | 11.50 | 100.00 | 30.00 | 0.33 | 1.00 |
| 7 | 45.00 | 20.00 | 20.00 | 30.00 | 0.33 | 1.00 |
| 8 | 45.00 | 20.00 | 100.00 | 30.00 | 0.34 | 1.00 |
| 9 | 45.00 | 20.00 | 60.00 | 10.00 | 1.01 | 2.00 |
| 10 | 45.00 | 20.00 | 60.00 | 50.00 | 0.20 | 1.00 |
| 11 | 45.00 | 11.50 | 20.00 | 10.00 | 1.02 | 3.00 |
| 12 | 45.00 | 11.50 | 100.00 | 10.00 | 1.03 | 2.00 |
| 13 | 45.00 | 11.50 | 20.00 | 50.00 | 0.20 | 1.00 |
| 14 | 45.00 | 11.50 | 100.00 | 50.00 | 0.21 | 1.00 |
| 15 | 45.00 | 11.50 | 60.00 | 30.00 | 0.34 | 2.00 |
| 16 | 45.00 | 11.50 | 60.00 | 30.00 | 0.33 | 2.00 |
| 17 | 45.00 | 11.50 | 60.00 | 30.00 | 0.34 | 1.00 |
| 18 | 45.00 | 3.00 | 20.00 | 30.00 | 0.34 | 1.00 |
| 19 | 45.00 | 3.00 | 100.00 | 30.00 | 0.34 | 1.00 |
| 20 | 45.00 | 3.00 | 60.00 | 10.00 | 1.05 | 2.00 |
| 21 | 45.00 | 3.00 | 60.00 | 50.00 | 0.35 | 2.00 |
| 22 | 25.00 | 3.00 | 60.00 | 30.00 | 0.34 | 1.00 |
| 23 | 25.00 | 20.00 | 60.00 | 30.00 | 0.34 | 1.00 |
| 24 | 25.00 | 11.50 | 60.00 | 10.00 | 1.03 | 2.00 |
| 25 | 25.00 | 11.50 | 60.00 | 50.00 | 0.21 | 1.00 |
| 26 | 25.00 | 11.50 | 20.00 | 30.00 | 0.34 | 1.00 |
| 27 | 25.00 | 11.50 | 100.00 | 30.00 | 0.34 | 1.00 |

**Table B.17: Raw data for TPC values for first replication.**

| ABS1 | TPC (mg GAE/mL) | TPC (mg GAE /g DW) | ABS2 | TPC (mg GAE/mL) | TPC (mg GAE /g DW) | TPC average |
|---|---|---|---|---|---|---|
| 0.84 | 0.97 | 29.60 | 1.03 | 1.17 | 35.95 | 32.77 |
| 0.88 | 1.01 | 30.95 | 1.02 | 1.17 | 35.62 | 33.28 |
| 1.28 | 1.44 | 13.84 | 1.32 | 1.49 | 14.29 | 14.06 |
| 0.54 | 0.65 | 32.05 | 0.62 | 0.73 | 35.86 | 33.95 |
| 0.81 | 0.93 | 28.99 | 0.84 | 0.97 | 30.22 | 29.61 |
| 0.61 | 0.72 | 22.53 | 0.62 | 0.74 | 22.93 | 22.73 |
| 0.84 | 0.97 | 30.18 | 0.81 | 0.93 | 28.94 | 29.56 |
| 0.40 | 0.49 | 14.86 | 0.50 | 0.61 | 18.19 | 16.52 |
| 1.20 | 1.35 | 13.83 | 1.23 | 1.39 | 14.20 | 14.02 |
| 0.57 | 0.68 | 35.22 | 0.61 | 0.72 | 37.28 | 36.25 |
| 0.81 | 0.93 | 9.49 | 0.77 | 0.90 | 9.12 | 9.31 |
| 0.59 | 0.70 | 7.01 | 0.58 | 0.69 | 6.99 | 7.00 |
| 0.52 | 0.62 | 31.84 | 0.53 | 0.64 | 32.61 | 32.22 |
| 0.34 | 0.43 | 21.26 | 0.39 | 0.48 | 23.75 | 22.51 |
| 0.51 | 0.62 | 18.47 | 0.56 | 0.67 | 20.08 | 19.28 |
| 0.49 | 0.60 | 18.66 | 0.52 | 0.62 | 19.36 | 19.01 |
| 0.87 | 1.00 | 30.04 | 0.98 | 1.12 | 33.79 | 31.92 |
| 0.79 | 0.91 | 27.57 | 0.94 | 1.08 | 32.59 | 30.08 |
| 0.48 | 0.59 | 18.00 | 0.54 | 0.65 | 19.88 | 18.94 |
| 1.22 | 1.37 | 13.54 | 1.29 | 1.45 | 14.30 | 13.92 |
| 0.48 | 0.59 | 17.33 | 0.49 | 0.59 | 17.61 | 17.47 |
| 0.84 | 0.97 | 29.29 | 0.96 | 1.09 | 33.16 | 31.23 |
| 0.83 | 0.95 | 29.38 | 0.91 | 1.04 | 32.03 | 30.71 |
| 1.14 | 1.30 | 13.00 | 1.21 | 1.37 | 13.70 | 13.35 |
| 0.60 | 0.71 | 36.63 | 0.65 | 0.77 | 39.50 | 38.07 |
| 0.71 | 0.83 | 25.20 | 0.74 | 0.86 | 26.01 | 25.61 |
| 0.39 | 0.49 | 15.03 | 0.48 | 0.58 | 17.73 | 16.38 |

**Table B.18: Raw data for TPC conditions for second replicate.**

| Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|
| 65 | 3 | 60 | 30 | 0.3364 | 1 |
| 65 | 20 | 60 | 30 | 0.3394 | 1 |
| 65 | 11.5 | 60 | 10 | 1.0728 | 1 |
| 65 | 11.5 | 60 | 50 | 0.2033 | 1 |
| 65 | 11.5 | 20 | 30 | 0.3485 | 1 |
| 65 | 11.5 | 100 | 30 | 0.341 | 1 |
| 45 | 20 | 20 | 30 | 0.3496 | 1 |
| 45 | 20 | 100 | 30 | 0.337 | 1 |
| 45 | 20 | 60 | 10 | 1.0241 | 1 |
| 45 | 20 | 60 | 50 | 0.2011 | 1 |
| 45 | 11.5 | 20 | 10 | 1.025 | 3 |
| 45 | 11.5 | 100 | 10 | 1.0651 | 2 |
| 45 | 11.5 | 20 | 50 | 0.2047 | 1 |
| 45 | 11.5 | 100 | 50 | 0.2037 | 1 |
| 45 | 11.5 | 60 | 30 | 0.3437 | 2 |
| 45 | 11.5 | 60 | 30 | 0.3447 | 2 |
| 45 | 11.5 | 60 | 30 | 0.3474 | 1 |
| 45 | 3 | 20 | 30 | 0.3417 | 1 |
| 45 | 3 | 100 | 30 | 0.332 | 1 |
| 45 | 3 | 60 | 10 | 1.0821 | 2 |
| 45 | 3 | 60 | 50 | 0.2078 | 2 |
| 25 | 3 | 60 | 30 | 0.3322 | 1 |
| 25 | 20 | 60 | 30 | 0.3413 | 1 |
| 25 | 11.5 | 60 | 10 | 0.9908 | 1 |
| 25 | 11.5 | 60 | 50 | 0.2004 | 1 |
| 25 | 11.5 | 20 | 30 | 0.344 | 1 |
| 25 | 11.5 | 100 | 30 | 0.335 | 1 |

**Table B.19: Raw data for TPC values for second replicate**

| ABS1 | TPC (mg/mL) | TPC(mg/g DW) | ABS2 | TPC (mg/mL) | TPC(mg/g DW) |
|---|---|---|---|---|---|
| 1.09 | 1.00 | 30.77 | 1.07 | 0.98 | 30.24 |
| 1.08 | 0.99 | 30.25 | 1.22 | 1.11 | 33.95 |
| 2.32 | 2.12 | 20.44 | 2.60 | 2.37 | 22.89 |
| 1.60 | 1.46 | 75.39 | 1.71 | 1.56 | 80.66 |
| 1.06 | 0.97 | 28.92 | 0.97 | 0.89 | 26.48 |
| 0.62 | 0.57 | 17.33 | 0.69 | 0.63 | 19.10 |
| 0.64 | 0.59 | 17.41 | 0.77 | 0.71 | 21.00 |
| 0.47 | 0.44 | 13.39 | 0.52 | 0.48 | 14.76 |
| 0.98 | 0.90 | 9.11 | 1.10 | 1.01 | 10.21 |
| 0.51 | 0.47 | 24.22 | 0.62 | 0.57 | 29.34 |
| 0.74 | 0.68 | 6.88 | 0.76 | 0.70 | 7.05 |
| 0.51 | 0.47 | 4.52 | 0.99 | 0.91 | 8.85 |
| 0.49 | 0.45 | 22.83 | 0.44 | 0.40 | 20.39 |
| 0.27 | 0.25 | 12.80 | 0.32 | 0.29 | 14.93 |
| 0.43 | 0.40 | 11.92 | 0.47 | 0.44 | 13.16 |
| 0.39 | 0.36 | 10.87 | 0.45 | 0.42 | 12.57 |
| 0.91 | 0.84 | 24.88 | 0.99 | 0.90 | 26.89 |
| 0.77 | 0.71 | 21.43 | 0.77 | 0.70 | 21.30 |
| 0.32 | 0.30 | 9.33 | 0.37 | 0.34 | 10.72 |
| 1.32 | 1.21 | 11.59 | 1.37 | 1.26 | 12.03 |
| 0.30 | 0.27 | 13.68 | 0.20 | 0.19 | 9.28 |
| 0.71 | 0.65 | 20.34 | 0.78 | 0.72 | 22.30 |
| 0.79 | 0.73 | 22.04 | 0.93 | 0.85 | 25.72 |
| 0.49 | 0.45 | 4.72 | 0.49 | 0.45 | 4.74 |
| 0.65 | 0.60 | 30.36 | 0.69 | 0.63 | 32.22 |
| 0.66 | 0.60 | 18.14 | 0.67 | 0.61 | 18.47 |
| 0.22 | 0.21 | 6.35 | 0.26 | 0.24 | 7.47 |

**Table B.20: Raw data for TPC conditions for third replicate.**

| Temp(°C) | Time(min) | SolvConc(%) | LSRatio(mL/g) | Weight(g) | DF |
|---|---|---|---|---|---|
| 65 | 3 | 60 | 30 | 0.3325 | 1 |
| 65 | 20 | 60 | 30 | 0.3477 | 1 |
| 65 | 11.5 | 60 | 10 | 1.016 | 1 |
| 65 | 11.5 | 60 | 50 | 0.2067 | 1 |
| 65 | 11.5 | 20 | 30 | 0.3256 | 1 |
| 65 | 11.5 | 100 | 30 | 0.3444 | 1 |
| 45 | 20 | 20 | 30 | 0.3395 | 1 |
| 45 | 20 | 100 | 30 | 0.3483 | 1 |
| 45 | 20 | 60 | 10 | 0.9987 | 1 |
| 45 | 20 | 60 | 50 | 0.2173 | 1 |
| 45 | 11.5 | 20 | 10 | 0.9952 | 3 |
| 45 | 11.5 | 100 | 10 | 1.072 | 2 |
| 45 | 11.5 | 20 | 50 | 0.2006 | 1 |
| 45 | 11.5 | 100 | 50 | 0.2068 | 1 |
| 45 | 11.5 | 60 | 30 | 0.3345 | 2 |
| 45 | 11.5 | 60 | 30 | 0.3321 | 2 |
| 45 | 11.5 | 60 | 30 | 0.3472 | 1 |
| 45 | 3 | 20 | 30 | 0.3402 | 1 |
| 45 | 3 | 100 | 30 | 0.3356 | 1 |
| 45 | 3 | 60 | 10 | 1.006 | 2 |
| 45 | 3 | 60 | 50 | 0.208 | 2 |
| 25 | 3 | 60 | 30 | 0.336 | 1 |
| 25 | 20 | 60 | 30 | 0.3353 | 1 |
| 25 | 11.5 | 60 | 10 | 0.9958 | 1 |
| 25 | 11.5 | 60 | 50 | 0.2059 | 1 |
| 25 | 11.5 | 20 | 30 | 0.342 | 1 |
| 25 | 11.5 | 100 | 30 | 0.3333 | 1 |

**Table B.21: Raw data for TPC values for third replicate.**

| ABS1 | TPC (mg/mL) | TPC (mg/g DW) | ABS2 | TPC(mg/mL) | TPC(mg/gDW) |
|---|---|---|---|---|---|
| 0.91 | 0.84 | 26.00 | 0.99 | 0.91 | 28.27 |
| 0.88 | 0.81 | 24.13 | 0.76 | 0.70 | 20.85 |
| 2.02 | 1.85 | 18.80 | 2.20 | 2.02 | 20.52 |
| 0.61 | 0.57 | 28.43 | 0.65 | 0.60 | 29.89 |
| 0.72 | 0.66 | 20.99 | 0.79 | 0.73 | 23.19 |
| 0.53 | 0.49 | 14.78 | 0.61 | 0.56 | 16.83 |
| 0.81 | 0.74 | 22.63 | 0.82 | 0.75 | 22.99 |
| 0.59 | 0.55 | 16.24 | 0.57 | 0.53 | 15.72 |
| 2.19 | 2.00 | 20.71 | 2.10 | 1.92 | 19.93 |
| 0.63 | 0.58 | 27.41 | 0.67 | 0.62 | 29.54 |
| 0.68 | 0.62 | 6.48 | 0.67 | 0.61 | 6.38 |
| 0.56 | 0.51 | 4.96 | 0.69 | 0.64 | 6.15 |
| 0.59 | 0.55 | 28.10 | 0.57 | 0.53 | 27.30 |
| 0.47 | 0.44 | 21.78 | 0.44 | 0.41 | 20.27 |
| 0.58 | 0.53 | 16.51 | 0.59 | 0.54 | 16.82 |
| 0.63 | 0.58 | 18.02 | 0.57 | 0.53 | 16.38 |
| 0.93 | 0.85 | 25.31 | 1.04 | 0.96 | 28.49 |
| 0.85 | 0.78 | 23.72 | 0.76 | 0.70 | 21.28 |
| 0.43 | 0.40 | 12.21 | 0.46 | 0.42 | 12.97 |
| 1.38 | 1.26 | 13.00 | 1.39 | 1.27 | 13.06 |
| 0.33 | 0.31 | 15.26 | 0.32 | 0.30 | 14.76 |
| 0.92 | 0.84 | 25.96 | 1.04 | 0.96 | 29.41 |
| 0.92 | 0.85 | 26.18 | 1.05 | 0.96 | 29.73 |
| 2.20 | 2.01 | 20.90 | 2.29 | 2.10 | 21.76 |
| 0.60 | 0.55 | 27.54 | 0.64 | 0.59 | 29.51 |
| 0.86 | 0.79 | 24.01 | 0.81 | 0.74 | 22.50 |
| 0.41 | 0.38 | 11.87 | 0.44 | 0.41 | 12.64 |