Spring 4-24-2014

CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC-RICH ANTIOXIDANTS FROM BLUE GREEN ALGAE (SPIRULINA) USING RESPONSE SURFACE APPROACHES

Ahmad Salamatullah
University of Nebraska-Lincoln, salamhahmad@hotmail.com

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by

Ahmad Salamatullah

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science & Technology

Under the Supervision of Professor Vicki Schlegel

Lincoln, Nebraska

May, 2014

CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC-RICH ANTIOXIDANTS FROM BLUE GREEN ALGAE (SPIRULINA) USING RESPONSE SURFACE APPROACHES

Ahmad Salamatullah, M.S

University of Nebraska, 2014

Adviser: Vicki Schlegel

Blue green algae (spirulina) is a dietary system that is widely consumed as a whole food or as a supplement in many Asian countries where populations are mostly unaffected by many of the diseases currently afflicting western societies, such as cancer, heart disease and arthiritis. Indeed, spirulina is a rich source of antioxidants with the phenolic compounds playing a significant role. As components of a complex dietary system, phenolic compounds can act alone or through synergistic mechanisms to impart a greater biologic effect than can be elicited by a sum of the individual parts. Therefore, an understanding of the antioxidative mechanism(s) of spirulina's polyphenols can only be achieved by a thorough knowledge of their chemical profiles (types and quantities). As phenolic compounds are a richly diverse set of molecules, different extraction methods are therefore needed to thoroughly characterize the different types and amounts present in a whole system or complex matrix.

As an initial first step in fulfilling this need, the objective of this project was to apply a response surface approach for obtaining highly potent antioxidative spirulina extracts in terms of their ability to scavenge free radicals and to correlate this health benefit to their phenolic content (total phenols / flavonoids). This objective was achieved by using six different extraction solvents, including methanol, ethanol, and acetone, with or without 1.2 N HCL and varying the solvent water ratio, mixing time, and solid / volume ratio. A three factor three-level face centered cube design was used for the response surface approach resulting in 16 samples per solvent system, which were prepared in triplicate. Each were then tested for total phenols, total

flavonoids, and for their ability to scavenge a free radical. It was determined that optimal responses required different approaches. For example, the highest total phenols and flavonoids were obtained with acetone and methanol, respectively. Although acidified solvents were not needed for extracting the optimal TP and TF rich extracts, HCl was required for potent antioxidative extracts. In this case, ethanol + HCl produced the extracts with the highest antioxidative capacities. These results demonstrate that total amounts of TF and TP do not correlate to antioxidative capacities, and the effect is probably due to the different types of phenols and flavonoids present as well, which was evidenced by the compositional analysis of select extracts. This project is therefore significant in that critical information was obtained to characterize the extraction process to ultimately identify the spirulina's polyphenols responsible for the antioxidative benefits and understand how they interact to deliver optimal protection against oxidative stress.

ACKNOWLEDGEMENTS

First and foremost, I want to thank God, whose many blessings have made me who I am today. I am forever grateful to Dr. Schlegel for her guidance, understanding, patience, and most importantly, her friendship during my graduate studies. Her unlimited background, patience, charisma and effort were essential to the birth of this document and to my formation as a future researchers. She encouraged me to not only grow as an experimentalist but also as an instructor and an independent thinker. Additionally, I want to thank Dr. Flores for giving me an opportunity to perform my graduate study at UNL, his first advice on my journey to get a Master degree " for sure you will graduated but the deal is how much knowledge you gain during your degree" has motivated me throughout the years.

I would like to gratefully and sincerely thank my committee members, Dr. Wehling and Dr. Carr for their time, efforts, guiding me through conceptions and reviewing my thesis and giving helpful feedback.

I want to express special thanks to all members of Dr. Schlegel's lab, especially Richard and Mohammed, with whom I worked closely and puzzled over many of the same problems. These two friends provided for some much needed humor and entertainment in what could have otherwise been a somewhat stressful laboratory environment. On a different note, I would like to thank King Saud University (KSU) for its financial support throughout my Master degree. Additionally, All faculties in Food Science and Nutrient Department at KSU.

Finally, and most importantly, I want to express my deepest love and thanks to my loving wife Aisheh for her support, encouragement, quiet patience and unwavering love. Thanks my parents for allowing me to be as ambitious as I wanted, thanks my sister, brothers and Aisheh's parents for their faith in me and warm supported words. Lastly, thank my lovely son Owhyce for your sweet smile and patient during my busy time. My education would have not been completed without my family support and prayer.

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A. LITERATURE SEARCH

Blue-green algae (BGA), technically known as "cyanobacteria", are microscopic organisms naturally present in lakes and streams but are lower in numbers compared to other marine algae (Wisconsin Division of Public Health, 2004). BGA growth is promoted by fresh, warm, shallow, and undisturbed surface water that receives abundant sunlight. BGA also grows relatively easily on by-product or waste sources of nutrients (Romay et al., 1998). Under such conditions, BGA propagate quickly as floating rafts that appear as "scum on the water" (Henrikson, 2010). Although abundant in many water bodies, BGA are generally not consumed by other aquatic organisms, and thus are not an important part of the aquatic food chain (Dietrich and Biggins, 1971). However, human consumption of freshwater BGA has been recognized as an excellent dietary food source due primarily to the presence of high levels of protein and its overall good digestibility (Gilani and Sepehr, 2003). In particular, spirulina, a species of cyanobacteria, has been used for food in Japan and China for centuries but to a lesser extent in Europe, North America, and other parts of the world (Romay et al., 1998). For example, the Asian population currently consume approximately 1 and 2 tablespoons (3–13 g) of sprirulina in a powder or tableted form on a daily basis.

A.1 Chemical Composition of Spirulina:

Spirulina is a rich source of many chemically diverse components, as shown in Table 1. Of these components, protein is the predominant component accounting for 60% of the total amount. For example, a typical serving size of 3 g (teaspoon) of spirulina

Table 1: Nutritional component used during the research's assays. (The Vitamin Shoppe. www.vsconneect.com; Tokusoglu and Unal., 2006).

Nutrient	Unit	Value per 1 teaspoon (3g)
Main Components		
Calories	Kal	10
Total Carbohydrate	G	0.5
Protein	Gm	2
Phycocyanin	Mg	420
Sulfolipids	Mg	40
Lipid	G	0.21
Lipids		
Palmatic acid	Mg	57.1
Oleic acid	Mg	71.4
Linoleic acid	Mg	25.2
Linolenic acid	Mg	9.6
Alpha linolenic acid	Mg	1.4
Eicosapentaenoic acid	Mg	5.2
Docosahexaenoic acid	Mg	6.3
Arachidonic acid	Mg	0.75
Minerals		
Calcium	Mg	7
Iron	Mg	1.5
Sodium	Mg	15
Potassium	Mg	40
Magnesium	Mg	12
Phosphorus	Mg	24
Zinc	Mg	0.09
Manganese	Mg	0.15
Vitamins		
Vitamin A(As Beta Carotene)	IU	5000
Lutein	Mcg	300
Zeaxanthin	Mg	2.5
Vitamin K	Mcg	17
Vitamin B12	Mcg	2.8
Vitamin B2	Mg	0.12
Vitamin B3	Mg	0.39
Vitamin B6	Mg	0.18
Vitamin E	Mg	0.3
Vitamin B1	Mg	0.105
Enzymes		
SOD (super oxide dismutase)	Units	5000

contains approximately 2 g of protein. Protein is an essential nutrient for building, repairing, and supporting muscle and tissues (Eriksen, 2008; Phillips et al., 2013). Phycocyanin is a protein complex that indirectly provides the blue and green color by absorbing the sun light and converting it to chlorophyll. Phycocyanin is also an antioxidant and a nitrogen source for cells (Boussiba and Richmond, 1980). Super oxide dismutase is an important protein also present in spirulina (Table 1) due to its ability to catalyze the reaction that converts the more reactive free radical, super oxide, into the less harmful hydrogen peroxide molecule (Halliwell, 1992).

Moreover, spirulina contains high levels of vitamin B12 with 47% of the daily recommended allowance obtained from only 3 g of the product (supplement fact, the vitamin shoppe) (Table 1). As a water soluble vitamin, also referred to as cobalamin (Albert et. al, 1980), B12 is essential for normal function of the brain**,** nervous system, and blood formation (Oh and Brown, 2003). In addition to vitamin B12, several other B vitamins are present in spirulina but at levels below 0.1% of the daily recommended intake. However, these B vitamins include B3, (regulates digestive process) (Xu et al., 2010)**,** B6 (produces energy for the brain) (Bourre, 2006)**,** and B9 (prevents many types of anemia) (Andrès et al., 2008) to name a few (Table 1).

Vitamin K is yet another abundant vitamin present in spriulina at relatively high levels when compared to other dietary sources (Tokusoglu and Unal, 2006). Vitamin K plays a significant role in preventing bone loss through urinary calcium excretion in postmenopausal women (Knapen et al., 1989; Price and Williamson, 1981). Sprirulina contains approximately 5000 IU of beta carotene for every teaspoon, which supplies 100% of the daily recommended level (Table 1). Studies have shown that carotene has

multiple properties beyond those required for our daily nutritional needs. For example, beta-carotene plays a role in the protection of skin against sunlight (Greenberg et al, 1990). It is also able to protect high density lipo protein from converting to its oxidative low density lipoprotein counterpart, which has strongly been correlated to coronary heart diseases (Carroll et al., 2000). Beta-carotene acts as an antioxidant by quenching singlet oxygen, which is involved in cholesterol oxidation, due to its highly conjugated structure.

Additionally, spirulina contains only ~7% crude lipid with palmitic acid and oleic acid accounting for approximately 50% of this fraction. However, this fraction is composed of also contain the omega 3 fatty acids, linolenic (~5% of the lipid fraction) and eicosapentaenoic acid (3%), which have been linked to preventing or remediating chronic inflammation (Lordan and Stanton, 2011; Tokusoglu and Unal, 2006). If left unchecked, chronic inflammation can lead to multiple diseases, including cancer, diabetes, arthritis, and heart disease (Centers for Disease Control and Prevention, 2011; Bastard et al., 2006). Lastly, a diverse group of minerals is also present in spirulina (Table 1) that are required for the body's functions, such as bone health, metabolism, and water balance (Tokusoglu and Unal, 2006). Table 2 summarizes other health related studies that have been directly linked to blue green algae and/or spirulina consumption.

A.2 <u>Cellular Oxidative Stress:</u>

Most of the diseases cited in Table 2 have been associated in some manner to unchecked cellular oxidative stress (Centers for Disease Control and Prevention, 2011; Bastard et al., 2006). Cellular oxidative stress is defined as an imbalance between the production of highly reactive molecules (frequently referred to as free radicals) and a biological system's ability to readily

Table 2: List of health related studies using BGA or Spirulina.

Stress-disease	Model	Treatment	Dosage	Main Outcomes	References
Cancer	20 hamsters	Phycotene in BGA	250 micrograms in 0.1 ml MEM; DMBA-induced carcinomas in 20 animals. The various agents were injected into the tumor bearing right buccal pouches twice-weekly for four weeks	Total tumor regression was found in 30% of phycotene animals, 20% of beta carotene animals and 15% of canthaxanthin animals after four weeks. Partial tumor regression was found in the remaining 70% of phycotene animals, 80% of beta carotene animals and 85% of canthaxanthin animals.	Schwartz J, Shklar G.1987
Immune system	Mouse	Spirulina	The BGA diet was a mixture of BGA (20%) and the normal diet (80%) feeding 4 weeks after the experiment.	Delayed-type hypersensitvity caused by difocyanate suppressed in mice fed with BGA.	Nagao et al., 1991.
	Mouse	Spirulina	Mice sensitized with 1% toluene-2, 4-diisocyanate (TDI).	Primary immune response against thymus-dependent and independent antigens enhanced.	Hayashi et al., 1992
Hyper lipidemia	Male Wistar rats 3 weeks old (body weight 54 g)	Spirulina	5, 10, and 15% of diet, respectively, for 4 weeks.	Rate of increase of triglycerides level in serum and liver suppressed by lipase activity.	Iwata et al., 1987-1990.
	Human 30 patients	Spirulina	4.2 grams of BGA per day for eight weeks and group B subjects were given the same amount of BGA for the first four weeks and for the next four weeks, they were observed without giving the agent.	LDL-cholesterol, and AI (Athero- genic index) lowered. HDL-cholesterol increased concurrently.	Nakaya et al., 1988.
Radio protictive effect	Mouse	Spirulina	Micronucleus test in polychromatic erythrocytes of bone marrow of mice	Ethanol extract of BGA result in significant reduceion of micronucleus frequencies by gamma- radiation.	Oishen et al., 1989

Table 2 continued: List of health related studies using BGA or Spirulina.

Stress-disease	Model	Treatment	Dosage	Main Outcomes	References
Hypercholestrolemia	Rabbits	Spirulina	0.5 g/d measuring after 30 days and 60 days	Total-cholesterol (TC), triacylglycerols (TAG) decreased, while high-density lipoprotein (HDL-cholesterol) increased.	Colla et al., 2008
Adipo hepatosis	Rat	Spirulina		Condition suppressed, and recovery stimulated.	Kato et al., 1984.
Renal Toxicity	Rat	Spirulina		Spirulina diet showed protective effect against renal failure induced by inorganic mercurt and 3 known pharmaceuticals: para-aminophenol, gentamicin, and cisplatin. Phycocyanin showed protective effect against renal failure induced by mercuty and cisplatin.	Yamane et al., 1998
Anticlastogenic	Tradescantia biosssay	Spirulina	100 mg/ml of the algae	Spirulina maxima reduce the genotoxic damage induced by maleic hydrazide (MH) using the Tradescantia biosssay	Ruiz et al., 2003
Intestinal flora	Rat	Spirulina	5% of the diet	Population of lactobacillus and Bifidobacteria in the intetine notably increased.	Tsuchihashi et al., 1987
Diabetes	15 diabetic patients	Spirulina	2 g of BGA for 21 days	Decrease serum glucuse at fasting by fedding BGA water-insluble fraction.	Takai et al., 1991
Obesity	Human	Spirulina	2.8 g of BGA 3 times over 4 weeks	Signifcantly decreasing of body wieght in obese outpatients.	Becker et al., 1986
Hyperchol esterolemic	30 ischemic heart disease patients	Spirulina	Divided the 30 patients to three group A, B, and C 2 g, 4 g per day, respectively for three months, group C as control.	BGA play significant role to decrease the blood cholestrol levels, weight reduction, and increasing the lipid profile of patients.	Ramamoorthy and Premakumari, 1996
Heavy metals toxicity	Rat	Spirulina	30% of the diet	Significant reduction in mercury levels, urea and creatinine levels.	Yamane et al., 1998

eliminate the reactive intermediates (Henrikson, 2010). To understand the oxidation state in biological systems, it is important to differentiate between the reactive species that are produced in cells. The "reactive oxygen species" (ROS) include the oxygen centered radicals (e.g., superoxide anion, hydroxyl radical, peroxyl radical, and alkoxyl radical), and the oxygen –centered and the oxygen – centered radicals non radical, (e.g., hydrogen peroxide and singlet oxygen). Reactive nitrogen species (RNS) consist of nitric oxide, nitric dioxide, and peroxynitrite. Most of these species are generally unstable and highly reactive as they exist with one or more unpaired electrons (Halliwell et al., 1995).

These intracellular free radicals are formed when certain catalytic enzymes are activated in various biological mechanisms throughout the cells. However, the mitochondria is the primarily source of reactive oxygen species (90%), which are produced during normal respiration as oxygen is converted to water (Figure 1). The reaction begins when the oxidative enzyme xanthine oxidase initially converts oxygen to the superoxide anion (Brash, 1999). This potent reactive species is kept in check by superoxide dismutase resulting in hydrogen peroxide. Hydrogen peroxide is then converted into oxygen and water by catalase; or water, oxygen, and glutathione disulfide by glutathione peroxidase. If all is in check, the potent reactive species produced during respiration are converted to much less aggressive molecules (Muzykantov, 2001) (Figure 1).

Still, if superoxide dismutase is not activated, degraded, or otherwise unavailable, the superoxide anion is available to react with nitric oxide (formed by arginine by nitrogen oxidase synthase) to produce the even more reactive peroxynitrite. Peroxynitrite

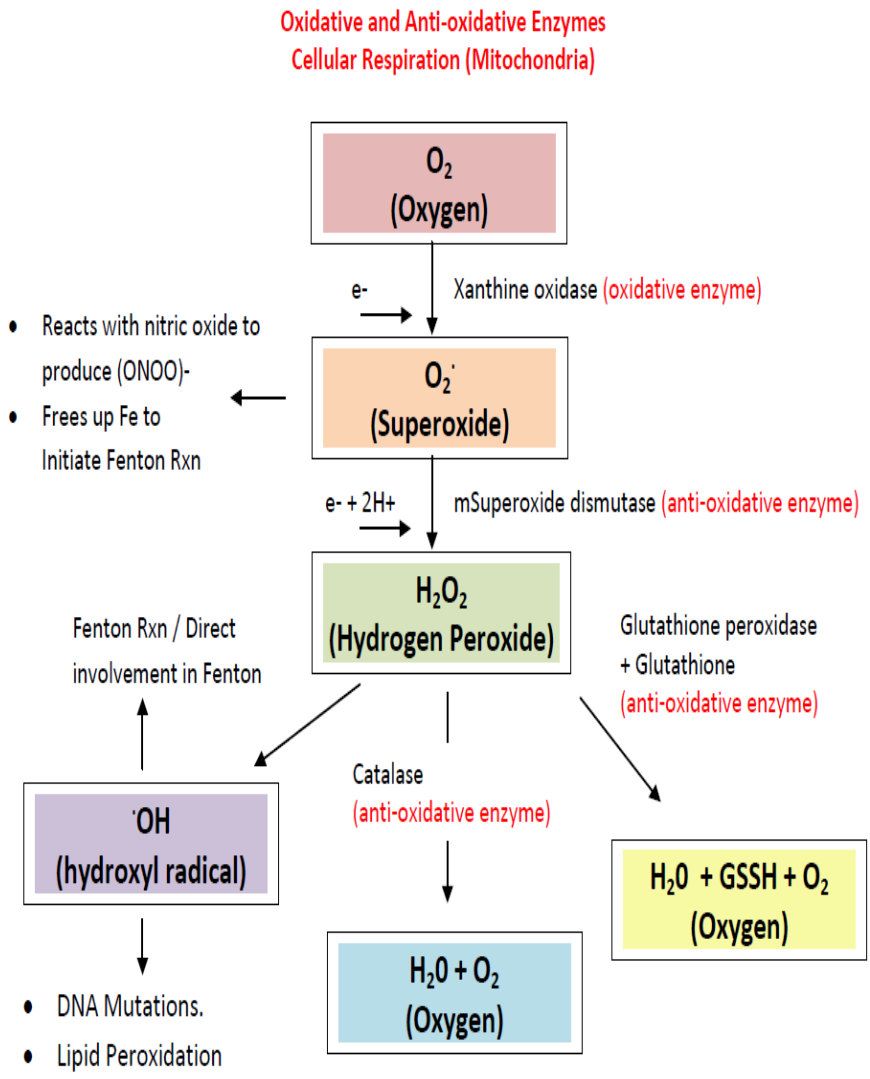


Figure 1: Oxidative and anti-oxidative enzymes involved in mitochondria respiration.

can cause cellular protein / DNA oxidation (McVean et al., 1999) and directly damage tissue at sites of inflammation (Papas, 1999). The conversion of superoxide to hydrogen peroxide also requires the reduced form of iron (Fe^{2+}). If the reaction does not occur for any given reason, Fe^{2+} is free to participate in the Fenton and Haber Weiss reaction (Figure 2). The relatively stable hydrogen peroxide molecule can enter the Fenton pathway and react with Fe^{2+} resulting in highly reactive hydroxyl radicals, which can cause critical damage to important cellular molecules, particularly DNA molecules (Ames et al., 1993).

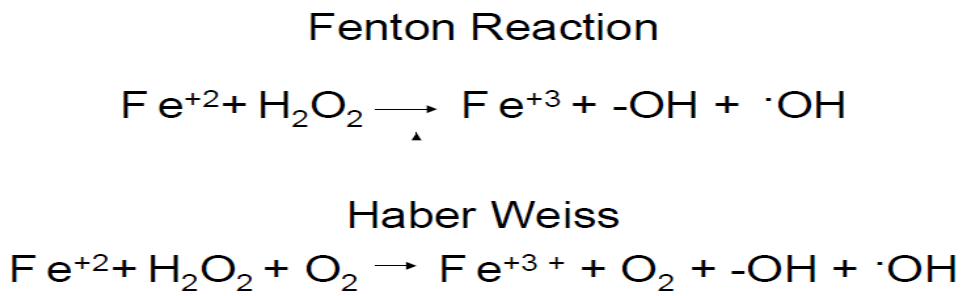


Figure 2: Chemical equations for the Fenton and Haer Weiss reactions.

Many studies have shown that RNS and ROS participate in a diverse array of biological processes, such as normal cell growth, induction and maintenance of the transformed state, programmed cell death and cellular senescence (Finkel, 2003; Dickinson and Chang, 2011). Despite these protective mechanisms, the balance of the oxidative state of a cell can not only be negatively offset by prolonged illness or infections, but also by lifestyle. Because of the increasing concern over serious health

problems caused by oxidative damages, identifying natural approaches that can reduce this stress has become a major emphasis of study by many researchers (Manna et al., 1997; Kuntz et al., 1999; Bestwick and Milne, 2001). In addition, the increasing public preference for natural and herbal medicines has prompted the discovery of natural anti-oxidative agents and the development of food systems rich in these agents.

A.3 <u>Phenolic Compounds as Dietary Antioxidants</u>:

When our body's natural defenses fail to protect against oxidative stress, balance may be restored by the consumption of nutraceuticals that have antioxidant properties. According to Wildman (2001), nutraceuticals include isoprenoid derivatives (i.e. carotenoids and tocopherols); phenolic compounds (tannins, anthocyanins, flavonoids); amino acid-based (isothiocyanates, folate, choline); carbohydrates (oligosaccharides, ascorbic acid); fatty acids (polyunsaturated fatty acids, lecithin, and conjugated linoleic acid); minerals (Ca, K, Zn); and microbiols (pre- and pro- biotics).

In particular, the phenolic compounds have been shown to exert potent antioxidant capacities, particularly the flavonoids, (Wildman, 2001; Biesalski, 2001) to protect against many diseases (Ishige et al., 2001; Casaburi et al., 2013). As secondary metabolites in plants, phenolic compounds are not directly involved with respiration, photosynthesis, or nutrient assimilation (Blumberg and Block, 1994) but rather play important roles in protecting a plant from insects, disease, and herbivores. Phenols also attract pollinators and thus help to ensure the propagation of the next generation in plant to plant competition (Wildman, 2001).

Phenols are present in all plant based systems with approximately 8000 different types categorized by their structure (Pescarmona, 2007), which in all categories include

the aglycones (phenols that are not bonded to another molecule) or the conjugated phenols (e.g., glycosylated or esterified with a single or multiple sugar units or other phenols, etc.). As shown in Figure 3, the phenolic compounds include phenolic acids and various classes of flavonoids, including flavonols, isoflavones, anthocyanins, etc.

Phenols protect against oxidation in both plants and humans by various mechanisms. First, a phenol is able to transfer its H-atom to a radical directly, which is termed the H-atom transfer. Second, phenols transfer an electron to deactivate a free radical followed by proton. Third, phenols transfer an electron to deactivate a free radical followed by proton abstraction via proton-coupled electron transfer. Fourth, the sequential proton- loss electron transfer can occur where deprotonation of phenol is followed by electron transfer from its phenoxyl anion to a free radical (Klein and Lukes, 2006). Phenolic compounds become resonance- stabilized radicals after donation making the free radical stable (Shahidi et al., 1992) (Figure 4). The quantity and the type of phenols present control the efficiency of phenolic rich natural systems to act as potent free radical scavengers (Musialik et al., 2009).

The antioxidant activity of a flavonoid in particular is dependent on the arrangement of the functional groups about the nuclear structure, which consists of three aromatic rings labeled A, B, and C (Figure 5a), as well as subtle changes in the nuclear structure itself. The structure that provides the most stability after hydrogen donation favors higher antioxidative activities. Studies have shown that flavonoids containing OH groups at C-3’ and C-4’ on the B ring and at C-3, C-5 and C-7 on the AC ring, as well as a double bond at C-2,3 and a carbonyl at C-4, are the most effective scavengers of oxygen and nitrogen radicals. Methylation of any hydroxyl results in drastically reduced

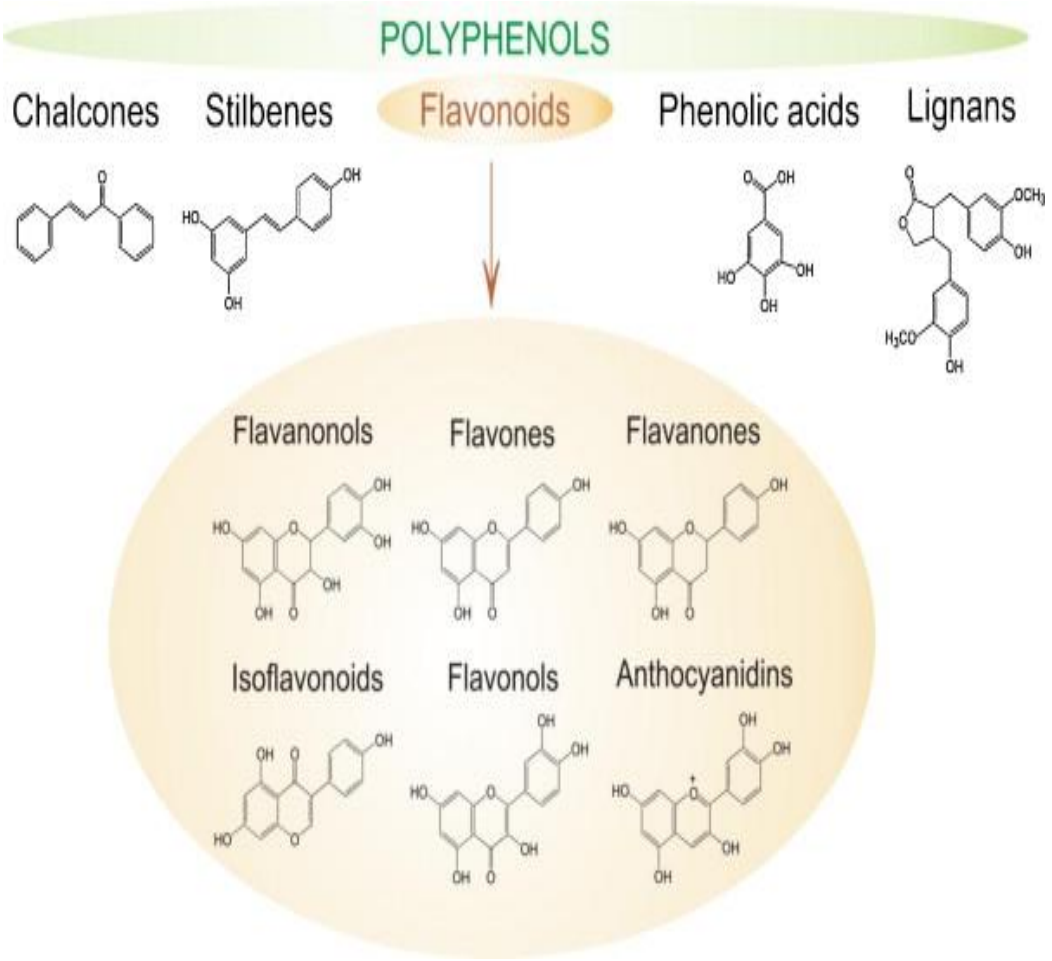


Figure 3: Differentiation of different phenols, with an emphasis of the various flavonoid groups (Pescarmona, 2007)

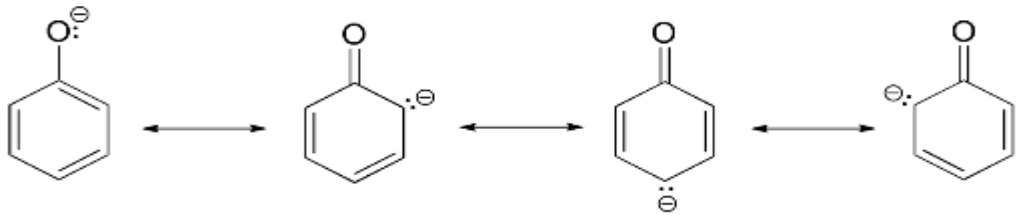


Figure 4: Antioxidant radical are stabilized through resonance structure.

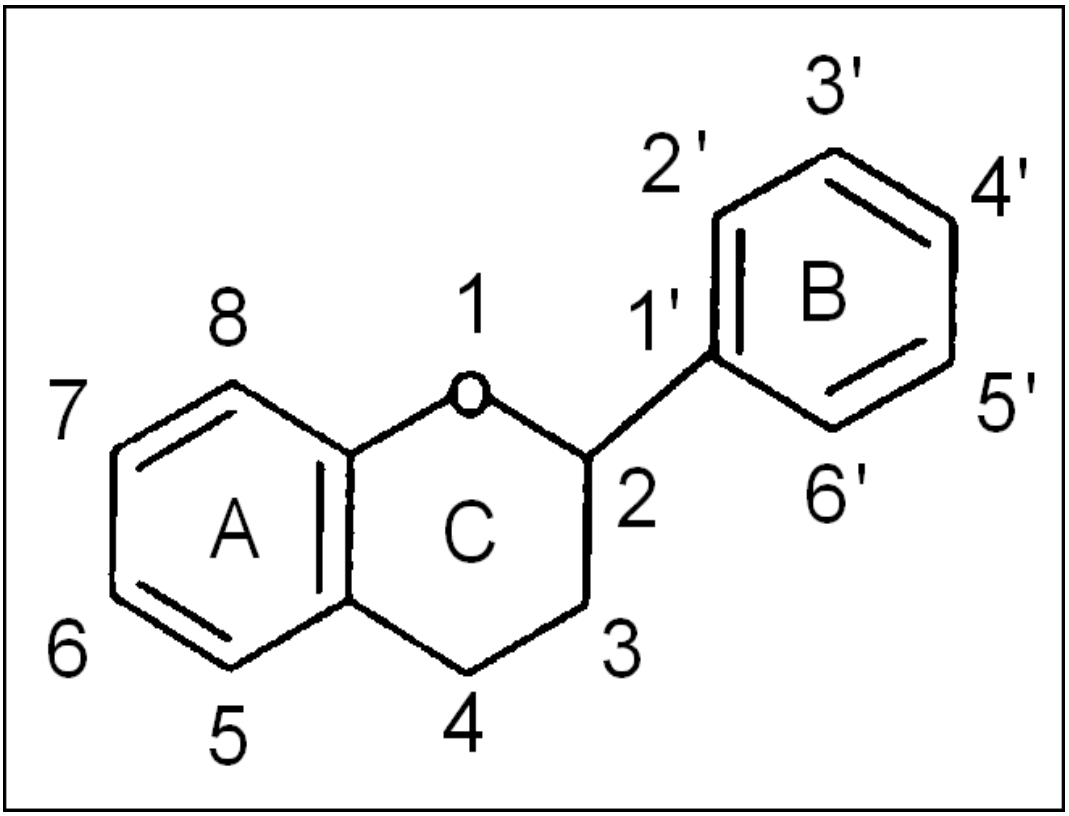


Figure 5a: A, B, C rings of flavonoid structure (Heim et al, 2002)

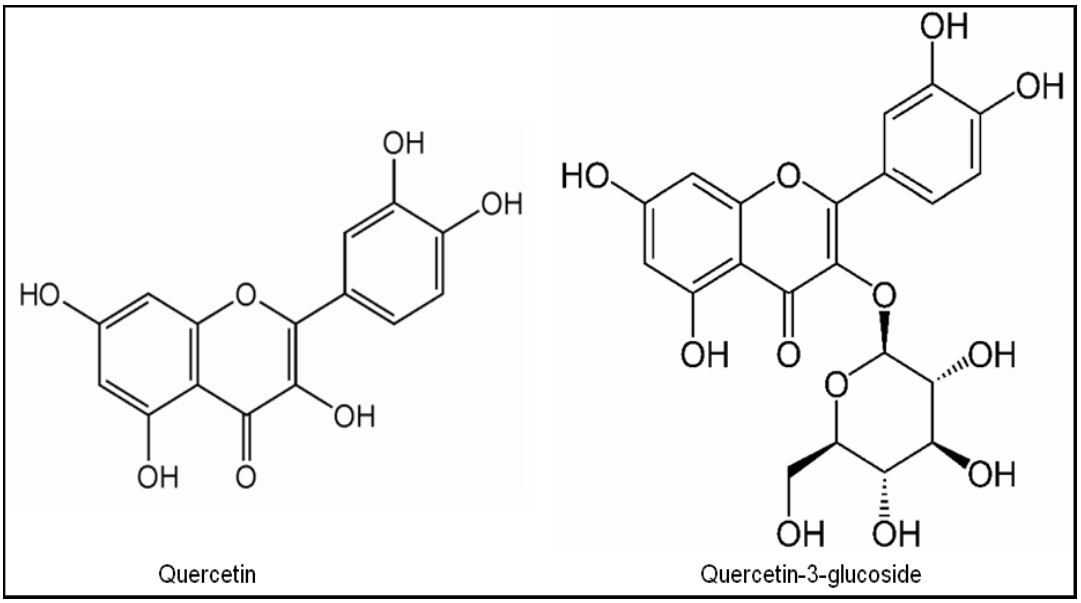


Figure 5b: Quercetin aglycone and Quercetin-3-glucoside
(biotec.or.th; http://www.chemicalbook.com)

scavenging activities (Figure 5b) (Heijnen et al., 2001; Heim et al., 2002; Gee et al., 2002). The antioxidant activity of phenolic compounds is positively correlated with pH as the electron donating capacity of the phenolic OH increases with dissociation (Mukai et al., 1997).

Lastly, phenols can act as inhibitors or activators of the oxidative enzymes described previously (Casella, 2006). The balance between both the activities and the intracellular levels of these antioxidants are essential for the survival of organisms and their health (Mate´ et al., 1999). The effects of such antioxidative responses may result from different interactions of the chemically diverse phenols that impart greater protective properties on one biological response relative to another. As such, different types and ratios may be responsible for different health promoting properties.

A4. <u>Phenols and Spriulina</u>:

Although the health benefitting properties of spirulina (Table 2) have been partially attributed to the chemically rich and diverse phenolic compounds interacting together as potent antioxidants (Kulshreshtha et al., 2008; Miranda et al., 1998), research remains limited on spirulina in term of overall phenol yields and types. El-bakey et al. (2009) determined that phenolic and flavonoid yields change in accordance to the growth media

Table 2. Effect of growth media ($NaNO_3$ / Phenylalanine) concentration on the amount of phenol and flavonoid (El-Baky et al. (2009).

$NaNO_3$ / Phenylalanine Content	**Total phenol (mg g^{-1} DW)**	**Total flavonoid (mg g^{-1} DW)**
2.5 g/L $NaNO_3$	4.51 +/- 0.23	1.32 +/- 0.03
2.5 g/L $NaNO_3$ + 50 mg/g phenylalanine	5.68 +/- 0.33	1.54 +/- 0.08
2.5 g/L $NaNO_3$ + 50 mg/g phenylalanine	7.36 +/- 0.36	1.94 +/- 0.06
3.125 g/L $NaNO_3$	5.19 +/- 0.35	1.45 +/- 0.07
3.125 g/L $NaNO_3$ + 50 mg/L phenylalanine	8.64 +/- 0.27	2.25 +/- 0.13
3.125 g/L NaNO +100 mg/L phenylalanine	10.95 +/- 0.47	3.31 +/- 0.21
3.77 g/L $NaNO_3$	6.54 +/- 0.54	1.93 +/- 0.05
3.77 g/L $NaNO_3$ + 50 mg/L phenylalanin	12.94 +/- 0.93	4.65 +/- 0.14

Extraction of phenol and flavonoid by $NaNO_3$ / Phenylalanine, all values show the mean of three replicates, ± standard deviation.Values are significant at ($P < 0.001$).

used. As shown in Table 2, the amounts varied from 4.51–12.94 mg/g and 1.32-4.65 mg/g for the phenols and flavonoids, respectively. Moreover, The High Performance Liquid Chromatograph (HPLC-DAD) profile of phenolic extracts of spirulina showed the presence of different phenolic acids and flavonoids in variable levels (Appendix). Gallic, chlorogenic, cinnamic, pinostrobin and p-OH-benzoic acids were the most abundant constituents among the extracts. Colla et al. (2007) also showed that phenol levels were affected by different growth temperatures with the largest yields of 5 mg/g obtained at 35 ^{o}C and a sodium nitrate level of 2.5 g/l. Wu et al. (2005) demonstrated that spirulina had five times higher amounts of phenolic compounds (6.86 +/- 0.58) compared to chlorella (1.44 +/- 0.04). It must be noted that most of these studies used either water only or aqueous methanol to extract the phenols without any further comparison with other extraction solvents.

As solvent composition, extraction time, temperature, particles, mix time, etc., can all affect final levels and types of phenolics, a single extract obtained from a one factor extraction cannot be used to understand the antioxidative properties of the whole system.

Yet, many studies have used a single extract and projected the potential health benefitting properties to the entire natural system **(**Jakopič et al., 2009; Mohammedi, 2011). Instead, a multi-extraction process must be developed to ultimately characterize the entire phenolic milieu present in spirulina. Without this knowledge, it will be difficult, if not impossible, to identify the responsible phenols (types and ratio) and their possible synergistic / additive / potentiate interactions for eliciting an antioxidative benefit.

A.5 Response Surface:

Response Surface Methodology (RSM) is a "collection of mathematical and statistical techniques used for modeling and analyzing problems in which a response of interest (output) is influenced by several variables (input)" (Montgomery et al., 2005). The effective use of RSM design can result in reduced variability and closer conformance to the target optimization parameters. Other advantages of RSM compared to the one variable input model include the accumulation of large amounts of information from small numbers of samples, understanding potential interactions of input variables on the response through the construction of contour maps, and determining a model equation (Bas and Boyac, 2007).

For this study, the Box-Wilson design, also called central composite design (CCD), was used with a face centered experimental approach (Figure 6) to study the effects of three variables at three levels (solvent composition, 25, 50, 75%; solid to volume ratio, 5, 10, 15%; time, 60, 120, 180 min) on three responses (total phenols, total flavonoids, and antioxidative capacity) (Box and Draper, 1987). The face-centered central composite response surface method (FCCC-RSM) used two center points resulting in 16 experiments for the entire design. As shown by Figure 6, the black balls represent the

extreme points, grey balls represent the face-centered points and the white balls represent the centerpoints (two replicates) (Kolmert et al., 2000).

Although RSM has limitations, this optimization approach has been widely used for a number of applications, including the hydrolysis of pectic substrates (Suresh et al., 2009), enzymatic synthesis of fatty esters (Lee, 2010), lipase-catalyzed incorporation of docosahexanenoic acid into borage oil (Senanayake and Shahidi, 2002), alkaline protease production from *Bacillus mojavensis* in a bioreactor (Beg, 2003), butylgalactoside synthesis by galactosidase from *Aspergillus oryzae* (Deniz Baş and Boyacı, 2007), and biotransformation of 2-phenylethanol to phenylacetaldehyde in a two-phase fed-batch system (Çelik et al.,2004). Moreover, RSM has been extensively used for determining optimal extraction methods of polyphenols from various natural systems, which are summarized in Table 4.

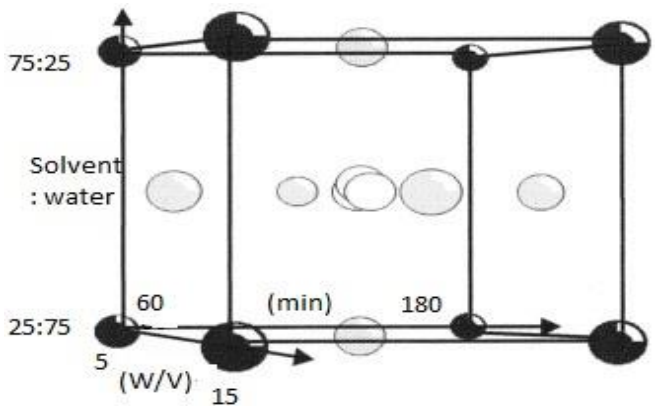


Figure 6. Three factors and three levels central composite face centered design

Table 4: RSM design used on each of the cited natural systems and outcomes in terms of amounts and experimental parameters.

Natural Systems	Design	Best Solvent of Extraction	Analysis and Ultimate Outcome	Reference
Grape skin, seed, and pulp	Doehlert	Acidified mixture of acetone/water/ methanol	Proanthocyanidins were the major P5 and henolic compounds in each part (60-93%)	Mane et al., (2007)
Evening primrose	Different factor combinations	56% acetone at 71 °C for 47 min	304 mg/g of phenolics	Wettasinghe and Shahidi,(1999)
Pistachio green hull	Central Composite Design	Maceration Extraction 20(v/w), 65°C, 45 minutes	53.5 ± 0.2 of phenol	Rajaei et al., (2010)
Black Currants	Central Composite Design	SO_2 1000−1200 ppm, 30−35 °C, and 19 L of solvent/kg	Maximum extraction of phenol 79 mg/g	Cacace and Mazza, 2002
Wheat	Face-centered cubic	Ethanol 54%, 61 °C, 64 min	Total phenol 54.7 ± 3.2 and 61.3 ± 1.9 for whole grain and bran of soft wheat	Liyana-Pathirana et al., 2005
Peanut Skins	Central Composite Design	Ethanol (90% microwave power, 30 s irradiation time and 1.5 g skins)	143.6 mg gallic acid equivalent (GAE)/g skins	Ballard et al., 2010
Black mulberry leaves	Box-Behnken	Ethanol/water 40–80 %, (40–80 °C), and (10–30 mL/g)	48.7 mg/g	Radojkovic et al., 2012
Grape cane	Central Composite Design	Ethanol 40.4% and 55.4%, 83.6 °C, and 70 ml/g	8.91 mg resveratrol/g	Erkan Karacabey et al., 2010

B. PROJECT OBJECTIVE

The objective of this project is to characterize the extraction procedures that recover the highest amounts of phenols / flavonoids, and those that exert the most potent antioxidative capacity using FCCC-RSM. It was expected that different extracts, and thereby extraction conditions, would produce different responses due to the chemical levels and diversity of the phenolic compounds present in spirulina. It should be noted that all of the phenols / flavonoids in a given extract may not be responsible for the antioxidative capacity, but this project sets the foundation for developing predictive equations to further fractionate and determine possible synergists, additives or potentiates in spirulina for this effect (in the short term) and linking to other biological responses in the long term. The objective of this project was satisfied by completing the following Specific Aims.

Specific Aim 1: *To determine the optimal procedures to recover phenolic / flavonoid rich spirulina antioxidative extracts with 6 solvent systems (methanol, ethanol, acetone +/- HCl) by using a RSM approach that incorporated a three level face center cubed design for solvent polarity, mixing time, and solid/solvent ratio.*

Specific Aim 2: *To determine predictive equations from the RSM data (Specific Aim 1) and develop contour plots to gain information on the effects of different extraction processes on the response cited in Specific Aim 1.*

Specific Aim 3: *To characterize the phenolic compositional profiles of select extracts that contained either the highest levels of phenols or flavonoids (Specific Aim 1) and/or exhibited the greatest anti-oxidative responses (Specific Aim 2).*

C. MATERIALS and METHODS

C.1 Chemicals and Reagents:

Extraction solvents including methanol, ethanol, and acetone were purchased from Fisher Scientific. (Fair Lawn, New Jersey). Other reagents used for the study were provided by Fisher Scientific and included sodium carbonate, sodium nitrite, andpotassium phosphate. Reagents that were acquired from other vendors consisted of Folin-Ciocalteu (MP Biomedical Inc.; Solon, OH), aluminum chloride (Acros Organic Inc.; Fair Lawn, NJ), sodium hydroxide (BD, West Chester, PA), fluorescein (J.T. Baker: Center Valley, PA), and 2-2′-azobis (2-amino-propane) dihydrochloride (AAPH) (Sigma-Aldrich., ST. Louis, MO). The standards used for the phenolic (gallic acid), flavonoid (catechins) and oxygen radical absorbance capacity (Trolox) assays were obtained from Sigma-Aldrich, ST. Louis, MO. Lastly, spirulina powder was purchased from The Vitamin Shoppe and maintained at room temperature 25 ^{o}C until prepared for analysis.

C.2 Extraction Procedures:

The effects of six different solvent systems (methanol, ethanol, and acetone +/- 1.2 N HCl) on phenols, flavonoids and antioxidative capacity were monitored while also adjusting for solvent polarity ratio (25-75%), solid / solvent ratio (5-15%), and mix time (60-180 min). The optimization procedures were based upon a response surface design i.e., three-factor-three-level face-centered cube design. The cited solvent systems and other parameters were selected because multiple studies have used combinations of these conditions, but as single factor extractions, to obtain phenols from various natural systems (Karacabey and Mazza, 2010; Silva et al., 2007; Liyana-Pathirana and

Fereidoon Shahidi, 2004). For this study, the solid amounts were adjusted accordingly to maintain a 3-5 ml final extraction volume. The suspension was mixed horizontally under steady rocking for the designated time period at room temperature. The samples were then centrifuged at 8000 rpm at 25 C for at least 10 minutes and supernatant collected. The supernatant was collected and analyzed for total phenols, flavonoids, and antioxidant capacity. Each extraction was performed in triplicate.

C.4 Total Phenolic Assay:

The Folin-Ciocalteu method was used to determine total phenol levels in the collected supernatant is described by Singleton and Rossi **(**1965). Briefly, a sample aliquot (100 µL) was reacted with 100 µL of Folin-Ciocalteu reagent and 4.5 mL of nanopure water. After 3 minutes of shaking at room temperature, 0.3 mL of 2% (w/v) sodium carbonate was added to the samples, followed by a reaction time of 2 hours at room temperature with intermittent shaking. Detection of the phenols was achieved spetroscopically (Beckman Coulter, Brea, CA) at a wavelength of 760 nm. A standard calibration curve using gallic acid was plotted to calculate the results. Total phenols were thus expressed in mg gallic acid / g spirulina powder.

C.5 Total Flavonoid Assay:

To quantify total flavonoids, 125 µL of the sample supernatant prepared was combined with 37.5 µL of 5% (w/v) sodium nitrite and 0.625 mL of nanopure water according to Adom and Liu (2002). After allowing the mixture to react for 4-6 minutes at room temperature, 75 µL of 10% (w/v) aluminum chloride was added to each sample, followed by 0.25 mL of 1.0 M sodium hydroxide. Nanopure water (0.4 mL) was added after allowing the sample to mix by 5-7 minutes. The mixture was than vortexed and

than measured spectroscopically at 510 nm. Total flavonoids were expressed as mg catechin / g spirulina powder.

C.6 Oxygen Radical Absorbance Capacity:

To measure antioxidant capacity, the oxygen radical absorbance capacity (ORAC) was completed as described by Huang et al. (2002). A standard stock solution was prepared by dissolving 0.010 g of Trolox (a water soluble derivative of Vitamin E) in 10 mL of 75 mM potassium phosphate buffer, pH 7.4. Standard dilution concentrations were prepared that ranged from 0.46–62.50 µg/mL. Fluorescein (8.16×10^{-5} mM) was incubated with the diluted standards and test samples for 10 minutes. The reaction was then activated by adding the radical initiator, 153 mM 2, 2'-azobis (2-amidinopropane) hydrochloride to generate peroxyl radicals. All samples/standards were prepared in 96 well plates and monitored with a fluorescent microplate reader (BMG LABTECH GmbH, Offenburg, Germany). Fluorescence was measured every 1.5 minutes at excitation and emission wavelengths of 485 nm and 520 nm, respectively, until the values plateaued. The area under the curve (AUC) and Net AUC were calculated to plot Net AUC vs. Trolox (µg/mL) calibration curves. The results were expressed as µmol Trolox/g spirulina powder.

C.7 Composition Analysis of Select Extracts:

Phenolic compositional analyses were completed on select samples by initially passing the extracts through a 0.45 µm filter before injection onto a 5 µm, 4.6 mm x 250 mm C18 reverse phase column (Vydac, Deerfield, IL). Separation was achieved using a Waters 600 HPLC system set at a flow rate of 1.0 ml/min and gradient elution (t = 0 min 100% A, t = 4min 92% A and 8% B, t = 10 min 14% B and 86% C, t = 22.5 min 16.5% B

and 83.5% C, *t* = 27.5 min 25% B and 75% C, *t* = 50 min 80% B and 20% C, *t* = 55 min 100% A and *t* = 60 min 100% A) with 50 mM ammonium phosphate at pH 2.6 (A), 80:20 (v:v) acetonitrile, 50 mM ammonium phosphate at pH 2.6 (B) and 200 mM phosphoric acid at pH 1.5 (C) serving as the mobile phases. Detection of the phenols and flavonoids was accomplished with a Waters 2996 photodiode array detector at 280 nm, 320 nm and 370 nm. Samples were identified and quantitated using external standards.

C.8 Experimental Design for RSM Analysis:

A three-factor and three level face center central composite design consisting of 16 extracts for total phenols, flavonoids, and antioxidative capacity were employed including two replicates at the center point (Table 5). The experimental design is presented in Table 6. The behavior of each solvent system was explained by the following second degree polynomial equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ji} X_i X_j$$

where Y is the response, b_o is the constant coefficient, b_i are the linear coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the interaction coefficients, and X_i and X_j are the coded values of the independent variables. To perform this operation, the StatsGraphic, Centerium, (version 26, Warrenton, VA) was used to develop regression equation between extraction variables and total phenols, total flavonoids, and antioxidative capacity.

Table 5: Levels of independent variables for extraction process based on central composite design.

Independent Variable	Units	Factor	Coded Levels		
			-1	**0**	**+ 1**
Organic Solvent : Water	(v:v)	X_1	25:75	50:50	75:25
Solid : Volume	(%)	X_2	5%	10%	15%
Time	(min)	X_3	60	120	180

*** Methanol, Ethanol, or Acetone +/- HCl to Water Ratio**

Table 6: Three factor, three- level face-centered cube design with 2 center points used for RSM, (coded and uncoded) parameters.

Standard Order	Factor X_1	Factor X_2	Factor X_3	Solvent Ratio	Solid:Vol (%)	Time (min)
1	1	0	0	75-25	10	120
2	0	0	1	50-50	10	180
3	1	-1	-1	75-25	5	60
4	0	0	-1	50-50	10	60
5	0	1	0	50-50	15	120
6	0	-1	0	50-50	5	120
7	-1	-1	1	25-75	5	180
8	0	0	0	50-50	10	120
9	-1	1	1	25-75	15	180
10	1	1	-1	75-25	15	60
11	-1	-1	-1	25-75	5	60
12	-1	1	-1	25-75	15	60
13	0	0	0	50-50	10	120
14	-1	0	0	25-75	10	120
15	1	1	1	75-25	15	180
16	1	-1	1	75-25	5	180

C.9 Statistical Analysis and Verification of the Model:

As started prevouisly extractions were carried out in triplicate and the experimental results were expressed as the mean +/- SD. The statistical analysis was performed using Stats Graph Centerium (version 26, Warrenton, VA). The experimental data were analyzed by multiple regression analysis through the least squares method. The model and the regression coefficients involved in the model and their effect were analyzed by Pareto ANOVA and were considered significant at a $p < 0.1$. The fitness of the model was further evaluated by determining the correlation coefficient for the model R^2 (>75), whereas the adequacy of the model to fit the experimental data was assessed by a lack of fit test ($p>0.05$).

C.10 Response Surface Curves:

Regression equations were formulated based on the whether the data obtained from each solvent system complied to the criteria stated in C.9. Response surface plots based on the "accepted" equations were generated with R2 software (version 3.0.2, Kurt Hornik, 2013) by using the function of two factors and keeping the other constant.

D. RESULTS and DISCUSSION

D.1 Selection of Independent Variables:

The extraction of phytochemicals from permeable solid natural systems using solvents is an important first step in characterizing phenolic-rich natural systems. Phenolic solubility is critically influenced by their chemical structure, which can vary from small molecules that contain various types and numbers of functional groups (OH, O, or COOH) or conjugated (glycosides, aldehydes, alcohols, methyl group) components to large polymerized molecules (tannins, lignins) (Mutlib and Abbott, 1992; Naczk and Shahidi, 2006; Jung et al., 2000). Additionally, phenols can interact with other substances, such as proteins and carbohydrates, leading to insoluble complexes (Hagerman and Butler, 1978; Kang et al., 2004; Jung et al., 2000). Considering that phenolics are a highly diversified class of phytochemicals with overall compositional profiles that are plant specific (Naczk and Shahidi, 2006), extraction optimization must be completed on a system by system basis.

The single most important extraction variable for obtaining optimal phenolic yields and possibly a biological response is the type of solvent used. Therefore, for this study, the effects of three different solvent systems (methanol, ethanol, and acetone) on three responses (total phenol, total flavonoid, and antioxidative capacity) were evaluated. These solvents were selected as a review of the literature showed their successful application to a number of different natural systems (Table 4). As it has also been shown that improved phenolic extraction efficiencies occur by the addition of water to the extraction solvent (Rostango et al., 2004), the solvent to water ratio of the three cited

solvents was varied by increasing the water content by 25% increments for 3 levels, starting at 25:75 water:solvent (v:v) ratio (Table 5).

Soluble phenolic compounds are mainly distributed in the cell wall vacuoles, while most flavonoids and insoluble phenols are loosely bound to the cell by hydrogen or hydrophilic bonds. Others are linked together throughout the lignin polymer by both ether or ester bonds (Dixen and Paiva, 1995). Acidified aqueous solvents have thus been used by a number of researchers to rupture cells walls and break these bonds to release the phenols from the solid matrix (Pompeu, et al., 2009; Kim et al., 2004, Ju and Howard, 2003). For these studies, another set of FCCC-RSM experiments were completed using each of the cited solvent systems, but acidified with 1.2 N HCl. Other studies have shown that elevated temperatures result in significantly higher phenolic level yields (Liyana-Pathiran and Shahidi, 2005; Pompeu et al., 2009). Considering that elevated temperatures could result in phenolic degradation, the effects of mixing time and liquid:solid ratios were instead evaluated for this project (Iverson, 1999: Skrede, et al., 2000). The ranges of the all the selected independent variables were again based on those cited in the literature (Table 4).

D.2 Total Phenols (TP).

D.2.1. *Total phenol results obtained from FCCC-RSM:* The levels of TP in response to each solvent system (methanol, ethanol, and acetone +/- 1.2 N HCl) while adjusting for water levels, solid to solvent ratio, and mix time, were evaluated, using a three factor, three level faced centered composite design (FCCC). This design resulted in 16 extractions, two of which were center points. The results (in mean +/- standard deviation of three replicates) for each of the coded extractions (Table 6) are shown in Tables 7 and

Table 7: Experimental data for TP response (in mg/g) of spirulina extracts under different extraction conditions and solvent systems.

Standard Order	Methanol	Ethanol	Acetone
1	1.48 ± 0.03	3.34 ± 0.09	1.81 ± 0.02
2	1.19 ± 0.00	1.59 ± 0.13	6.84 ± 1.28
3	3.62 ± 0.00	1.84 ± 0.09	2.10 ± 1.07
4	1.15± 0.02	1.05 ± 0.03	8.75 ± 0.82
5	1.46± 0.05	2.08 ± 0.12	1.79 ± 0.22
6	1.35± 0.02	2.14 ± 0.02	1.55 ± 0.12
7	0.97±0.01	1.57 ± 0.07	1.24 ± 0.00
8	0.93± 0.12	2.46 ± 0.20	6.02 ± 0.07
9	2.45± 0.03	1.55 ± 0.03	1.27± 0.12
10	4.39 ± 0.00	2.34 ± 0.20	1.87 ± 0.01
11	1.15 ± 0.00	2.66 ± 0.015	4.72 ± 0.50
12	1.07 ± 0.04	1.33 ± 0.01	1.64 ± 0.02
13	1.15 ± 0.00	2.26 ± 0.02	8.61 ± 0.44
14	1.15 ± 0.03	4.74 ± 0.00	6.04 ± 0.30
15	1.33 ± 0.01	3.32 ± 0.09	1.65 ± 0.11
16	1.57 ± 0.05	1.06 ± 0.17	2.06 ± 0.02

* Results are shown as the mean +/- standard deviation of n = 3.

Table 8: Experimental data for TP response (in mg/g) of spirulina extracts under different extraction conditions and solvent systems + 1.2 N HCl.

Standard Order	Methanol + HCl	Ethanol + HCl	Acetone + HCl
1	1.27 ± 0.02	2.39 ± 0.00	2.03 ± 0.00
2	1.06 ± 0.01	1.38 ± 0.10	3.45 ± 0.19
3	2.21 ± 0.02	2.61 ± 0.26	0.92 ± 0.03
4	1.53 ± 0.42	0.87 ± 0.04	0.85 ± 0.02
5	1.97 ± 0.04	1.19 ± 0.00	1.13 ± 0.00
6	1.08 ± 0.06	1.10 ± 0.23	1.24 ± 0.02
7	1.23 ± 0.01	1.71 ± 0.10	1.75 ± 0.04
8	1.41 ± 0.15	0.83 ± 0.10	0.25 ± 0.01
9	2.82 ± 0.18	0.98 ± 0.01	1.74 ± 0.02
10	3.15 ± 0.12	2.15 ± 0.06	3.97 ± 0.11
11	3.42 ± 0.00	1.35 ± 0.04	2.88 ± 0.29
12	2.44 ± 0.02	1.81 ± 0.10	2.24 ± 0.01
13	1.31 ± 0.02	0.92 ± 0.02	0.02 ± 0.01
14	2.32 ± 0.10	1.51 ± 0.02	4.34 ± 0.02
15	1.27 ± 0.03	1.23 ± 0.00	1.15 ± 0.03
16	1.79 ± 0.01**	1.98 ± 0.09	3.38 ± 0.01

* Results are shown as the mean +/- standard deviation of n = 3.

** Eliminated when building model as an outlier.

Table 9: Ranges of TP for each solvent system.

Extraction Solvent	High and Low Total Phenol (mg/g)	Range (mg/g)
Methanol	0.93 – 4.39	3.46
Ethanol	1.06 – 3.34	2.28
Acetone	1.24 – 8.61	7.37
Methanol + HCl	1.08 – 3.42	2.34
Ethanol + HCl	0.83 – 2.61	1.78
Acetone + HCl	0.25 – 4.34	4.09

8, and the ranges of phenols ranges obtained for each solvent system are shown in Table 9. The results show that acetone with was able to extract the highest overall TP yields, suggesting that the majority of the phenol present are non-polar, or lack a polar conjugate. However, the overall range for acetone was high, indicating that the method was sensitive to small changes in the extraction process. Acetone + HCl also yielded high levels of TP, second only to acetone only, but it was approximately 2X less than when using acetone alone. The solvent system that yielded the second highest levels was methanol followed by methanol + HCl. The ethanol extracts contained even lower TP than the previously cited solvents, with ethanol + HCl extracting the lowest amounts, which was particularly unexpected as many studies have reported ethanol as the extraction solvent of choice for phenolic compounds (Radojkovic et al., 2012; Ballard et al., 2009; Theodora, et al., 2013; Maja et al., 2013). A comparison of the results in terms of using HCl, suggests that acidified solutions are not necessary if the long-term scope of the research was only about total yields.

D.2.2. *Fitting the TP models*: Multiple regression coefficients obtained by employing a least squares technique to predict a quadratic polynomial model for TP are summarized in Tables 10-11 for each solvent system (with and without HCl). (The

Table 10: Regression coefficients (coded) predicted by the quadratic polynomial model for phenols when extracted with the cited solvent systems.

Coefficient	Methanol	Ethanol	Acetone
b_o	0.98	2.73	6.36
Linear			
b_1 (SP)	0.561**	0.006	-0.541
b_2 (S:S)	0.209	0.128	-0.369
b_3 (MT)	-0.387**	-0.013	-0.602
Quadratic			
b_{11} (SP x SP)	0.371	1.365^	-1.96**
b_{22} (S:S x S:S)	0.490	-0.597	-4.35^
b_{33} (MT x MT)	0.224	-1.351^	1.90**
Cross product			
b_{12} (SP x S:S)	-0.110	0.513	0.299
b_{13} (SP x MT)	-0.790*	0.133*	0.447
b_{23} (S:S x MT)	0.068	0.384	0.366
R^2	88.5	87.6	86.3
p values			
Model	0.0296	0.0366	0.0421
Lack of Fit	0.2544	0.0621	0.7208

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 10%

Table 11: Regression coefficients (coded) predicted by the quadratic polynomial models for phenols when extracted under the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	1.35	1.08	1.23
Linear			
b_1 (SP)	-0.544*	0.307*	-0.148
b_2 (S:S)	0.482*	-0.145**	0.005
b_3 (MT)	-0.749*	-0.145**	0.062
Quadratic			
b_{11} (SP x SP)	0.339	0.778*	1.400*
b_{22} (S:S x S:S)	0.178	-0.036	-0.600
b_{33} (MT x MT)	-0.050	-0.048	0.363
Cross product			
b_{12} (SP x S:S)	0.424**	-0.132	0.183
b_{13} (SP x MT)	-0.448*	-0.119	0.160
b_{23} (S:S x MT)	0.500*	-0.202**	-0.583
R^2	90.4	83.9	41.31
p values			
Model	0.0647	0.0712	0.8520
Lack of Fit	0.1179	0.1170	0.0700

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 10%

coefficients are related to coded variables.) Analysis of variance analyses (ANOVA) of the quadratic models for all the solvent systems without HCl were significant ($p < 0.05$), indicating that the quadratic model adequately represents the data.

Alternatively, the methanol + HCL, and ethanol + HCl showed slightly lower correlations with p values of 0.0647 and 0.0712, or in other words, a slightly higher risk of the model not representing the data. Yet, the R^2 values for methanol / ethanol + HCl were well above that recommended by Le, Beheara, and Park (2010) and Chauhan and Gupta (2004) who showed that any model with $R^2 > 75$ could be accepted. (A high R^2 coefficient provides assurance for low dispersion of the experimental data.) The R^2 values for the solvents without HCl were also well above this level. Nonetheless, the quadratic model for acetone + HCl solvent had a high probability p value (0.8529) for the model and low R^2 value (41.31).

D.2.3. *Adequacy of the TP models and corresponding regression equations***:** In general, ensuring the model exhibits a good fit with experimental data is essential to avoid poor or misleading results (Maran et al., 2013). Therefore, the adequacy of the model for each solvent system was evaluated by applying a lack of fit test that compares the differences between the residuals of the current model with that of observed data (Maren et al., 2013). As shown in Tables 10 and 11, the p values were > 0.05 for methanol, ethanol (+/- HCl) and acetone (-HCL) confirming the adequacy of the models for these solvents. Once again, the acetone + HCl model failed this test, as the p value was < 0.05 (Table 11). As most of the models, with the exception of acetone + HCl showed an insignificant lack of fit, the responses can be sufficiently explained by the regression equations shown in Table 12. The equations were composed by evaluating the

p values of the individual regression coefficients (Table 10) and eliminating insignificant variables.

Table 12: Regression equations that fit the model and passed lack of fit test

$\mathbf{TP_{methanol}}$	$= 0.980 + 0.256Xsp - 0.387Xmt - 790XspXmt$
$\mathbf{TP_{ethanol}}$	$= 2.73 + 1.365XspXsp - 1.351XmtXmt$
$\mathbf{TP_{acetone}}$	$= 6.36 - 1.96XspXsp - 4.35XssXss + 1.90XmtXmt$
$\mathbf{TP_{methanol + HCl}}$	$= 1.35 - 0.544Xsp + 0.482Xss - 0.749Xmt + 0.488XspXsp + 0.339XspXss - 0.424XspXmt$
$\mathbf{TP_{ethanol + HCl}}$	$= 1.08 + 0.307Xsp - 0.145Xss - 0.778XspXsp - 0.119XspXmt$

sp – Solvent Polarity, ss – Solid:Solvent, mt – Mix Time

D.2.4. *TP Response Surface Plots:* The regression equations (Table 12) predicted the effects (positive: negative), each factor played in the mass transfer of the TPs from the solid spirulina matrix to the solvent. For the solvents without HCl, solvent polarity and mixing time were the largest effectors on the TP response, but solid to solvent ratio also played a role in the acetone system. Figures 7-9 show the influence of the input variables on TP levels. (Note: Response curves were only generated for those solvents that passed the criteria described in Sections D.2.2. and D.2.3). The polynomial nature of the extraction conditions can be easily observed in most cases.

- **D.2.4.1. Methanol (Figure 7a-f)**: At a constant mixing time of 112.5 min, total phenol levels increased proportionately with methanol:water ratios, but these levels were dependent upon the solid:solvent ratio (Figure 7a). High TP levels were obtained at ~ 10% solid:solvent ratio and 30% methanol. As the methanol concentration increased, the TP levels decreased regardless of the solid% but began increasing again at ~50% methanol for solid:solvent ratios less than 10-12%. Alternatively, TP yields were higher at low methanol + HCl concentrations regardless of solid:solvent ratio, but dropped

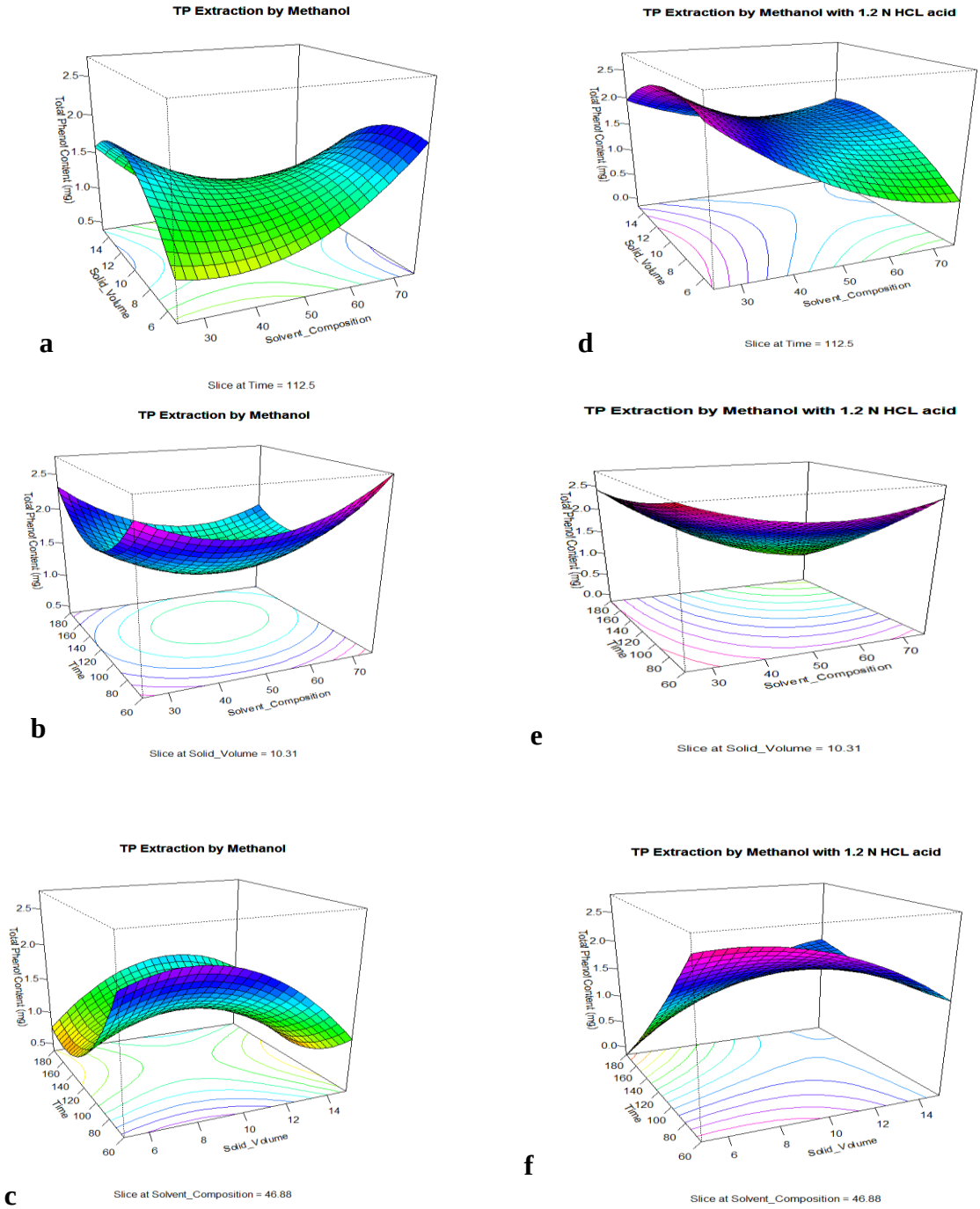


Figure 7: Response surface plots of the TP extracted with methanol +/-HCl as a function of time, solvent composition and solid volume: (a,d) time of extraction was kept constant at 112.5 min, (b,e) solid volume of extraction was kept constant at 10.31 %, (c,f) solvent composition was kept constant at 46.88%.

substantially as the solvent concentration increased and the solid:solvent ratios decreased (Figure 7d). When the solid:solvent ratio was kept constant (10.3%), optimal yields were obtained for both the mixing times and methanol concentration extremes, with the lowest levels corresponding close to the zero coded values (100-140 mix time and 40-60% methanol concentration) (Figure 7b). A similar pattern occurred for methanol + HCl with the notable exception that lower TP levels were produced at the highest mix times (180 min) at a solvent concentration of ~75%) (Figure 7e).

By maintaining a methanol composition of 46.88%, a concave curve was generated for the time vs solid plot (Figure 7c). Higher yields were obtained at times on either side of the zero coded value (100-140 min) and were maintained at these levels over the solid:solvent continuum. However, higher TP levels were obtained at the solid:solvent mid points and solvent compositions of 30-50% (Figure 7d). As shown in Figure 7f, a unique pattern was generated by the methanol + HCl extraction process. When the methanol:water ratio (46.88%) was maintained, both high and low TP yields were obtained at the processing extremes. For example, the lowest TP levels were extracted with the lowest solid:solvent ratio (5%) and the highest mixing time (180 min), while the highest TP levels were obtained at the lowest mixing time (60 min) and lowest solid:solvent ratio (75%) (Figure 7f). At low mix times (60 min) and high solid:solvent ratio (15%), TP levels were lower than those obtained at high mix times and high solid:solvent ratios and high mix times and low solvent to solid ration.

In summary, different 3D plot shapes were produced for methanol +/- HCl, indicating that methanol extraction system is not only affecting the TP levels, but possibly targeting other types of phenols or constituents that could react with the assay.

Based on these results, the acidified solution is able to efficiently extract certain phenols at low mix times but as the latter parameter increases, TP levels drop substantially, as shown by the main effect plot (data not shown). This effect could be due to degradation of the phenolics being exposed to the acidified solution for longer periods of time. However, it is more likely that both the solvent and pH negatively affect TP levels considering that yields decreased with higher mix times and methanol concentration in the absence of HCl (Figure 7a). The same negative response occurred when the concentration of methanol + HCl increased (Figure 7d), albeit not as drastic.

TP levels increased with higher methanol concentration but only where short mixing times had to be used (Figure 7a). Lower mixing time may be required to prevent separation of solute molecules by solute particles, which is more predominant with higher solvent:water compostion, as discussed by Cacace and Mazza (2003). High TP levels were also extracted at the low solid:solvent ratios (Figure 7a, b, c, f), which is consistent with mass transfer principles. Basically, the solvent is not saturated with other components thereby allowing higher solubility of more phenols. Such results have been reported for black mulberry leaves (Radojkovic et al. 2012), but the incline of TP levels after reaching a low in response to solid:solvent ratio has not been shown to our knowledge (7 c).

- **D.2.4.2. Ethanol (Figure 8 a-f)**: Extraction of TP with ethanol +/- HCl using the FCCC-RSM generated three saddle type plots (Figures 8a,b,d). When time was kept constant (Figures 8a), TP levels decreased to a low at ~40-60% ethanol and then increased with solid:solvent of 10% followed by a decrease in TP with increasing solid:volume ratios (Figures 8a,b). However, low (25%) and high (75%) ethanol

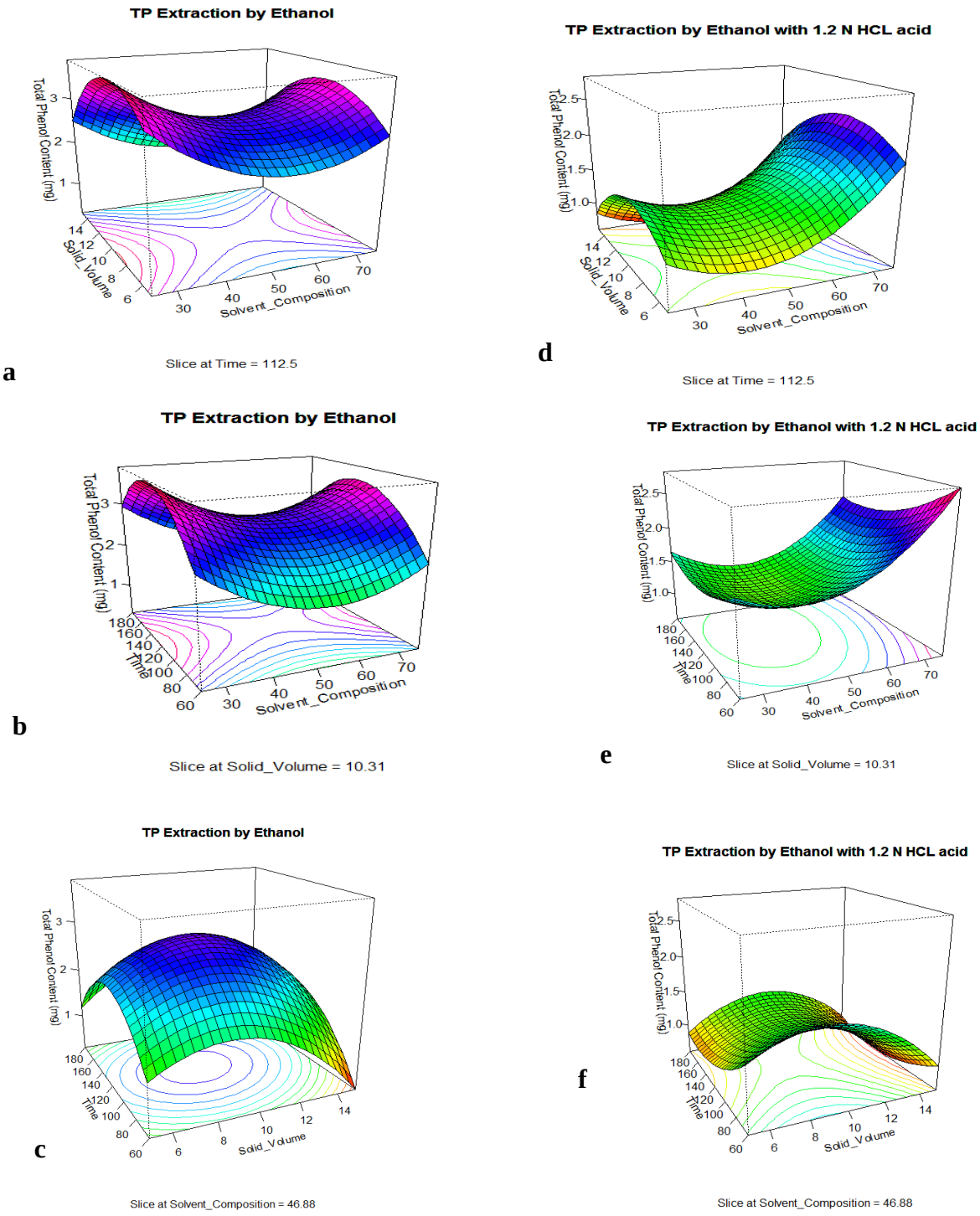


Figure 8: Response surface plots of the TP extracted with ethanaol +/- HCl as a function of time, solvent composition and solid volume: (a,d) the time of extraction was kept constant at 112.5 min, (b,e) the solid volume of extraction was kept constant at 10.31 %, (c,f) the solvent composition was kept constant at 46.88%.

composition produced the higher TP levels across the solid:solvent plot. The same contour was obtained when the solid:solvent ratio was maintained (Figure 8b). As stated previously, another saddle type plot was produced based using ethanol + HCL as the extraction solvent (Figure 8d). At a constant mix time (112.5 min), TP increased substantially with solvent composition start at 40%, and maximized when the solid:solvent ratio was ~ 10%. Compared to the saddle plots generated by the methanol +/- HCl (Figures 7a,b), these results differed primarily in the overall height and torque of the saddle plots.

Nonetheless, further inspection of the saddle plots indicates that ethanol extractions are more rugged in that higher overall TPs levels can be obtained at many of the extraction points, with the notable exception of methanol + HCl concentrations below 40% (Figure 8d). At constant solid:solvent percentage (Figure 8e), TPs declined with ethanol + HCl concentration again at ratios below ~40% . A convex plot (Figure 8c) was generated for TP methanol extractions at a constant solvent composition (46.66%) whereas an inverse saddle plot (Figure 8f) was produced by the methanol + HCl extractions. It can be clearly seen from these plots that maximal TP can be obtained using the mid center ethanol composition and mix times. However, the lowest TP levels were obtained with low mix times (60 min), high solid:solvent ratios (15%), using a 50% ethanol:water mixture. Based solely on yields, removing HCl from the extraction process is preferable as the data indicate lower TP levels and lower overall ruggedness using acidified ethanol extraction solutions.

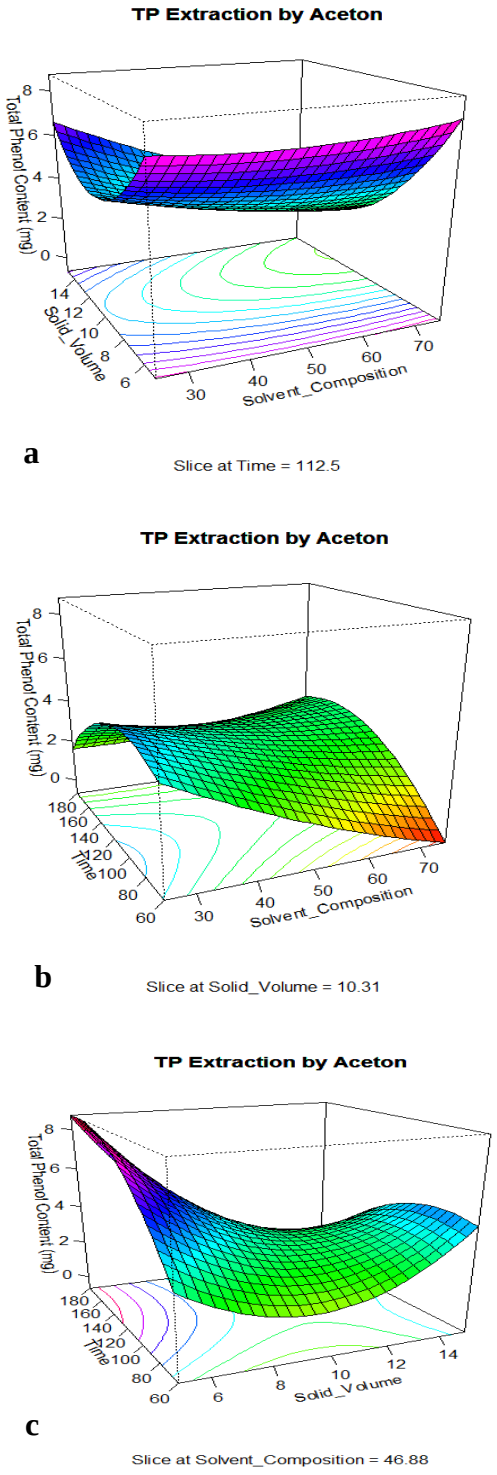


Figure 9: Response surface plots of the TP extracted with acetone as a function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 112.5 min, (b) solid volume of extraction was kept constant at 10.31%, (c) solvent composition was kept constant at 46.88%.

D.2.4.3. Acetone (Figure 9 a-c): A review of comparison Figure 9a-c with the other solvent system plots (Figures 7-8) immediately confirms that the highest TP extraction efficiencies were achieved with acetone, as was also noted previously (refer to Section C.2.2.). However, the different contours of the plots and the quadratic contribution of each indicate that the extractions have to be precise to obtain the yields desired. For example, mix times must be approximately 180 min for low solid:solvent ratio (5-6%) based extractions or below 120 min for solid:solvent ratio of 12-15 % (Figure 9c). Moreover, solvent composition must be approximately 50% to achieve high optimal levels for a mix time of 120 min and solid:volume of 10% (Figure 9b).

D.3 Total Flavonoids (TF):

D.3.1. *TF results obtained from FCCC-RSM:* The TF results (in mean +/- standard deviation of three replicates) obtained from the FCCC-RSM (Table 6) are shown in Tables 13 and 14, whereas Table 15 lists the low and high TF levels extracted with each solvent system and the overall ranges. Similar to the TP results, the acetone + HCl and ethanol extractions again resulted in significantly lower yields (0.005, 0.09, respectively) when compared to the other solvents. But, the lowest values over the entire range were produced by the ethanol + HCl extraction. The methanol extracts (with and without HCl) resulted in the highest TF levels for spirulina, but also had the largest range indicating that this extraction was sensitive to differences in the experimental parameters. The addition of HCl to the acetone positively impacted TF yields when compared to acetone alone, as the higher values were comparable. Additionally, ethanol was negatively impacted by the addition of HCl as the high value of 1.15 mg/g was more than 3X less without HCl (4.88 mg/g).

Table 13: Experimental data for TF response (in mg/g) of spirulina extracts under different extraction conditions and solvent systems.

Standard Order	Methanol	Ethanol	Acetone
1	0.61 ± 0.10	1.01 ± 0.00	1.38 ± 0.08
2	0.19 ± 0.06	0.34 ± 0.08	0.13 ± 0.00
3	6.30 ± 0.10	4.88 ± 0.57	0.97 ± 0.24
4	0.35 ± 0.04	0.73 ± 0.09	0.45 ± 0.03
5	0.35 ± 0.09	0.35 ± 0.00	0.34 ± 0.00
6	0.25 ± 0.02	0.25 ± 0.00	0.18 ± 0.05
7	0.52 ± 0.00	0.85 ± 0.10	0.77 ± 0.00
8	0.25 ± 0.00	0.98 ± 0.33	0.30 ± 0.03
9	0.64 ± 0.03	0.73± 0.33	0.99 ± 0.10
10	3.55 ± 0.09	1.04 ±0 .06	0.44 ± 0.03
11	0.25 ± 0.03	0.32 ± 0.10	3.19 ± 0.34
12	0.65 ± 0.03	0.65 ± 0.00	0.95 ± 0.06
13	0.38 ± 0.04	0.09 ± 0.00	0.30 ± 0.02
14	0.29 ± 0.02	0.77 ± 0.12	0.76 ± 0.05
15	1.04 ± 0.02	0.96 ± 0.09	0.62 ± 0.21
16	0.96 ± 0.00	1.64 ± 0.29	2.08 ± 0.06

* Results are shown as the mean +/- standard deviation of n = 3.

Table 14: Experimental data for TF response (in mg/g) of spirulina extracts under different extraction conditions and solvent systems.

Standard Order	Methanol + HCl	Ethanol + HCl	Acetone + HCl
1	0.65 ± 0.19	0.80 ± 0.01	1.15 ± 0.10
2	0.21 ± 0.00	0.52 ± 0.00	0.04 ± 0.00
3	6.58 ± 0.05	1.97 ± 0.02	0.01 ± 0.00
4	0.40 ± 0.01	0.19 ± 0.00	0.21 ± 0.00
5	1.26 ± 0.02	0.27 ± 0.00	0.26 ± 0.02
6	0.87 ± 0.03	0.93 ± 0.01	0.16 ± 0.00
7	0.58 ± 0.01	0.89± 0.07	1.12 ± 0.01
8	0.50 ± 0.02	0.58 ± 0.00	0.24 ± 0.03
9	2.84 ± 0.38	0.17 ± 0.00	0.26 ± 0.00
10	3.24 ± 0.30	1.51 ± 0.03	0.36 ± 0.00
11	0.26 ± 0.02	0.44 ± 0.02	0.06 ± 0.02
12	0.49 ± 0.03	0.35 ± 0.02	1.05 ± 0.00
13	0.25 ± 0.02	1.12 ± 0.00	0.16 ± 0.02
14	1.18 ± 0.29	0.48± 0.33	0.20± 0.01
15	0.52 ± 0.01	0.25 ± 0.00	0.20 ± 0.02
16	1.23 ± 0.00	1.20 ± 0.07	2.70 ± 0.45

* Results are shown as the mean +/- standard deviation of n = 3.

Table 15: Ranges of TF for each solvent system.

Extraction Solvent	High and Low Total Flavonoid (mg/g)	Range (mg/g)
Methanol	0.25 – 6.30	6.05 mg/g
Ethanol	0.09 – 4.88	4.70 mg/g
Acetone	0.13 – 3.19	2.98 mg/g
Methanol + HCl	0.21 – 6.58	6.37 mg/g
Ethanol + HCl	0.17 – 1.15	0.98 mg/g
Acetone + HCl	0.005 – 2.70	2.70 mg/g

D.3.2. *Fitting the TF models.* The regression coefficients for TF extracted from each solvent system are summarized in Tables 16 (without HCl) and 17 (with HCl). ANOVA of the quadratic models for the solvent systems with and without HCl were all significant ($p < 0.10$). Methanol, ethanol, and acetone solvent systems + HCL had R^2 of 92.3, 83.1, and 88.6, respectively. The R^2 values for the other solvents without HCl were also above the acceptable level of $p > 75$, except for the acetone without HCl, which had an R^2=69.2.

D.3.3. *Adequacy of the TF models and corresponding regression equations.* The methanol, ethanol (+/- HCl) and acetone + HCL were also acceptable for the lack of fit test as each had p value > 0.05. Considering that acetone without HCl also failed this test ($p = 0.0038$), it was deemed unacceptable for further analysis. However, analysis of more points or center points may account for the low R^2 value and failure of the lack of fit test. Table 18 shows the regression equations for the solvents that complied to the criteria for the model (refer to Section C.9).

D.3.4. *TF response surface plots and contours*: For the solvents without HCl, mixing time and solvent polarity were the largest effectors on the TF response, but solid to solvent ratio also played a role in the acetone system, as shown in Table 18.

Table 16: Regression coefficients (coded) predicted by the quadratic polynomial model for TF when extracted with the cited solvent systems.

Coefficient	Methanol	Ethanol	Acetone
b_o	-0.090	0.311	0.250
Linear			
b_1 (SP)	0.787**	0.625*	-0.580
b_2 (S:S)	-0.107	-0.090	-0.411
b_3 (MT)	-0.874*	-0.641*	-0.113
Quadratic			
b_{11} (SP x SP)	0.753	0.796	0.853
b_{22} (S:S x S:S)	0.585	0.203	0.042
b_{33} (MT x MT)	0.568	0.438	0.070
Cross product			
b_{12} (SP x S:S)	-0.678	-0.586**	0.077
b_{13} (SP x MT)	-0.736**	-0.496	0.385
b_{23} (S:S x MT)	0.193	-0.076	0.226
R^2	82.1	83.6	69.2
p values			
Model	0.0934	0.0743	0.0550
Lack of Fit	0.0626	0.3158	0.0038

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 10%

Table 17: Regression coefficients (coded) predicted by the quadratic polynomial model for TF when extracted with the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	0.263	0.622	0.176
Linear			
b_1 (SP)	0.686*	0.304*	0.176
b_2 (S:S)	-0.117	-0.342*	-0.193
b_3 (MT)	-0.557**	-0.105	0.267**
Quadratic			
b_{11} (SP x SP)	0.716	0.131	0.519**
b_{22} (S:S x S:S)	0.832	0.183	0.052
b_{33} (MT x MT)	0.108	-0.157	-0.033
Cross product			
b_{12} (SP x S:S)	-0.731**	-0.120	-0.279**
b_{13} (SP x MT)	-1.343^	-0.244**	0.280**
b_{23} (S:S x MT)	0.667	-0.157	-0.583^
R^2	92.5	83.1	88.6
p values			
Model	0.0009	0.0803	0.0290
Lack of Fit	0.1495	0.7528	0.0961

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 10%

Table 18: Regression equations that fit the model and passed lack of fit test for TF.

$\mathbf{TF_{methamol}}$	$= -0.090 + 0.787Xsp - 0.874Xmt + 0.753XspXsp - 0.736\ XspXmt$
$\mathbf{TF_{ethanol}}$	$= 0.311 + 0.625Xsp - 0.641Xmt - 0.586XspXss$
$\mathbf{TF_{methanol + HCl}}$	$= 0.263 + 0.686Xsp - 0.557Xmt - 0.817XspXss - 1.343XspXmt$
$\mathbf{TF_{ethanol +HCL}}$	$= 0.622 + 0.304Xsp - 0.342Xss - 0.244XspXmt.$
$\mathbf{TF_{acetone + HCl}}$	$= 0.176 + 0.267Xmt + 0.519XspXsp - 0.279XspXss + 0.280XspXmt - 0.583XssXmt.$

sp – Solvent Polarity, ss – Solid:Solvent, mt – Mix Time

Additionally, Figures 10-12 show the influence of these factors on TF. Unlike the TP plots, the more linear nature (more planar contour) of extraction conditions of TF are exhibited by these plots, as predicted by the equations (Table 18).

D.3.4.1. Methanol (Figure 10a-f): At a constant mixing time of 105 min, methanol extractions of TF levels increased slightly with higher solvent:water ratios, and were not affected significantly by solid:solvent ratio (Figure 10a). Optimal TF levels were obtained at ~ 5% solid:solvent ratio and 75% methanol, whereas the lowest levels occurred at ~ 40% methanol and 12% solid:solvent ratio. When the solid:solvent ratio was kept constant (10.62%), optimal yields were again obtained at the highest methanol concentration, with the lowest values starting at ~ 25% (Figure10b). Levels were higher with short mix times and high methanol composition, but decreased quite dramatically for longer mix times. By maintaining a methanol composition of 46.88%, (Figure 10c), a unique twist pattern was generated. Higher yields were obtained at 10% solid and 60 min but dropped substantially as the solid:solvent ratio decreased to 5% at a comitant time of 180 min. However, the TF levels increased at the solid:solvent mid points

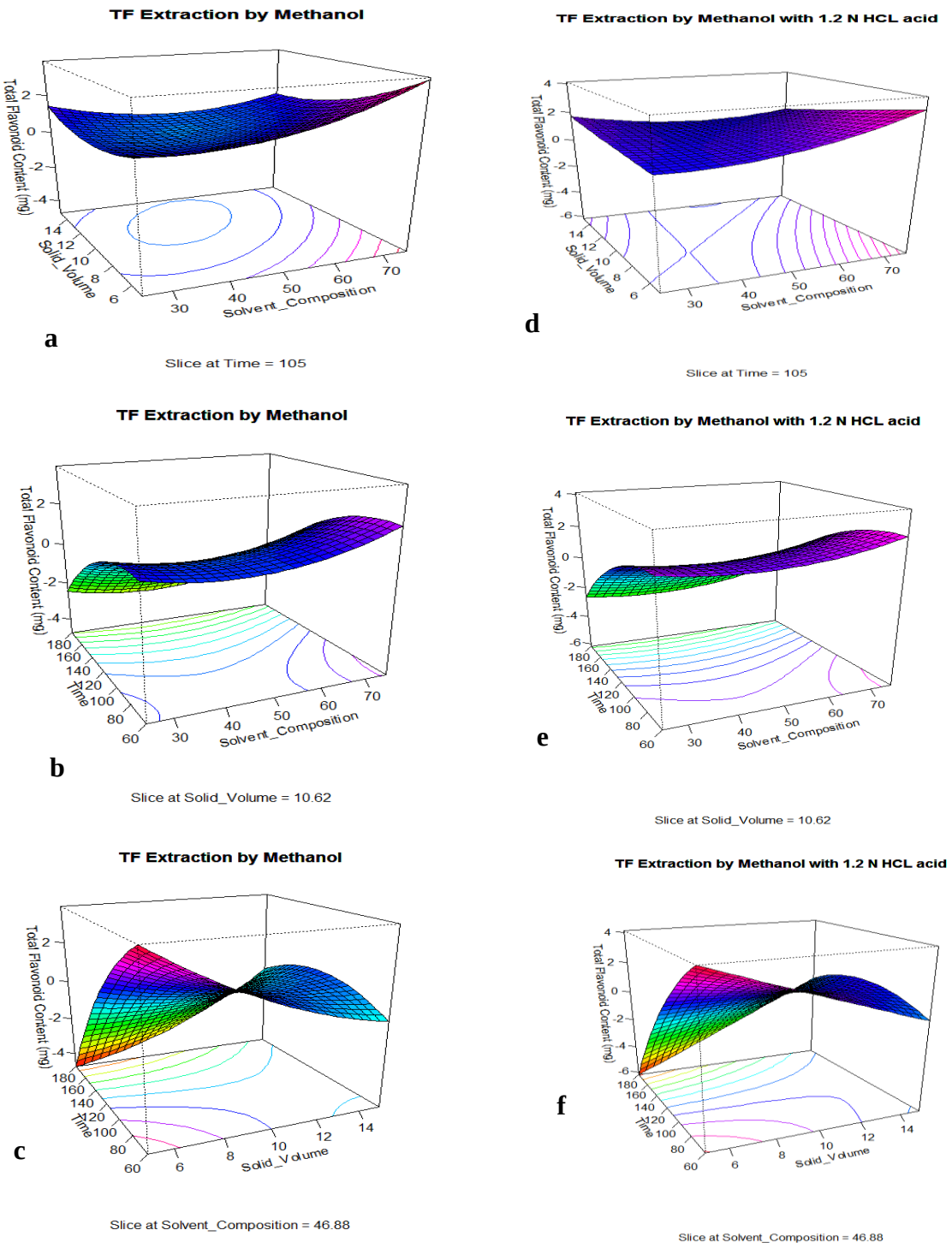


Figure 10: Response surface plots of the TF extracted with methanol +/-HCl function of time, solvent composition and solid volume: (a, d) time of extraction was kept constant at 105 min, (b, e) solid volume of ethanol +/- HCl extraction was kept constant at 10.62 %, (c, f) solvent composition was kept constant at 45.66%.

and maintained at approximately the same level with increasing solid:solvent ratio with slight decreases at the extremes of mixing times.

In summary, different 3D plot shapes were produced when a given factor was held constant, indicating that methanol extraction system is not only affecting the TF levels, but possibly targeting other types of flavonoids or constituents that could react with the assay. The similarities between the methanol +/- HCl further suggest that HCl is not needed, at least in terms of extracting total yields. This is further supported by the similarities of the +/- HCL plots. It must be emphasized that TF levels increased with higher concentrations of methanol when solid:solvent ratio and time were both kept constant (Figure 10a, c). This effect indicates that the flavonoids in this natural system are more non-polar or are not bonded to a hydrophilic conjugate, such as a glycoside. The same response occurred when the concentration of methanol +/- HCl increased (Figure 10 b, d). In both of the last cases, high TF levels were extracted at the low solid:solvent ratios, which is actually more consistent with mass transfers principles, as described previously (Figure 10 e,f). Basically, the solvent is not saturated with other components thereby allowing higher solubility of more flavonoids, as described previously in the TP section (Cacace and Mazza, 2003).

Comparison of the TP methanol plots (Figure 7a-e) with the TF plots (Table 10 e-f) show very different contours. The results indicate that a different extraction process may be needed to obtain optimal flavonoid levels. In the literature, a one factor extraction process is typically applied to a sample and a variety of tests are completed on that single extract (Jakopič, et al, 2009; Mohammedi 2011; Bucić-Kojić, et al., 2013). Yet, these results show that one extraction parameter may be optimal for TP, but

incompatible for TF, in terms of yields, further substantiating the need for this type of research for fully understanding the composition of spirulina or any type of natural system.

- **D.3.4.2. Ethanol (Figure 11 a-f)**: Because the ethanol plots shown as Figures 11a, c, and d, have the same pattern, they will be discussed together. Total flavonoids levels increased as solvent composition increased to a high of ~ 70% ethanol +/- HCl when time or solid:solvent were held constant at 105 min and 10.62%, respectively. Alternatively, the lowest level of TF occurred at 25% ethanol and 5% solid:solvent at a constant mix time of 105 min (Figure 11a). Comparably low TF levels were extracted with ~25 % ethanol +/- HCL and ~ 180 min mix time when the solid:solvent ratio was held constant (10.62%) (Figure 11 b and e), albeit the ethanol only extraction produced higher overall yields (Figure 11b). Twist type plots were produced when extracting TF with ethanol +/-HCL while holding the solid:solvent ratio (Figures 11c, f). For both systems, the lowest TF level occurred at 180 min and 5% solid:solvent ratio for both solvent systems, while the highest levels occurred at 5% solid:solvent ratio and 70% ethanol composition, with the ethanol + HCl being slightly lower. Of all the solvent process parameters, highest level of TF resulted at a 5% solid:solvent. When the time was held constant (Figure 11d), the highest TF levels were obtained at 70% ethanol + HCL and a 5% solid:solvent ratio, while the lowest TF levels obtained from 30% ethanol + HCL with 10 % solid:solvent ratio, as reflected by the concave curve.

Based solely on yields, removing HCl from the ethanol extraction process is again preferable as the data indicate higher TF levels, but also higher overall ruggedness of the

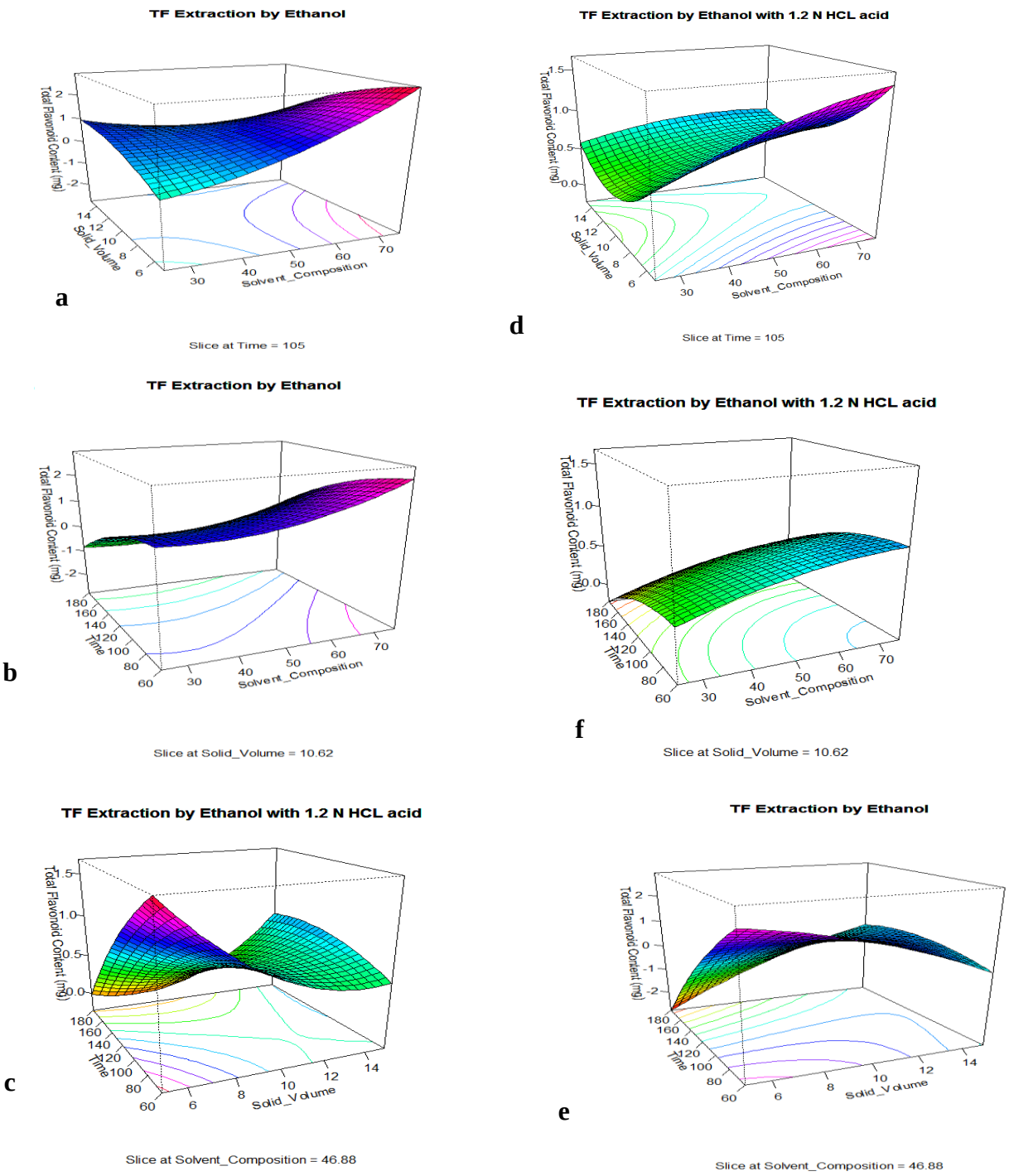


Figure 11: Response surface plots of the TF extracted with ethanol +/- HCl function of time, solvent composition and solid volume: (a,d) time of extraction was kept constant at 105 min, (b, e) solid volume of ethanol +/- HCl extraction was kept constant at 10.62 %, (c, f) solvent composition was kept constant at 45.66%.
process (Figures 11, a-f).

According to a main effects plot, TF levels were positively impacted by methanol and ethanol without HCl when the solvent compositions were at the extreme high (75%), and mix time and solid:solvent ratio are at the lowest points of 60 min and 5%, respectively.

Nonetheless, comparison of the methanol +/- HCL plots with the ethanol +/- HCL plots indicate that the latter extractions are more rugged in that higher overall TFs levels can be obtained at most points, with the exception of extracting spirulina with methanol +/- HCl concentrations higher 50% (Figure 10d). Moreover, closer inspection of the TF methanol +/- HCL plots, specifically Figures 12a,b, suggest that the ethanol +/- HCL plots are highly rugged in terms of obtaining a consistent TF level. A comparison of two plots indicates that the response was not critical in affecting the experimental parameters. Relatively high, but not the highest TF levels, were obtained at most points, with the exception of methanol +/- HCl concentrations lower than 50%.

When developing a processing system, manufacturers will base a successful process not only on optimal yields, but also on consistent yields. The lower amounts of resources required, and the ability to have a wider range of experimental range without affecting other processes must be considered to save time and money for a company. However, for the purposes of this project, we are also interested in characterizing the extract for the long term, so although these extracts may have the same TF levels, it must be acknowledged that their composition profiles could still be different.

D.3.4.3. Acetone (Figure 12 a-c): A review of Figures 12 a-c immediately confirms that the lowest TF extraction efficiencies were obtained with acetone + HCl. Based on the contour plots, with one factor being held constant, two plot patterns

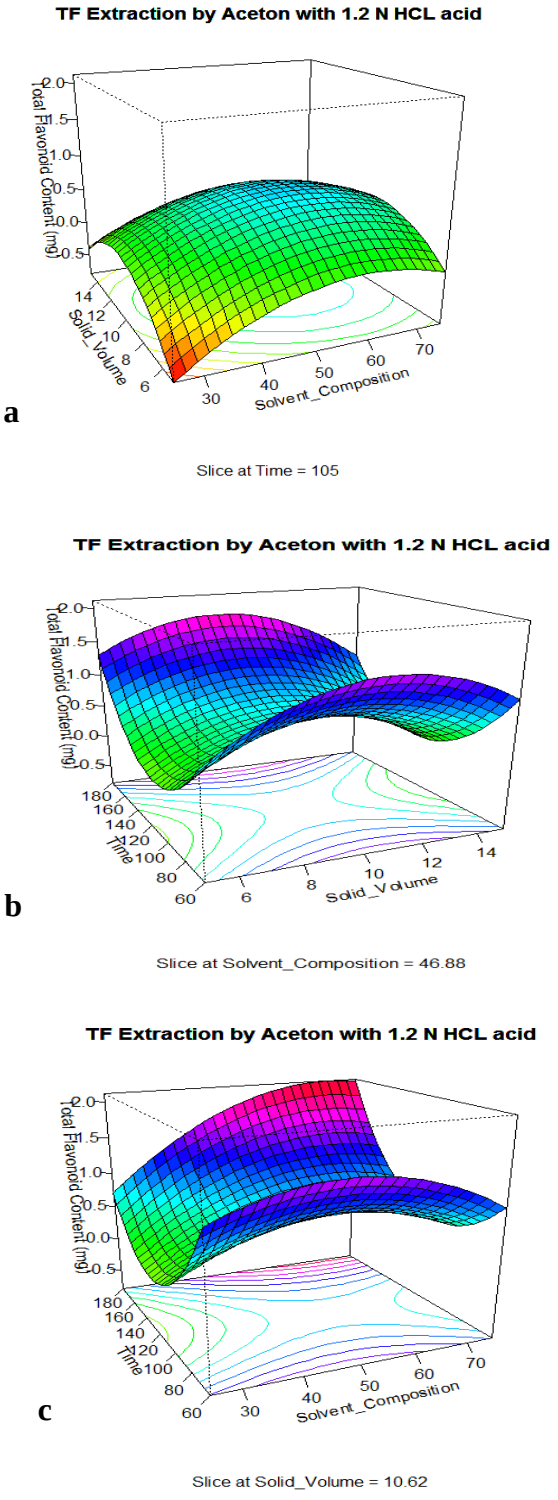


Figure 12: Response surface plots of the TF extracted with acetone + HCl function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 105 min, (b) the solid volume of ethanol +/- HCl extraction was kept constant at 10.62 %, (c) solvent composition was kept constant at 45.66%.

emerged. As shown in Figure 12a, a convex plot was generated at a constant time (105 min), resulting in higher yields at the mid of solvent composition (50%) and a solid:solvent ratio of 10%. At a constant solid:solvent ratio (Figure 12b and Figure 12c, substantially higher TF levels were extracted at the extreme ends of the mixing times (60 min and 180 min) across both solid:solvent ratio and solvent plot to the mid point of each and then decreased again in most cases. As shown in Figure 12c, the highest solvent composition (75%) and longest mixing time resulted in the highest yields, which could be due to the crossproduct interaction of these two factors, as evidenced by the regression equation (Table 18).

Again, different contour plots and regression equations were generated from the acetone + HCl data compared to the methanol (Figure 10) and ethanol extractions (Figure 11), demonstrating that different solvent systems will affect spirulina TF extractions differently. One set of parameters will produce different TF values for a given time than another based on the solvent system. Still the highest yields were obtained from the methanol at most of the extraction points compared to ethanol and acetone, suggesting that the flavonoids were more polar in nature. These plots (Figure 12) were also different in contour compared to the acetone contour plots for TP extractions (Figure 9), again indicating that the presence of HCl does affect the extraction process.

D.4 Antioxidative Capacity (AC)

D.4.1. *AC results obtained from FCCC-RSM:*

The AC results (in mean +/- standard deviation of three replicates) for each of the coded extractions are shown in Tables 19 and 20 relative to the solvents with and without HCl, whereas AC high and low values and overall ranges are shown in Table 21.

The highest AC value (147.9 μmole Trolox/g) was obtained by the methanol + HCl, which also had the highest range considering the lowest value of 7.66 umole Trolox/g of product. Acetone and ethanol + HCl produced extracts with relatively high AC values, but at more than 50% less than methanol + HCl. Yet, these two solvent systems produced the lowest "low" AC values as well. Interesting for, the methanol without HCl produced the overall lowest "high" AC values, even though the highest TF yields were obtained with this solvent system (Table 15) and had similar yields and plot contours as the TF methanol + HCl extractions (Figure 10). Although the HCl did not drastically improve total TP and TF yields for most of the solvent systems (Section D2 and D3), the addition of HCL to methanol and ethanol improved the antioxidative capacities. These results are probably due to the types of phenols being extracted, in that each had components with more potent scavenging properties.

D.4.2. *Fitting the AC models:* The AC values from the FCCC-RSM extraction for each solvent system, are summarized in Tables 22 (without HCl) and 23 (with HCl). ANOVA of the quadratic models for each solvent system showed that the methanol, ethanol + HCl and acetone + HCl alone fit the second order polynomial models as each had corresponding p values < 0.10. Additionally, each of these solvent systems had high correlation coefficients well above the 75 compliance level (Table 22, and 23). Despite the high AC values obtained for the methanol + HCl system, the corresponding quadratic model had a high probability p value (0.9190) and low R^2 value (34.97). More extraction points, or even more of the replicates of the same point may improve these values considering that the ORAC assay is a highly variable assay, and even more so at higher

Table 19: Experimental data for AC (in μmole Trolox/g) of spirulina extracts under different extraction conditions and solvent systems.

Standard Order	Methanol	Ethanol	Acetone
1	9.66 ± 0.01	24.23 ± 2.00	14.25 ± 1.00
2	12.50 ± 0.30	14.67 ± 0.29	11.25 ± 1.00
3	12.23 ± 1.97	13.35 ± 0.29	0.23 ± 0.10
4	10.01 ± 2.06	24.93 ± 14.42	6.36 ± 0.16
5	11.20 ± 2.95	12.58 ± 0.71	7.24 ± 0.98
6	12.38 ± 0.41	18.24 ± 1.99	5.13 ± 0.12
7	18.77 ± 4.25	30.23 ± 1.99	5.37 ± 1.04
8	9.51 ± 0.35	20.24 ± 1.32	42.28 ± 3.03
9	14.00 ± 0.60	25.67 ± 2.69	6.26 ± 1.00
10	13.22 ± 4.38	13.83 ± 0.16	61.92 ± 7.63
11	6.29 ± 2.00	6.35 ± 0.17	43.26 ± 1.73
12	12.41± 0.21	24.41 ± 0.21	6.46 ± 1.06
13	12.7 ± 0.45	6.29 ± 0.43	14.13 ± 5.37
14	8.55 ± 1.15	14.40 ± 0.25	13.21 ± 2.61
15	15.26 ± 5.51	29.23 ± 1.01	7.41 ± 1.13
16	17.23 ±1 .75	30.93 ± 1.52	12.13 ± 0.20

* Results are shown as the mean +/- standard deviation of n = 3.

Table 20: Experimental data for AC (in μmole Trolox/g) of spirulina extracts under different extraction conditions and solvent systems.

Standard Order	Methanol + HCl	Ethanol + HCL	Acetone + HCl
1	10.60 ± 0.31	32.93 ± 2.51	7.49 ± 0.21**
2	36.27 ± 3.95	20.18 ± 4.91	1.41 ± 0.17
3	72.27 ± 1.04	18.25 ± 0.01	1.17 ± 0.05
4	20.26 ± 4.95	7.48 ± 0.13	5.48 ± 0.86
5	16.27 ± 3.93	29.29 ± 2.95	5.45 ± 1.43
6	47.25 ± 3.00	20.33 ± 4.93	4.99 ± 0.03
7	10.25 ± 1.99	32.27 ± 0.041	7.35 ± 2.15
8	10.30 ± 5.00	12.26 ± 1.99	9.34 ± 0.86
9	7.67 ± 2.36	11.72 ± 2.21	4.24 ± 0.02
10	114.25 ± 30.70	60.24 ± 5.00	8.25 ± 1.98
11	36.01 ± 5.65	9.17 ± 4.90	10.10 ± 1.53
12	13.25 ± 2.01	27.81± 10.70	6.28 ± 0.78
13	56.28 ± 4.00	14.68 ± 2.62	14.94 ± 1.30
14	147.90 ± 2.49	8.25 ± 0.02	55.26 ± 4.99
15	9.32 ± 3.00	13.34± 0.22	6.83 ± 0.50
16	18.27 ± 1.95	60.27 ± 2.03	12.39 ± 1.83

* Results are shown as the mean +/- standard deviation of n = 3.

** Eliminated when building model as an outlier.

Table 21: Ranges of AC for each solvent system.

Extraction Solvent	High and Low Antioxidative Capacity (μmole Trolox/g)	Range (μmole Trolox/g)
Methanol	6.29 – 18.77	12.48
Ethanol	6.35 – 30.93	24.58
Acetone	0.23 – 61.92	61.69
Methanol + HCl	7.67 – 147.9	140.23
Ethanol + HCl	7.48 – 60.27	52.79
Acetone + HCl	1.17 – 55.26	54.09

numbers. Nonetheless, this system or the others that did not fit the model were not evaluated any further.

D.4.3 *Adequacy of the AC Models and corresponding regression equations:*

As shown in Table 22 and 23, the lack of fit values were > 0.05 for methanol, ethanol + /-HCl and acetone +/- HCl passed the lack of fit test as each had p values > 0.05, and thus their regression equations are shown in Table 24 confirming the adequacy of the models for these solvents.

D.4.4 *AC response surface plots and contours:* As supported by the equations, mixing time had the largest effect on the AC response in the methanol system, but solid to solvent ratio and solvent polarity also played a role, as it did in the ethanol, and acetone systems (Table 24). However, solvent polarity had the most positive impact on the ethanol / methanol + HCl extractions, followed by solid:solvent ratio, which exerted a negative influence as did the mixing time. However, the equation indicates an negative interaction between mixing and solid:solvent ratio, as did methanol extraction. The linear and quadratic nature of the relation of the extraction conditions on these solvent systems is easily distinguishable in the contour plots (Figures 13, 14, 15).

Table 22: Regression coefficients (coded) predicted by the quadratic polynomial model for antioxidative capacity when extracted with the cited solvent systems.

Coefficient	Methanol	Ethanol	Acetone
b_o	10.05	14.89	17.05
Linear			
b_1 (SP)	0.772	1.025	2.259
b_2 (S:S)	-0.097	0.631	2.196
b_3 (MT)	2.37^	4.760	-7.460
Quadratic			
b_{11} (SP x SP)	-0.432	3.597	3.791
b_{22} (S:S x S:S)	2.251**	-0.576	-3.759
b_{33} (MT x MT)	1.712	4.079	-1.132
Cross product			
b_{12} (SP x S:S)	-0.272	-1.870	11.76
b_{13} (SP x MT)	-0.900*	1.001	-0718
b_{23} (S:S x MT)	-1.712	4.079	-3.431
R^2	87.5	51.2	42.3
p values			
Model	0.0371	0.6971	0.8386
Lack of Fit	0.7086	0.2808	0.4860

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 10%

Table 23: Regression coefficients (coded) predicted by the quadratic polynomial model for antioxidative capacity when extracted with the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	44.23	13.66	19.85
Linear			
b_1 (SP)	0.699	9.580^	-0.878
b_2 (S:S)	-2.393	0.209	-0.495
b_3 (MT)	-17.093	0.987	0.111
Quadratic			
b_{11} (SP x SP)	29.51	6.839	26.838^
b_{22} (S:S x S:S)	-18.00	11.061**	-18.471*
b_{33} (MT x MT)	-19.51	-2.400	-20.169*
Cross product			
b_{12} (SP x S:S)	7.216	-0.378	1.056
b_{13} (SP x MT)	-16.030	-1.487	1.823
b_{23} (S:S x MT)	-4.173	-16.014^	-1.492
R^2	34.97	90.46	87.21
p values			
Model	0.9190	0.0180	0.0782
Lack of Fit	0.4127	0.1432	0.3312

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
^ Signficant at 1%, * Significant at 5%, ** Significant at 1

Table 24: Regression equations that fit the model and passed lack of fit test.

ORAC$_{methanol}$	= 10.05 + 2.37*Xmt* +*2.251XssXss* – *0.900XssXmt*.
ORAC$_{ethanol + HCl}$	= 13.66 + 9.580*Xsp* + 6.839*XspXsp* + 11.061*XssXss* – 16.014*XssXmt*.
ORAC$_{acetone + HCL}$	= 19.85 + 26.838 *XspXsp* -18.471 *XssXss* – *20.169XmtXmt*.

sp – Solvent Polarity, ss – Solid:Solvent, mt – Mix Time

D.4.4.1. Methanol (Figure 13a-c): At a constant mixing time of 105 min, a shallow convex curve was produced, resulting in high levels at mid of solvent composition (50%) and mid of solid:solvent ratio (10%) (Figure 13a). When the solid:solvent ratio was kept constant (10.62%) (Figure 13b), the highest scavenging values were obtained at the highest solvent composition (75%) and mixing time (180 min), while the lowest level occurred at the opposite side of the plot, i.e., 75% methanol composition and 60 min mixing time. At a constant solvent composition of 46.88%, (Figure 13c), a similar concave curve was obtained but not as sharp at the higher and lower levels. It must be pointed out that the highest value obtained from the methanol extraction was still lower than the other extractions. The data further show that the phenolic targeted samples extracted with methanol did not have high AC, even though the highest flavonoid yields (6.05 mg/g) were extracted with methanol (Table 15), whereas 3.46 mg/g of phenolics fell mid range compared to TP obtained from the other systems (Table 9). Yet, the similarities between the two plots (Figure 13 a and c) support that this is a rugged process as the time parameter will provide similar outcomes pending a solvent composition of 50% or the solid volume of 10%.

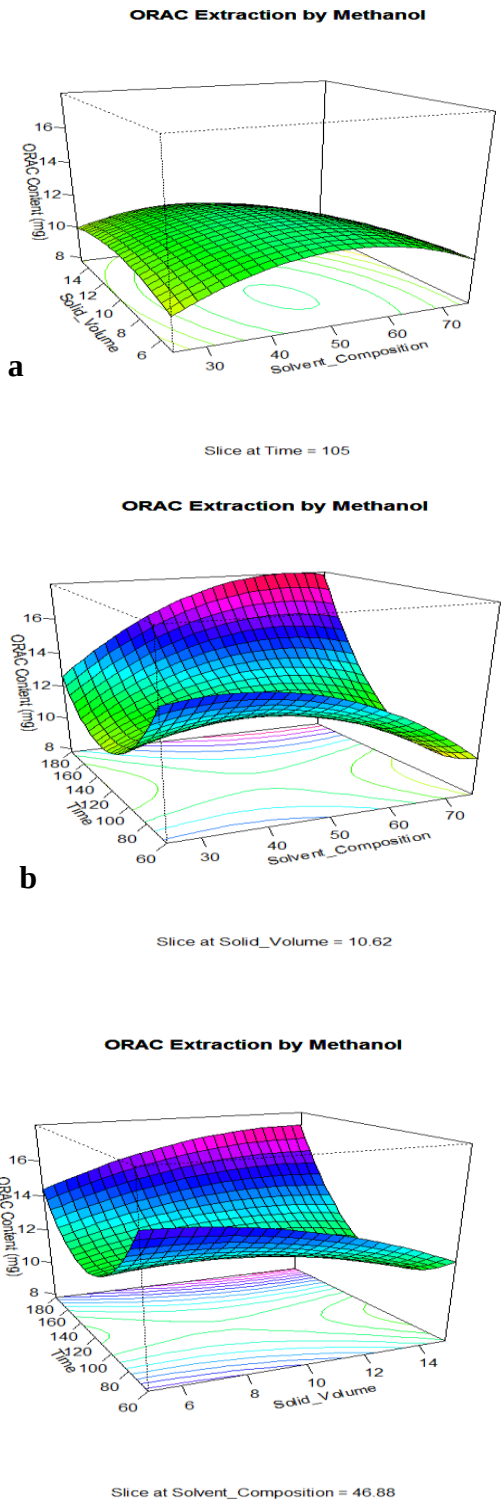
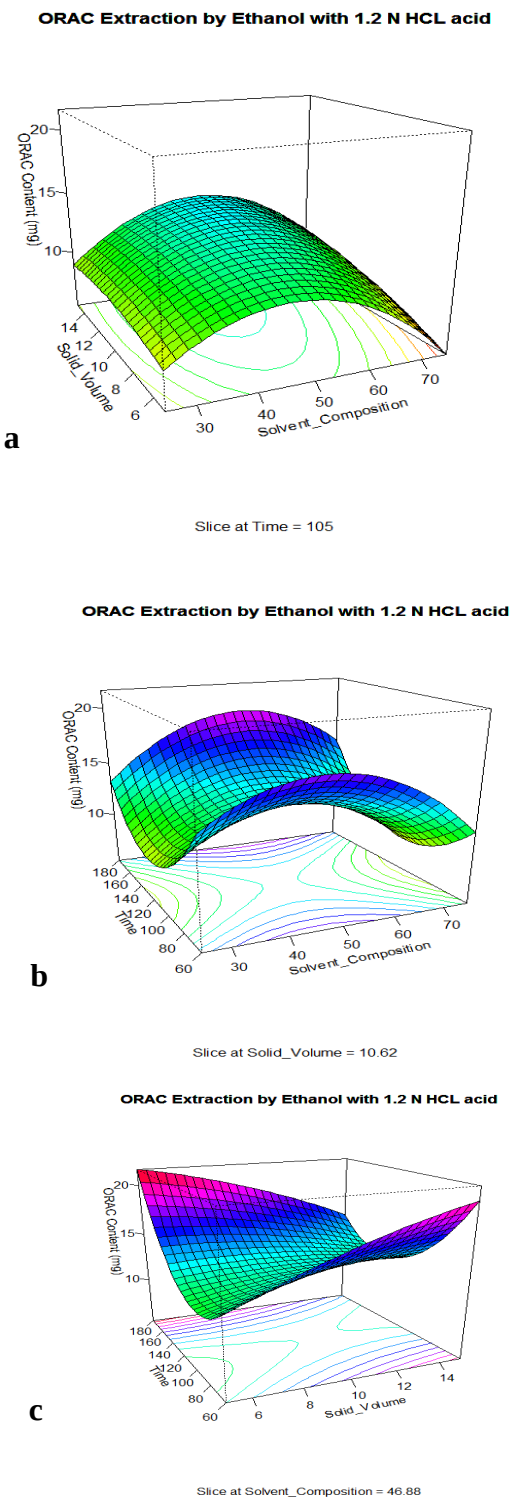


Figure 13: Response surface plots of the antioxidative response of methanol extractions extraction as a function of time, solvent composition and solid volume: (a) the time of extraction was kept constant at 105 min, (b) the solid volume of extraction was kept constant at 10.62 %, (c,) the solvent composition was kept constant at 46.88%.

- **D.4.4.2. Ethanol + HCl (Figure 14 a-f)**: Evaluation of the AC data obtained from the ethanol + HCL extraction generated a shallow convex curve (Figure 14a), similar to that shown in Figure 13a, and inverse saddle type plots (Figure 14b, c). As is typical for convex curves (13a), higher AC values were obtained at the center points. In this case, the solvent composition was 50 % and solid:solvent ratio was 10% while the time was held at is center point of 120 min. For the inverted saddle plot (Figure 13b), antioxidative capacity levels decreased to a low at ~ 30% ethanol and a mix time at the center point of 120 min and then increased with shorter and longer times. As with any inverted saddle plot higher values were obtained along the extremes of the mixing times, increased with solvent composition (50%) and then started to decrease again with further increasing solvent composition. Inspection of Figure 14c confirms that the highest AC were achieved with ethanol+ HCL. AC levels were highest when the mixing and the solid:solvent ratio were held at their lowest levels and the solvent composition was 50%. Any direction from that point resulted in lower AC values with the lowest occurring at 5% solid:solvent ratio and 180 min mix time. As mixing time greatly influenced AC, the results suggest that low levels of solids with potent AC could be exhibited but only when mixing time was kept to a minimum. Higher mixing times negatively affected AC values, which could be due longer exposure to the acidified solution. Although values increased with higher solid:solvent ratio while maintaining a mix time of 180 min, the AC were still lower than when short mixing times were applied.

Evaluation of the methanol results (Figure 13) with those of the ethanol + HCL (Figure 14) plots show comparable pattern plots between. Still, higher AC were achieved



a

b

c

Figure 14: Response surface plots of the AC of ethanol +1.2HCL extractions extraction as a function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 105 min, (b) solid volume of extraction was kept constant at 10.62 %, (c,) the solvent composition was kept constant at 46.88%.

with the ethanol + HCL suggesting that extracts contained higher levels of phenols / flavonoids or the different, but more potent components were extracted. As shown by phenol and flavonoid data presented in Tables 9 and 15, respectively, the latter hypothesis is probably correct as the highest phenolic value was 3.42 mg/g and flavonoid value was 1.15 mg/g. These results are lower than those determined from the methanol extractions (Table 9 and 15), dispelling the hypothesis that higher TF or TP levels caused the higher AC levels.

D.4.4.3. Acetone + HCL (Figure 15 a-c): Figure 15a-c show that the highest AC values for the acetone + HCl extractions was achieved using a maximum mixing time of 180 min and minimum solvent composition of ~30% (Figures 15 a,b,c). However, the different contours of the plots and the quadratic contribution of the each of the factors indicate that the extractions have to be precise to obtain the yields desired. Based on the plots, there was substantial drop in AC values as mixing time decreased, which is reversed with all previous solvents (Figure 15b,c).

D.5. Composition Analysis of Select Extracts:

Select extracts were analyzed via HPLC to determine qualitatively the extent of the differences (if any) in their composition profiles. These extracts were selected primarily on extracts that exhibited highiest phenol amount, as compared to their solvent counterparts that used the same parameters. For example Sample 10 for the methanol + HCl extraction, exhibited the highest AC (114.25 μmole/Trolox) (Table 20), and among the highest TPs (3.15 mg/g) and TFs (3.24 mg/g) (Tables 13 and 14). Similarly, sample 10 for methanol without HCl contained among the highest TP (4.39 mg/g) and TF (3.55

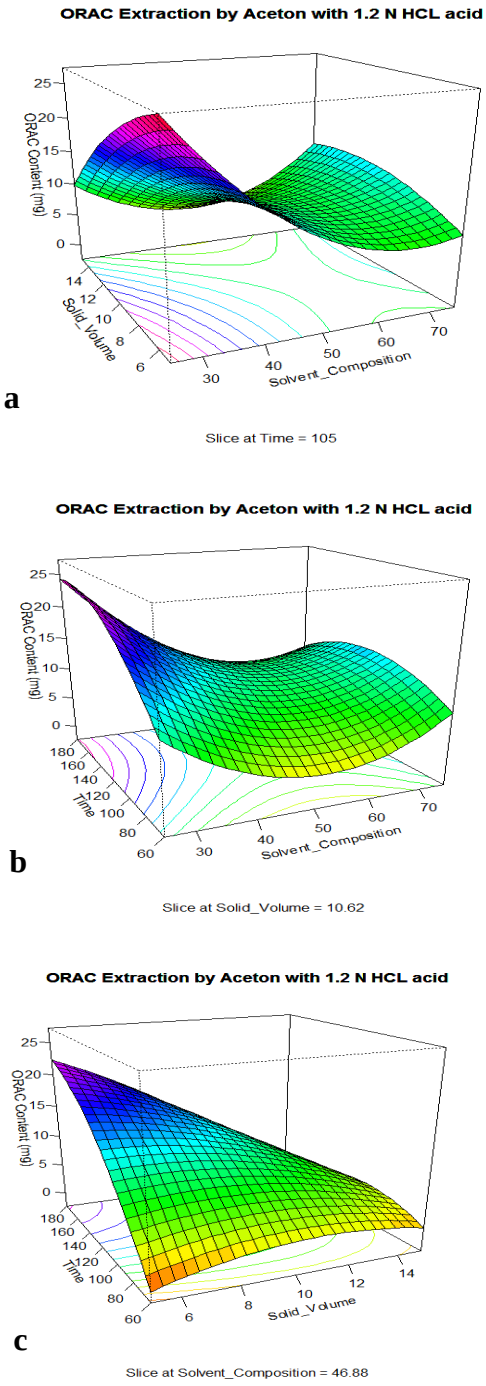


Figure 15: Response surface plots of the AC of acetone + 1.2 N HCl extraction as a function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 105 min, (b) solid volume of extraction was kept constant at 10.62 %, (c) the solvent composition was kept constant at 46.88%.

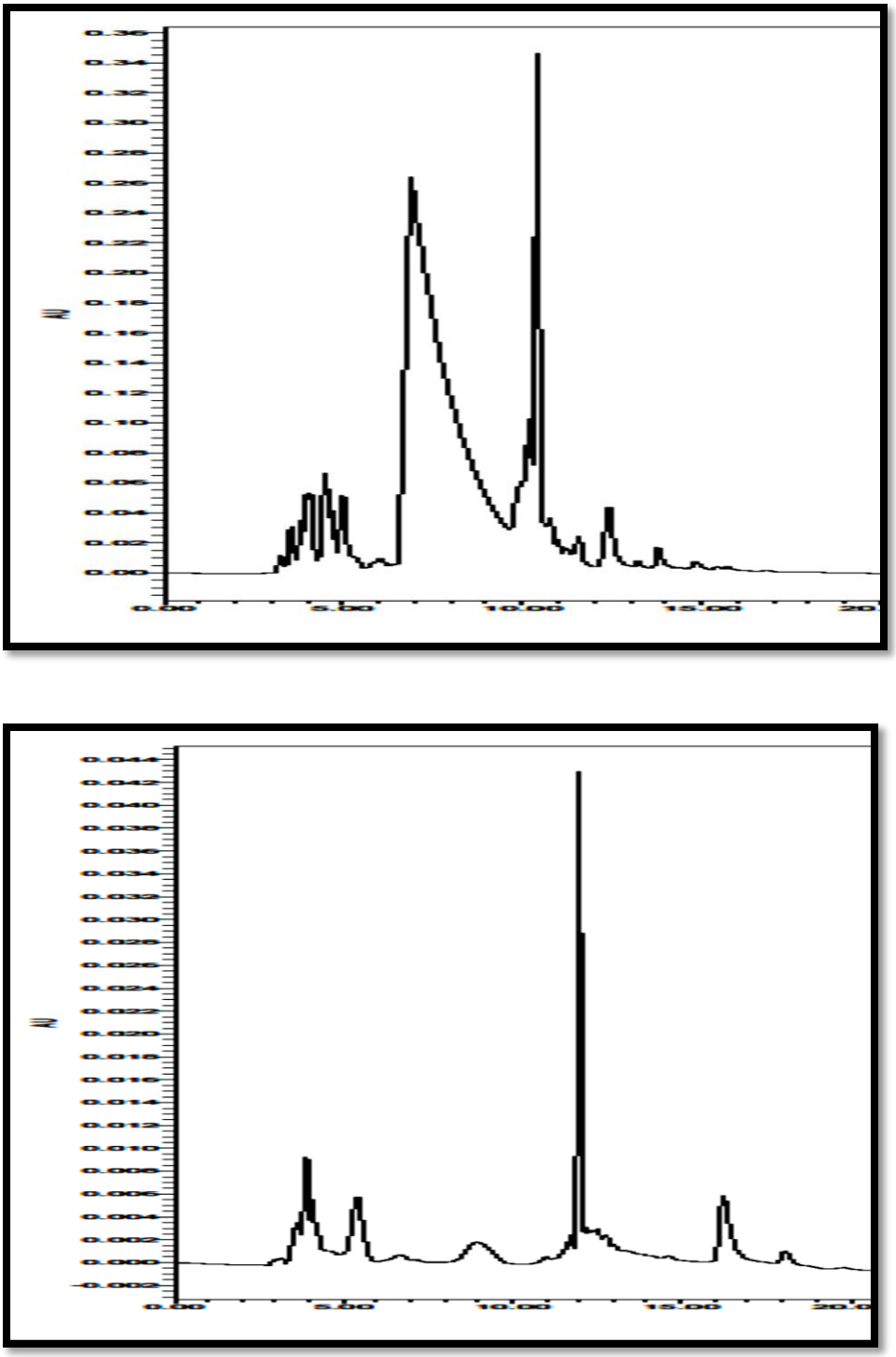


Figure 16: Reverse phase chromatograms of extracts (a) methanol + HCl, (b.) methano - HCl obtained with 75:25 solvent:water, 15% solid:solvent, and 60 min mix time

mg/g), but had a comparatively lower antioxidative capacity (13.22 μmole/Trolox). Figures 16 a and b show the chromatograms from both of the extractions.

As can be seen by these chromatograms, different compositional profiles were obtained, demonstrating that extractions with similar process factors, but slight different solvent systems resulted in different HPLC profiles and ultimately unusually different AC values. As TP and TF values were similar between these two extracts, the results also show that further compositional profiles are needed to link a biological activity to the overall profiles. Additional examples of the HPLC compositional profiles are shown in the Appendix Section of this document to illustrate difference in compositional profiles of select samples obtained with major changes to a process (solvent) or minor (extraction process). Also shown in the Appendix section are the optimal process extractions predicted by each accepted model to obtain the highest TF / TP / AC.

E. CONCLUSIONS

The aim of this study was to characterize the extraction conditions that would not only provide phenol and flavonoid rich extracts, but also the link these extracts to a biological activity, i.e., in this case AC. The following bullet points summarize the main conclusions determined from this project. Note, this list is based on the data that fit the second order quadratic model.

- The optimal solvent for obtaining TP rich extracts was acetone, indicating the phenols in this system are primarily non-polar. Acidified acetone is not needed to improve this response.

- The optimal solvent for obtaining TF rich extracts was methanol, indicating the flavonoids were more polar in nature. Acidified methanol is not needed to improve this response.
- The optimal solvent for obtaining high AC extracts was ethanol + HCl, indicating that high total phenols / flavonoids are not only playing a role in this biological system but also different types of compounds.
- From the above bullet statements, more than one type of solvent and process parameters are required to access different types of antioxidants present in spirulina and its associated biological activity. Other process extractions will most likely be required if another biological activity is targeted.
- In most cases, a low solid:solvent ratio is needed to obtained high TP / TF / AC, with the exception of AC, which required high solid:solvent ratio.

In future work, other parameters should be accessed, such as particle size, temperature, and mix agitation. Nonetheless, the results from this study provide an understanding of the processing parameters needed for obtaining certain select extracts and the next step of understanding the phenols responsible for delivering optimal protection against oxidative stress.

F. APPENDIX:

The following tables provide the optimal conditions provided by the accepted models to obtain the optimal amounts. Experiments have yet to be completed to compare these conditions with those obtained at the bench.

Optimum value for Methanol – HCL = 3.83 mg/g TP

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	1.0
Mix	-1.0

Optimum value for Ethanol-HCl = 4.13 mg/g TP

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	0.577454
Mix	0.126448

Optimum value for Acetone-HCl = 9.03 mg/g TP

Factor	***Optimum***
Solvent Polarity	-0.25869
Solid:Solvent Ratio	-0.0935707
Mix	-1.0

Optimum value for Methanol + HCL = 3.15 mg/g TP

Factor	***Optimum***
Solvent Polarity	-1.0
Solid:Solvent Ratio	-1.0
Mix	-1.0

Optimum value for Ethanol + HCl = 2.42 mg/g TP

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	-0.64194

Optimum value for Methanol - HCL= 5.19 mg/g TF

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	-1.0

Optimum value Ethanol-HCl = 4.11 mg/g TF

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	-1.0

Optimum value for Methanol + HCl = 5.99 mg/g TF

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	-1.0

Optimum value for Ethanol + HCl = 1.80 mg/g TF

Factor	**Optimum**
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	-0.809735

Optimum value for Acetone + HCl = 2.49472 TF

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	1.0

Optimum value for Methanol – HCl = 33.15 μmole Trolox/g

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	-1.0
Mix	1.0

Optimum value for Ethanol + HCl = 55.08 μmole Trolox/g

Factor	***Optimum***
Solvent Polarity	1.0
Solid:Solvent Ratio	1.0
Mix	-1.0

Optimum value for Acetone + HCl = 47.62 μmole Trolox/g

Factor	***Optimum***
Solvent Polarity	-1.0
Solid:Solvent Ratio	-0.0413631
Mix	-0.0398903

The chromatograms shown on the following pages show difference in select extracts prepared with different parameters.

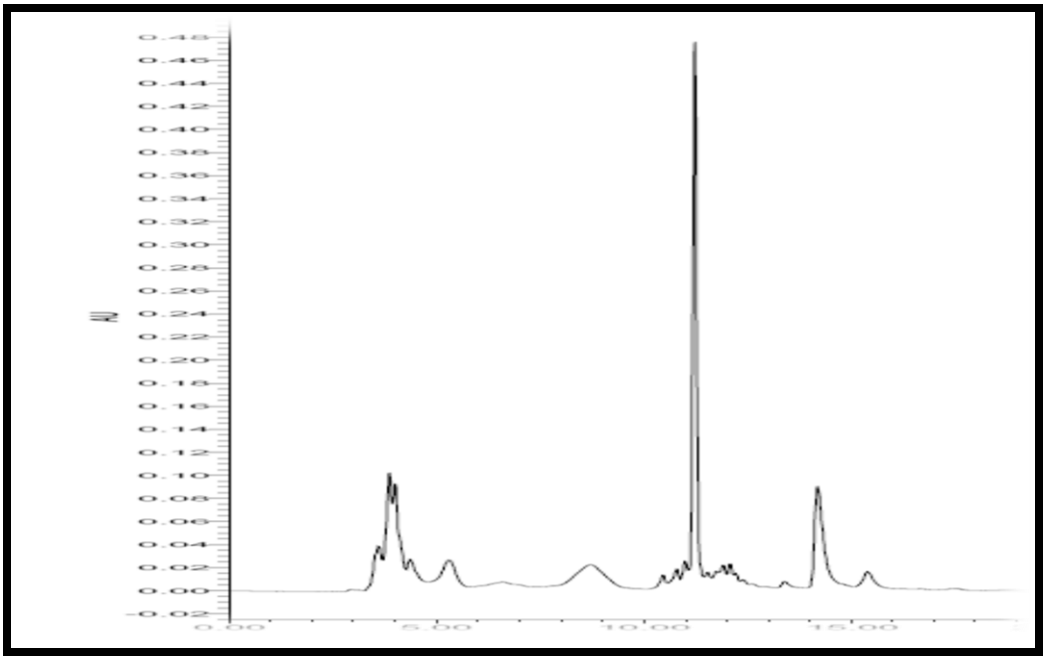


Figure 17: Reverse phase chromatograms of extracts methanol + HCl. obtained with 50:50 solvent:water, 10% solid:solvent, and 60 min mix time.

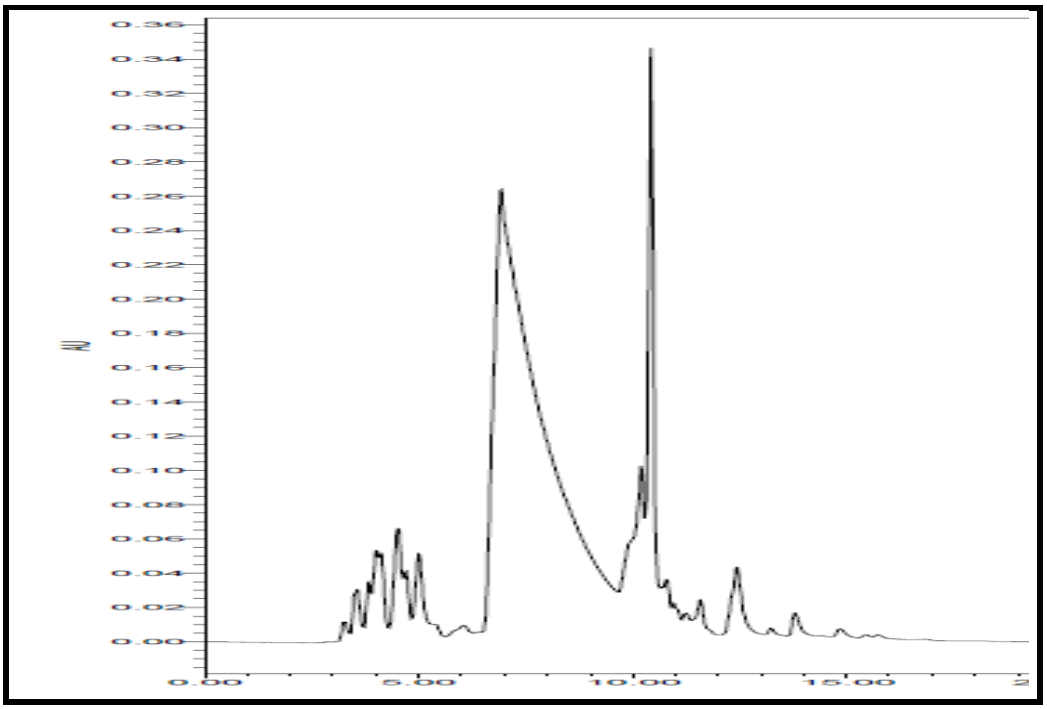


Figure 18: Reverse phase chromatograms of extracts methanol - HCl. obtained with 75:25 solvent:water, 5% solid:solvent, and 60 min mix time

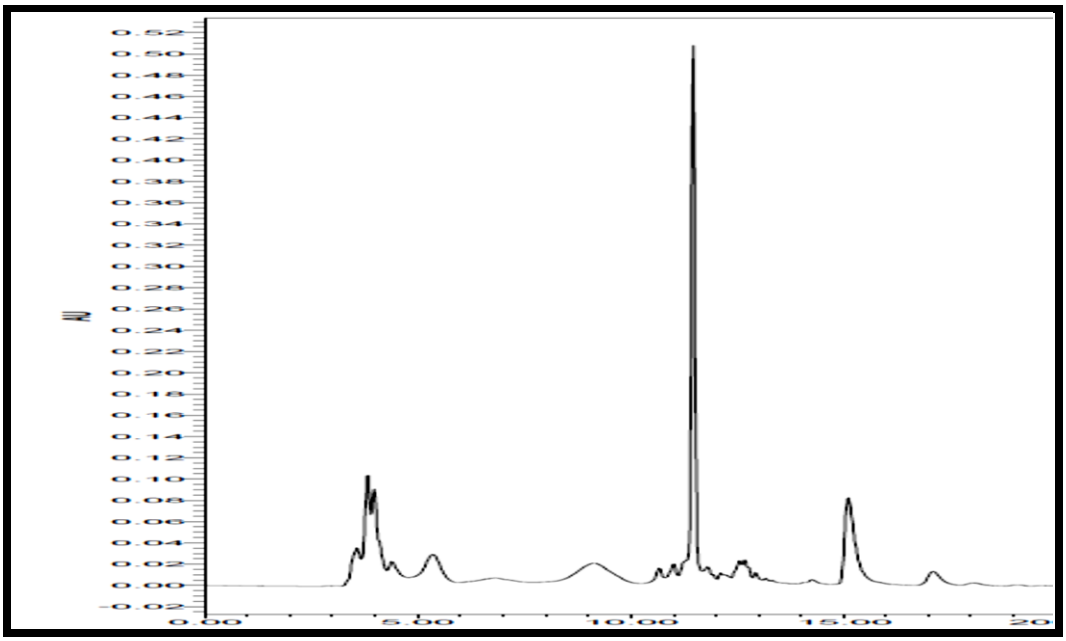


Figure 19: Reverse phase chromatograms of extracts methanol - HCl. obtained with 50:50 solvent:water, 5% solid:solvent, and 120 min mix time.

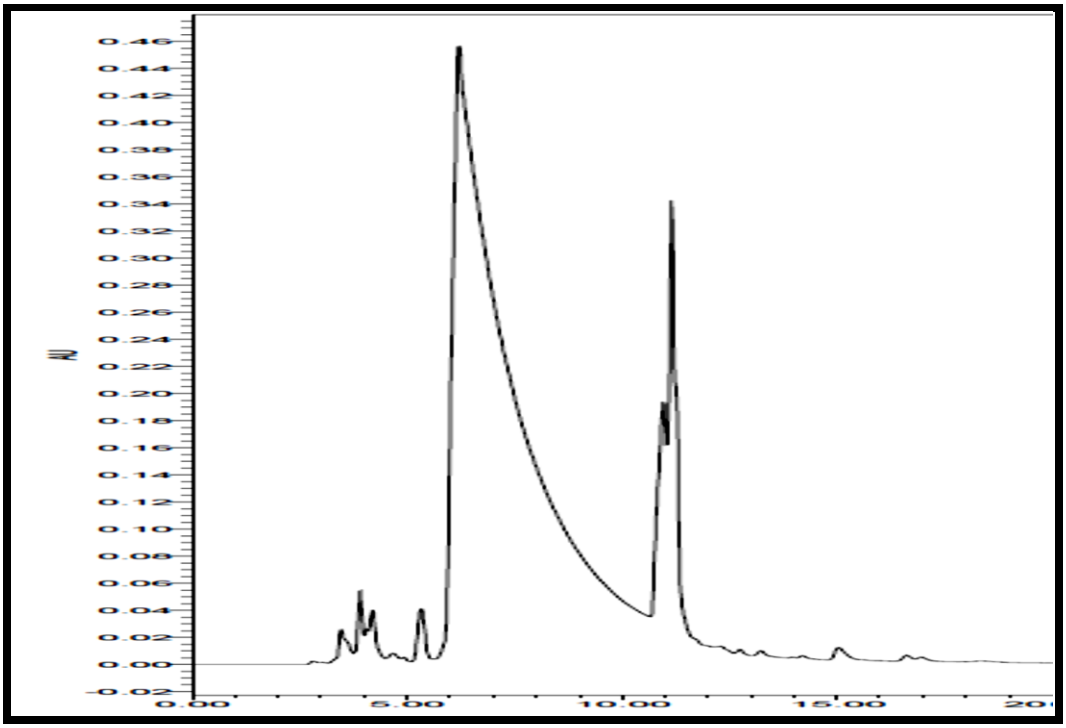


Figure 20: Reverse phase chromatograms of extracts methanol + HCl. obtained with 25:75 solvent:water, 5% solid:solvent, and 60 min mix time.

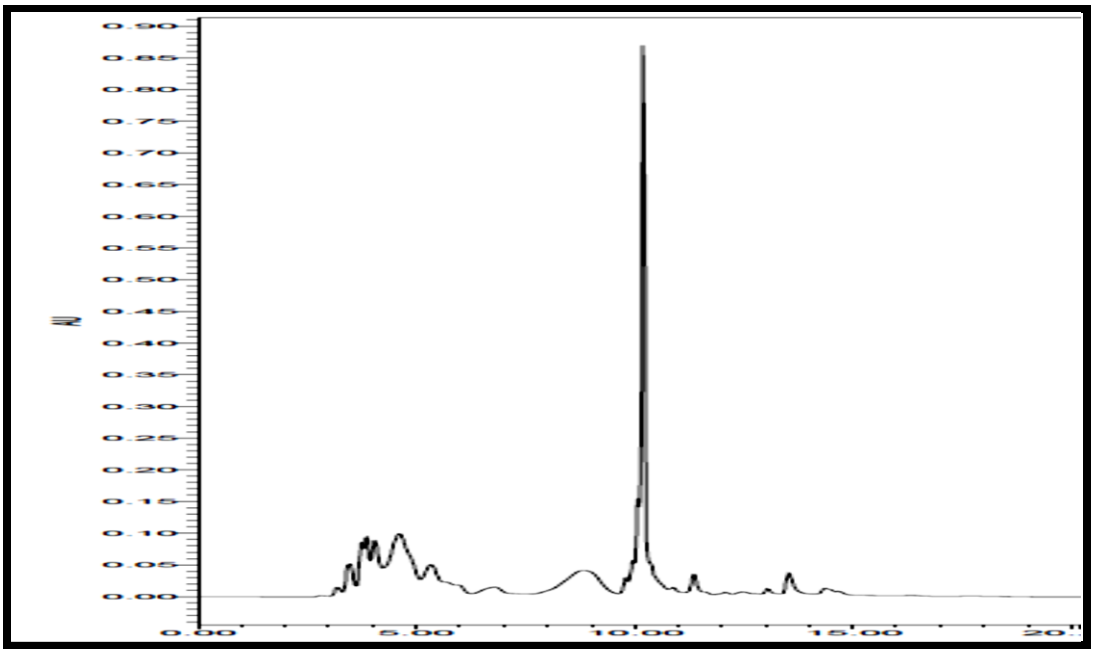


Figure 21: Reverse phase chromatograms of extracts ethanol + HCl. obtained with 75:25 solvent:water, 5% solid:solvent, and 60 min mix time.

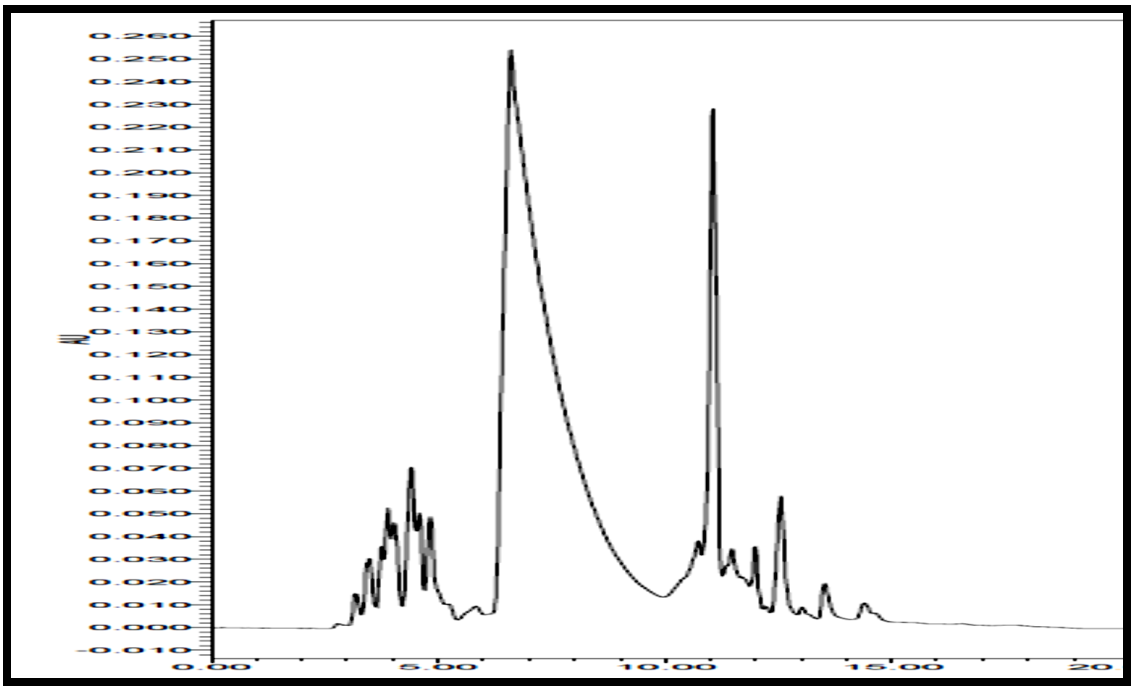


Figure 22: Reverse phase chromatograms of extracts ethanol + HCl. obtained with 75:25 solvent:water, 15% solid:solvent, and 60 min mix time.

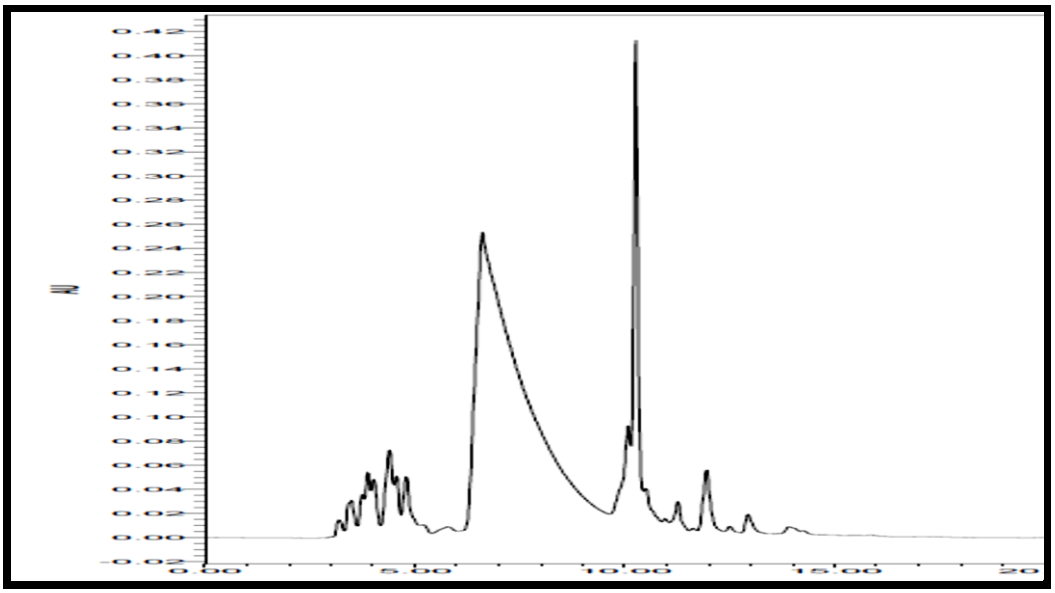


Figure 23: Reverse phase chromatograms of extracts ethanol - HCl. obtained with 75:25 solvent:water, 15% solid:solvent, and 60 min mix time.

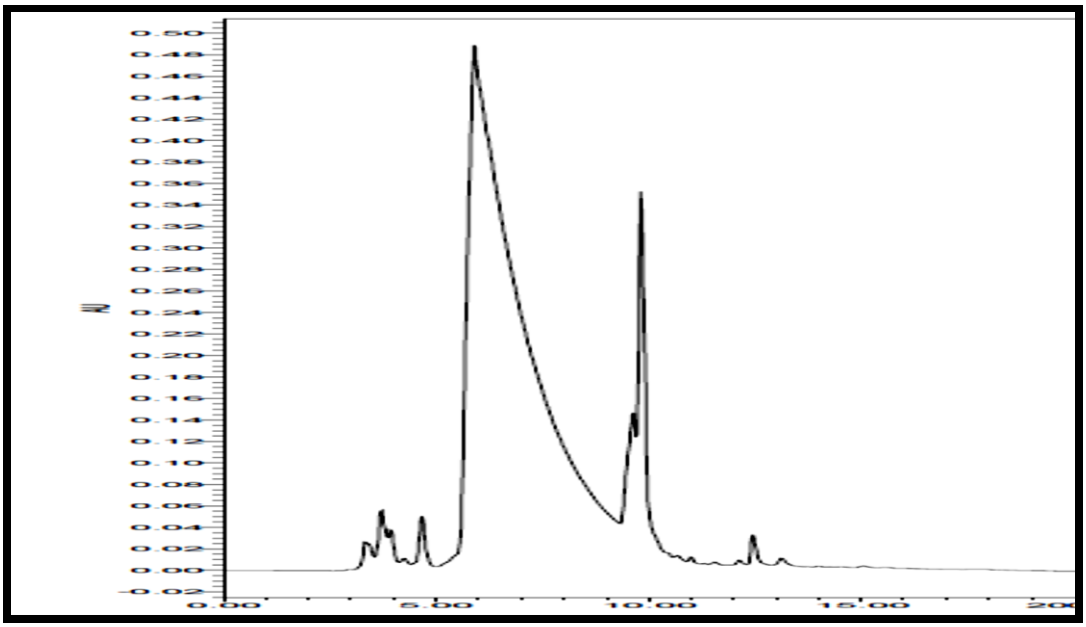


Figure 24: Reverse phase chromatograms of extracts ethanol - HCl. obtained with 25:75 solvent:water, 5% solid:solvent, and 60 min mix time.

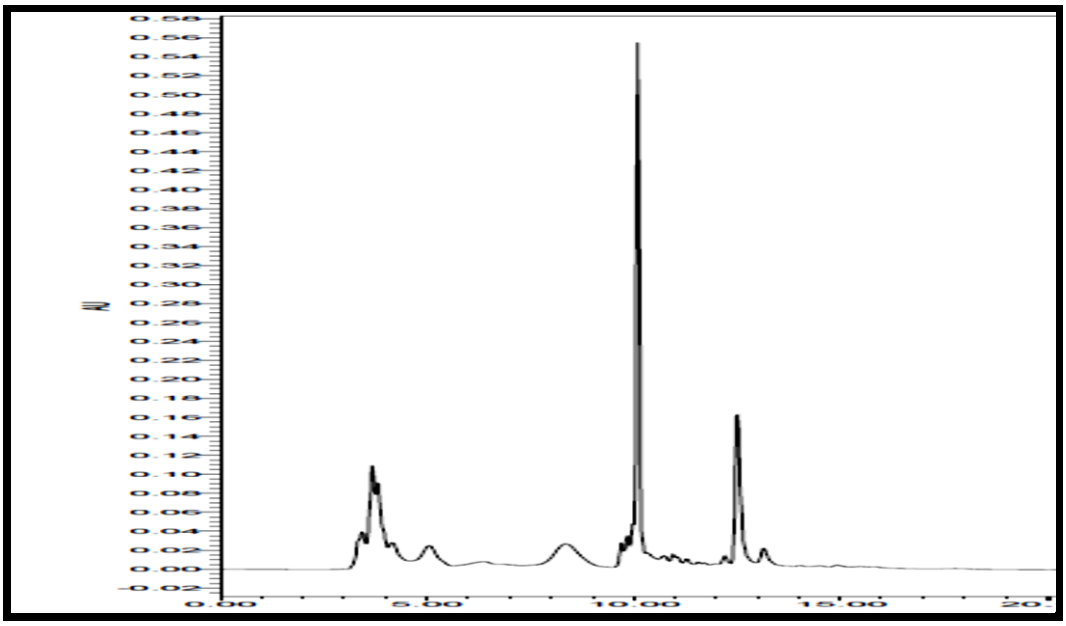


Figure 25: Reverse phase chromatograms of extracts acetone - HCl. obtained with 50:50 solvent:water, 10% solid:solvent, and 60 min mix time.

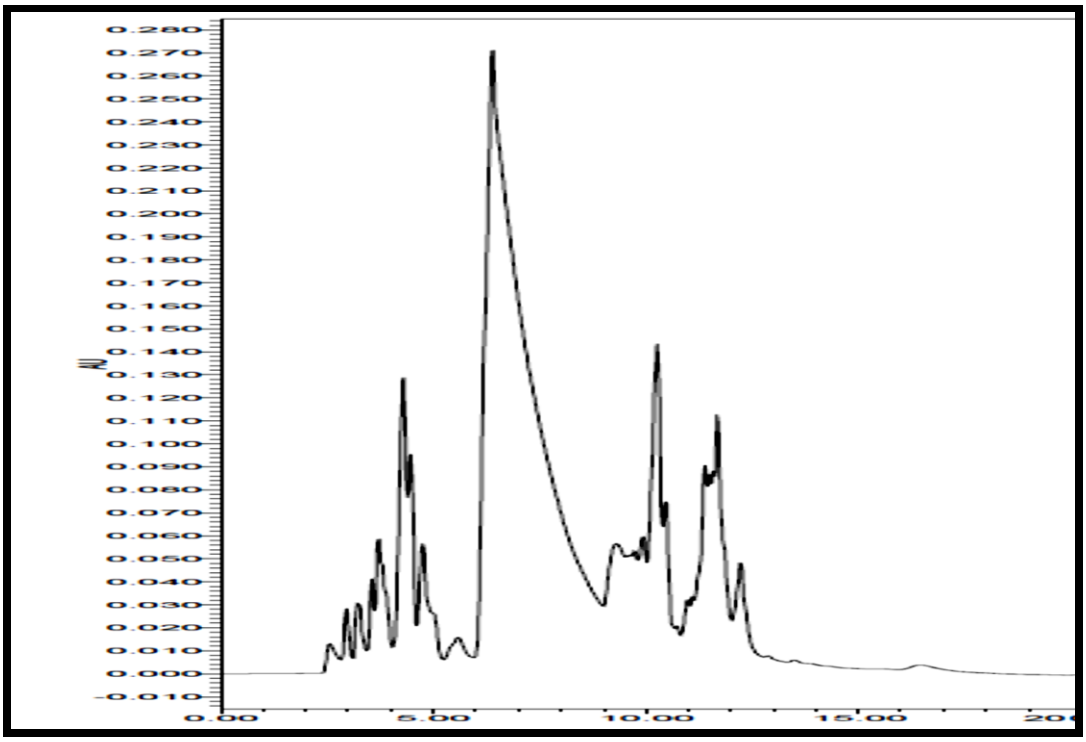


Figure 26: Reverse phase chromatograms of extracts acetone - HCl. obtained with 50:50 solvent:water, 15% solid:solvent, and 120 min mix time.

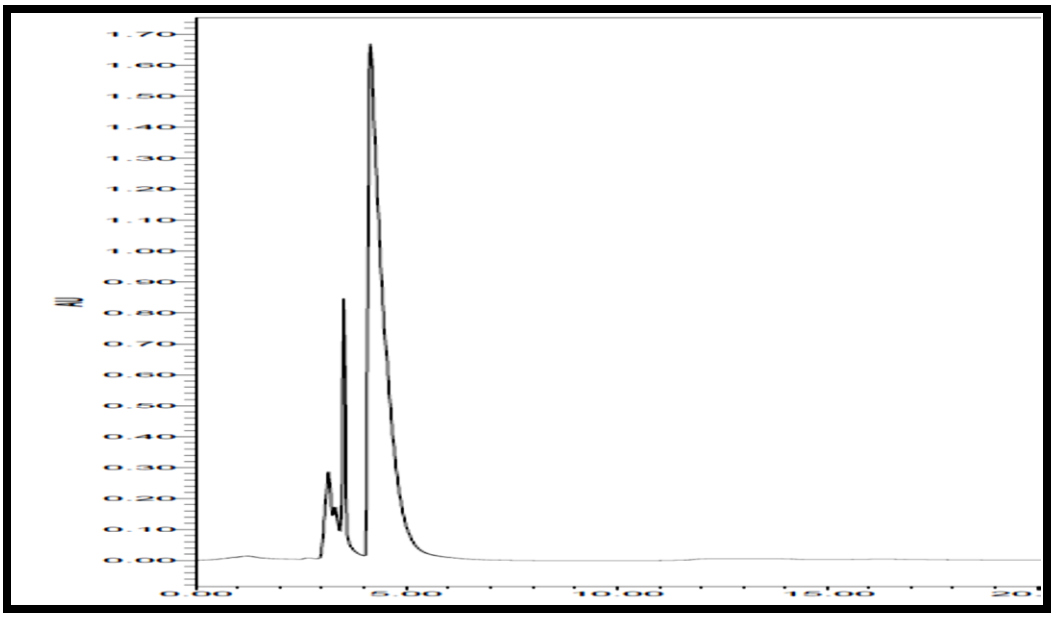


Figure 27: Reverse phase chromatograms of extracts acetone + HCl. obtained with 25:75 solvent:water, 15% solid:solvent, and 180 min mix time.

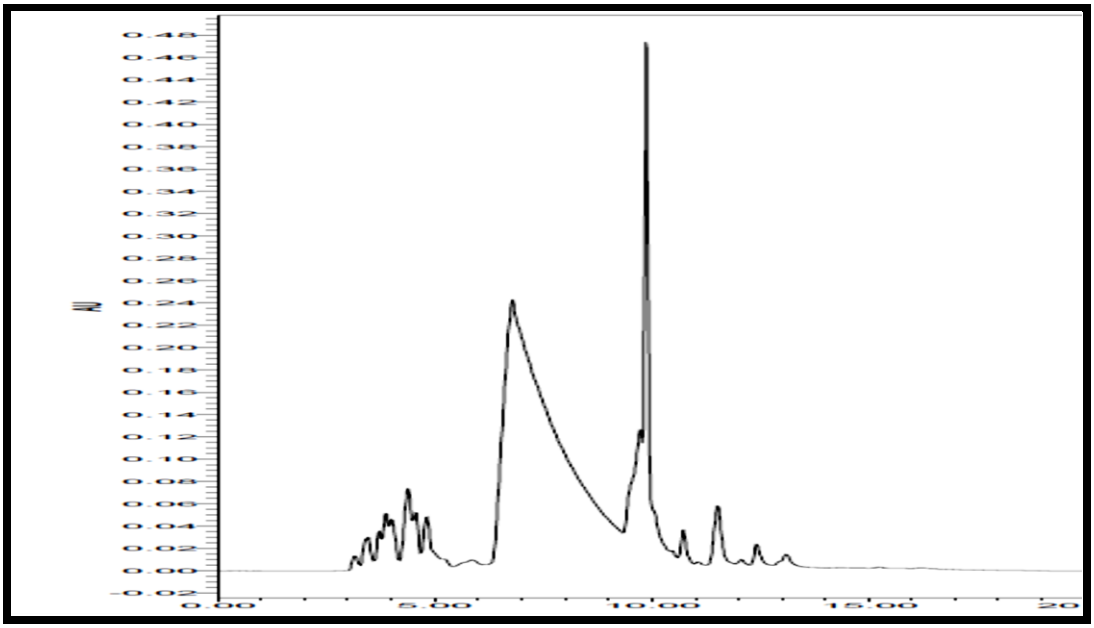


Figure 28: Reverse phase chromatograms of extracts acetone + HCl. obtained with 75:25 solvent:water, 15% solid:solvent, and 60 min mix time.

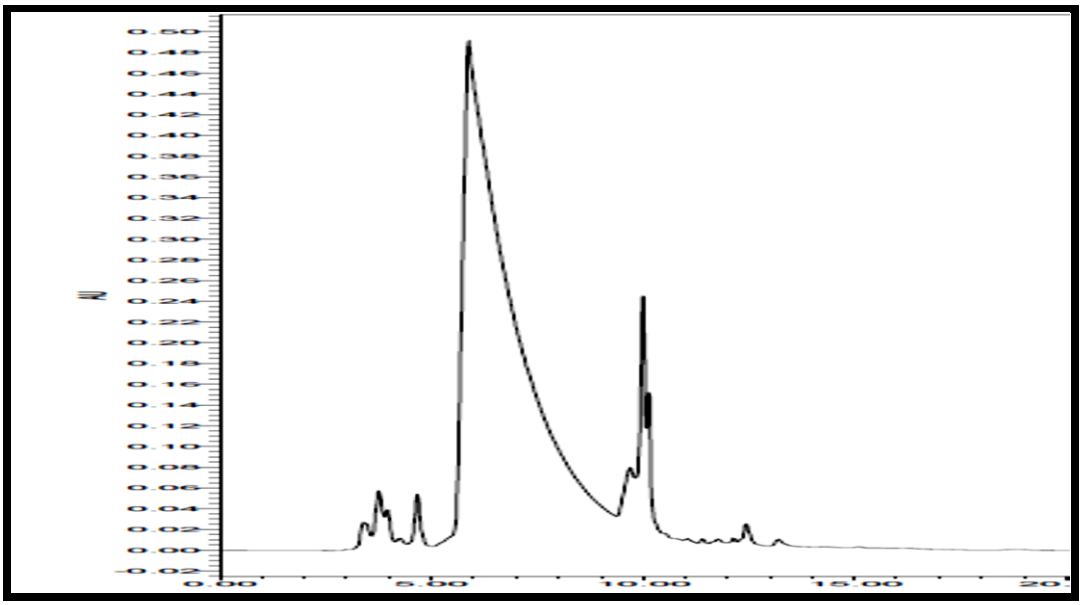


Figure 29: Reverse phase chromatograms of extracts acetone + HCl. obtained with 25:75 solvent:water, 5% solid:solvent, and 60 min mix time.

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