# MICROWAVE ASSISTED EXTRACTION OPTIMISATION OF INDUSTRIALLY APPLICABLE LIPIDS FROM GRAIN AMARANTH

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## DEDICATION

*This thesis is dedicated to my beloved grandparents,* ***Dr. Sita Ram Joshi*** *&* ***Mrs. Madhuri Joshi****; my lovely parents,* ***Mr. Pradeep Kumar Joshi*** *&* ***Mrs. Sangeeta Joshi****; my dear sibling,* ***Miss. Saumya P Joshi*** *and to all my* ***friends*** *who endured me with great fortitude*

# MICROWAVE ASSISTED EXTRACTION OPTIMISATION OF INDUSTRIALLY APPLICABLE LIPIDS FROM GRAIN AMARANTH

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**MASTER OF SCIENCE** **BIORESOURCE ENGINEERING**

## ABSTRACT

Recently, the scientific community has recognized the potential application of lipophilic compounds of grain amaranth as an active ingredient in nutraceutical supplements and cosmeceutical formulations specifically squalene, sterols, tocols and polyphenols. This research study has actualised the optimisation of a novel methodology for eluting bioactive lipophilic compounds from grain amaranth by employing microwave assisted extraction (MAE). The study developed an approach to exploit the lipids of this unconventional pseudo-cereal for industrial applications.

Experimentation of this study was carried out in three phases. In the first phase, pre-optimisation studies were conducted to identify the optimum parameters such as sample particle size, extraction solvent and microwave input power, which determined the experimental domain required to optimise the extraction of oil and its constituents, squalene & stigmasterol. For this purpose a full factorial screening design was operationalized. The second phase accomplished the optimisation process of extracting amaranth grain oil and its constituents, squalene & stigmasterol, by augmenting two consecutive central composite designs, considering factors which weren't included in the factorial design such as sample to solvent ratio, solvent to solvent ratio and extraction time, keeping the constant optimum values for the identified factors. The third phase compared the outcomes of optimised MAE process with optimised Soxhlet extraction quantitatively (oil yield %) and qualitatively (squalene, stigmasterol, polyphenols, α – tocopherol and DPPH free radical-scavenging activity). The optimised MAE method achieved the highest extraction yield of 9.1% amaranth grain oil containing 59.53, 3.17, 1.052 and 0.72 mg/g of squalene, stigmasterol, α - tocopherol and total polyphenol respectively with DPPH free radical scavenging activity of 86.24%, whereas, the optimised Soxhlet extraction achieved its highest extraction yield of 8.8% amaranth oil containing 58.78, 3.06, 0.983 and 0.407 mg/g of squalene

stigmasterol, α – tocopherol and total polyphenol, respectively with a DPPH free radical scavenging activity of 70.11%. This attests MAE as a more industrially viable methodology for extracting lipids from amaranth grain over standard Soxhlet extraction methodology not just limited to higher oil yield with reduced extraction time and solvent consumption but also for preserving the best quality of its high value industrially applicable thermostable compounds, squalene & stigmasterol as well as the thermolabile compounds α–tocopherol and total polyphenol, maintaining higher antioxidant scavenging activity.

# RÉSUMÉ

Les composés lipidiques issus de l'amarante sont maintenant reconnus posséder une bioactivité d'intérêt nutraceutique et cosméceutique, particulièrement pour la squalène, les stérols, tocols et polyphénols. Cette étude a visé l'optimisation de l'extraction microonde des composés bioactifs lipophiles de la graine d'amarante. Cette étude a voulu développer une raison industrielle aux composés lipidiques d'une pseudo-céréale non-conventionnelle.

Les expériences de cette étude ont été menées en trois phases. La première étape s'est concentrée sur l'identification des paramètres importants tels la granulométrie, le type de solvant d'extraction, et la puissance microonde, jouant sur l'extraction de l'huile et de ses constituants, la squalène et le stigmastérol. Pour cette étude, un plan expérimental factoriel complet a été utilisé. Pour la seconde étape, l'optimisation de l'extraction a été visée en augmentant le plan expérimental en prenant en considération le ratio échantillon et solvant, le ratio solvant et solvant, et le temps d'extraction, tout en maintenant optimisé les paramètres étudiés à la première étape. La troisième étape a comparé les résultats des conditions optimales d'extraction microonde aux résultats des conditions optimales d'extraction Soxhlet, quantitatif (% rendement en huile) et qualitatifs (composition en squalène, stigmastérol, polyphénols, α-tocophérol et DPPH (capacité phagocyte des radicaux libres). L'extraction optimale microonde de la graine d'amarante a extrait 9.1% d'huile contenant 59.53, 3.17, 1.052 et 0.72 mg/g de squalène, stigmastérol, α-tocophérol et polyphénols totaux avec une capacité phagocyte des radicaux libres de 86.24%. L'extraction optimale Soxhlet a extrait quant à elle, 8.8% d'huile contenant 58.78, 3.06, 0.983 et 0.407 mg/g de squalène, stigmastérol, α-tocophérol et polyphénols totaux avec une capacité phagocyte des radicaux libres 70.11%. Ceci confirme la meilleure performance de l'extraction micro-onde pour l'extraction des lipides de la graine d'amarante sur l'extraction conventionnelle Soxhlet, autant pour le rendement que pour la conservation de la qualité de l'huile, et ses composés thermostable (tels la squalène) et thermolabiles (tels l'α-tocophérol), maintenant une meilleure capacité phagocyte des radicaux libres.

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Heart filled with everlasting love, I dedicate everything that I have accomplished including this thesis to my beloved grandparents, parents and my sister for their mellifluous love, support and affection showered upon me that fosters my educational ambitions.

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## NOMENCLATURE

| | |
|---|---|
| *AACC* | *American Association of Cereal Chemists* |
| *ACS* | *American Chemical Society* |
| *AGF* | *Amaranth grain flour* |
| *AIU* | *Amylase inhibitory activity* |
| *ALT* | *Alanine aminotransaminase* |
| *ANOVA* | *Analysis of variance* |
| *AOCS* | *American Oil Chemists' Society* |
| *AST* | *Aspartate aminotransaminase* |
| *ASTM* | *American Society for Testing and Materials* |
| *°C* | *Degree centigrade* |
| *Ca* | *Calcium* |
| *CCRD* | *Central composite rotatable design* |
| *CIDA* | *Canadian International Development Agency* |
| *CIU* | *Chymotrypsin inhibitor activity* |
| *cm* | *Centimeter* |
| *Cps* | *The specific heat of solvent* |
| *Cpv* | *the specific heat of borosilicate glass* |
| *Cu* | *Copper* |
| *cv.* | *Cultivar* |
| *DMAE* | *Dynamic microwave-assisted extraction* |
| *DPPH* | *2,2-diphenyl-1-picrylhydrazyl* |
| *ε'* | *dielectric constant* |
| *ε"* | *dielectric loss* |
| *EtOH* | *Ethanol* |
| *FAO* | *Food and Agriculture Organisation* |
| *Fe* | *Iron* |
| *FFSD* | *Full factorial screening design* |
| *g* | *Gram* |
| *GC-MS* | *Gas chromatography – mass spectroscopy* |
| *GHz* | *Gigahertz* |
| *HDL* | *High density lipoprotein* |
| *HMG – CoA* | *3-hydroxy-3-methylglutaryl-coenzyme A* |
| *HPMAE* | *High pressure microwave-assisted extraction* |
| *HPLC* | *High-performance liquid chromatography* |
| *IDRC* | *International Development Research Center* |
| *IR* | *Infrared* |
| *IU* | *International units* |
| *K* | *Potassium* |
| *Kg* | *Kilogram* |
| *LDL* | *Low density lipoprotein* |
| *MAE* | *Microwave assisted extraction* |
| *Mg* | *Magnesium* |
| *mg* | *Milligram* |

| | |
|---|---|
| *MHz* | *Megahertz* |
| *min* | *Minutes* |
| *ml* | *Milliliter* |
| *mm* | *Millimeter* |
| *Mn* | *Manganese* |
| *Ms* | *The mass of solvent* |
| *MtOH* | *Methanol* |
| *Mv* | *The mass of the borosilicate glass vessel* |
| *Na* | *Sodium* |
| *Ni* | *Nickel* |
| *NPMAE* | *Nitrogen-protected microwave-assisted extraction* |
| *P* | *The actual microwave power output* |
| *Pa* | *Pascal* |
| *PSE* | *Pressurized solvent extraction* |
| *psi* | *Per square inch* |
| *rpm* | *Revolutions per minute* |
| *RS* | *Resistant starch* |
| *RSC* | *Royal Society of Chemistry* |
| *S* | *Second* |
| *SD* | *Standard deviation* |
| *SFE* | *Supercritical fluid extraction* |
| *SFME* | *Solvent-free microwave-assisted extraction* |
| *ΔT* | *The temperature difference* |
| *t* | *The time of heating* |
| *Tan δ* | *Dissipation factor* |
| *TBARS* | *Thiobarbituric acid reactive substances* |
| *TIU* | *Trypsin inhibitor activity* |
| *µg* | *Microgram* |
| *µl* | *Microliter* |
| *µm* | *Micrometer* |
| *UMAE* | *Ultrasonic microwave-assisted extraction* |
| *UV* | *Ultraviolet* |
| *v/v* | *Volume/ volume* |
| *Vis* | *Visible* |
| *VLDL* | *Very low density lipoprotein* |
| *VMAE* | *Vacuum microwave-assisted extraction* |
| *W* | *Watt* |
| *wb* | *Wet basis* |
| *WHO* | *World Health Organisation* |
| *w/v* | *Weight/ volume* |
| *Zn* | *Zinc* |

# CHAPTER 1: **GENERAL INTRODUCTION**

Amaranth (*Amaranthus* spp.), has been consumed throughout history by Inca, Maya and Aztec civilisations of America. It is a rediscovered crop, which observed an increase in interest since the 1980s, when research by the United States National Academy of Sciences acknowledged the high nutritional value and agronomic potential of this pseudo-cereal (Monteros et al., 1998; Ulbricht et al., 2009). Currently, it is cultivated in central and south America as well as in some parts of Asia and Africa. This increase in its acceptance in countries where its consumption has not been traditional can be attributed to its quick growth rate, commendable tolerance to growth stresses like drought, salinity, alkalinity acidity or poor soils, and noteworthy nutritional content. From a botanical or nutritional viewpoint, amaranth shares the facets of both cereal and leguminous seed, due to its protein composition and amino acid profile which are placed almost between a cereal and a bean. Its protein content and quality is labelled as nutritionally favourable due to elevated levels of lysine as compared to other grains whereas, due to the waxy nature of its starch, it has potential to be used as a stabiliser or thickener and emulsifier in food products (Singhal, and Kulkarni, 1988; Caselato-Sousa and Amaya-Farfán, 2012).

The lipophilic content of grain amaranth is greater (6 - 9%) in comparison to other cereal grains. Recently it has been a major cause of interest by the scientific community due to its chemopreventive properties pertaining to its antidiabetic, cholesterol-lowering & antilipemic, antihypertensive, immunomodulatory, hepatic health improving, anticarcinogenic effects and antioxidant characteristics. These effects can be accredited to its high nutraceutical and cosmeceutical value ingredients including squalene, sterols, α-tocopherol and polyphenols (Czaplicki et al., 2011). The squalene content of amaranth grain oil is exceptionally high (Escudero et al., 2006), just next to shark liver oil, which makes it a potential candidate for cosmetic formulations as squalene possesses emollient properties which assists in rejuvenating the suppleness and flexibility of the skin and also guards the human skin surfaces from lipid peroxidation caused by UV light exposure and other oxidative damage sources (Kohno et al., 1995).

The extraction technique used for eluting out these important bioactive ingredients from plants plays a key role that determines the best quality of the extract obtained at lowest cost so that it can be adopted for industrial purposes.

Until present, Soxhlet has been the most prominently adopted conventional extraction technique used for leaching out the lipophilic constituents from amaranth grain (Lyon and Becker, 1987; Singhal and Kulkarni, 1988; Jahaniaval et al., 2000; León-Camacho et al., 2001; He et al., 2003; Gamel et al., 2007). However, it is a very time and solvent consuming technique which leads to an incomplete extraction from the sample matrix (Luque de Castro and Garcia-Ayuso, 1998). Therefore, there has been an increase in the exploration of novel extraction technologies for this purpose. Microwave assisted extraction (MAE) is a potential alternative to conventional extraction technologies as it takes shorter time, consumes less solvent, coupled with higher extraction rates with better product quality (Delazar et al., 2012).

## 1.1 HYPOTHESIS

There are diverse research studies that have compared MAE with conventional extraction methodologies and attested MAE as a more commercially feasible extraction technique (Hao et al., 2002; Lucchesi et al., 2004; Talebi et al., 2004; Barbero et al., 2006; Wang et al., 2006; Hemwimon et al., 2007). It is consequently hypothesised that MAE would be better or a comparably viable process for extracting lipids from grain amaranth than standard conventional extraction, not just limited to higher oil yield with reduced extraction time and solvent consumption but also preserving the best quality of the high value constituents including squalene, stigmasterol, α – tocopherol and total tolyphenol with greater free radical-scavenging activity.

## 1.2 OBJECTIVES

The principal objective of this research was to study and design an optimised method for eluting lipophilic components of grain amaranth. The following specific objectives involved:

- To conduct pre-optimisation studies for the selection of operating parameters, namely sample particle size [whole amaranth grain and amaranth grain flour (AGF)], extraction solvent and microwave input power. A full factorial design was employed for screening to identify the key parameters which can determine the experimental domain required to optimise the extraction of oil and its constituents, squalene & stigmasterol by central composite rotatable design (CCRD). The microwave absorbing capabilities for solvents were examined by calculating the microwave output power, for a better understanding of the effects of solvents on the overall efficiency of MAE.

- To augment two consecutive CCRDs for optimising the extraction yield of oil and it's constituent's squalene and stigmasterol from AGF. The scope of the study was to optimise the combination of factors which results in maximising responses.

- To compare the outcomes of optimised MAE process with optimised Soxhlet extraction quantitatively (oil yield %) and qualitatively (squalene, stigmasterol, polyphenols, α – tocopherol and DPPH free radical-scavenging activity).

## PREFACE TO CHAPTER 2

In **Chapter 1**, scientific illustrations were made that led to the study presented in this thesis. In **Chapter 2**, a review of literature is presented that brings attention to the amaranth's general characteristics, its composition and nutritional properties; health benefits and industrial applicability of amaranth grain oil; pioneering conventional techniques for extracting lipids from grain amaranth; and an overview of microwave heating, the mechanisms of microwave assisted extraction and its instrumentation.

# CHAPTER 2: **REVIEW OF LITERATURE**

## 2.1 CHARACTERISTICS OF AMARANTH

### 2.1.1 TAXONOMY

Amaranth belongs to the class *Dicotyledoneae*, subclass *Caryophyllidae*, order *Caryophyllales*, amaranth family *Amaranthaceae*, sub family *Amaranthoideae*, genus *Amaranthus*. There are more than 60 species within the genus *Amaranthus*, most of which are cosmopolitan weeds and cultivated amaranth species which can be used as food grain, leafy vegetables, forage and ornamentals (Sauer, 1967). The cultivated amaranth species which can be utilized for human consumption are divided into grain and vegetable amaranths. Even though it produces cereal-like and starch-rich seeds, it is not considered as a part of the cereal family (Saunders and Becker, 1984).

#### 2.1.1.1 GRAIN AMARANTH

The four principal *Amaranthus* species which are considered as a group of grain or pseudo-cereal, include:

- *Amaranthus caudatus L.* (subsp. *caudatus*; subsp. *mantegazzianus* (Passer) Hanelt, syn. *Amaranthus edulis* Spegazzini, named love liebleeding and Inca wheat, respectively.
- *Amaranthus cruentus L.* (syn. *A. paniculatus L.*) – bush greens, red amaranth.
- *Amaranthus hypochondriacus L.* (syn. *Amaranthus leucocarpus*, *Amaranthus frumentaceous*) – prince's feather.
- *Amaranthus hypochondriacus L.* x *Amaranthus hybridus L.* (Plainsman)

Within these species many more varieties and subspecies exist (Bale and Kaufmann, 1992; Williams and Brenner, 1995; Grobelnik Mlakar et al., 2010).

### 2.1.1.2 VEGETABLE AMARANTH

Most of the *Amaranthus* species have edible leaves which have a mild spinach-like flavour. These are normally used as potherbs and principally consumed as boiled greens. In some parts of humid tropics of Asia and Africa, *Amaranthus* greens are a widely eaten vegetable because of their high yields, ability to grow in hot weather and their high nutritive value. Commonly found species are *Amaranthus tricolor L.* (Chinese salad); *Amaranthus blitum L.*; *Amaranthus creuntu L.*; *Amaranthus dubius L.*; *Amaranthus lividus L.* [subsp. *Amaranthus ascendens* (kitchen amaranth); subsp. *Amaranthus oleraceus* (vegetable amaranth, Chinese spinach; subsp. *Amaranthus lividus*)]; *Amaranthus palmeri*; *Amaranthus hybridus*; *Amaranthus viridis L.*; *Amaranth gracilis* Desf.; *Amaranthus gangeticus L.*; (Franke, 1989; Grobelnik Mlakar et al., 2010; Mburu et al, 2011).

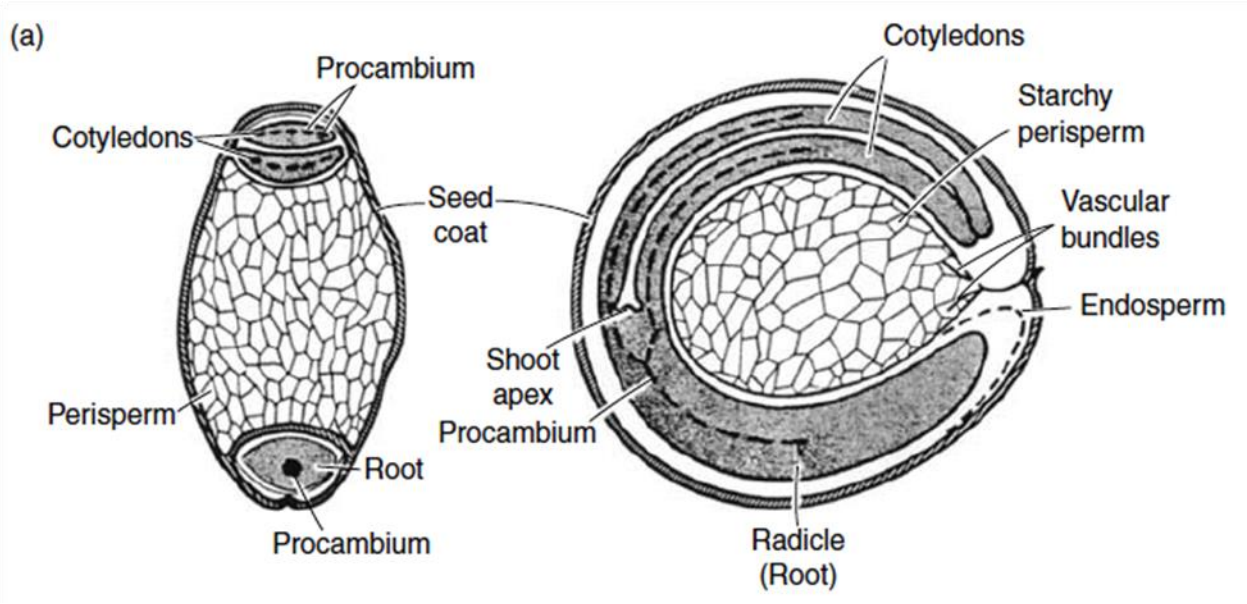
### 2.1.2 PHYSIOLOGY

Grain amaranth is an annual herbaceous plant, which uses the C4 photosynthesis pathway by using carbon dioxide very efficiently under a wide range of temperature (from 25 to 40°C), higher light intensity and moisture stress environmental conditions, by fixing carbon dioxide in the chloroplasts of specialised cells surrounding the leaf's vascular bundles. This characteristic is responsible for lower water losses by transpiration through the stomata. Amaranth being a C4 plant also has the capacity to photosynthesise at high rates and at high temperatures, through osmotic adjustments, which helps it in tolerating lack of water to some extent without wilting (Bressani, 1993; Kigel, 1994). These characteristics make amaranth a crop of choice to adapt to climate change and ensure food security.

### 2.1.3 MORPHOLOGY

The stature of the amaranth plant varies notably depending upon species and environment. For example, individual cultivars can vary in height from 91 to 274 cm and have stem diameters from 2.54 to 15 cm, depending upon plant stand density and available soil moisture. Additionally, seed heads have varied from 30 to 112 cm in diameter at the base and varied in

height from 13 to 61 cm (Irving et al., 1981). Grain type species of amaranth plants possess a main stem axis that terminates in a large apical branched inflorescence. The flowers of amaranth are unisexual, developed on branched flower clusters (glomerules) and found in varied colours such as purple, orange, red or gold. A glomerule is described as a dichasial cyme that forms large flowering panicles. Vegetable species of amaranth usually have a smooth leaf, with an uncertain growth habit which produces new luscious axillary growth. The leaves are usually elliptical, with an acute tip and a cuneate base, whereas the size of the leaf varies considerably between and within species. The flowers are indefinite inflorescences. The buds of flower emanate directly in the leaf axils (Brenner and Hauptli, 1990). The seeds are very small (0.9 to 1.7 mm diameter), with 0.5 - 1.2 g per 1000 seeds or 1,000 to 3,000 seeds/g, approximately 30 - 70 times smaller than a typical wheat grain. Seed coat colour varies from black, brown, yellow, and white. As depicted in **Figure 2.1**, the seed embryo is campylotropous, circular, with the ends nearly touching and enclosing the perisperm (Irving et al., 1981; Becker 1994).




Cross-section Longitudinal section

**(b)**

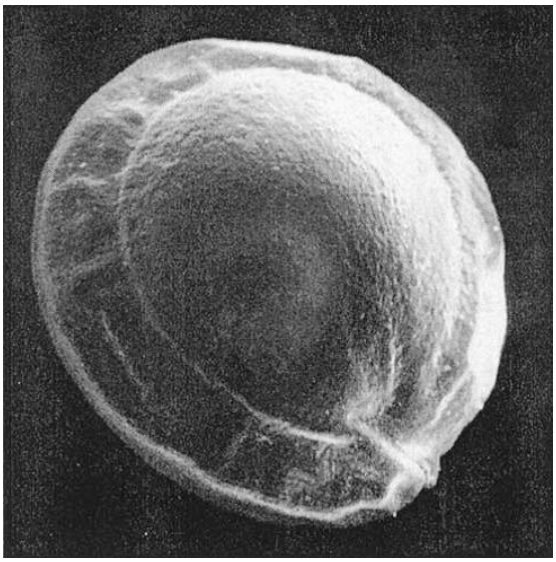


**Figure 2.1: (a) Illustration of *Amaranthus cruentus* seed in cross and longitudinal sections (b) Scanning electron micrograph of a seed of *Amaranthus cruentus* Adapted from Irving et al., (1981) and Becker (1994).**

## 2.2 COMPOSITION AND NUTRITIONAL PROPERTIES OF GRAIN AMARANTH

### 2.2.1 CARBOHYDRATE

#### 2.2.1.1 STARCH

Starch is the main carbohydrate component of grain amaranth which is located in the cells of the perisperm (Wu and Corke, 1999). Starch is the main component of grain amaranth seeds accounting between 48 and 69 % (Qian and Kuhn, 1999) in the form of starch granules, therefore it is the starch which dominates the characteristics of amaranth products. The size of starch granules are small varying between 0.5 - 3.0 µm in diameter and are spherical, angular or polygonal in shape usually of uniform size (Lopez et al., 1994; Uriyapongson and Rayas-Duarte, 1994; Radosavljevic, 1998). The starch granules located in the cells of the cotyledon region form greater agglomerates with a size of about 80 µm, consisting of several single granules (Zhao and Whistler, 1994; Walkowski et al, 1997).

The two types of starch are amylose and amylopectin. Amylose is a linear biopolymer of α-d-(1,4)-glucosyl units with few branches connected by α-d-(1,6)-glucosyl linkage, while amylopectin has much higher density of branching attached by α-d-(1,6)-glucosyl linkage in the main α-d-(1,4)-glucosyl chains (Tang et al., 2006). Amaranth starch contains higher amylopectin 92 - 95% (Choi et al., 2004) and lower amylose at 1 - 8% contents (Capriles et al., 2008; Kong et al., 2009) depending on different genotypes (Hoover et al., 1998). Due to its high amylopectin content, amaranth starch is classified as a "waxy starch" (Lopez et al., 1994). The waxy starch possesses a high water binding capacity, high swelling power, easy gelatinisation, high enzyme susceptibility, good freeze–thaw and retrogradation stability (Baker and Rayas-Duarte, 1998A). These properties support the use of amaranth starch as a stabiliser and thickener in food products and as an emulsifier for salad dressings (Jobling, 2004).

There are many publications (Paredes – Lopez et al., 1994; Lopez et al., 1994; Uriyapongson & Rayas-Duarte, 1994; Wu et al., 1995; Baker and Rayas-Duarte, 1998B; Hoover et al., 1998; Radosavljevic et al., 1998; Choi et al., 2004; Marcone, 2001; Kong et al., 2008; Kong et al.,

2009) that discuss the viscosity properties of amaranth starch with varying results difficult to compare. Nonetheless, a common characteristic indicates that the viscosity and gelatinisation behavior of amaranth starch differs from most of the cereal starches and is comparable with waxy maize starch (Paredes – Lopez et al., 1994; Lopez et al., 1994 and 1998B).

Digestion of amaranth starch shows efficient absorption in the human intestinal tissues, which is beneficial for formula-fed infants and seniors having reduced digestive capacity (Dreher et al., 1984). According to Yanez et al., (1986) the digestibility of gelatinised amaranth starch (63.5%) is much higher than that of maize starch (35%). Capriles et al., (2008) found that the digestible starch, hydrolysis index, and predicted glycemic index are significantly increased by popping, roasting, and flaking processes, suggesting that processed amaranth, would have fast and complete starch digestion. Capriles et al., (2008) also reported that the cooked, popped, and extruded amaranth seeds had starch digestibility similar to that of white bread, while flaked and roasted seeds had a slightly greater capacity to increase glycemic response.

#### 2.2.1.2 RESISTANT STARCH

Resistant starch (RS) is a non-naturally occurring starch product that is produced due to starch degradation as a result of processing and that escapes digestion in the small intestine of healthy individuals as it is not susceptible to human digestive enzymes and thus reaches the colon, where it is fermented by the bacterial biota along with dietary fiber (Champ et al., 2003). Like other dietary fiber, it has been found to have similar health benefits, such as lowering blood lipids or lowering the risk of colon cancer. The RS content depends on the starch attributes present in food, type of granule, amylose/amylopectin ratio and crystallinity of starch, and the analytical method used.

Pederson et al., (1987) recorded a slight increase in the dietary fiber and a decrease in the starch content following the heat treatment (toasting, popping) of amaranth, however they couldn't explain the reason behind this rise in dietary fibre, but later studies attributed it to the formation of resistance starch by these treatments. The study by Gonzalez et al., (2007), revealed an increase in RS content of amaranth from 0.65% (untreated) to 5.95% (at a treatment

temperature of 150°C with 120 g/kg wb moisture content) by fluidised bed heat treatment. On the other hand, Gamel et al., (2005) observed a low RS content as a result of cooking and popping of amaranth as compared to untreated samples of amaranth. Mikulikova and Kraic (2006) estimated the RS content in amaranth using an enzymatic method (AACC Method 32 - 40) studying 18 genotypes and determined a wide variation in the resistant starch content of the seeds. Linsberger-Martin et al., (2012) also found an increase in resistant starch content of amaranth under the effect of high hydrostatic pressure as compared to untreated ones.

#### 2.2.1.3 FIBER

Fiber is responsible for health benefits irrespective of its type including dietary fiber, soluble and insoluble. Information from the literature reports great variations in fibre content in different species of amaranth which are comparable with other cereals. The amaranth bran fraction dominates over the perisperm in terms of dietary fibre content (Betschart et al., 1981). The dietary fibre content of amaranth is between 9 to 16%. The fraction of soluble dietary fiber varies between 20 and 28% in *Amaranthus cruentus* and 33 and 49% in *Amaranthus hypochondriacus* (Bressani et al., 1990).

### 2.2.2 PROTEINS

The nutritional value of pseudocereals is considered superior to cereal grains due to their higher protein content. The protein of grain amaranth is mainly concentrated in the germ and seed coat, which accounts for 65% of the total proteins, while the rest, 35%, is found in the starch-rich endosperm (Saunders and Becker, 1984).

**Table 2.1: Protein distribution in physical fractions of grain amaranth and some cereal grain (%) (Bressani, 1989)**

| Grain | Germ | Endosperm |
|---|---|---|
| Amaranth | 65.0 | 35.0 |
| Maize | 18.5 | 81.5 |
| Sorghum | 15.2 | 84.8 |
| Rice | 12.5 | 87.5 |

As shown in **Table 2.1**, the germ fraction of the seed has a higher protein concentration than in the endosperm. Since the germ proteins are the significant sources of essential amino acids, as compared to the storage proteins of the endosperm, this yields a higher concentration of lysine in amaranth grain compared to cereal grains in general (Bressani, 1989).

The protein content of grain amaranth varies between 11 to 18% dry matter, which is more than most of the common grains except soybean and it is well comparable with protein and energy requirements as stated in the report of a Joint FAO/WHO (1973) Ad Hoc Expert Committee (Bressani et al., 1987A; Bressani et al., 1987B; Imeri et al., 1987).

The digestibility of raw amaranth protein ranges between 74 - 80%. However, protein efficiency ratio and digestibility can be considerably enhanced if the grain is heat processed (Garcia et al., 1987). Heat processing also results in the denaturation of trypsin inhibitors and other anti- nutritional substances (Imeri et al., 1987).

#### 2.2.2.1 AMINO ACIDS

The amino acid profile of amaranth includes 5% lysine, and 4% sulphur amino acids, which are the limiting amino acids in other grains. The significant presence of lysine is the main factor which determines the high quality of amaranth protein. The sulphur amino acid content of amaranth is significantly higher than that of soybean (Saunders and Becker, 1984; Teutonico and Knorr, 1985). The essential amino acid composition of three major *Amaranthus* species is presented in **Table 2.2**.

**Table 2.2: Essential amino acid contents of major grain species of amaranth (g/100 g protein) (Saunders and Becker, 1984; Segura-Nieto, 1994; Senft, 1979)**

| Amino Acid | *Amaranthus caudatus* | *Amaranthus cruentus* | *Amaranthus hypochondriacus* | Amaranth (Mean) | FAO/WHO (1973) (Requirements) |
|---|---|---|---|---|---|
| Isoleucine | 3.6 - 4.1 | 3.4 - 3.7 | 2.8 - 3.8 | 3.0 | 4.0 |
| Leucine | 5.9 - 6.3 | 4.8 - 5.9 | 5.0 - 5.8 | 4.7 | 7.0 |
| Lysine | 5.7 - 6.4 | 4.8 - 5.8 | 3.2 - 6.0 | 5.0 | 5.5 |
| Methionine | 2.4 - 3.3 | 1.8 - 2.6 | 0.6 - 1.6 | | |
| Phenylalanine | 3.4 - 4.0 | 3.2 - 4.5 | 3.8 - 4.5 | | |
| Threonine | 3.8 | 3.2 - 4.2 | 2.6 - 4.3 | 2.9 | 4.0 |
| Tryptophan | 1.1 | | 1.1 - 4.0 | 1.3 | 1.0 |
| Tyrosine | 2.8 | 2.4 - 4.0 | 3.1 - 4.0 | | |
| Valine | 4.1 - 4.7 | 3.9 - 4.3 | 3.2 - 4.2 | 3.6 | 5.0 |
| Methionine + Cysteine | 4.7 | 3.8 - 5.4 | 2.6 - 5.5 | 4.4 | 3.5 |
| Phenylalanine + Tyrosine | 6.2 | 5.6 - 8.5 | 6.9 - 8.5 | 6.4 | 6.0 |

### 2.2.2.2 STORAGE PROTEINS

Proteins within seeds are mainly categorised into four types based on their solubility: albumin, globulin, prolamin, and glutelin. Amaranth protein accounts for albumin as a major fraction (48.9 - 65%), followed by glutelins (22.4 - 42.3%), globulins (13.7 - 18.1%), and a minor amount of prolamins (1.0 - 3.2%). Albumins and globulins account for about 60% of the total nitrogen. The major amaranth protein is probably globulin – P (Konishi et al., 1985). As per the Nutritionist's Protein Value Chart proposed by FAO/WHO: a score of 100 is ideal, among which amaranth protein scored highest (score = 75) as compared to cow's milk (score = 72) followed by soybean (score = 68), wheat (score = 60), peanuts (score = 52) and corn (score = 44) (FAO/WHO, 1973).

The study by Gorinstein et al., (2004) reported a close association between protein fractions of amaranth and soybeans. The prolamin content demonstrates dissimilarities with cereals, whereas the glutelin fraction showed some similarities to maize (Gorinstein et al., 2001; 2004). The globulins can be differentiated into two categories: 7S and 11S globulins based on their sedimentation coefficient. Amaranth grains do have similar 7S (conamaranthin) and l1S

(amaranthin) storage globulins (Marcone et al., 1994; Martinez et al., 1997; Marcone, 1999). Thermal treatments cause a reduction in the water-soluble protein fraction (albumins and globulins) and alcohol-soluble fraction (prolamins) (Gamel et al., 2005). It can be concluded that the amaranth proteins are similar to the seed proteins in other dicotyledonous crops such as legumes, and have no relationship to the major prolamins of cereals.

## 2.2.3 LIPIDS

Another component of much interest in grain amaranth is its lipid content, which is present in higher quantity (5 - 8%) than in cereal grains; moreover it is the richest source of squalene among plant sources (Singhal and Kulkarni, 1988). The lipid content of amaranth is characterised by a high content of unsaturated fatty acids, with a very high content of linoleic acid. The lipid content is higher in the germ and seed coat than in the perisperm (Betschart et al., 1981; Becker, 1994). **Tables 2.3** and **2.4** present the crude fat percentage of major grain species of amaranth and the lipid profile of amaranth oil and grain respectively.

**Table 2.3: Crude fat percentage of major grain species of amaranth (Budin et al., 1996)**

| Amaranth Specie | Crude Fat Percentage[a] |
|---|---|
| *Amaranthus caudatus* | 6.6 – 6.8 |
| *Amaranthus cruentus* | 6.3 – 7.8 |
| *Amaranthus hypochondriacus* | 4.7 – 7.0 |
| *Amaranthus hypochondriacus* x *Amaranthus hybridus* (Plainsman) | 7.2 |

**[a]Standard deviation is considered while calculating the crude fat percentage**

**Table 2.4: Lipid profile of amaranth grain oil and amaranth grain (g/100 g) (Amaranth Institute, 2001[a]; USDA, 2012[b])**

| Lipid | Amaranth Grain Oil[a] | Amaranth Grain[b] |
|---|---|---|
| Fatty acids, saturated | 23.2 | 1.459 |
| 14:0 | 0.2 | |
| 16:0 | 20.2 | |
| 18:0 | 3.0 | |
| Fatty acids, monosaturated | 22.5 | 1.685 |
| 16:1 | | |
| 18:1 | 25.5 | |
| 20:1 | | |
| Fatty acids, polysaturated | 49.8 | 2.778 |
| 18:2 | 49.5 | |
| 18:3 | 0.2 | |
| Cholesterol | 0.0 | 0.0 |
| Squalene | 6.5 | |
| Vitamin E | 11.0 | |

### 2.2.3.1 FATTY ACID PATTERN

Amaranth grain has high levels of unsaturated lipids, which account for 76% of the total fatty acids present, with the saturated/unsaturated fatty acid ratios ranging from 0.12 to 0.50. Studies have revealed that due to the high unsaturated fatty acid composition of amaranth grain oil it is capable of controlling hyperlipidemia, hypertension, obesity, and glucose intolerance (Martirosyan et al., 2007).

The major fatty acids in amaranth oil are linoleic, oleic and palmitic acids. The ranges in fatty acid profiles of amaranth oil for different species are as follows: linoleic (25 - 62%), oleic (19 - 35%), palmitic (12 - 25%), stearic (2 - 8.6%), and linolenic (0.3 - 2.2%) acid (Cai et al., 2004; Grobelnik Mlakar et al., 2009). **Table 2.5** presents the fatty acids profiles of major amaranth species.

**Table 2.5: Average fatty acid profiles of amaranth grain species, compared with means of seven other grains (Budin et al., 1996)**

| Amaranth Species & Other Grains | Fatty Acid (%) | | | | | | S/U Ratio[a] |
|---|---|---|---|---|---|---|---|
| | Palmitic 16:0 | Stearic 18:0 | Oleic 18:1 | Linoleic 18:2 | Linolenic 18:3 | All Others | |
| *Amaranthus caudatus* | 18.3 | 3.1 | 28.0 | 35.6 | 0.3 | 14.7 | 0.34 |
| *Amaranthus cruentus* | 15.8 - 20.1 | 3.2 - 3.8 | 20.9 - 28.3 | 37.0 - 43.0 | 0.0 - 0.7 | 6.1 - 20.7 | 0.32 - 0.34 |
| *Amaranthus hypochondriacus* | 17.9 - 21.8 | 2.8 - 3.5 | 16.3 - 29.8 | 41.1 - 52.5 | 0.0 - 0.3 | 5.1 - 16.8 | 0.34 - 0.36 |
| *Amaranthus hypochondriacus* x *Amaranthus hybridus* (Plainsman) | 19.1 | 3.2 | 24.7 | 43.0 | 0.5 | 9.5 | 0.33 |
| Amaranth (mean) | 18.5 | 3.2 | 22.0 | 44.8 | 0.2 | 11.3 | 0.33 |
| Barley HW | 21.0 | 0.0 | 15.6 | 52.4 | 5.6 | 5.4 | 0.29 |
| Barley Robust | 18.5 | 0.0 | 15.2 | 54.0 | 5.5 | 6.8 | 0.25 |
| Buckwheat | 18.2 | 0.0 | 36.4 | 34.8 | 0.0 | 10.6 | 0.26 |
| Corn | 10.3 | 0.6 | 21.4 | 62.9 | 0.5 | 4.3 | 0.13 |
| Lupin | 9.0 | 0.0 | 57.5 | 16.7 | 10.9 | 5.9 | 0.11 |
| Oats | 17.3 | 0.0 | 39.8 | 38.5 | 0.0 | 4.4 | 0.22 |
| Wheat | 15.4 | 0.0 | 22.3 | 54.2 | 3.5 | 4.6 | 0.19 |

**[a]S/U ratio = saturated/unsaturated ratio = (16:0 + 18:0)/ (18:1 + 18:2 + 18:3)**

### 2.2.3.2 PHOSPHOLIPIDS

Phospholipids component of amaranth grain oil accounts for around 5% (Becker, 1994). In a study conducted by Opute (1979), the phospholipids were evaluated at 4% in amaranth oil, in which the fractions of cephalin, lecithin and phosphoinositol were found to be 13%, 16% and 8% respectively. However, lecithin is a mixture of many phopholipids that acts as an emulsifier, enabling oils to form a colloid with water and emulsions made from lecithin are primarily used as stabilisers for drug delivery and intravenous nutrition in pharmaceutical industries. Currently lecithin from egg-yolk and soybeans are used as a food additive in many products and can be purchased as a dietary supplement (Dickinson et al., 1993).

### 2.2.3.3 TOCOLS

The tocols have been admitted to have antioxidant (protects against various pollutants, peroxides, and free radicals), anti-inflammatory (aids in treatment of asthma and arthritis), antithrombolytic (dissolves blood clots), anticancerous (retards growth and/or proliferation of some types of cancer cells) properties and prevents from cardiovascular diseases (checks cholesterol biosynthesis) (Constantinides et al., 2006). The tocol (tocopherols and tocotrienols) content of amaranth varies from one variety to another. It has been reported that the tocol content of amaranth is approximately equal to that present in olive oil (191 mg/kg oil) (Becker, 1994), while others have evaluated the tocol content at about 1465.15 mg/kg (Qureshi et al., 1996). Budin et al. (1996) found an even higher content of about 2000 mg/kg tocols in amaranth oil. As presented in **Table 2.6**, it is evident that the vitamin E content of amaranth is not unique in relation with other grains; however, the total tocol content of amaranth is only lower than that of lupin and wheat (Budin et al., 1996).

**Table 2.6: Tocopherol profiles of amaranth grain species and other grains (mg/100 g seed, wb) (Budin et al., 1996)**

| Amaranth Species & Other Grains | α Tocopherol | α Tocotrienols | β Tocopherol | β Tocotrienols/ γ Tocopherol | γ Tocotrienols | δ Tocopherol | δ Tocotrienols | Total |
|---|---|---|---|---|---|---|---|---|
| *Amaranthus caudatus* | 1.47 | 0.09 | 1.65 | 0.16 | 0.00 | 0.37 | 0.00 | 3.74 |
| *Amaranthus cruentus* | 1.81 - 2.95 | 0.00 - 0.05 | 1.01 - 2.14 | 0.14 - 0.40 | 0.00 | 0.11 - 0.77 | 0.00 – 0.03 | 3.15 - 5.75 |
| *Amaranthus hypochondriacus* | 0.78 - 2.11 | 0.00 - 0.11 | 1.50 - 2.68 | 0.17 - 0.68 | 0.00 - 0.06 | 0.25 - 2.05 | 0.00 - 0.03 | 2.81 - 6.91 |
| *Amaranthus hypochondriacus* x *Amaranthus hybridus* (Plainsman) | 2.02 | 0.00 | 1.31 | 0.39 | 0.00 | 0.99 | 0.00 | 4.71 |
| Amaranth (mean) | 1.66 | 0.04 | 2.17 | 0.29 | 0.00 | 0.78 | 0.00 | 4.94 |
| Barley HW | 0.89 | 0.85 | 0.18 | 0.35 | 0.46 | 0.07 | 0.00 | 2.80 |
| Barley robust | 0.93 | 0.67 | 0.14 | 0.36 | 0.35 | 0.03 | 0.00 | 2.48 |
| Buckwheat | 0.46 | 0.04 | 0.00 | 2.89 | 0.00 | 0.15 | 0.00 | 3.54 |
| Corn | 0.89 | 0.24 | 0.18 | 2.85 | 0.47 | 0.10 | 0.00 | 4.73 |
| Lupin | 0.62 | 0.05 | 0.25 | 6.12 | 0.17 | 0.18 | 0.00 | 7.39 |
| Oats | 1.00 | 0.54 | 0.15 | 0.42 | 0.00 | 0.03 | 0.00 | 2.14 |
| Wheat | 1.13 | 0.12 | 0.43 | 6.20 | 0.00 | 0.00 | 0.00 | 7.88 |

### 2.2.3.4 SQUALENE

Squalene is a highly unsaturated all-trans linear terpenoid hydrocarbon with shark, whale, deep-sea dogfish liver oil as its traditional sources (Trautwein et al., 1997; Cai et al., 2004; Naziri et al., 2011). In addition to its presence in fish liver oil, it is a constituent part of human skin surface lipids, particularly sebum (~500 µg/g dry weight), as well as abundant in adipose tissue (~300 µg/g dry weight), levels being dependent on age. While the richest plant source of squalene is amaranth seed oil, which contains approximately 3 to 7% of squalene, a considerably higher amount than usually found in oils from other cereal grains. Squalene has an application in pharmaceutical and cosmetics industries as well as uses as an oxidation-resistant industrial lubricant (Becker et al., 1981; Lyon and Becker, 1987; Becker, 1994; Qureshi et al., 1996; Bruni

et al., 2001; Escudero et al., 2004; Escudero et al., 2006). **Table 2.7** presents the oil content as well as squalene in oil content of different species of amaranth.

**Table 2.7: Oil and squalene in oil percentage of some amaranth species and other seeds (Lyon and Becker, 1987; Becker, 1994; He et al., 2002; He and Corke, 2003; Gamel et al., 2006)**

| Amaranth Species and Others | Oil (%) | Squalene in Oil (%) |
|---|---|---|
| *Amaranthus caudatus* | 7.1 | 4.8 |
| *Amaranthus cruentus* | 1.9 – 8.5 | 3.32 – 4.93 |
| *Amaranthus Hypochondriacus* | 3.03 – 5.97 | 4.74 – 6.98 |
| *Amaranthus tricolor* | 5.08 | 6.14 |
| Corn | 4.0 | 0.03 |
| Cottonseed | 7.0 | 0.01 |
| Rice | 1.0 - 3.0 | 0.3 |
| Olive | 36.0 | 0.4 |
| Peanut | 47.0 | 0.03 |

### 2.2.4 VITAMINS

Usually, amaranth is not considered as a significant source of vitamins (**Table 2.8**). It was stated by Souci et al., (1994), that amaranth has higher thiamine content than wheat, whereas Bressani, (1994) reported a lower content of thiamine in amaranth than in most common cereals. However, undoubtedly amaranth is a good source of riboflavin (vitamin $B_2$), ascorbic acid (vitamin C), folic acid and tocopherol (vitamin E) (Dodok et al., 1994; Gamel et al., 2006). Higher vitamin E content leads to enhanced anti-oxidative effects, which in turn increases the stability of the oil.

**Table 2.8: Vitamin composition of grain amaranth**
**USDA, (2012)**

| Vitamins | Value/100 g |
|---|---|
| Vitamin A (International units) | 2.0 IU |
| Vitamin $B_1$ | 0.116 mg |
| Vitamin $B_2$ | 0.200 mg |
| Vitamin $B_3$ | 0.923 mg |
| Vitamin $B_6$ | 0.591 mg |
| Vitamin $B_{12}$ | 0.0 µg |
| Folic acid (Dietary folate equivalent) | 82 µg |
| Vitamin C (Total ascorbic acid) | 4.2 mg |
| Vitamin D ($D_2$ + $D_{3)}$ (International units) | 0.0 µg 0.0 IU |
| Vitamin E (α-tocopherol) | 1.19 mg |
| Vitamin K (Phylloquinone) | 0.0 µg |

### 2.2.5 MINERALS

Studies show that amaranth is a good source of minerals compared with other grains and cereals. Saunders and Becker, (1984) proposed that the bran and germ have higher ash contents than the perisperm, which accounts for 66% of the total minerals present in grain amaranth. The mineral content of amaranth is dominated by calcium, magnesium, iron, potassium, and zinc (Saunders and Becker, 1984; Pederson et al., 1987; Bressani, 1994; Yanez et al., 1994; Gamel et al., 2006). Nutritionists recommend a calcium/phosphorus ratio (Ca:P) of 1:1.5, while Bressani, (1994) showed a good value of 1:(1.9 - 2.7) in amaranth. Pederson et al., (1987) witnessed a lot of variation in the mineral content of different varieties of amaranth which were popped, flaked and toasted. The pattern of responses for each variety was different under same type of processing. **Table 2.9** presents a comparison of the mineral composition of some grain amaranths with other food grains.

**Table 2.9: Mineral composition of some grain amaranths compared with other food grain (mg/100 g) (Singhal and Kulkarni, 1988; USDA, 2012)**

| Source | Ca | P | Fe | Mg | Zn | Cu | Mn | Ni | Na | K |
|---|---|---|---|---|---|---|---|---|---|---|
| *Amaranthus Caudatus* | 36 - 170 | | 3.1-8.42 | 289 | 4.0 | 0.8 | 2.22 | 0.24 | 37.0 | 580 |
| *Amaranthus Cruentus* | 24.8-389 | 525-602 | 3.8-17.4 | 244-332 | 3.7-3.96 | 0.79-1.2 | 1.92-2.60 | 1.4-2.4 | 22-45 | 290-460 |
| *Amaranthus hypochondriacus* | 137-249 | 341-647 | 7.5-21.7 | 292-363 | 3.6-3.9 | 0.6-0.8 | 1.9-2.9 | | 6.7-100 | |
| Amaranth (uncooked) | 159 | 557 | 7.61 | 248 | 2.87 | | | | 4 | 508 |
| Wheat flour | 48 | 355 | 11.5 | 128 | 2.0 | 0.5 | | | 3.0 | 370 |
| Oat | 50 | 380 | 3.8 | | | | | | | 352 |
| Rice | 10 | 160 | 3.1 | 48 | | 0.72 | | | 8.0 | 70 |
| Maize | 10 | 348 | 2.0 | 144 | | 0.19 | | | 15.9 | 286 |
| Sorghum | 25 | 222 | 5.8 | 140 | | 1.78 | | | 7.3 | 131 |
| Soybean | 240 | 690 | 11.5 | | | | | | | 167.7 |
| Bengal lentil | 56 | 331 | 9.1 | 138 | | 0.98 | | | 73.2 | |

### 2.2.6 SECONDARY METABOLITES

Unlike primary metabolites such as starch, fat, or proteins, which are mainly involved in the energy metabolism and synthetic pathways, secondary metabolites are found in minute quantity and are produced for specific functions by plants. Certain secondary metabolites have been found to have either positive or negative impact on human health through their pharmacological effects.

#### 2.2.6.1 TOTAL PHENOLIC COMPOUNDS

Many researchers have determined the polyphenolic compounds in terms of tannic acid or tannins. Tannins are the polyphenolic secondary metabolites of higher plants and are either galloyl esters and their derivatives, or oligomeric and polymeric proanthocyanidins. The tannins are mostly concentrated in the hulls of cereals and legumes. Tannins negatively affect the digestion as well as absorption processes by forming complexes with various nutrients or digestive enzymes and thus are often referred to as anti-nutritional compounds. Dark amaranth grains are found to contain more tannins than light ones (104 - 116 mg/100 g vs. 80 - 120 mg/ 100 g) (Bressani, 1994). In a study by Becker et al., (1981), ten different samples were tested and tannins were found ranging from 80 - 420 mg/ 100 g, whereas Breene (1991) established an

average value of 40 - 120 mg/100 g. However, higher values (410 - 520 mg/100 g) were recorded when acidified methanol was used as the extraction medium instead of aqueous methanol in various amaranth species (Bejosano and Corke, 1998).

Klimczak et al., (2002) evaluated the total phenolics in grain amaranth, in terms of ferulic acid (an alkali-extractable phenolic compound), between 39.17 to 56.22 mg/100 g. *Amaranthus caudatus* seeds were reported to have 27% free phenolic acids. The major compounds were caffeic acid (55.79 μg/g seeds), p-hydroxybenzoic acid (20.89 μg/g), and ferulic acid (18.41 μg/g). Low amounts of protocatechuic acid and salicylic acid were detected as well. However, thermal treatment or germination has been shown to decrease the content of phenolic compounds (Klimczak, 2002). Bunzel et al., (2005) examined the amounts of ferulic acid in amaranth's insoluble fiber and non-starch polysaccharides. Alkaline hydrolysis liberated 62 mg/100 g trans-ferulic acid and a high content (20.3 mg/ 100g) of cis-ferulic acid. Three compounds of feruloylated oligosaccharides were recognized, which implied that ferulic acid is essentially bound to pectic arabinans and galactans in the amaranth insoluble fiber.

Czerwinski et al., (2004) reported the total phenolics in terms of gallic acid equivalent (Folin-Ciocalteu reagent), anthocyanins, and flavonoids (spectrophotometrically) in two amaranth samples. The amounts of polyphenols in the amaranth samples were found to be between 14.72 to 14.91 mg/100 g seeds, that of anthocyanins from 59.6 to 62.5 mg/100 g seeds, and that of flavonoids from 13.4 to 14.3 mg/100 g seeds.

#### 2.2.6.2 SAPONINS

Saponins are strongly bitter tasting, surface active agents (surfactants), which can cause intensive foaming activity in aqueous solutions. Saponins have an ability to form complexes with proteins, lipids (e.g. cholesterol), mineral like zinc & iron and possess a hemolytic effect. Very little amount of saponins are absorbed in the human body due to their ability to form complexes. Therefore, saponins are considered as antinutrients as they reduce the bioavailability of proteins and some minerals which are beneficial for the human body (Chauhan et al., 1992).

As far as health benefiting effects are concerned, saponins are anticarcinogenic, anti-microbial, cholesterol decreasing, immune modulating and anti-inflammatory. However, prolonged ingestion of some saponins can cause intestinal corrosion, which further enhances the absorption of subsequent doses of saponin, which may cause severe health problems, potentially fatal, due to systematic toxicity (Price, et al., 1987). The amounts of saponins in grain amaranth are relatively low. Several amaranth species detected an average of 0.09% (aescin equivalents) (Dobos, 1992) and these results have been supported by studies conducted by Oleszek et al., (1999) and Caudrado et al., (1992). These studies proved the low concentration of saponins in amaranth seeds, thus ensuring the low toxicity of amaranth-derived products.

### 2.2.6.3 PHYTIC ACID

Phytic acids are considered as antinutritional compounds because, they are responsible for forming complexes with basic protein residues, causing inhibition of enzymatic digestive reactions and interference with the adsorption of minerals, in particular with zinc. Phytates are present in grain amaranth in the range of 0.2 - 0.6% (Breene, 1991; Bressani, 1994; Escudero et al., 2004; Gamel et al., 2006). As far as health benefits are concerned, phytic acid has been shown to help in reducing blood cholesterol (Thompson, 1993). The study by Bressani, (1994) showed that phytic acid in seeds is uniformly distributed and thus makes its removal from the seeds difficult. However, recent studies have demonstrated that phytate content is reduced by cooking (approximately 20%), popping (15%) and germination for 48 hour (22%), which signifies that these approaches can be applied to reduce the phytate content of amaranth (Gamel et al., 2006).

### 2.2.6.4 ENZYME INHIBITORS

The protease inhibitors, e.g. chymotrypsin or trypsin, are considered anti-nutritive as they are responsible for restricting the activity of proteolytic enzymes by competitive inhibition within the gastrointestinal tract of animals, thereby diminishing the digestibility of proteins (Liener, 1980). The content of protease inhibitors in amaranth is very low compared with other cereals. Gamel et al., (2006) detected trypsin inhibitor activity (TIU) ranging from 3.05 to 4.34 TIU/mg,

chymotrypsin inhibitor activity (CIU) ranging from 0.21 to 0.26 CIU/mg, and amylase inhibitor activity (AIU) ranging from 0.23 to 0.27 AIU/mg.

#### 2.2.6.5 PIGMENTS

The red-violet pigment hues present in the leaves, stalk/stems, flower as well as grains of various amaranth species is a result of betacyanins, which belongs to betalain pigment group. In amaranth, the betacyanin compounds are recognised as amaranthin and iso-amaranthin (Dixit et al., 1991; Stintzing et al., 2004; Repo-Carrasco-Valencia et al., 2010). The amaranthin is an intermediate compound involved in the conversion of nitrogen compounds in the cell (Gins et al., 2002). Amaranthine has the same basic structure, betanidin (aglucone), as the betacyanines from red beet. The stability of betanin is higher than amaranthine which can be important if such pigments are isolated and used as colouring agents in food (Cai et al., 1998). The literatures until now presents it as a potential colouring agent only.

## 2.3 INDUSTRIAL APPLICATIONS OF AMARANTH GRAIN OIL

### 2.3.1 AMARANTH GRAIN OIL: FOR NUTRACEUTICAL INDUSTRIES

#### 2.3.1.1 ANTIDIABETIC EFFECTS

Amaranth oil has been reported to reduce blood sugar levels in animals. The studies by Kim et al., (2006a; 2006b) have revealed that supplementing diet with amaranth oil may reduce serum glucose levels and enhance serum insulin levels in streptozotocin-induced diabetic rats and that these effects may be due to the normalization of liver function enzymes.

#### 2.3.1.2 CHOLESTEROL LOWERING AND ANTI LIPEMIC EFFECTS

Several studies (Berger et al., 2003; Shin et al., 2004; Gonor et al., 2006b; Kulakova et al., 2006; Martirosyan et al., 2007) have reported that amaranth oil may have beneficial effects in reducing blood cholesterol. Amaranth is a rich source of tocotrienols (form of vitamin E),

squalene, and plant stanols, which has been accepted to have cholesterol-lowering effects in mammals. Shin et al., (2004) and Kulakova et al., (2006) studied the effect of consuming amaranth oil by rats which exhibited a decrease in total blood cholesterol, triacylglycerol and liver cholesterol concentration. Experiments on hamster by Berger et al., (2003) showed a decrease in non-HDL cholesterol (high density lipoprotein; HDL, often called good cholesterol prevents the occurrence of cardiovascular diseases).

Gonor et al., (2006b) studied the influence of a diet containing amaranth oil on the lipid profiles and the erythrocyte fatty acid profiles of cardiac ischemia patients. The diets supplied squalene amounts of 100, 200, 400, and 600 mg/d, and the diet containing 600 mg showed significant positive changes in the cholesterol and triacylglycerol levels as well as in the composition of fatty acids of erythrocyte membranes. In addition to that, Martirosyan et al., (2007) concluded from their study that human diet containing 18 ml of amaranth oil per day contributes to an increase in the concentration of polyunsaturated fatty acids, particularly, long-chain acid of omega 3 families and decrease in total cholesterol, triglycerides, LDL (low density lipoprotein; LDL, called bad cholesterol, supports the occurrence of cardiovascular diseases) and VLDL (Very-low-density lipoprotein) in patients suffering from hypertension and coronary heart disease. Like Gonor et al., (2006b), Martirosyan et al., (2007) also proved the concentration dependent cholesterol lowering effect of amaranth oil. The interesting feature of the study by Shin et al., (2004) revealed the fact that amaranth's squalene results in hypolipidemic effect in the blood and liver, an increase in fecal and bile acid cholesterol excretion and inhibition of the HMG-CoA reductase activity, these effects were not observed with shark squalene. Therefore there has been emerging concern over the unknown role of squalene from amaranth or another associated substance present in the amaranth oil. The enzyme squalene monooxygenase has been identified to act as a regulatory site for cholesterol synthesis, and whose inhibition ultimately results in a decrease of the synthesis of cholesterol (Belter et al., 2011). If diminishing the activity of squalene monooxynase can help regulate the synthesis of cholesterol, then it is possible that an inhibitor, or inhibitors (whether direct or indirect), are being co-extracted with several of the amaranth seed fractions, including squalene itself. Should this new view be proven correct, it would also explain the intriguing observation made by Shin et al., (2004) that the squalene from amaranth is not equivalent to shark squalene.

#### 2.3.1.3 ANTIHYPERTENSIVE EFFECTS

Studies have revealed that amaranth can assist in reducing blood pressure in patients with coronary heart disease (Gonor et al., 2006b; Martirosyan et al., 2007). This may occur because amaranth oil is said to influence the fluidity of cell membranes, thereby increasing the movement of sodium and potassium ions across the cell membrane, which leads to a decrease in blood pressure (Martirosyan et al., 2007).

#### 2.3.1.4 IMMUNOMODULATORY EFFECTS

Amaranth oil was demonstrated to induce immunomodulatory effects in patients with ischemic heart disease and hyperlipoproteinemia (Gonor et al., 2006a). Recent studies have indicated that squalene, a major constituent of amaranth grain oil acts as an immunoadjuvant (Carlson et al., 2000), suggesting that amaranth may have some intrinsic immunostimulatory properties.

#### 2.3.1.5 HEPATIC HEALTH IMPROVING EFFECTS

Apart from the anti-diabetic effects of amaranth oil cited by Kim et al., (2006a; 2006b), their research also reported that feeding rats with amaranth oil resulted in a decrease (normalisation of liver function enzymes) in AST (aspartate aminotransferase) and ALT (alanine aminotransferase) enzymes. In addition to that, it improved (reduction) the TBARS (thiobarbituric acid reactive substances, lipid peroxidation and oxidative stress indexes) levels in the liver cytosol. Furthermore, diabetic animals, fed amaranth oil, also witnessed a substantial increase in fecal excretion of cholesterol, triacylglycerols, and bile acids signifying the extensive role of amaranth oil in improving hepatic health. However, human trials are required to understand its implication on human liver function.

### 2.3.1.6 ANTICARCINOGENIC EFFECTS

During the past few years, some studies have looked at the anticarcinogenic activities of squalene, a major component of amaranth oil (Senthilkumar et al., 2006). The chemoprotective effects of squalene in humans have not been studied; however animal models have been reported to show inhibitory effects of squalene on carcinogenesis (Yamaguchi et al., 1985; Murakoshi et al., 1992; Desai et al., 1996; Rao et al., 1998; Smith et al., 1998). The study by Rao et al., (1998) highlighted squalene as an effective inhibitor against colon cancer, whereas, Smith (2000) showed a reduction in tumour multiplicity in a lung tumourigenesis induced mice. Similarly, Desai et al., (1996) reported significant reduction in the incidence of skin tumour in skin-tumor induced mice by treatment with squalene doses.

This chemoprotective effect is attributed to squalene, α – tocopherol and phenolic compounds (Escrich et al., 2011; Lou-Bonafonte et al., 2012), as these bioactives are available in amaranth oil and olive oil (Czaplicki et al., 2011). It is evident from many studies (Reddy, 1992; Gerber, 1994; Martin-Moreno et al., 1994; La Vecchia et al., 1995; Trichopoulou et al., 1995; La Vecchia and Negri, 1997; Newmark, 1997 and Bartsch et al., 1999) that olive oil has a chemoprotective effect against breast, colon and pancreatic cancers.

### 2.3.1.7 ANTIOXIDANT EFFECTS

Research studies have revealed the presence of antioxidants like phenolics (Czaplicki et al., 2011); tocopherols (Berger et al, 2003; Czaplicki et al., 2011) and squalene (Lyon and Becker, 1987; Becker, 1994; He et al., 2002; He and Corke, 2003; Gamel et al., 2006) in amaranth oil. The polyphenol and tocopherols are well established antioxidants whereas squalene's antioxidant properties have only been identified in the last two decades (Saint-Leger et al., 1986; Aioi et al., 1995; Kohno et al., 1995). Due to the presence of these bioactive compounds in amaranth oil, it is hypothesised to have antioxidant properties.

### 2.3.2 AMARANTH GRAIN OIL: POTENTIAL COSMECEUTICAL INGREDIENT

Amaranth oil has a great potential to be used in cosmetic formulation due to its high concentration of the bioactive ingredient squalene. Squalene appears not to be very susceptible to peroxidation as it engulfs singlet oxygen in skin and thereby protects human skin surfaces from lipid peroxidation caused by UV light exposure and other oxidative damage (Kohno et al., 1995).

Squalene is identified as a naturally occurring emollient, which can be absorbed deep into the skin effectively to rejuvenate the suppleness and flexibility of the skin and not leaving an oily residue (Kohno et al., 1995). Blasco et al., (2006) learned about the optimal composition of squalene in an oil-in-water emulsion mixture to determine the possibility of finding new cosmetic emulsion whereas, Rissmann et al., (2008) researched on developing a skin hydrator similar to vernix caseosa [waxy or cheese-like white substance found coating the skin of newborn human babies as it facilitates passage through birth canal, moisturises infant's skin, is antibacterial in nature, conserves heat and protects the delicate newborn skin from environmental stresses (Marchini et al., 2002)], for which different lipid fractions were isolated from lanolin and consequently mixed with squalene, triglycerides, cholesterol, ceramides, and fatty acids to generate semi-synthetic lipid mixtures that mimic the lipid composition of vernix caseosa. The results showed that the rate of barrier recovery increased and was comparable to a vernix caseosa lipid treatment.

## 2.4 AMARANTH GRAIN OIL EXTRACTION

### 2.4.1 CONVENTIONAL EXTRACTION

"Solid–liquid extraction or leaching is a separation process affected by a fluid involving the transfer of solutes from a solid matrix to a solvent. From an engineering viewpoint, solid-liquid extraction of foods is a multicomponent, multiphase, un-steady state mass transfer operation" (Aguilera, 2003). This operation may be associated with the transfer of more than one chemical species – the solute like sucrose, lipids, proteins, phytochemicals and functional hydrocolloids

from a solid like cane or beets, oilseeds, oilseed meals, plants and algae respectively to a solvent (Aguilera, 2003).

Soxhlet extraction was developed in 1879 (RSC, 2007) and is still a very well-known, trusted solid-liquid conventional extraction method and remains the main reference for evaluating the performance of other solid-liquid extraction methods (Luque de Castro and Garcia-Ayuso, 1998). Later Folch et al., (1957) & Bligh and Dyer, (1959) proposed another solid-liquid extraction technique for lipids using chloroform/methanol (2:1 v/v) and water phase system. Opute, (1979) used extraction method by Folch et al., (1957) for studying the fatty acid profile of various species of amaranth [*Amaranthus hybridus, Amaranthus spinosus, Amaranthus tricolor, Amaranthus arthropurpureus*], whereas, Dhellot et al., (2006) compared Soxhlet with the methods by Folch et al, (1957) & Bligh and Dyer, (1959) to study the chemical composition and nutritional characterisation of two varieties of *Amaranthus hybridus* and found variable results. But in general Soxhlet extraction was largely used to study the lipid profiles of different amaranth species like *Amaranthus caudatus* (Gamel et al., 2007), *Amaranthus cruentus* (Lyon and Becker, 1987; Singhal and Kulkarni, 1988; Jahaniaval et al., 2000; León-Camacho et al., 2001; Gamel et al., 2007) and *Amaranthus hypochondriacus* (Jahaniaval et al., 2000). He et al., (2003) determined the oil and squalene contents of 20 different species of *Amaranthus* including *Amaranthus cruentus* and *Amaranthus hypochondriacus* using the Soxhlet technique.

Traditional extraction methods are very time and solvent consuming coupled with incomplete extraction from the sample matrix (Luque de Castro & Garcia-Ayuso, 1998). Therefore, in the past two decades, several novel extraction techniques have been developed and investigated, most of which were claimed to be better in terms of efficiency, extraction time and solvent consumption (Ondruschka and Asghari, 2006; Wang and Weller, 2006; Herrero, 2010). Microwave assisted extractions (MAE), supercritical fluid extraction (SFE), and pressurized solvent extraction (PSE) are some of the novel techniques which are available.

### 2.4.2 MICROWAVE ASSISTED EXTRACTION (MAE)

Over the years, microwave-assisted extraction has drawn significant attention among the research community in various fields, specifically plant extracts with nutraceutical significance, due to its special heating mechanism, moderate capital cost and its good performance under atmospheric conditions (Sparr Eskilsson and Björklund, 2000). Microwaves are non-ionising electromagnetic radiations with a frequency band between 300 MHz to 300 GHz, causing molecular motion through migration of ions and rotation of dipoles without altering molecular structure. Microwaves are stationed between the x-rays and infrared rays in the electromagnetic spectrum (Letellier and Budzinski, 1999) and propagate with a time interval between peaks during oscillations, ranging from $3 \times 10^{-8}$ to $3 \times 10^{-11}$ seconds (Venkatesh and Raghavan, 2004). The heating effect of microwaves depends on their frequency and on the applied power.

#### 2.4.2.1 PRINCIPLE OF MICROWAVE HEATING

The dielectric properties of materials have a major role in the heating effect of microwaves, which are characterised by two parameters, namely the dielectric constant, ε' and the dielectric loss, ε". The dielectric constant, ε', is defined as the ability of a molecule to be polarised by the electric field which at low frequencies, boost up at the amplified amount of energy that can be stored by the material. The dielectric loss, ε", describes the degree of efficiency with which the energy of the electromagnetic radiation can be converted into heat. The dielectric loss is inversely proportional to the dielectric constant (Kingston and Haswell, 1997). The dissipation factor, tan δ, is defined as the ratio of the dielectric loss of a material, also known as the "loss factor," to its dielectric constant, and measures the ability of the sample to absorb microwave energy and dissipate it as heat (Zuloaga et al., 1999).

$$\tan \delta = \varepsilon'/\varepsilon \qquad \textbf{(2.1)}$$

When a sample is exposed to a microwave, its energy penetrates the sample and is absorbed at a rate dependent on its dissipation factor. Materials that are transparent to microwave energy are considered to have infinite penetration, while zero for materials such as metals which reflect

microwave. Absorptive samples have a finite dissipation factor because microwaves dissipate into the sample; hence the energy is rapidly absorbed and dissipated as heat (Smith, 1984). The higher the dissipation factor of the sample, the lower the microwave energy will penetrate into it, at a given frequency (Zuloaga et al., 1999).

Heat is generated as a result of microwave application via two mechanisms, ionic conduction and dipole rotation, which occur simultaneously. When charged particles are allowed to move without any obstruction through a material, a current will travel in phase with the field, which is termed as ionic polarisation or ionic conduction [**Figure: 2.2 (a)**]. Contrary, if the charged particles are bound within the material, the electric field component will cause them to move until opposing forces balance the electric force. This leads in dipolar polarisation within the material. The molecular movement is extremely rapid due to the high frequency of the field, for example at microwave frequency of 2450 MHz, the polarity changes 2.45 billion cycles/ second [**Figure: 2.2 (b)**]. At this frequency, it creates intense heat that can rise as quickly as 10°C per second. Water is a dipolar molecule and a prominent component of biological materials, its content directly influences heating. The dielectric heating leads to volumetric heating of the product (Lew et al., 2002).

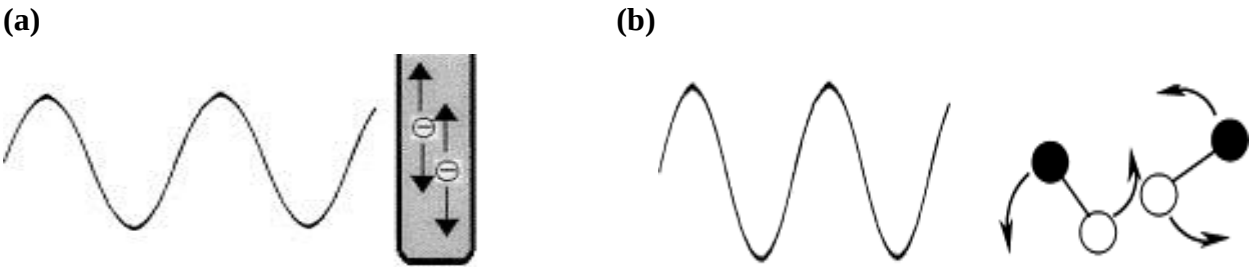



**Figure 2.2: (a) Charged particles in a solution will follow the applied electric field**
**(b) Dipolar molecules which try to align with an oscillating electric field**
**Adopted from Lidström et al., (2001)**

The heating effect caused by the ionic conduction and dipole rotation rely upon the mobility and concentration of the sample ions and on the relaxation time of the sample. When ion mobility and concentration of the sample ions both are low, in that case the heating of the sample will be exclusively influenced by dipole rotation. On the contrary, microwave heating will be governed by ionic conduction, if there is an increase in sample mobility and concentration,

furthermore ionic conduction will influence microwave heating and the heating time will not be aligned with the relaxation time of the solution. Whereas, increase in ionic concentration causes, the dissipation factor to increase and heating time to decrease. The heating time depends not only on the dielectric absorptivity of the sample but also on the particular microwave system design and sample size (Kingston and Howard, 1988).

### 2.4.2.2 MECHANISM OF MAE

The principle behind microwave assisted extraction (MAE) is different from those of conventional extraction methodologies because MAE causes changes in cell structure as a result of electromagnetic waves.

The reason behind the enhanced extraction process and yield in case of MAE may be due to the co-adjuvant of both transport phenomena (**Figure 2.3**) heat and mass gradients working in the same directions (Chemat et al., 2009).

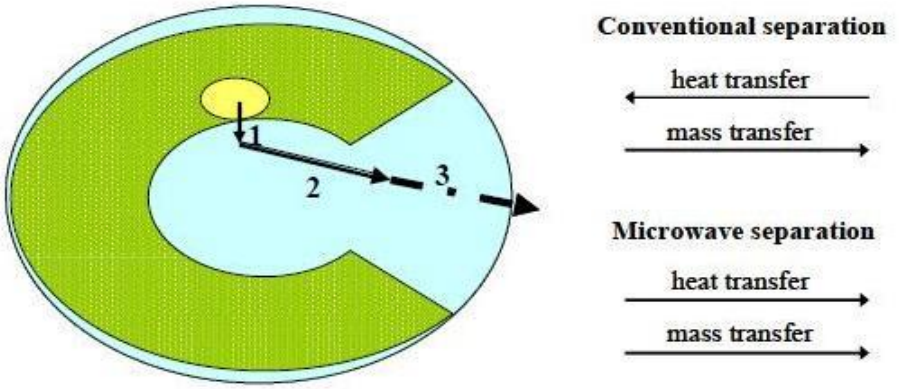



**Figure 2.3: (1) Desorption (2) Internal diffusion (3) External diffusion**
**Basic heat and mass transfer mechanisms in microwave and conventional extraction methods**
**Adopted from Périno-Issartier et al., (2011)**

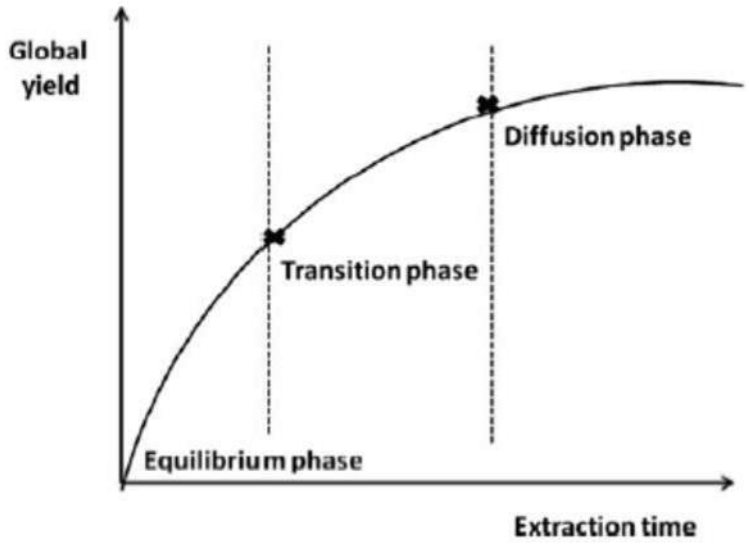



**Figure 2.4: The extraction process takes place in three different steps**
**Adopted from Raynie, (2000)**

The extraction process takes place in three different steps (**Figure 2.4**). The extraction process starts with the equilibrium phase, where the phenomena of solubilisation and partition mediate, in which the substrate is removed from the outer surface of the particle at an approximately constant velocity (Raynie, 2000). The equilibrium phase is followed by an intermediary stage called the transition phase. At the solid–liquid interface, resistance to mass transfer starts to arise; mass transfer by convection and diffusion exists (Raynie, 2000). Being the last stage of the extraction process, in the diffusion stage the solute has to break the interactions that bind it to the matrix and diffuse into the extracting solvent. The rate of extraction in this period is low, characterised by the removal of the extract through the diffusion mechanism. This point is an irreversible step of the extraction process; it is often regarded as the limiting step of the process (Raynie, 2000).

### 2.4.2.3 MAE MECHANISM IN RELATION TO BIOLOGICAL SYSTEMS

Usually, biological materials are dried prior to extraction, however, even following drying, the plant cells still contain minute microscopic traces of moisture that serve as the target for microwave heating. When a plant cell is microwave treated, the moisture inside the cell heats up,

evaporates and generates tremendous pressure on the cell wall, with swelling of the cell. The resulting pressure pushes the cell wall from inside which causes stretching and ultimately rupturing which facilitates leaching of the active constituents from the ruptured cells to the surrounding solvent thus improving the yield of extraction (Zhou and Liu, 2006). This effect can even be accentuated, if the plant matrix is permeated with solvents with higher heating efficiency under microwave. Higher temperature accomplished by microwave radiation can hydrolyse ether linkages of cellulose, which is the main constituent of plant cell wall, and can convert into soluble fractions within 1 to 2 min. During MAE, cell wall achieves higher temperature, intensifies the dehydration of cellulose and reduces its mechanical strength and this in turn helps solvent to reach compounds inside the cell (Latha, 2006). Furthermore, studies on extraction of essential oils from plant materials (Luque de Castro et al., 1999), demonstrated that MAE causes desorption of compounds of interest out of the plant matrix, this happens due to the targeted heating of the free water molecules present in the gland and vascular systems; results in drastic expansion due to localised heating followed by rupture of their walls, admitting the flow of essential oil towards the organic solvent (Garcia-Ayusa et al., 2000).

#### 2.4.2.4 MAE INSTRUMENTATION

##### 2.4.2.4.1 PRINCIPAL ELEMENTS OF MAE DEVICE

The microwave devices consist of four major components; the microwave generator: In general terms, it's referred as the "magnetron"; this component produces the electromagnetic wave at microwave frequencies. The electromagnetic wave is transported by the Waveguide; this component is responsible for the propagation of microwaves from the magnetron to the microwave cavity/applicator. The applicator is a site where the sample is placed for the microwave treatment. The circulator permits the microwaves to pass only in the forward direction (reducing damage to the microwave generator) (Mandal et al., 2007).

A fan shaped blade is used to achieve uniform randomisation of microwave radiation by reflecting and mixing the energy entering the microwave cavity from the waveguide.

This distribution of incoming energy helps the sample to be free from position restriction for heating (Luque-García and Luque de Castro, 2003).

In general, MAE systems are classified into multi-mode system and focused-mode system (mono-mode). Multi-mode systems facilitate random dispersal of microwave radiation in a cavity by a mode stirrer whereas focused systems (mono-mode) concentrate the microwave radiation on a confined section of the cavity. Generally, multi-mode systems are operated at high pressure whereas mono-mode systems operate principally under atmospheric pressure. MAE systems can operate as 'closed systems' and 'open systems' to refer to the systems that operate above atmospheric pressure and at atmospheric pressure, respectively (Dean and Xiong, 2000). Schematic diagrams of both the closed and open systems are depicted in **Figure 2.5**.

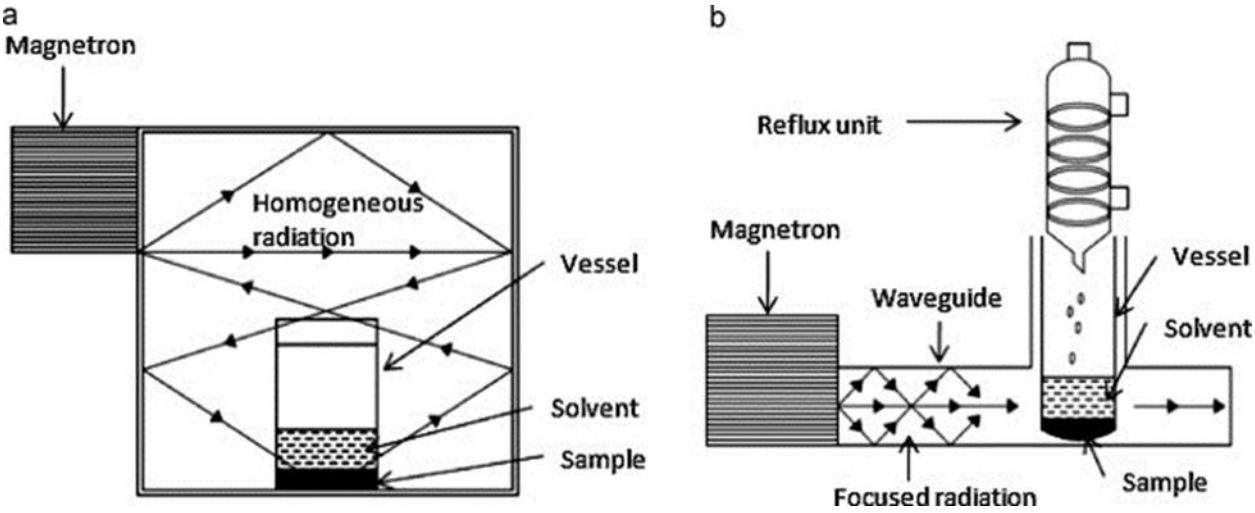



**Figure 2.5: (a) Closed type microwave system and (b) open type microwave system Adapted from Mandal et al., (2007)**

### 2.4.2.4.2 CLOSED MAE SYSTEM

The systems in which the process of extraction is carried out in a sealed-vessel, under uniform microwave heating, with different modes of microwave radiations are defined as closed MAE system. The extraction process in this system is carried out under high working pressure and temperature which results in fast and efficient extraction due to the ability of the extraction solvent to absorb microwave energy. However, the pressure inside the extraction vessel is regulated in such a way that it would not exceed the working pressure, whereas temperature is

controlled above the normal boiling point of the extraction solvent. Although, a closed system facilitates fast and efficient extraction with less solvent consumption, the thermo labile compounds with limited sample throughput are more susceptible to degradation with this system. Recent advancements in the closed system have led to the development of high pressure microwave-assisted extraction (HPMAE) (Chan et al., 2011).

#### 2.4.2.4.3 OPEN MAE SYSTEM

In open MAE systems, the process of extraction is carried out under atmospheric pressure and only a part of the vessel is directly exposed to the propagation of microwave radiation (mono mode). Unlike closed MAE system, it operates under milder conditions and hence, less safety issues and more appropriate for extracting thermo labile compounds. The upper part of the system vessel can be linked to a reflux unit to condense any vaporised solvent. It has higher sample throughput and more solvent can be added to the system at any time during the process. It is widely accepted for its application in the extraction of bioactive compounds and in analytical chemistry. Furthermore, recent development has led to the discovery of more efficient MAE systems like Nitrogen-protected microwave-assisted extraction (NPMAE), Vacuum microwave-assisted extraction (VMAE), Ultrasonic microwave-assisted extraction (UMAE), Dynamic microwave-assisted extraction (DMAE), Solvent-free microwave-assisted extraction (SFME), which can enhance the extraction yield by reducing thermal degradation and oxidation of some active compounds (Chan et al., 2011).

## PREFACE TO CHAPTER 3

A comprehensive review of literature, presented in **Chapter 2**, conferred the present state of knowledge about general characteristics of *Amaranthus* including their composition and nutritional properties; nutraceutical and cosmeceutical attributes of amaranth grain oil and its industrial potential; conventional techniques adopted for extracting lipids from grain amaranth and an overview of microwave heating, mechanism of microwave assisted extraction and its instrumentation.

**Chapter 3**, exhibits the pre-optimisation studies for the selection of operating parameters, namely sample particle size, extraction solvent and microwave input power. The key parameters which can determine the experimental domain required further optimising for the extraction of oil and its constituents, squalene & stigmasterol by central composite rotatable design (CCRD) in the later study (**Chapter 4**) which components were identified by a full factorial screening design. The microwave absorbing capabilities for different solvents combinations are also examined by calculating the microwave output power.

Part of the research study presented in **Chapter 3**, has been used to prepare a conference presentation, as follows.

**Joshi, S.P., Orsat, V. (2012). Extraction from Grain Amaranth. Northeast Agricultural and Biological Engineering Conference (NABEC), Orillia, Ontario, Canada, July 15 – 18.**

**Chapter 3** is formulated in manuscript style and is soon to be submitted for publication as follows.

**Joshi, S.P., Orsat, V., Gariépy, Y., Raghavan, G.S.V. Pre-Optimisation Studies of Microwave Assisted Extraction of Lipids from Grain Amaranth.**

The first author of the manuscript and the conference presentation, Mr. Siddhartha P Joshi, Master's candidate, Department of Bioresource Engineering, McGill University, conducted the reviews of literatures, the design of experiment, the practical experimental work in the laboratory, the statistical analysis and interpretation of results, the writing and the preparation of this manuscript for publication.

The second author, Dr. Valérie Orsat, Associate Professor and Chair, Department of Bioresource Engineering, McGill University is the thesis advisor, provided her technical expertise, and guided the first author of this manuscript throughout all the stages of planning and executing the experimental work, analysing and interpreting the results, correcting and preparing the manuscripts for publication.

The third author, Mr. Yvan Gariépy, Research Engineer, Department of Bioresource Engineering, McGill University, shared his technical expertise in relation to GC-MS.

The fourth author, Dr. G.S. Vijaya Raghavan, James McGill Professor and Graduate Program Director, Department of Bioresource Engineering, McGill University, shared his technical expertise at various levels of this study.

# CHAPTER 3: **PRE-OPTIMISATION STUDIES OF MICROWAVE ASSISTED EXTRACTION OF LIPIDS FROM GRAIN AMARANTH**

## 3.1 ABSTRACT

The research presents the studies conducted prior to process optimisation of microwave assisted extraction (MAE) of lipids from grain amaranth. It identifies the experimental domain required for resourceful optimisation. Parameter screening studies proved the significance of wisely choosing sample particle size, extraction solvent and microwave input power. Based on the outcomes of the parameter screening studies, the experimental domains of interest for a full factorial design were identified. A 4x3 full factorial design was employed for screening the following factors: microwave input power with four levels - 80, 120, 160 and 200 W; and the extraction solvent with three categories – A (MeOH:Hexane = 20:80% v/v), B (EtOH:Hexane = 20:80% v/v) and C (100% Hexane). The microwave absorbing capabilities for solvents were examined by calculating the microwave output power, for a better understanding of the effects of solvents on the overall efficiency of MAE.

*Key words: Experimental domain, screening studies, microwave input power, extraction solvents, microwave output power.*

## 3.2 INTRODUCTION

Plant cell material comprises a wide spread of bioactive compounds such as lipids, phytochemicals, flavors, fragrances and pigments, that find numerous applications in food, pharmaceutical and cosmetics industries. Extraction methodologies used for eluting out these valuable bioactive ingredients from plants, play an important role in the determination of the best quality of the extract obtained at the lowest cost possible to ensure its commercial potential.

The genus *Amaranthus* is an ancient crop which is still cultivated as a minor crop in Central and South America and some areas of Asia and Africa. There is a renewed interest in grain amaranth as it produces grains that contain greater levels of high nutritional quality protein,

unusual quality of starch, and high-value oil. The oil content of amaranth is high (6-9%) as compared with cereal grains and is a major source of interest due to its commercially exploitable constituents specifically squalene (Singhal, and Kulkarni, 1988; Caselato-Sousa and Amaya-Farfán, 2012). The richest source of squalene is shark liver oil, while amaranth oil, just next to shark liver oil is the richest plant source of squalene. Interest in more sustainable sources of squalene has increased recently due to the ban on shark hunting (Fox, 2009). Squalene is a hydrocarbon and triterpene. It acts as an intermediate in cholesterol, steroid hormones & Vitamin D biosynthesis in the human body. It is found in humans under the skin and inside the adipose tissue (Amarowicz, 2009). Squalene has been found to act as an anticancer agent and it has hypo-cholesterolemic effects. It has a significant commercial use in cosmetics as a moisturising agent and as a nutraceutical ingredient (Amarowicz, 2009). Another constituent which is of major importance is stigmasterol, which may significantly reduce the risk of coronary heart diseases by lowering plasma cholesterol levels without causing noticeable side effects. Stigmasterol is also reported to have anti-cancer properties and can play an important role in helping the absorption of fat soluble vitamins & antioxidants (Moreau et al., 2002).

The most accepted and trusted conventional method of lipid extraction is Soxhlet extraction, which has been used for a long time as the main reference for evaluating the performance of other solid–liquid extraction methods. It is a very time and solvent consuming process with incomplete extraction from the sample matrix (Luque de Castro & Garcia-Ayuso, 1998). To improve efficiency and reduce the environmental footprint of solvent use, there has been an increase in demand for new extraction technologies. For that purpose, microwave assisted extraction (MAE) has been considered as a potential alternative to conventional extraction technologies for its shorter extraction time, consuming less solvent coupled with higher extraction rates with better product quality (Delazar et al., 2012). For efficient MAE, the operating factors of the extraction process must be first optimised. The experimental factors which determine the performance of MAE processes using a mono-mode open microwave system include the sample matrix characteristics & size, solvent nature & volume, extraction time, microwave power and temperature (Tatke and Jaiswal, 2011). The sample matrix characteristics, including the sample particle size and the condition of the sample as it is prepared

for MAE, play an extensive role in the effective recovery of desired compounds (Wang and Weller, 2006).

For this purpose, parameter screening experiments were carried out to investigate the effect of particle size on MAE process using hexane as a solvent with a microwave input power of 80 W; for which whole and ground samples were considered. The particle size that obtained higher oil yield was chosen for further studies.

Correct solvent choice is a fundamental requirement for the optimisation of MAE as the solvent type will affect the solubility of the target compounds, interaction between solvent & sample and microwave absorbing properties of the solvent (Letellier et al., 1991). Hexane is the primary used solvent in the industry for extracting oil from oil seeds and has one of the best solubility for non-polar compounds like oil (Kim and Yoon, 1990; Köseolğua and Engelgau, 1990; Bhagya and Srinivas, 1992; Srinivas et al., 1992; Wanasundara and Shahidi, 1994; Dominquez et al., 1995; Lawson, 1995; Proctor and Bowen, 1996), however, it is transparent to microwave energy hence does not heat-up sufficiently for an effective mass transfer during MAE process, whereas polar solvents like methanol & ethanol have higher microwave absorbing capacity as compared to hexane and heat up much better (Kiss, 2000). Previous MAE studies (Zhou and Liu, 2006) have demonstrated that mixing polar solvents with hexane enhances the microwave extraction process.

The preliminary trials of this study have confirmed that hexane is an inefficient MAE solvent for releasing oil from amaranth grain matrix in terms of its yield as compared to conventional Soxhlet extraction.

Similarly the intensity of the microwave input power also acts as a deciding factor for an effective MAE process. The microwave input power and the temperature developed during MAE processes are closely related to each other. To some extent, the rise in microwave input power is directly proportional to the rise in temperature of the extraction mixture of solvent and sample, per unit time. This rise in temperature promotes better leaching efficiency of target compounds from the active sites of the sample matrix, since higher temperatures result in a decrease of

surface tension & solvent viscosity as well as cause an enhancement of the solubility of the extract in the solvent (Tatke and Jaiswal, 2011). However, high temperatures may cause detrimental effects, such as deterioration of thermo-labile compounds present in the extract and safety concerns associated with the high pressure development inside the microwave reactor which can lead to bursting of the microwave reactor and/or its content; therefore greater care should be taken to carefully select the microwave input power to control the rise in temperature and avoid detrimental excess temperatures (Sihvonen et al., 1999).

Moreover, in the last decade there have been various studies on MAE (Chemat et al., 2005; Hu et al., 2008; Xiao et al., 2008; Nemes and Orsat, 2011; Routray and Orsat, 2011, Singh et al., 2011; Krishnaswamy et al., 2012), that established the significance of regulating the microwave power level during the development and the optimisation of MAE processes for the extraction of natural compounds. The successful control of microwave power during MAE processes depends on using proper calibration and in understanding how the instrumentation can control the incident power level. The actual power absorbed by the system as it relates to the output power can be calibrated by knowing the value of the function $\Delta T$ (temperature difference developed in the system due to microwave heating). It enables the calculation of the microwave absorbed power using a calorimetric equation $P = (MC_p\Delta T)/t$ [where P is the absorbed microwave power by the system (W); M is the weight of the system (g); $C_p$ is the heat capacity (J $g^{-1}$ $°C^{-1}$); T is the temperature rise (°C); t is the heating time (s)].

For most of the focussed mono-mode microwave systems, the reaction temperature is generally measured by a calibrated external infrared (IR) sensor, unified into the cavity that identifies the surface temperature of the reaction vessel from a predefined distance. It is assumed that the measured temperature on the outside of the reaction vessel will be more or less in agreement with the temperature of the reaction mixture contained inside but this cannot be applied in every case. Contrary, due to heat reflux system, the reactor walls are typically the coldest spot of the reaction system as compared to conventional heating as the energy conversion using microwave energy takes place directly within the reaction mixture (Leadbeater et al., 2005; Nüchter et al., 2005; Kremsner and Kappe, 2006; Hosseini et al., 2007; Herrero et al., 2008).

In addition, an external IR sensor cannot record actual rise in temperature of the reaction mixture unless, the mixture has been sufficiently agitated. Inefficient agitation can lead to temperature gradients within the reaction mixture due to field non-homogeneities in the high density mono-mode microwave cavities (Herrero et al., 2008). Moreover, these discrepancies will be further aggravated in recording real temperature of reaction mixture by external IR sensor if the composition of reaction medium includes a mixture of material and/or solvents, where one of it is strongly microwave absorbing while the other weakly absorbing or transparent (Kremsner and Kappe, 2006). Similar situation existed in this study where hexane (microwave transparent/weakly absorbing) was mixed with methanol or ethanol (strongly microwave absorbing) to be used as the extraction solvent and experiments were carried out in a focussed mono-mode microwave unit which lacked a stirring system.

Hence the above mentioned factors were considered in the determination of the value of the $\Delta T$ function, by measuring the temperature of the solvent mixture before and after the microwave treatment with the help of a type K thermocouple following brief and vigorous stirring, to calibrate the amount of power absorbed.

This paper presents the pre-optimisation study which was conducted using a full factorial design with preliminary experiments to identify the experimental domain of interest, such as optimum solvent composition as well as microwave input power required for future resourceful optimisation using response surface experimentation.

## 3.3 MATERIALS AND METHODS

### 3.3.1 GRAIN AMARANTH

*Amaranthus* (cv. Plainsman) was obtained from the Emile A. Lods Agronomy Research Centre, McGill University, Ste-Anne-de-Bellevue, QC, Canada. It was manually cleaned by winnowing method to remove dust particles and fine straws. The cleaned grain amaranth was coarsely ground in a coffee grinder (Bodum, Model: 10903) for 1.5 minutes to achieve fine quality powder, the process was stopped at 15 seconds interval for 1 minute to avoid over

heating of the sample. To ensure uniform particle size of the amaranth grain flour (AGF), it was passed through an ASTM E-11 (No: 35; Tyler equivalent: 32 mesh) standard test sieve (W.S. Tyler, St. Catharines, ON, Canada) and confirmed 500 µm particle size. The AGF was then stored in an air tight glass bottle (wrapped with aluminium foil) at -4°C until further analysis. The moisture content (12.39% wet basis, SD = 0.23) was determined in triplicate by drying 10 g of AGF at 105°C for 24 hours.

### 3.3.2 STANDARDS AND SOLVENTS

Squalene (neat) and stigmasterol (95% pure) standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Hexane & methanol of ACS HPLC grade were procured from Fisher Scientific (Fair Lawn, NJ, USA) and anhydrous ethanol was obtained from Commercial Alcohols (Brampton, ON, Canada)

### 3.3.3 SAMPLE PREPARATION

Prior to each extraction, AGF (moisture content 12.39% wb, SD = 0.23) was freeze dried for 24 hours to maintain constant moisture content of the sample throughout the extraction experiment. The freeze dried AGF (moisture content 5.1% wb, SD = 0.06) was sealed immediately with Para film (Bemis Flexible Packaging, Neenah, WI, USA), after taking it out of the freeze dryer to avoid alteration in moisture content and was incubated to reach room temperature before the extraction process.

### 3.3.4 HEATING PATTERN STUDIES AND MICROWAVE OUTPUT POWER CALIBRATION OF MAE SYSTEM FOR DIFFERENT SOLVENT COMBINATIONS

The output power in the mono-mode (focused) cavity of MAE system (Star System 2, CEM, Matthews, USA; operating at 800 W maximum power and 2,450 MHz frequency) was calculated in triplicate for different solvents (A – MeOH:Hexane = 20:80 %v/v; B – EtOH:Hexane = 20:80 %v/v; C – 100% Hexane) irradiated for 30 s at microwave input power of 80, 120, 160, 200, 240

and 280 W. A modified version of the calorimetric method developed by Nemes, (2012) was adopted, for which fresh loads of 50 ml of solvent (weight: A = 164.27 g; B = 164.19 g; C = 162.86 g) in 250 ml volume borosilicate glass vessel (Montreal Glass Blowing Inc, Notre-Dame-De-L'Ile-Perrot, QC, Canada) with the initial temperature of 21 ± 3°C was used. The solvent temperature was recorded before and after irradiating the solvent with a type K thermocouple after briefly and vigorously stirring the solvent with a glass rod. To minimise the errors due to heat loss to the surroundings, the mass of the container that was in contact with the solvent was taken into consideration [as opposed to not accounting for the mass of the container (Cheng et al., 2006; Costa et al., 2001; Kingston and Jassie, 1986)], and by decreasing the heating time [30 s in this experiment as opposed to 1 min used by Costa et al. (2001) and 2 min used by Kingston and Jassie (1986)]. The absorbed power was calculated using the following **Equation 3.1**:

$$P = \frac{(Ms*Cps+Mv*Cpv)*\Delta T}{t} \quad \textbf{(3.1)}$$

Where, P = the actual microwave power output [W]

Ms = the mass of solvent [g]

Mv = the mass of the borosilicate glass vessel [g]

Cps = the specific heat of solvent [J/(g °C)]

Cpv = the specific heat of borosilicate glass [0.75 J/(g °C)]

ΔT = the temperature difference [°C]

t = the time of heating [s]

### 3.3.5 MAE OF LIPIDS FROM AGF

All MAE experiments were carried out in triplicate in the batch mode with a mono-mode (focused) open – vessel microwave system (Star System 2, CEM, Matthews, USA) operating at 800 W maximum power and 2,450 MHz frequency. The freeze dried AGF (10 g) along with 50 ml solvent were placed inside 250 ml volume borosilicate glass vessel (Montreal Glass Blowing Inc, Notre-Dame-De-L'Ile-Perrot, QC, Canada). The reaction mixture was vigorously stirred for 3 minutes to evenly distribute the solvent through the AGF matrix. The vessel was introduced

into the microwave cavity equipped with a reflux system. All the microwave extractions were performed under a set input microwave power (80, 120, 160 and 200 W) for a known period of time (10 min) in 50 ml of solvent (A – MeOH:Hexane = 20:80 %v/v, B – EtOH:Hexane = 20:80 %v/v, C – 100% Hexane). The power levels used for the experiments were expressed as a percentage of the power supplied within the microwave cavity as per the cavity's calibration. Immediately after taking out the borosilicate glass vessel from the microwave cavity, the reaction mixture was once again vigorously stirred for 3 minutes to aid proper heat distribution as well as mass transfer between the AGF and the solvent. The extract thus obtained was separated from the AGF using vacuum filtration with 70 mm (diameter) circular filter paper (Whatman, Buckinghamshire, UK). To ensure complete separation of the extract from the AGF, the AGF was further rinsed with solvent of HPLC grade. Finally, the oil was separated from the solvent using a Rotavap (Buchi R-205) operating at 100 rpm, coupled with a heating water bath (Buchi B- 490) set at 80°C.

### 3.3.6 SOXHLET EXTRACTION OF LIPIDS FROM AGF

All Soxhlet extraction experiments were carried out in triplicate in the batch mode using an extraction unit composed of Supelco Soxhlet apparatus united with a condenser at its top and a 250 ml distillation flask with magnetic stirrer at its bottom placed on heating plate. The cellulose extraction thimble (Whatman International Ltd, Maidstone, England) filled with known quantity of freeze dried AGF (36 g), was placed inside the thimble - holding the cavity of the Soxhlet apparatus. During the operation, fresh solvent condensate from the distillation flask falls over AGF placed inside the thimble, which gradually assists in desorption of the lipids from AGF into the solvent condensate pool surrounding it within the thimble-holding cavity of the Soxhlet apparatus. When the liquid reached the overflow level, a siphon aspirated the solution (AGF lipids – solvent mixture) of the thimble holding cavity and unloaded it back into the distillation flask carrying the extracted analytes into the bulk liquid. This operation was repeated for the known time and the oil was separated from the solvent using a Rotavap (Buchi R-205) operating at 100 rpm, coupled with a heating water bath (Buchi B- 490) set at 80°C.

### 3.3.7 CALCULATION OF OIL YIELD (%)

The yield percentage of oil was determined gravimetrically by the following expression (**Equation 3.2**); where $M_{AGF}$ is the mass of AGF (g) and $M_O$ the mass of extracted oil (g) for both MAE and Soxhlet.

$$Oil\ Yield\ \% = (M_O / M_{AGF})100 \quad \textbf{(3.2)}$$

### 3.3.8 PARAMETER SCREENING STUDY

The parameter screening study was carried out in triplicate to investigate the effect of particle size on oil yield only, through MAE using Soxhlet as control technique, for which whole and ground (500 µm particle size) samples were considered. For better comparability both MAE and Soxhlet extraction were conducted with same sample to solvent ratio (MAE: 10 g AGF/50 ml solvent; Soxhlet: 36 g AGF/180 ml solvent) using solvent C (hexane). Time of extraction for MAE was 10 min whereas for Soxhlet it was 90 min [time for Soxhlet extraction was selected based on Lopez-Avila et al., (1994); Pan et al., (2002)].

### 3.3.9 EXPERIMENTAL DESIGN

A 4x3 full factorial screening design (FFSD) as presented in **Table 3.1**, was formulated to identify the key parameters which can determine the experimental domain required to optimise the oil extraction from AGF by central composite rotatable design (CCRD) in the later study (**Chapter 4**) using JMP10 software by SAS (SAS Institute Inc., Cary, NC, USA). For this purpose, a design was developed with 12 combinations consisting of one continuous factor: microwave input power with four levels - 80, 120, 160 and 200 W; another categorical factor: extraction solvent with three categories – A, B and C.

**Table 3.1: Independent variables in MAE FFSD**

| Independent Variables | Variable Types | Values | | | |
|---|---|---|---|---|---|
| Microwave Input Power (W) | Continuous | 80 | 120 | 160 | 200 |
| Solvent | Categorical | A | | B | C |

**Solvent (A–MeOH:Hexane = 20:80% v/v; B–EtOH:Hexane = 20:80% v/v; C–100% Hexane)**

### 3.3.10 SAMPLE PREPARATION FOR GC-MS ANALYSIS

All the samples for GC-MS analysis were prepared as follows: 50 ml of HPLC grade Hexane solvent was added to 500 mg of amaranth grain oil under magnetic stirring for 10 min and was then kept for 4 hours without stirring or moving at room temperature to allow all the solid phase to settle down. The liquid phase was then filtered using syringe filters into capped vials and was analysed by GC-MS.

### 3.3.11 GC-MS ANALYSIS FOR SQUALENE AND STIGMASTEROL QUANTIFICATION

The quantification was carried out in six replicates per sample by employing an Agilent Technologies 6890 network GC system coupled with Agilent Technologies 5973 network mass selective detector and Agilent Technologies 7683 auto-injector in split mode. A vial of each sample was injected into the gas chromatograph at a split ratio of 50:1. Separation of samples was achieved on a HP-5 MS capillary column that was 30 m in length, 0.25 mm in internal diameter, and had a 250 μm coating thickness. Helium was used as a carrier gas at a flow rate of 1.0 ml/min and pressure 72394.98 Pa (10.5 psi). The injector and transfer line temperatures were both set at 300°C. The oven temperature program was as follows: initial temperature 100°C held for 3 min and was increased to 250°C at 10°C/min. From 250°C it was further increased to 300°C at 5°C/min where it was held for 15 min. The concentrations of squalene and stigmasterol were calculated in all samples by means of their peak areas. The calibration curve for squalene standard was obtained by using concentrations of 0.25, 0.50, 0.75 and 1.0 mg/ml whereas the calibration curve for stigmasterol standard was obtained by using concentrations of 0.01, 0.02, 0.03 and 0.04 mg/ml. The regression equation obtained from the calibration curves were utilised to quantify the amount of squalene (**Equation 3.3**; $R^2 = 0.9947$) and stigmasterol (**Equation 3.4**; $R^2 = 0.9924$) in the amaranth grain crude oil in mg/g of oil.

$$Squalene\ (mg/g\ of\ oil) = (4 \times Peak\ Area \times 10^{-6}) + 0.0323 \quad \textbf{(3.3)}$$

$$Stigmasterol\ (mg/g\ of\ oil) = (4 \times Peak\ Area \times 10^{-6}) + 0.0007 \quad \textbf{(3.4)}$$

### 3.3.12 EFFECT OF HIGH TEMPERATURE (100°C) ON SQUALENE AND STIGMASTEROL CONTENTS OF AMARANTH GRAIN OIL

In this study, stability of squalene and stigmasterol present in the amaranth grain oil was determined in triplicate by exposing 500 mg of oil to a constant high temperature of 100°C in presence of oxygen in a hot air oven. The period of exposure was varied at 10, 20, 30, 40, 50, 60 min.

### 3.3.13 EFFECT ON MICROWAVE ABSORBING CAPACITY OF METHANOL – HEXANE MIXTURE BY VARYING THE CONCENTRATION OF METHANOL IN HEXANE

Methodology which was discussed in the section 3.3.4 was adopted to study the microwave power absorption for different concentrations of MeOH in hexane at a fixed microwave input power of 120 W.

## 3.4 RESULTS AND DISCUSSION

### 3.4.1 HEATING PATTERN OF SOLVENTS A, B AND C UNDER MICROWAVE WITH ACTUAL MICROWAVE POWER OUTPUT CALIBRATION

A couple of reported studies (Baghurst and Mingos, 1992; Chen and Spiro, 1994) have been conducted in the past to examine the heating pattern of various solvents under microwave heating.

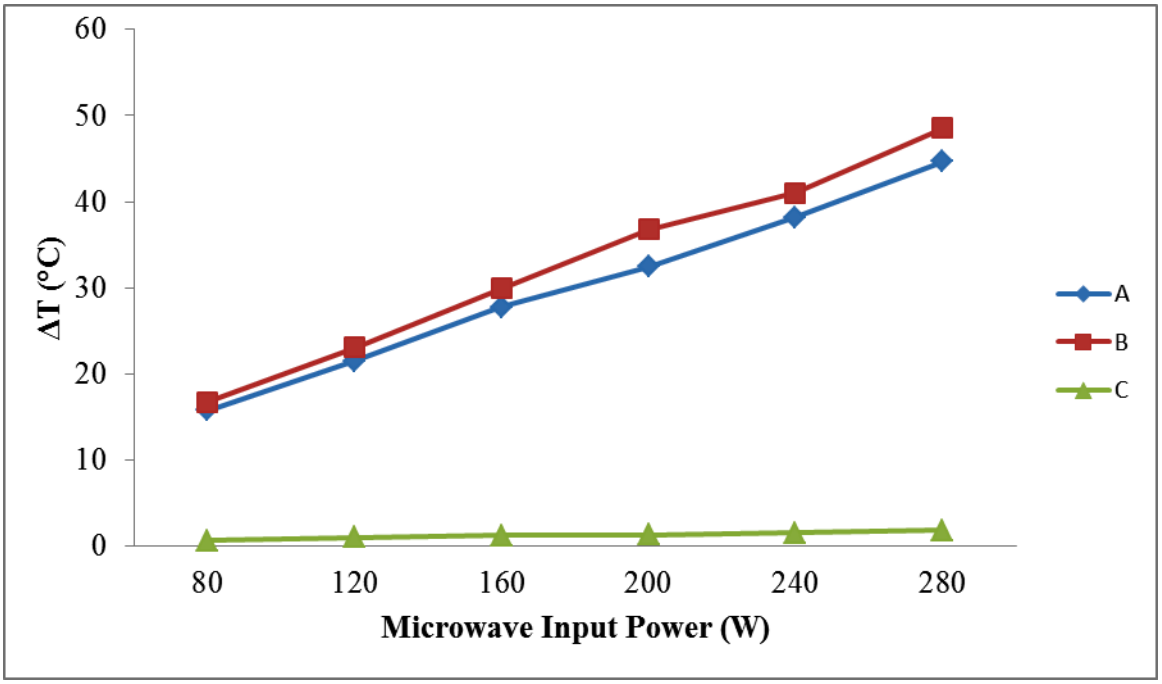



**Figure 3.1: Microwave heating profiles of solvents A, B and C at various microwave powers**

**Figure 3.1** clearly illustrates that value of ΔT continues to rise with an increase in power level for all the three solvents. The highest growth was seen in case of solvent B as it was composed of a strong microwave absorbing solvent, EtOH [loss tangent tan δ = 0.941 at 2.45 GHz, 20°C; Hayes (2002)] with hexane while solvent A was placed second as it was composed of a moderately microwave absorbing solvent, MeOH [loss tangent tan δ = 0.659 at 2.45 GHz, 20°C; Hayes (2002)] with hexane whereas, solvent C which was purely hexane [loss tangent tan δ = 0.020 at 2.45 GHz, 20°C; Hayes (2002)] saw a very slight increase in temperature. Drastic increases in temperature rise were observed with an addition of just 20% MeOH (solvent A) or EtOH (solvent B) in hexane as compared to pure hexane (solvent C). This linear relationship between microwave input power and ΔT in case of solvent A and B was noticed until microwave input power of 280 W, when the microwave input power was raised beyond 280 W, both solvent A and B under went powerful internal explosion which resulted in lifting of the condensing/reflux system. Even Saillard et al., (1995) reported similar but mild explosions under microwave heating of EtOH but not in the case of MeOH. The reason behind this phenomenon was over heating of pockets of solvents. This rapid increase of temperature under microwaves caused a quick increase of the internal pressure, which could be potentially dangerous in the absence of a safety apparatus (Peterson, 1993). Hence due to safety as well as repeatability

concerns associated with these experiments, actual microwave output power values were calibrated not beyond microwave input power level of 280 W. Hence the regression equations of microwave output power for all the three solvents A, B and C were obtained at input microwave powers of 80, 120, 160, 200, 240 and 280. The $R^2$ values of regression equation for solvent A (**Equation 3.5**), B (**Equation 3.6**) and C (**Equation 3.7**) are 0.9986, 0.9966 and 0.9841 respectively.

**Table 3.2: Microwave output power regression equations for solvent A, B and C**

| Solvent | Regression Equation | |
|---|---|---|
| *A* | *Microwave Output Power = 0.5060 (Microwave Input Power) + 15.5170* | **(3.5)** |
| *B* | *Microwave Output Power = 0.5533 (Microwave Input Power) + 15.4960* | **(3.6)** |
| *C* | *Microwave Output Power = 0.0187 (Microwave Input Power) + 0.67540* | **(3.7)** |

If the complexity of the heating mechanism for dielectric materials is taken into consideration, the results obtained in these equations might not reflect the true microwave power output because heating efficiency under microwave irradiation is influenced by many factors including specific heat, thermal conductivity and structure of the material. Specific heat is an important property in the thermal behavior of the material subjected to microwaves as it keeps changing with the rise in temperature of the material (Peyre et al., 1997). However, this calorimetric method is widely used by manufacturers (Cheng et al., 2006; Swain et al., 2006; Tanongkankit et al., 2013) and accepted due to its simplicity and practicality.

### 3.4.2 OUTCOMES OF PARAMETER SCREENING STUDY

Results obtained from the preliminary study acknowledged the importance of choosing optimum sample particle size, extraction solvent type and microwave input power for MAE processes. The ground sample showed better recovery than whole sample, but there was an interesting observation which was noticed while carrying out MAE using whole grains; most of the experiments could not succeed or move forward after 3 min of process, due to the formation of high cavitation, bubbling of the sample which led to the entry of the reaction mixture into the condenser. Even though some of the experiments worked well using whole sample, due to safety

concerns, loss of repeatability and low oil yield compared to ground sample, further experiments were carried out using only ground samples.

Even ground samples could not achieve convincing results with MAE operating at 80 W microwave input power using hexane as the extraction solvent, when compared with conventional Soxhlet extraction. The oil yield recovery from MAE was 5.9% whereas from Soxhlet it was 7.2% which means that the efficiency of MAE was almost 18% lower than Soxhlet.

Such observations from MAE forced the study to look for and identify the experimental domain within which an optimum combination of factors would maximise the extraction yields of oil. Alfaro et al. (2003) reported similar results while conducting MAE of ginger using hexane, which gave a lower yield than Soxhlet, on the other hand the use of EtOH, as the extraction solvent, gave significantly higher yield than for Soxhlet - EtOH extraction. This can be explained in reference to the difference in dielectric properties of the solvents (refer to section 3.4.1). MeOH and EtOH are relatively good absorbers of microwave energy but they are not good extraction solvents for oils while hexane is a good extraction solvent for oils but not a good microwave absorber. Therefore it was decided that the best way to gain both efficacy and selectivity for the MAE of oil, it is necessary to mix the solvents and hence three different solvent combinations were considered - A (MeOH:Hexane = 20:80% v/v), B (EtOH:Hexane = 20:80% v/v) and C (100% Hexane). Moreover, it was determined that microwave input power of 80 W was not sufficient for resourceful extraction, hence four levels of microwave input power were selected: 80 W, 120 W, 160 W and 200 W. A full factorial screening design (FFSD) was operationalized to screen out the optimum solvent composition as well as the microwave input power required to maximise the yield of oil, squalene and stigmasterol during MAE processes.

### 3.4.3 INTERPRETATION OF MAE FFSD

FFSD was employed as described in the experimental design section to scrutinise the best process factors combinations required to maximise the yield of oil, squalene and stigmasterol. The layout of the design, with mean (triplicate) values of responses, is presented **Table 3.3**. The

analysis of variance (ANOVA), regression plot, factorial surface plot and actual by predicted plot for each response are presented individually.

**Table 3.3: MAE FFSD with observed response for oil, squalene & stigmasterol**

| Design Points | Microwave Input Power (W) | Solvent | Oil Yield (%) | Squalene Yield (mg/g of oil) | Stigmasterol Yield (mg/g of oil) |
|---|---|---|---|---|---|
| 1 | 80 | A | 8.67 | 57.63 | 2.415 |
| 2 | 80 | B | 7.76 | 55.32 | 2.337 |
| 3 | 80 | C | 5.9 | 49.98 | 2.056 |
| 4 | 120 | A | 8.79 | 57.75 | 2.45 |
| 5 | 120 | B | 7.89 | 55.45 | 2.31 |
| 6 | 120 | C | 6.68 | 43.68 | 1.63 |
| 7 | 160 | A | 8.78 | 56.71 | 1.88 |
| 8 | 160 | B | 7.89 | 51.46 | 1.56 |
| 9 | 160 | C | 6.76 | 38.91 | 1.07 |
| 10 | 200 | A | 8.7 | 49.29 | 1.27 |
| 11 | 200 | B | 7.8 | 44.28 | 1.12 |
| 12 | 200 | C | 6.74 | 32.6 | 0.57 |

### 3.4.3.1 MAE FFSD OIL YIELD (%)

The ANOVA (**Table 3.4**) revealed that tested factors were significant for oil yield (%) at P value < 0.0001 with a $R^2$ value of 0.9809, which was in agreement with the adjusted $R^2$ of 0.9649. This implied that the model was fitted well and could be used to predict the oil yield from grain amaranth by MAE.

**Table 3.4: MAE FFSD ANOVA for oil yield (%)**

| Oil Yield (%) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9809; $R^2$ Ajusted = 0.9649 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob >F |
| Microwave Input Power | 1 | 0.1307 | 0.1307 | 3.9130 | 0.0953 |
| Solvent | 2 | 9.9273 | 4.9636 | 148.6414 | <0.0001 |
| Microwave Input Power x Solvent | 2 | 0.2084 | 0.1042 | 3.1200 | 0.1178 |
| Model | 5 | 10.2663 | 2.0533 | 61.4872 | <0.0001 |
| Error | 6 | 0.2004 | 0.0334 | | |
| Total | 11 | 10.4667 | 0.9515 | | |

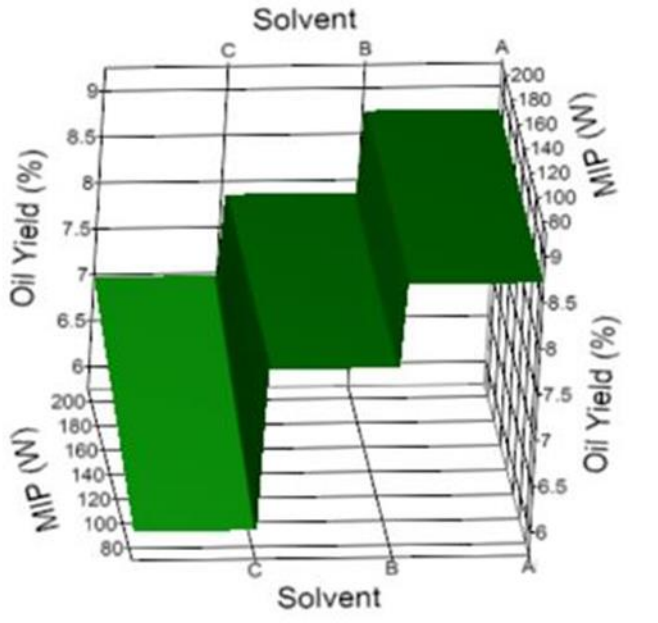



**Figure 3.2: MAE FFSD process factors effects on oil yield**

The model, factorial surface plot (**Figure 3.2**) and regression plot (**Figure 3.3; left**) confirmed that the extraction solvent (P value <0.0001) had a major implication on oil yield, specifically solvent A which showed significant positive impact, while on the other hand solvent C showed a significant negative impact on yield whereas, solvent B could not contribute to an extent to achieve the goal of maximising oil yield.

Solvent A resulted in almost 10 % more oil yield than solvent B for microwave input powers of 120 W and 180 W. Although the microwave input power did not have a compelling effect on yield, when the power was raised from 80 W to 120 W, there was a slight increase in yield; then the oil yield remained almost constant at 160 W however, further increase in power from 160 W to 200 W caused a little yield reduction in case of both solvent A and B, which indicates that there is limit to positive impact of increasing input power on oil yield. In case of solvent C, a linear increase in oil yield was noticed with an increase in power level until 120 W and then started reducing with further increase in power level. Additionally, at input power of 160 W, AGF was found to be scorched with the use of solvent C, while the extraction that took place at 200 W resulted in scorching of AGF for all the solvent categories i.e. A, B and C. The point to be noted was that the scorching didn't have massive detrimental effect on oil yield contrarily,

solvent C gave a better yield at 160 W than 120 W and a very slight decrease at 200 W as compared to 160 W. Based on visual observation, maximum scorching was discovered with the extraction which took place at 200 W with solvent C, the reason being that the solvent C is composed of only hexane which is microwave transparent so most of the microwave power was absorbed by AGF, at high microwave power of 200 W.

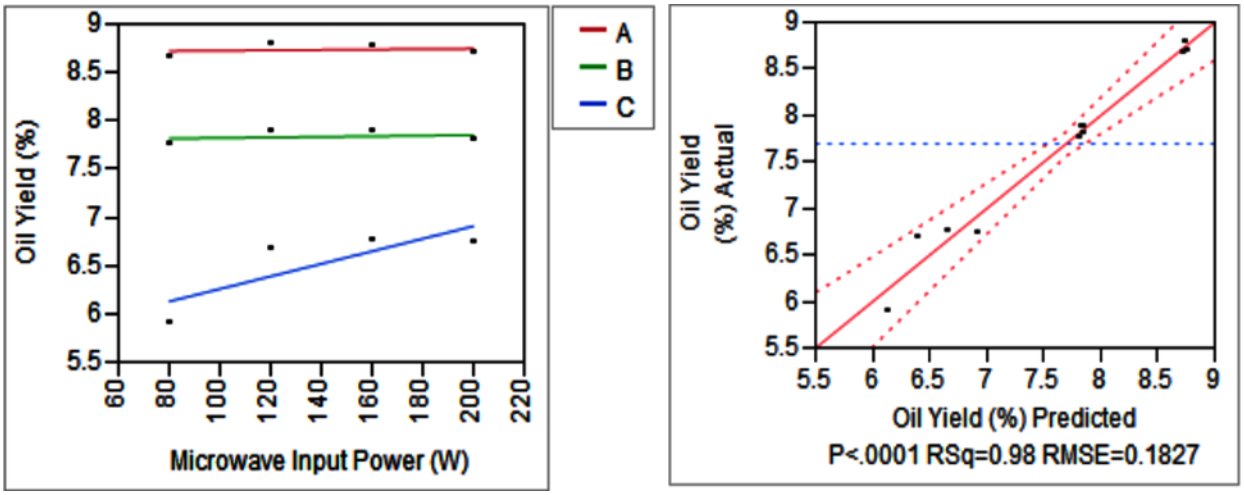



**Figure: 3.3 MAE FFSD Regression plot (left); Predicted vs actual plot (right) for oil yield (%)**

The mean value of oil yield for twelve response observations was 7.37 % (intercept value) with a standard error of 0.1733, which can be maximised if the extraction takes place at input power level of 120 or 160 W using solvent A. The $R^2$ value for predicted vs actual plot (**Figure: 3.3; right**) was 0.98 at P value < 0.0001. Hence this model can be used to predict oil yield (%) under different experimental conditions during MAE.

### 3.4.3.2 MAE FFSD SQUALENE AND STIGMASTEROL YIELD (mg/g of Oil)

The ANOVA revealed that the model was significant for squalene (**Table 3.5)** as well as for stigmasterol (**Table 3.6)** yields at P value = 0.0004 with a $R^2$ value 0.96 and P value = 0.0009 with a $R^2$ value = 0.95 respectively. Even the adjusted $R^2$ values for squalene (0.9248) and stigmasterol (0.9048) yields were in reasonable agreement with their respective $R^2$ values. This implied that the model was well fitted and could be used to predict the yields of squalene as well as stigmasterol from grain amaranth by MAE.

**Table 3.5: MAE FFSD ANOVA for squalene yield (mg/g of oil)**

| Squalene Yield (mg/g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.96; $R^2$ Adjusted = 0.9249 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| Microwave Input Power | 1 | 240.3201 | 240.3201 | 48.9635 | 0.0004 |
| Solvent | 2 | 424.1397 | 212.0699 | 43.2078 | 0.0003 |
| Microwave Input Power x Solvent | 2 | 24.4311 | 12.2156 | 2.4888 | 0.1633 |
| Model | 5 | 688.8909 | 137.778 | 28.0713 | 0.0004 |
| Error | 6 | 29.4489 | 4.908 | | |
| Total | 11 | 718.3398 | 65.3036 | | |

**Table 3.6: MAE FFSD ANOVA for stigmasterol yield (mg/g of oil)**

| Stigmasterol Yield (mg/g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.95; $R^2$ Adjusted = 0.9049 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| Microwave Input Power | 1 | 3.0034 | 3.0034 | 82.2041 | 0.0001 |
| Solvent | 2 | 0.9757 | 0.4878 | 13.3523 | 0.0062 |
| Microwave Input Power x Solvent | 2 | 0.0261 | 0.0130 | 0.3567 | 0.7139 |
| Model | 5 | 4.0051 | 0.8010 | 21.9244 | 0.0009 |
| Error | 6 | 0.2192 | 0.0365 | | |
| Total | 11 | 4.2243 | 0.3840 | | |

The model indicated both independent factors, microwave input power and solvent type, were significant as their P values were less than 0.05 and proved to have greater role on squalene as well as stigmasterol yield than the dependent factors.

The effect of process factors on squalene and stigmasterol yield is well illustrated in the following figure (**Figure 3.4**). The surface plot revealed that Solvent A, which is a statistically significant factor level, positively influenced the most, the yield of both squalene and stigmasterol whereas, solvent C caused a significant negative impact. Linear increase in squalene and stigmasterol yield was observed in case of solvent A & B with an increase in input power from 80 W to 120 W, in fact the highest yield was seen at 120 W and started diminishing with further increase in power.

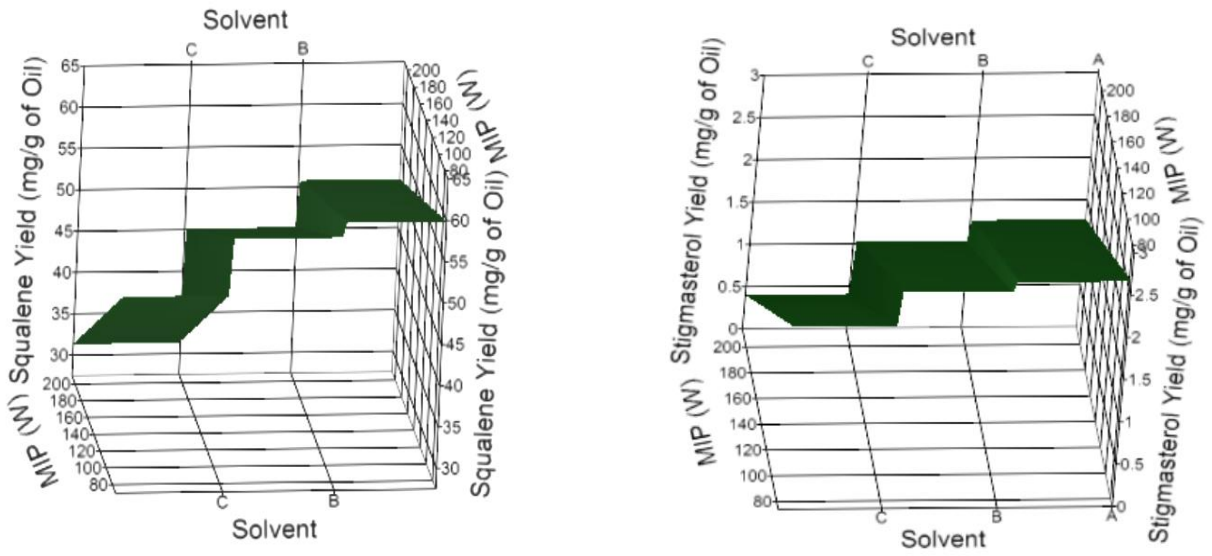



**Figure 3.4: MAE FFSD process factors effects on squalene yield (left) and stigmasterol yield (right)**

Whereas, when the extraction of amaranth grain oil was performed using solvent C, a linear decrease in squalene and stigmasterol yield was noticed with a linear increase in input power level. So unlike oil yield, scorching of AGF did affect the yield of both squalene as well as stigmasterol. This implies that the extract quality was degraded with an increase in the input power level after a certain limit which depended upon the solvent used.

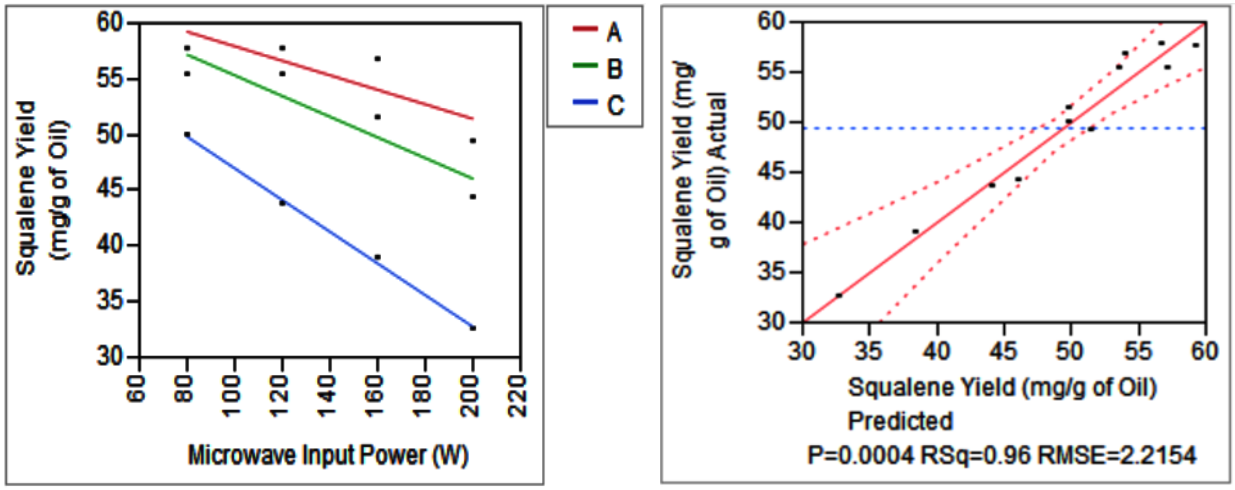



**Figure: 3.5 MAE FFSD Regression plot (left); Predicted vs actual plot (right) for squalene yield**

The mean value of squalene yield for twelve response observations was 63.431 mg/ g of oil (intercept) with standard error: 2.1017, which can be maximised if the extraction takes place at input power level of 120 W using solvent A (**Figure: 3.5; left**). The $R^2$ value for predicted vs

actual plot (**Figure: 3.5; right**) was 0.96 at P value = 0.0004. Hence this model can be used to predict squalene yield (mg/g of oil) under different experimental conditions during MAE.

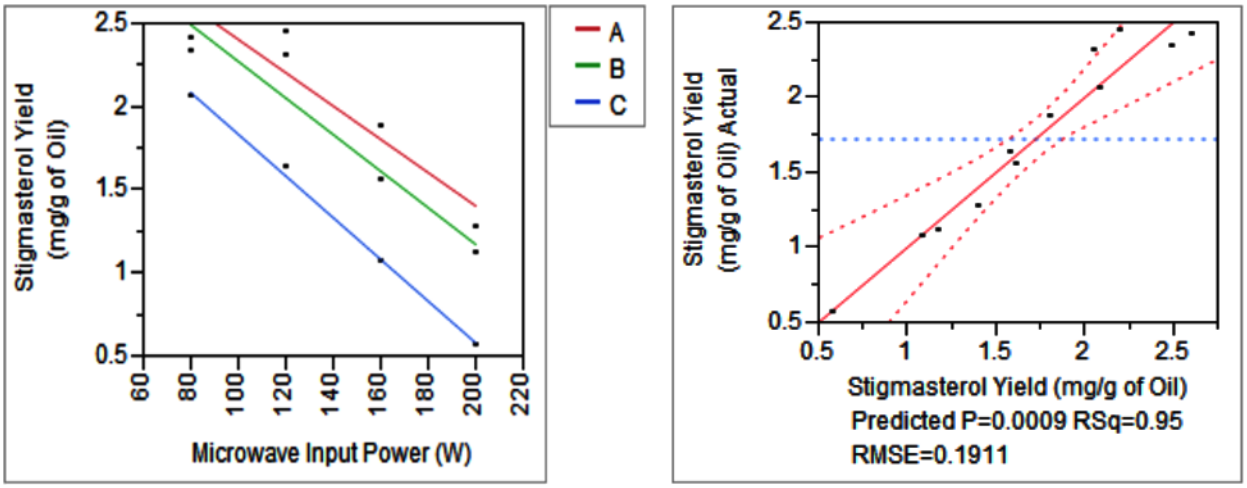



**Figure: 3.6 MAE FFSD Regression plot (left); Predicted vs actual plot (right) for stigmasterol yield**

The mean value of stigmasterol yield for twelve response observations was 3.2885 mg/g of oil (intercept) with standard error: 0.1813, which can be maximised if the extraction takes place at input power level of 120 W using solvent A (**Figure 3.6; left**). The $R^2$ value for predicted vs actual plot (**Figure 3.6; right**) was 0.95 at P value = 0.0009. Hence this model can be used to predict stigmasterol yield (mg/g of oil) under different experimental conditions during MAE.

Moreover, when the focus of attention was diverted towards the model's scaled estimates, the conclusion that could be made, by looking at the regression coefficient values for solvent A and C, proved solvent C as a powerful negative factor level for oil, squalene and stigmasterol yields.

### 3.4.4 EFFECT OF HIGH TEMPERATURE (100°C) ON SQUALENE AND STIGMASTEROL CONTENTS OF AMARANTH OIL

Prior studies (Sims et al., 1972; Malecka, 1991) have reported that the presence of squalene and sterols protects polyunsaturated oil from oxidative polymerisation during heating in presence of air at frying temperatures. Blekas and Boskou (1981), Mariod et al., (2006) and Thanh et al., (2006) demonstrated from their studies that sterol contents of oil from different sources were hampered during processing that involved heat treatment. Similarly Tikekar et al., (2008) proved

that the squalene content of amaranth flour was affected negatively contrarily to Gamel et al., (2006) who demonstrated that squalene content of amaranth remained almost the same by popping and drying processes. This has prompted us to further investigate the effects of high temperature (100°C) on squalene and stigmasterol contents of amaranth oil. **Table 3.7** illustrates the concentration of squalene and stigmasterol after different times of exposure at 100°C:

**Table 3.7: Responses of exposing amaranth oil to hot air oven (100°C) over time**

| Time (Min) | Squalene (mg/g of oil) | Stigmasterol (mg/g of oil) |
|---|---|---|
| 0 | 55.37 ± 2.38 | 2.36 ± 0.084 |
| 10 | 46.32 ± 2.43 | 1.96 ± 0.067 |
| 20 | 37.93 ± 1.65 | 1.28 ± 0.051 |
| 30 | 35.87 ± 1.47 | 1.25 ± 0.053 |
| 40 | 35.42 ± 1.32 | 1.24 ± 0.046 |
| 50 | 35.28 ± 1.48 | 1.23 ± 0.031 |
| 60 | 35.27 ± 1.54 | 1.23 ± 0.032 |

The data presented in the above table clearly indicates that both squalene and stigmasterol contents of crude amaranth oil were negatively affected upon exposing the crude amaranth oil to 100°C in presence of air inside the hot air oven. Stigmasterol content was found to be little more affected than squalene; as at the end of 60 min exposure stigmasterol content was reduced by almost 48% whereas the overall decrease in squalene content was around 36% of their initial concentrations. An important point to be noted is that there was not much reduction in content after 20 min of exposure; this implied that both squalene and stigmasterol underwent a significant reduction at the onset of the thermal treatment while both remained stable comparatively during the following 40 min of thermal treatment.

### 3.4.5 IDENTIFICATION OF EXPERIMENTAL DOMAIN

Certainly the high temperature extraction process can hamper the quality of the extract as it degrades its high value constituents, in addition to that, the main conclusions which can be drawn from the above model predictions is, too low microwave power level will cause too little energy to be dissipated as heat to promote mass transfer in the extraction process. It is important to have enough microwave energy to dissipate as heat, as the effects of ionic conduction and

dipolar polarisation increase with temperature. If the power is too high, energy is either wasted or may lead to the degradation of the constituents of the extract. The model also acknowledged the role of solvent A as a major factor level that played a significant role in maximising responses. In addition to that, while conducting experiments it was discovered that AGF particles swell in contact with solvent A only. Hence it was hypothesised that, the interaction between AGF particles and solvent A was the major reason for enhanced recovery. However, more studies are required to reach any firm conclusion.

The screening experimentations could have been stopped at this moment, as the study had given convincing results to carry out extraction using solvent A at 120 W, but the question then arose whether MAE of lipids from amaranth grain could be further enhanced. Since the FFSD couldn't predict how the responses will be affected outside the studied range of factors, a new design was needed for investigating the different levels. The factorial design in this study has used a fixed AGF to solvent ratio (w/v), concentration of MeOH in hexane (% v/v) and extraction time (min). However, before carrying out a response surface optimisation study using above mentioned factors, it was necessary to decide on the levels for these factors; hence the tested levels of AGF to solvent ratio (10 g/50 ml of solvent) and extraction time (10 min) were considered as central points for a proposed central composite rotatable (CCRD) design. This will enable the study to determine best level for maximising responses as it broadens the scope of the factor levels. Moreover, before selecting concentration ranges for MeOH in hexane that can be varied for further optimisation process, it was necessary to study how different concentrations of MeOH in hexane affect the microwave absorbing capacity of the whole solvent. Therefore the following study was conducted.

### 3.4.6 MICROWAVE OUTPUT POWER CALIBRATION FOR DIFFERENT CONCENTRATIONS OF METHANOL IN HEXANE

The actual output power pattern (**Table 3.8**) recorded from the study revealed that the microwave absorbing capacity of the MeOH - Hexane mixture was increased linearly until concentration of MeOH reached 30%, further increase in MeOH concentration caused a

**Table 3.8: Predicted vs actual values of microwave output power for varying concentration of MeOH in hexane**

| Concentration of MeOH in Hexane (% v/v) | Microwave Output Power (Actual) (W) | Microwave Output Power (Predicted) (W) | SD |
|---|---|---|---|
| 0 | 3.71 | 6.0781 | 1.6745 |
| 20 | 76.2 | 73.0914 | 2.1980 |
| 25 | 86.27 | 81.3902 | 3.4505 |
| 30 | 90.41 | 87.0458 | 2.3788 |
| 35 | 88.44 | 90.4277 | 1.4055 |
| 40 | 84.16 | 91.9053 | 5.4767 |
| 60 | 81.83 | 86.1597 | 3.0615 |
| 80 | 87.19 | 79.4946 | 5.4414 |
| 100 | 92.93 | 95.5499 | 1.8526 |

linear decrease in microwave absorbing capacity until MeOH concentration reached 60%. Further increase in MeOH concentration caused again a linear increase in microwave absorbing capacity of the solvent mixture until 100% MeOH. So while fitting the data in the polynomial model, it was observed that if the degree of the model was raised from two (quadratic) to three (cubic) and from three (cubic) to four (quartic) there was an enhancement in the $R^2$ value from 0.7448 to 0.9679 and from 0.9679 to 0.9953 respectively. Similar trends were observed in case of the predictability of the model. As the predictability of the polynomial quadratic model was too low to mirror actual data hence it was assumed that it wouldn't be a wise decision to consider the quadratic model, whereas quartic model had higher predictability with higher $R^2$ value of 0.9953, but its applicability for industrial purposes is beyond reality. Therefore, the values mentioned in **Table 3.8** were fitted into a bivariate cubic polynomial model (**Figure 3.7; left**) to generate **Equation 3.8**.

$$\textit{Microwave Output Power} = 94.551445 - 0.0585382 \times (\textit{Concentration of Methanol}) - 0.0257744 \times (\textit{Concentration of Methanol} - 43.3333)^2 + 0.0004925 \times (\textit{Concentration of Methanol} - 43.3333)^3 \quad \textbf{(3.8)}$$

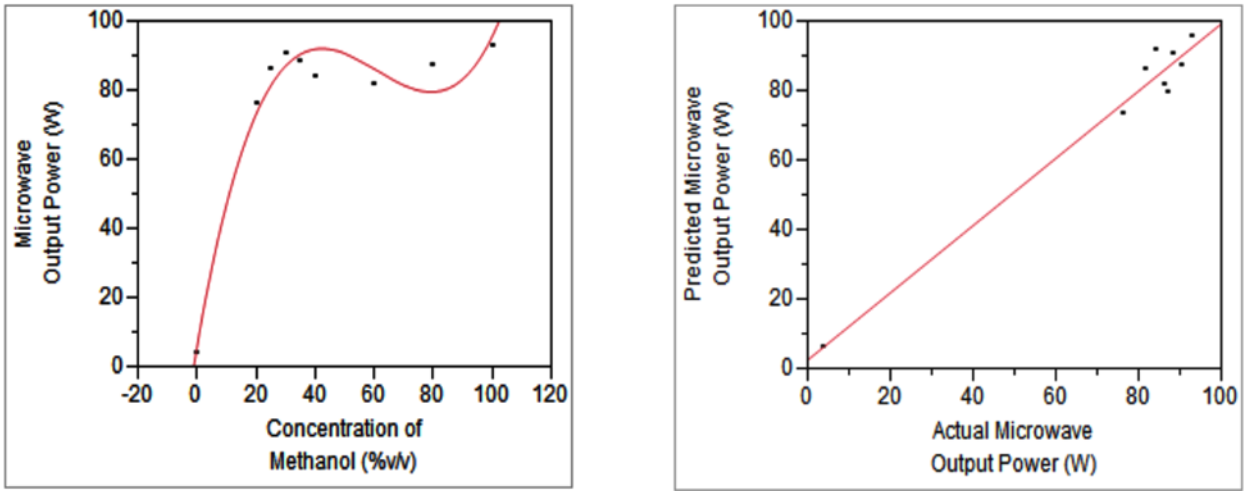



**Figure 3.7: Regression plot (left); Predicted vs actual plot (right) microwave output power for varying concentrations of methanol in hexane**

The model was significant at P value = 0.0004 with $R^2$ value of 0.9679 which was in agreement with adjusted $R^2$ of 0.9486. The ANOVA results for the model are represented in **Table 3.9**.

**Table 3.9: ANOVA for microwave output power for varying concentrations of methanol in hexane**

| Microwave Output Power (W) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9679; $R^2$ Adjusted = 0.9486 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| Model | 3 | 6001.3375 | 2000.45 | 50.2190 | 0.0004 |
| Error | 5 | 199.1723 | 39.83 | | |
| Total | 8 | 6200.5098 | 775.0637 | | |

The $R^2$ value for predicted vs actual plot (**Figure 3.7; right**) was 0.97 at P value < 0.0001. Hence this model can be used to predict the microwave output power for different concentrations of MeOH in hexane at a fixed microwave input power of 120 W under different experimental conditions during MAE.

If the complexity of the heating mechanism for dielectric materials is taken into consideration, the results obtained in these equations might not reflect the true microwave power output because the heating efficiency under microwave is influenced by many factors including specific heat, thermal conductivity and structure of the material. Specific heat is an important property in the thermal behavior of the material subjected to microwaves as it keeps changing with the rise in temperature of the material (Peyre et al., 1997). However, this calorimetric

method is widely used by manufacturers (Cheng et al., 2006; Swain et al., 2006; Tanongkankit et al., 2013) and accepted due to its simplicity and practicality.

This study proved that the microwave absorbing capacity of the MeOH - Hexane mixture was increased linearly until concentration of MeOH reached 30% initially, hence this strongly supports to choose to test 20% MeOH in hexane (% v/v) as the recommended central point for a proposed central composite rotatable (CCRD) design experiment.

## 3.5 CONCLUSION

Microwave energy can accelerate the extraction of lipids from grain amaranth and enhance the responses with minimum use of solvents. Observations made from full factorial screening design along with microwave power absorbing calibration for different solvents has identified the experimental domain as well as factors levels required for future optimisation of the MAE process by response surface rotatable design.

## 3.6 ACHNOWLEDGEMENT

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## PREFACE TO CHAPTER 4

The study conducted in **Chapter 3**, endorsed AGF over whole amaranth grain; MeOH - Hexane mixture as an extraction solvent and microwave input power of 120 W for better recovery of lipophilic compounds from amaranth grain using full factorial screening design as a part of pre-optimisation studies. **Chapter 4** discusses the optimisation process of extracting amaranth grain oil and its constituents, squalene & stigmasterol, by augmenting two consecutive central composite designs, considering factors which weren't included in the factorial design such as sample to solvent ratio, solvent to solvent ratio and extraction time keeping the constant optimum values for the identified factors. The outcomes of optimised MAE process will be compared with optimised Soxhlet extraction quantitatively (oil yield %) and qualitatively (squalene, stigmasterol, polyphenols, α – tocopherol and DPPH free radical-scavenging activity).

Part of the research study presented in **Chapter 4**, has been used to prepare two conference presentations, as follows.

**Joshi, S.P., Orsat, V. (2013). Optimisation of Microwave Assisted Extraction of High Value Lipids from Grain Amaranth. Promoting Small Millets for Improved Rural Economy and Food Security. University of Agricultural Sciences, Dharwad, Karnataka, India, February 8 - 9.**

**Joshi, S.P., Orsat, V., Gariépy, Y., Raghavan, G.S.V. (2013). Optimisation of Microwave Assisted Extraction of High Value Industrial Lipids from Grain Amaranth. 104$^{th}$ American Oil Chemists' Society (AOCS) - Annual Meeting & Expo, Montréal, Québec, Canada, April 28 - May 1.**

**Chapter 4** is formulated in manuscript style and is soon to be submitted for publication as follows.

**Joshi, S.P., Orsat, V. Microwave Assisted Extraction of Lipids from Grain Amaranth - Method Development.**

The first author of the manuscript and the conference presentation, Mr. Siddhartha P Joshi, Master's candidate, Department of Bioresource Engineering, McGill University, conducted the reviews of literatures, the design of experiment, the practical experimental work in the laboratory, the statistical analysis and interpretation of results, the writing and the preparation of this manuscript for publication.

The second author, Dr. Valérie Orsat, Associate Professor and Chair, Department of Bioresource Engineering, McGill University is the thesis advisor, provided her technical expertise, and guided the first author of this manuscript throughout all the stages of planning and executing the experimental work, analysing and interpreting the results, correcting and preparing the manuscripts for publication.

# CHAPTER 4: **MICROWAVE ASSISTED EXTRACTION OF LIPIDS FROM GRAIN AMARANTH – METHOD DEVELOPMENT**

## 4.1 ABSTRACT

An optimised microwave-assisted extraction (MAE) method was developed for extracting lipids from grain amaranth. The research presents the optimisation of factors for maximising the extraction yield of oil and its constituents, squalene and stigmasterol from grain amaranth. This work was conducted using the experimental domain identified through a pre – optimisation study by means of a full factorial screening design operating at microwave input power with four levels - 80, 120, 160 and 200 W and extraction solvent with three categories – A, B and C (A– MeOH/Hexane = 20/80% ml/ml; B–EtOH/Hexane = 20/80% ml/ml; C–100% Hexane) at a constant amaranth grain flour (AGF) to solvent ratio and extraction time. The MAE responses were found to be maximised operating at 120 W using solvent A. Hence, for this study MAE process was optimised using a MeOH - Hexane mixture as the extraction solvent by amalgamation of two central composite rotatable designs (CCRD) with the following factors, that is, AGF/ solvent (5g - 15g/ 50 ml); MeOH/Hexane (10 - 50% ml/ml) and Extraction Time (5 - 25 min) operating at constant microwave input power of 120 W. Later, the outcomes of optimised MAE process were compared with optimised Soxhlet extraction quantitatively (oil yield %) and qualitatively (squalene, stigmasterol, polyphenols, α – tocopherol and DPPH free radical-scavenging activity).

*Key words: Full factorial screening design, central composite rotatable design, squalene, stigmasterol, α – tocopherol, free radicle – scavenging activity*

## 4.2 INTRODUCTION

The exploration of alternative crops which can be employed to increase the profitability of farming systems is of global concern. The plants of interest represent a resource having a current and a future potential application as a food additive, pharmaceutical, pigment, perfume and cosmetic ingredient. *Amaranthus* species are among such unconventional plants which have a

potential for gaining greater interest to both consumers and farmers, as a food and as an industrial crop.

There are scientific research studies (Singhal and Kulkarni, 1988) that examined the nutritional values of various species of pseudo cereal grain such as amaranth and their attributes pertaining to positive effects on human health. Food products such as bread, cookies, biscuits, candies, pancakes, noodles and soups made using edible amaranth grain varieties are already commercialised but not enough emphasis has been given for the industrial applicability and commercialisation of amaranth grain oil, even though bioactive ingredients such as squalene, sterols, tocopherols and polyphenols are abundantly present in its oil which find their major significance in nutraceutical and cosmetic industries (Caselato-Sousa and Amaya-Farfán, 2012; Rastogi and Shukla, 2012).

Microwave-assisted extraction (MAE) has received enormous attention as a promising alternative to conventional solid-liquid extraction methods, mainly due to its significantly lower solvent consumption and reduced processing time. The enhanced extract recovery by microwave has been characterised by its volumetric heating, which causes dipole rotation of the solvent/solid matrix in the microwave field leading to a rapid rise in temperature of the solvent. Specifically, microwave heating occurs when molecules of the polar solvent cannot align themselves quickly enough to the high-frequency electric field (typically 2450 MHz) of microwave, which in turn causes solvent molecules to dissipate the absorbed energy in the form of heat (Meda et al., 2005; Orsat et al., 2005; Raghavan et al., 2005; Hemwimon et al., 2007).

For the past decade, there have been numerous published articles (Routray and Orsat, 2011, Singh et al., 2011; Krishnaswamy et al., 2012; Kubra et al., 2013) on MAE used for polyphenolic antioxidants, however not much interest has been shown to find its industrial applicability for extracting oils from seeds.

The concept of experimental design and analysis of variance (ANOVA) was framed mainly in association with agricultural field research. Over the course of time its application in industrial and technological experimentation gathered interest and started growing. Indeed, most industrial

research use factorial design at the onset of research to screen out the experimental parameters required followed by an optimisation process using response surface methodology (Myers and Montgomery, 2002 A & B). Hence a full factorial screening design was operationalized (**Chapter 3**) and it identified the experimental domain required to optimise the extraction of lipids from AGF by CCRD in this chapter.

## 4.3 MATERIALS AND METHODS

The sample & its preparation, the MAE system & experimentation methodology, Soxhlet system & experimentation methodology, sample preparation & methodology adopted for chromatographic technique used in this study were identical with those reported in section 3.3 presented in **Chapter 3**, where their detailed description can be found.

### 4.3.1 STANDARDS, SOLVENTS AND REAGENTS

Squalene, stigmasterol (95% pure) and α - tocopherol standards were purchased from Sigma Aldrich (St. Louis, MO, USA). n-Hexane, methanol, xylenes and n-propanol of ACS HPLC grade were procured from Fisher Scientific (Fair Lawn, NJ, USA) and anhydrous ethanol was obtained from Commercial Alcohols (Brampton, ON, Canada). Folin & Ciocalteu's phenol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride, 2, 2'- bipyridyl reagents were acquired from Sigma Aldrich (St. Louis, MO, USA) and sodium carbonate anhydrous ACS HPLC grade was received from Fisher Scientific (Fair Lawn, NJ, USA).

### 4.3.2 OUTCOMES OF PRE-OPTIMISATION STUDIES

The MAE optimisation presented in this paper is built upon the outcomes of the pre-optimisation study (**Chapter 3)**. Pre-optimisation consisted of a parameter screening study followed by a full factorial screening design. The parameter screening study indicated the significance of using AGF over whole amaranth grain and conducting MAE using solvent mixtures composed of a strong and a weak microwave absorbing solvent operating above the minimum microwave input power of 80 W.

The design assisted in screening out the optimum combination of factors that maximised the yield of oil and its constituents squalene and stigmasterols at a fixed AGF to solvent ratio (10 g/50 ml of solvent) and extraction time (10 min).

The responses were found to be maximised when extraction was carried out using solvent A (MeOH:Hexane 20:80% v/v) at microwave input power of 120 W. However, this full factorial design could not predict how the responses will be affected if values of AGF to solvent ratio (g/ml), concentration of MeOH in hexane (% ml/ml) and extraction time (min) were varied, hence it was necessary to determine whether responses could be further enhanced using these factors to create a central composite rotatable design (CCRD) operating at a microwave input power of 120 W.

### 4.3.3 EXPERIMENTAL DESIGN

The design was constituted of two stages of experimentation; first, the process optimisation of MAE of lipids from grain amaranth was accomplished by amalgamation of two successive central composite rotatable designs (CCRD); second, the performance of the optimised MAE method was evaluated using optimised Soxhlet extraction as a reference technique. The experiments were carried in triplicate for each design points for both MAE and Soxhlet extraction.

The optimisation procedures for MAE started with a CCRD (CCRD - I), consisting of 20 design points including 6 central points as presented in **Table 4.1**:

**Table 4.1: Independent variables in MAE CCRD - I**

| Independent Variables | Variable Types | Values | | |
|---|---|---|---|---|
| AGF (g)/ 50 (ml) Solvent | Continuous | 5 | 10 | 15 |
| MeOH/ Hexane (% ml/ml) | Continuous | 10 | 20 | 30 |
| Extraction Time (min) | Continuous | 5 | 10 | 15 |

The above mentioned design was followed by an another CCRD - II, consisting of 15 design points including 7 central points which were operationalized at a fixed AGF to solvent ratio (5 g/50 ml of solvent) within the experimental domain as mentioned in **Table 4.2**:

**Table 4.2: MAE Independent variables in MAE CCRD - II**

| Independent Variables | Variable Types | Values | | |
|---|---|---|---|---|
| MeOH/ Hexane (% ml/ml) | Continuous | 30 | 40 | 50 |
| Extraction Time (min) | Continuous | 15 | 20 | 25 |

During the parameter screening study (**Chapter 3**), Soxhlet was studied as a control technique for which, level of AGF to solvent ratio (36 g/180 ml) was adopted to be same as MAE (10 g/180 ml). Nonetheless, when the focus of attention was taken over to the results reported (an increase in response with a decrease in sample to solvent ratio for Soxhlet) by Jadhav et al., (2009), it was consequently hypothesised that 36 g/180 ml is a large AGF to solvent ratio which needs to be decreased to enhance the responses, therefore to optimise the Soxhlet extraction using CCRD, the above tested level of combination for AGF to solvent ratio wasn't included in the design, moreover the minimum level of AGF size was reduced from 36 g to 20 g whereas, solvent volume was raised from 180 ml and was fixed at 200 ml. In this way we were able to mimic same minimum level of AGF to solvent ratio for Soxhlet (20 g/200 ml) and MAE (5 g/50 ml; CCRD - I) for better comparability. However, it was impossible to mimic all the levels of AGF to solvent ratio due to technical limitations associated with the Soxhlet apparatus [first, Soxhlet extraction cannot be performed efficiently if the quantity of solvent used is below 180 ml; second, the thimble specification which fits the Soxhlet apparatus does not allow a sample size above 36 g; therefore it was necessary to adjust both quantity of AGF (g) as well as solvent (ml) to obtain optimum AGF to solvent ratio which can be compared meaningfully with MAE].

The tested level of extraction time for Soxhlet extraction during parameter screen study (**Chapter 3**) was 90 min, hence to broaden the scope of the level of extraction time; it was adopted as a central point for Soxhlet CCRD, whereas for better comparability with MAE, concentration levels of MeOH in hexane were adopted to be the same as MAE.

Considering above mentioned aspects, the following Soxhlet CCRD optimisation design was formulated to be used as reference for estimating the efficiency of MAE, which consisted of 20 design points including 6 central points within experimental domain as mentioned in the **Table 4.3**:

**Table 4.3: Independent variables in Soxhlet CCRD**

| Independent Variables | Variable Types | Values | | |
|---|---|---|---|---|
| AGF (g)/200 (ml) Solvent | Continuous | 20 | 25 | 30 |
| MeOH/ Hexane (% ml/ml) | Continuous | 10 | 20 | 30 |
| Extraction Time (min) | Continuous | 60 | 90 | 120 |

All the design experimentation for MAE as well as Soxhlet were carried using freeze dried AGF (moisture content 5.1% wet basis, SD = 0.06) to maintain constant moisture content. After resolution of the best possible factor combinations for both MAE and Soxhlet, extractions were carried out at the best factor combination using non freeze-dried AGF (moisture content 12.39% wet basis, SD = 0.23), to know if there is any significant effect of moisture content on extraction yield.

MAE and Soxhlet were further compared at their best factor combination levels in terms of their polyphenolic and α-tocopherol content as well as their free radical-scavenging activity

### 4.3.4 SPECTROPHOTOMETRIC DETERMINATION OF TOTAL PHENOLIC COMPOUNDS USING FOLIN & CIOCALTEAU'S REAGENTS

A spectrophotometric method based on Slinkard and Singleton, (1977) was developed to quantify the total phenolic content of crude amaranth grain oil in terms of gallic acid equivalent (GAE) using Folin & Ciocalteau's reagent. The method developed by Slinkard and Singleton, (1977) cannot be directly applied to estimate total polyphenolic content of oil, hence it was modified. The determination of polyphenols was performed as follows: 100 mg of amaranth grain oil was dissolved in 0.5 ml of n-hexane and polyphenols were extracted from oil-hexane solution with 1 ml portion of MeOH/double distilled water (30/70% ml/ml). The mixture was shaken for 5 min at 2800 rpm using miniRoto Vortexers (Fisher Scientific, Ottawa, ON, Canada). The separation of oil and MeOH - water solution was achieved by centrifugation (International Equipment Company, Needham Heights, MA, USA) for 10 min at 3300 rpm. One milliliter of MeOH - water solution containing extract present at the bottom of the centrifuge tube was pipetted out and transferred to another centrifuge tube. The methanolic extract was further diluted with 5 ml double distilled water and followed by addition of 2 ml Folin & Ciocalteau's reagent. After 3 min, 6 ml of 7.5% $Na_2CO_3$ was added to it. The absorbance of the

sample was measured after 30 min of incubation in the dark, at 765 nm against a blank sample with UV/Vis spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., GE Healthcare, Cambridge, England). A calibration curve of gallic acid in MeOH/double distilled $H_2O$ (30/70% ml/ml) was constructed with a concentration range between 0.05 - 0.25 mg/ml. The regression presented in **Equation 4.1** ($R^2 = 0.9998$) was thus obtained as follows:

$$\textit{Total Phenolic Concentration (mg/ml)} = 0.116\,(\textit{Absorbance})_{765\,nm} + 0.0313 \qquad \textbf{(4.1)}$$

### 4.3.5 SPECTROPHOTOMETRIC DETERMINATION OF α - TOCOPHEROL USING EMMERIE -ENGEL REACTION

The colorimetric method used for determination of α – tocopherol in amaranth grain oil is based upon Emmerie - Engel Reaction (1938) as reported by Rosenberg (1945) and later by Baker and Frank (1968). The original method was developed to quantify α – tocopherol content of serum/plasma/tissue; hence the method was modified so that it can be applied directly to determine the concentration of α – tocopherol in amaranth grain oil.

The determination of α – tocopherol was performed as follows: 500 mg of amaranth grain oil was dissolved in 5 ml of n-hexane/EtOH (20/80% ml/ml). One milliliter of this oil – solvent mixture was added to 1 ml xylene. The mixture was shaken for 5 min at 2800 rpm using miniRoto Vortexers (Fisher Scientific, Ottawa, ON, Canada) to extract the α – tocopherol and carotenoids into the xylene. The extract (0.5 ml) was pipetted out into a cuvette; 0.5 ml of 2'2' bipyridyl reagent (120 mg/100 ml n-propanol) was added to it and mixed well. The absorbance was read in the dark at 460 nm to measure carotenoid as it interferes with α - tocopherol assay. In the same cuvette, 0.1 ml Ferric chloride solution (120 mg/100 ml absolute EtOH) was added, mixed well and was incubated for 15 min in the dark. The red colour developed due to the reduction of ferric ions to ferrous ions, was read exactly after 15 minutes in the dark at 520 nm (maximum absorbance range for α – tocopherol) using UV/Vis spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., GE Healthcare, Cambridge, England). The absorbance read at 460 nm (maximum absorbance range for carotenoids) was subtracted from the α – tocopherol absorbance at 520 nm by the mathematical expression [0.29 x $(\text{Sample Absorbance})_{460}$]; the carotenoid

absorbance at 520 nm is 29% of that at 460 nm. A calibration curve of α – tocopherol standard in n-hexane/EtOH (20/80% ml/ml) was constructed with a concentration range between 0.005 - 0.03 mg/ml. The regression ($R^2$ = 0.995) is as presented in **Equation 4.2**:

$$\alpha - Tocopherol\ Concentration\ (mg/ml) = 0.0913[(Sample\ Absorbance)_{520nm} - 0.29(Sample\ Absorbance)_{460nm}] - 0.0051 \quad \textbf{(4.2)}$$

### 4.3.6 DPPH FREE RADICAL - SCAVENGING ACTIVITY

The antioxidant capacity of the amaranth grain oil was studied through the evaluation of the free radical-scavenging effect on the DPPH radical. The method used by De Ancos et al., (2002) was mostly modified so that it can be applied directly to determine the free radical scavenging activity of amaranth grain oil. The determination was performed as follows: 1 g of amaranth grain oil was dissolved in 10 ml of n-hexane/EtOH (20/80% ml/ml). An aliquot of 100 µl oil – solvent mixture was added to 1.5 ml of DPPH [1 mg/100 ml n-hexane/EtOH (20/80% ml/ml)]. The mixture was shaken for 5 min at 2800 rpm using miniRoto Vortexers (Fisher Scientific, Ottawa, ON, Canada) and incubated in the dark for 30 min. The absorbance was measured later, at 515 nm, against a blank of n-hexane/EtOH (20/80% ml/ml) without DPPH. Results were expressed as percentage of inhibition of the DPPH radical. Percentage of inhibition of the DPPH radical was calculated according to **Equation 4.3**:

$$DPPH\ (\%\ Inhibition) = \frac{Control\ Abs\ (517nm) - Sample\ Abs\ (517nm)}{Control\ Abs\ (517nm)}\ x\ 100 \quad \textbf{(4.3)}$$

Where, Control Abs = Absorption of Blank Sample; Sample Abs = Absorption of amaranth grain oil sample.

## 4.4 RESULTS AND DISCUSSIONS

### 4.4.1 MAE OPTIMISATION DESIGNS

Optimisation of MAE of amaranth grain oil was achieved by amalgamation of two CCRDs. The CCRD - I was structured as a three level, three factors design, to learn about the direction and the magnitude of the factors effects on responses. The CCRD - I layout, with mean (triplicate) values of responses, is presented in **Table 4.4**:

**Table 4.4: MAE CCRD - I, with observed response for oil, squalene & stigmasterol**

| Design Points | AGF(g)/ 50 (ml) Solvent | MeOH/Hexane (% ml/ml) | Extraction Time (min) | Oil Yield (%) | Squalene Yield (mg/g of oil) | Stigmasterol Yield (mg/g of oil) |
|---|---|---|---|---|---|---|
| 1 | 5 | 10 | 5 | 7.51 | 56.95 | 2.15 |
| 2 | 5 | 10 | 15 | 7.53 | 57.02 | 2.33 |
| 3 | 5 | 30 | 5 | 8.74 | 59.53 | 3.17 |
| 4 | 5 | 30 | 15 | 9.1 | 59.61 | 3.21 |
| 5 | 15 | 10 | 5 | 6.66 | 56.82 | 2.05 |
| 6 | 15 | 10 | 15 | 6.92 | 56.89 | 2.07 |
| 7 | 15 | 30 | 5 | 7.64 | 59.32 | 2.67 |
| 8 | 15 | 30 | 15 | 7.44 | 59.46 | 2.71 |
| 9 | 1.59 | 20 | 10 | 8.91 | 57.77 | 2.46 |
| 10 | 18.41 | 20 | 10 | 7.02 | 56.48 | 1.92 |
| 11 | 10 | 3.18 | 10 | 6.7 | 55.79 | 1.88 |
| 12 | 10 | 36.82 | 10 | 8.5 | 58.56 | 2.76 |
| 13 | 10 | 20 | 1.59 | 7.05 | 55.90 | 1.72 |
| 14 | 10 | 20 | 18.41 | 8.11 | 57.73 | 2.44 |
| 15 | 10 | 20 | 10 | 8.89 | 57.80 | 2.46 |
| 16 | 10 | 20 | 10 | 8.88 | 57.75 | 2.47 |
| 17 | 10 | 20 | 10 | 8.83 | 57.78 | 2.48 |
| 18 | 10 | 20 | 10 | 8.41 | 57.78 | 2.45 |
| 19 | 10 | 20 | 10 | 8.85 | 57.74 | 2.46 |
| 20 | 10 | 20 | 10 | 8.8 | 57.82 | 2.44 |

#### 4.4.1.1 MAE CCRD - I OIL YIELD (%)

The ANOVA revealed that the model was highly significant for oil yield at P value = 0.0001 with a $R^2$ value of 0.9657, which was in reasonable agreement with its adjusted $R^2$ (0.9349)

accompanied with non-significant lack of fit. This implied that the model was fitted well and could be used to predict the value of oil yield from grain amaranth by MAE. The ANOVA results are presented in the **Table 4.5**:

**Table 4.5: MAE CCRD - I ANOVA for oil yield (%)**

| Oil Yield (%) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9657; $R^2$ Adjusted = 0.9349 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 50 (ml) Solvent (5, 15) | 1 | 4.0081 | 4.0081 | 83.4119 | < 0.0001 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 3.9312 | 3.9312 | 81.8106 | < 0.0001 |
| Extraction Time (min) (5, 15) | 1 | 0.3617 | 0.3617 | 7.5282 | 0.0207 |
| AGF(g)/ 50 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.2112 | 0.2112 | 4.3962 | 0.0624 |
| AGF(g)/ 50 (ml) Solvent x Extraction Time (min) | 1 | 0.0128 | 0.0128 | 0.2664 | 0.6170 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0018 | 0.0018 | 0.0375 | 0.8504 |
| AGF(g)/ 50 (ml) Solvent x AGF(g)/ 50 (ml) Solvent | 1 | 1.1147 | 1.1147 | 23.1988 | 0.0007 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 2.3892 | 2.3892 | 49.7211 | < 0.0001 |
| Extraction Time (min) x Extraction Time (min) | 1 | 2.4729 | 2.4729 | 51.4630 | < 0.0001 |
| Model | 9 | 13.5604 | 1.5067 | 31.3552 | < 0.0001 |
| Lack of Fit | 5 | 0.3137 | 0.0627 | 1.8820 | 0.2522 |
| Error | 10 | 0.4805 | 0.0480 | | |
| Total | 19 | 14.0489 | 0.7389 | | |

The linear impact of independent variables AGF (g)/50 (ml) Solvent, MeOH/Hexane (% ml/ml), Extraction Time (min) was significant; besides that quadratic terms [AGF (g)/50 (ml) Solvent]$^2$, [MeOH/Hexane (% ml/ml)]$^2$ and [Extraction Time (min)]$^2$ were also found to be significant. The predicted, second - order, polynomial model for oil yield of AGF by MAE, in terms of coded factors is provided in **Equation 4.4**

*Oil yield (%) = 8.7753 – 0.5417 [AGF (g)/ 50 (ml) Solvent] + 0.5365 [MeOH/Hexane (%ml/ml)] + 0.1628 [Extraction Time (min)] - 0.1625 [AGF(g)/ 50 (ml) Solvent MeOH/Hexane (% ml/ml)] – 0.04 [AGF (g)/ 50 (ml) Solvent × Extraction Time (min)] – 0.015 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] – 0.2781 [AGF(g)/ 50 (ml) Solvent x AGF(g)/ 50 (ml) Solvent] – 0.4072 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (% ml/ml)] – 0.4142 [Extraction Time (min) × Extraction Time (min)]* **(4.4)**

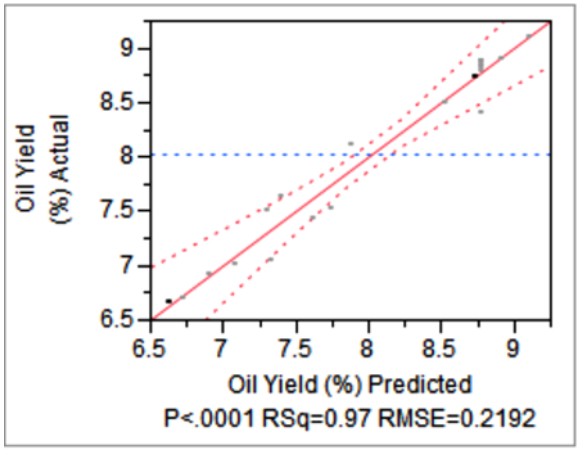



**Figure 4.1: MAE CCRD–I predicted vs actual plot for oil yield (%)**

The $R^2$ value for predicted vs actual plot **(Figure 4.1)** was 0.97 at P value < 0.0001. Hence this model can be used to predict oil yield (%) under different experimental conditions during MAE.

### 4.4.1.2 MAE CCRD - I SQUALENE AND STIGMASTEROL YIELD (mg/g of oil)

The ANOVA for squalene as well as stigmasterol revealed that the models were significant (P < 0.05) with a $R^2$ value of 0.7336 and 0.7905 respectively. The ANOVA results for squalene and stigmasterol are presented in **Tables 4.6** and **4.7**.

**Table 4.6: MAE CCRD - I ANOVA for squalene yield (mg/g of oil)**

| Squalene Yield (mg/g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.7336; $R^2$ Adjusted = 0.4938 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 50 (ml) Solvent (5, 15) | 1 | 0.5698 | 0.5698 | 0.8792 | 0.3705 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 16.2532 | 16.2532 | 25.0805 | 0.0005 |
| Extraction Time (min) (5, 15) | 1 | 0.8653 | 0.8653 | 1.3353 | 0.2747 |
| AGF(g)/ 50 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.0013 | 0.0013 | 0.0019 | 0.9658 |
| AGF(g)/ 50 (ml) Solvent x Extraction Time (min) | 1 | 0.0005 | 0.0005 | 0.0007 | 0.9795 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0008 | 0.0008 | 0.0012 | 0.9727 |
| AGF(g)/ 50 (ml) Solvent x AGF(g)/ 50 (ml) Solvent | 1 | 0.0080 | 0.0080 | 0.0124 | 0.9135 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 0.0246 | 0.0246 | 0.0379 | 0.8495 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.1065 | 0.1065 | 0.1644 | 0.6937 |
| Model | 9 | 17.8447 | 1.9827 | 3.0596 | 0.0481 |
| Lack of Fit | 5 | 6.4759 | 1.2952 | 1444.444 | < 0.0001 |
| Error | 10 | 6.4804 | 0.6480 | | |
| Total | 19 | 24.3251 | 1.2803 | | |

The predicted, second – order, polynomial model for squalene from AGF by MAE, in terms of coded factors is provided below as **Equation 4.5**

*Squalene yield (%) = 57.7394 – 0.2043 [AGF (g)/ 50 (ml) Solvent] + 1.0909 [MeOH/Hexane (%ml/ml)] + 0.2517 [Extraction Time (min)] - 0.0125 [AGF(g)/ 50 (ml) Solvent × MeOH/Hexane (% ml/ml)] + 0.0075 [AGF (g)/ 50 (ml) Solvent × Extraction Time (min)] + 0.01 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] + 0.0236 [AGF(g)/ 50 (ml) Solvent × AGF(g)/ 50 (ml) Solvent] + 0.0413 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (%ml/ml)] – 0.0859 [Extraction Time (min) × Extraction Time (min)]* **(4.5)**

**Table 4.7: MAE CCRD - I ANOVA for stigmasterol yield (mg/g of oil)**

| Stigmasterol Yield (mg/g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.7905; $R^2$ Adjusted = 0.6019 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 50 (ml) Solvent (5, 15) | 1 | 0.3767 | 0.3767 | 6.3465 | 0.0304 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 1.5765 | 1.5765 | 26.5594 | 0.0004 |
| Extraction Time (min) (5, 15) | 1 | 0.1628 | 0.1628 | 2.7421 | 0.1287 |
| AGF(g)/ 50 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.0512 | 0.0512 | 0.8626 | 0.3749 |
| AGF(g)/ 50 (ml) Solvent x Extraction Time (min) | 1 | 0.0032 | 0.0032 | 0.0539 | 0.8211 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0018 | 0.0018 | 0.0303 | 0.8652 |
| AGF(g)/ 50 (ml) Solvent x AGF(g)/ 50 (ml) Solvent | 1 | 0.0049 | 0.0049 | 0.0838 | 0.7781 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 0.0108 | 0.0108 | 0.1820 | 0.6787 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.0476 | 0.0476 | 0.8020 | 0.3915 |
| Model | 9 | 2.2393 | 0.2488 | 4.1919 | 0.0177 |
| Lack of Fit | 5 | 0.5926 | 0.1185 | 592.5568 | < 0.0001 |
| Error | 10 | 0.5936 | 0.0594 | | |
| Total | 19 | 2.8329 | 0.1491 | | |

The predicted, second – order, polynomial model for stigmasterol yield of AGF by MAE, in terms of coded factors is provided below as **Equation 4.6**

*Stigmasterol yield (%) = 2.4482 – 0.1660 [AGF (g)/ 50 (ml) Solvent] + 0.3398 [MeOH/Hexane (%ml/ml)] + 0.1092 [Extraction Time (min)] - 0.08 [AGF(g)/ 50 (ml) Solvent × MeOH/Hexane (% ml/ml)] - 0.02 [AGF (g)/ 50 (ml) Solvent × Extraction Time (min)] - 0.015 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] - 0.0186 [AGF(g)/ 50 (ml) Solvent × AGF(g)/ 50 (ml) Solvent] + 0.0274 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (%ml/ml)] – 0.0575 [Extraction Time (min) × Extraction Time (min)]* **(4.6)**

The models for squalene and stigmasterol yields indicated, highly significant influence of independent factors MeOH/Hexane (% ml/ml); in addition to that, the model also reported statistically significant influence of the AGF(g)/ 50 (ml) to solvent factor on stigmasterol yield. However, the lack of fit were highly significant for both squalene and stigmasterol yield models. Hence, it might not be appropriate to use these models for prediction purposes.

#### 4.4.1.3 OUTCOMES OF CCRD - I

The relationship between independent and dependent factors was well conveyed by CCRD - I. The maximum oil yield observed was 9.1% at a factor combination obtained using AGF (g)/ 50 (ml) solvent of 5 g/50 ml, MeOH/Hexane ratio of 30/70 (% ml/ml) and an extraction time of 15 minutes. Oil yield was found to be negatively affected at AGF (g)/50 (ml) solvent of 1.59 g/50 ml, even though there was an increase in oil yield with the decrease in the AGF (g)/50 (ml) solvent. This implied that there is a limit to positive impact on oil yield even if there is a decrease in AGF (g)/50 (ml) solvent. Although, in most analytical processes, low sample sizes are considered for higher efficiency, but if sample sizes are smaller than a certain limit, a considerable amount of microwave energy will be reflected rather than getting absorbed, which in turn will reduce the extraction output (Lebovka et al., 2011).

The experimental data presented a linear increase in oil, squalene and stigmasterol yields with an increase in MeOH/Hexane and extraction time at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml. The question arose whether MAE of amaranth grain oil and its constituents could be further enhanced or until what limit this positive relation between the responses and variables is going to last as yield cannot keep increasing after a certain limit, i.e. the linear increase should be succeeded either by a decrease or constant values after achieving its maximum limit. Hence it was hypothesised that models obtained using these experimental data may not be able to predict the effects of these independent variable correctly if levels of MeOH/Hexane and extraction time cross 30/70 (% ml/ml) and 15 min limits respectively.

Additional matter of concern was the significant lack of fit of squalene and stigmasterol models, as it was assumed that if further experiments were carried out, we might come across the

values of squalene and stigmasterol that will enable us to fit data in a model with non-significant lack of fit so that we would have reliable predictability. However, to determine whether significant lack of fit component can be eliminated from these models of squalene and stigmasterol yields, different statistical transformation mechanisms were applied to their responses; in addition to that almost all the non-significant factors were also neglected from the models to study the changes occurring in the model. However, no distinctness could be observed in the P value of lack of fit, except perhaps little changes noticed in the model's $R^2$ value. Therefore, the model was presented in its original form. Even though, lack of fit was highly significant, models were able to point out the direction of the factor's effects.

The conclusions drawn from the outcomes of CCRD - I forced the authors to investigate about the effects of factors - MeOH/Hexane and extraction time at their higher levels at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml. The best level obtained from CCRD - I for MeOH/Hexane and extraction time factors were also included in the new design to examine the change in pattern with better comparability of responses.

The CCRD - II was structured as a three levels, two factors design, to learn about the direction and the magnitude of the factors effects on responses. The CCRD – II layout with mean (triplicate) values of responses is presented in **Table 4.8**

**Table 4.8: MAE CCRD - II with observed response for oil, squalene & stigmasterol**

| Design Points | MeOH/ Hexane (% ml/ml) | Extraction Time (min) | Oil Yield (%) | Squalene Yield (mg/g of oil) | Stigmasterol Yield (mg/g of oil) |
|---|---|---|---|---|---|
| 1 | 30 | 15 | 9.18 | 59.58 | 3.16 |
| 2 | 30 | 25 | 9.16 | 59.53 | 3.15 |
| 3 | 50 | 15 | 7.38 | 53.71 | 1.87 |
| 4 | 50 | 25 | 7.37 | 53.69 | 1.85 |
| 5 | 25.86 | 20 | 8.88 | 56.71 | 2.98 |
| 6 | 54.14 | 20 | 6.74 | 51.98 | 1.8 |
| 7 | 40 | 12.93 | 7.84 | 55.89 | 1.98 |
| 8 | 40 | 27.07 | 8.36 | 55.80 | 1.97 |
| 9 | 40 | 20 | 8.48 | 55.87 | 1.98 |
| 10 | 40 | 20 | 8.17 | 55.88 | 1.95 |
| 11 | 40 | 20 | 8.29 | 55.88 | 1.94 |
| 12 | 40 | 20 | 8.73 | 55.79 | 1.97 |
| 13 | 40 | 20 | 8.33 | 55.81 | 1.98 |
| 14 | 40 | 20 | 8.39 | 55.85 | 1.99 |
| 15 | 40 | 20 | 8.41 | 55.88 | 1.95 |

#### 4.4.1.4 MAE CCRD - II Oil Yield (%)

The CCRD–II ANOVA affirmed that the model was highly significant for oil yield at P value = 0.0001 with a $R^2$ value of 0.9222, which was in reasonable agreement with its adjusted $R^2$ of 0.8789 accompanied with non-significant lack of fit. This implied that the model was fitted well and could be used to predict the value of oil yield from grain amaranth by MAE. The ANOVA results are presented in the **Table 4.9**.

**Table 4.9: MAE CCRD - II ANOVA for oil yield (%)**

| Oil Yield (%) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9222; $R^2$ Adjusted = 0.8789 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| MeOH/Hexane (% ml/ml) (30, 50) | 1 | 5.4721 | 5.4721 | 98.5093 | < 0.0001 |
| Extraction Time (min) (15, 25) | 1 | 0.0622 | 0.0622 | 1.1197 | 0.3176 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0000 | 0.0000 | 0.0005 | 0.9835 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 0.3591 | 0.3591 | 6.4650 | 0.0316 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.0385 | 0.0385 | 0.6936 | 0.4265 |
| Model | 5 | 5.9243 | 1.1849 | 21.3301 | < 0.0001 |
| Lack of Fit | 3 | 0.3145 | 0.1048 | 3.3931 | 0.0947 |
| Error | 9 | 0.4999 | 0.0555 | | |
| Total | 14 | 6.4243 | 0.4589 | | |

The linear impact of independent variable MeOH/Hexane (%ml/ml) and quadratic term [MeOH/Hexane (%ml/ml)]$^2$ were significant. The predicted, second – order, polynomial model for oil yield of AGF by MAE, in terms of coded factors is provided below in **Equation 4.7**.

$$\begin{aligned} \textit{Oil yield (\%)} = {} & \textit{8.4} - \textit{0.8271 [MeOH/Hexane (\%ml/ml)]} + \textit{0.0882 [Extraction Time (min)]} \\ & + \textit{0.0025 [MeOH/Hexane (\% ml/ml)} \times \textit{Extraction Time (min)]} \\ & - \textit{0.2156 [MeOH/Hexane (\% ml/ml)} \times \textit{MeOH/Hexane (\% ml/ml)]} \\ & - \textit{0.0706 [Extraction Time (min)} \times \textit{Extraction Time (min)]} \end{aligned} \quad \textbf{(4.7)}$$

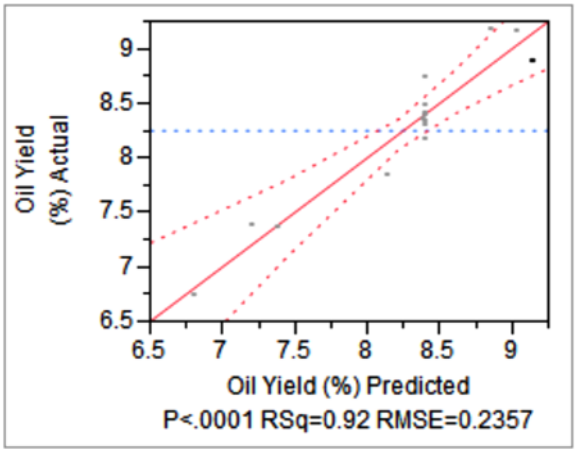



**Figure 4.2: MAE CCRD – II predicted vs actual plot for oil yield (%)**

The $R^2$ value for predicted vs actual plot (**Figure 4.2)** was 0.92 at P value < 0.0001. Hence this model can be used to predict oil yield (%) under different experimental conditions during MAE.

#### 4.4.1.5 MAE CCRD - II SQUALENE AND STIGMASTEROL YIELD (mg/g of oil)

The CCRD - II ANOVA for squalene as well as stigmasterol affirmed that the models were significant (P < 0.05) with $R^2$ values of 0.8501 and 0.9031 respectively. The ANOVA results for squalene and stigmasterol are presented in Tables **4.10** and **4.11** respectively.

**Table 4.10: MAE CCRD - II ANOVA for squalene yield (mg/g of oil)**

| Squalene Yield (mg/g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.8501; $R^2$ Adjusted = 0. 7669 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| MeOH/Hexane (% ml/ml) (30, 50) | 1 | 42.3165 | 42.3165 | 48.4735 | < 0.0001 |
| Extraction Time (min) (15, 25) | 1 | 0.0049 | 0.0049 | 0.0056 | 0.9421 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0002 | 0.0002 | 0.0003 | 0.9875 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 1.0579 | 1.0579 | 1.2119 | 0.2995 |
| Extraction Time (min) x Extraction Time (min) | 1 | 1.1148 | 1.1148 | 1.2771 | 0.2877 |
| Model | 5 | 44.5719 | 8.9149 | 10.2114 | < 0.0017 |
| Lack of Fit | 3 | 7.8485 | 2.6162 | 1894.475 | 0.0001 |
| Error | 9 | 7.8568 | 0.8729 | | |
| Total | 14 | 52.4287 | 3.7449 | | |

The predicted, second – order, polynomial model for squalene of AGF by MAE, in terms of coded factors is provided below in **Equation 4.8**.

*Squalene yield (%) = 55.8514 – 2.2999 [MeOH/Hexane (%ml/ml)] – 0.0247 [Extraction Time (min)] + 0.0075 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] – 0.3701 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (% ml/ml)] + 0.3799 [Extraction Time (min) × Extraction Time (min)]* **(4.8)**

**Table 4.11: MAE CCRD - II ANOVA for stigmasterol yield (mg/g of oil)**

| Stigmasterol Yield (mg/ g of oil) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9031; $R^2$ Adjusted = 0. 8492 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| MeOH/Hexane (% ml/ml) (30, 50) | 1 | 2.2671 | 2.2671 | 63.8561 | < 0.0001 |
| Extraction Time (min) (15, 25) | 1 | 0.0002 | 0.0002 | 0.0069 | 0.9358 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0000 | 0.0000 | 0.0007 | 0.9794 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 0.6649 | 0.6649 | 18.7272 | 0.0019 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.0569 | 0.0569 | 1.6050 | 0.2370 |
| Model | 5 | 2.9767 | 0.5953 | 16.7683 | < 0.0003 |
| Lack of Fit | 3 | 0.3174 | 0.1058 | 292.3090 | 0.0001 |
| Error | 9 | 0.3195 | 0.0355 | | |
| Total | 14 | 3.2962 | 0.2354 | | |

The predicted, second – order, polynomial model for stigmasterol yield of AGF by MAE, in terms of coded factors is provided in **Equation 4.9**.

$$\begin{aligned} \textit{Stigmasterol yield (\%)} = {} & 1.9657 - 0.5323\,[\textit{MeOH/Hexane (\%ml/ml)}] - 0.0055\,[\textit{Extraction Time (min)}] \\ & - 0.0025\,[\textit{MeOH/Hexane (\% ml/ml)} \times \textit{Extraction Time (min)}] \\ & + 0.2934\,[\textit{MeOH/Hexane (\% ml/ml)} \times \textit{MeOH/Hexane (\% ml/ml)}] \\ & + 0.0859\,[\textit{Extraction Time (min)} \times \textit{Extraction Time (min)}] \end{aligned} \quad \textbf{(4.9)}$$

The models for squalene and stigmasterol yields indicated the highly significant influence of the independent factor MeOH/Hexane (% ml/ ml); in addition to that, the model also reported the statistically significant influence of the quadratic term [MeOH/Hexane (%ml/ml)]$^2$ on stigmasterol yield. However, the lack of fit was highly significant for both squalene and stigmasterol yields so it might not be appropriate to use these models for prediction.

Same as with CCRD - I, to eliminate significant lack of fit component from the models of squalene and stigmasterol yields, different statistical transformation mechanisms were applied to their responses. In addition to that, almost all the non-significant factors were also neglected from the models to study the changes occurring in the models, but no distinctness could be

observed in the P values of lack of fit, with the exception of little changes noticed in the models' $R^2$ values. Therefore, the models were presented in their original form. Although the lack of fit was highly significant, the models were able to point out the direction of the factor's effects

#### 4.4.1.6 INTERPRETATION OF CCRD - I and CCRD - II

The purpose of the amalgamation of CCRD - I and CCRD - II was actualised as it satisfied the aspiration of optimising the MAE process. The amalgamation process witnessed a dramatic pattern of responses under various levels of extraction factors. The three dimensional response for oil yield is illustrated in **Figure 4.3**, presenting the interaction between MeOH/Hexane and extraction time at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml.

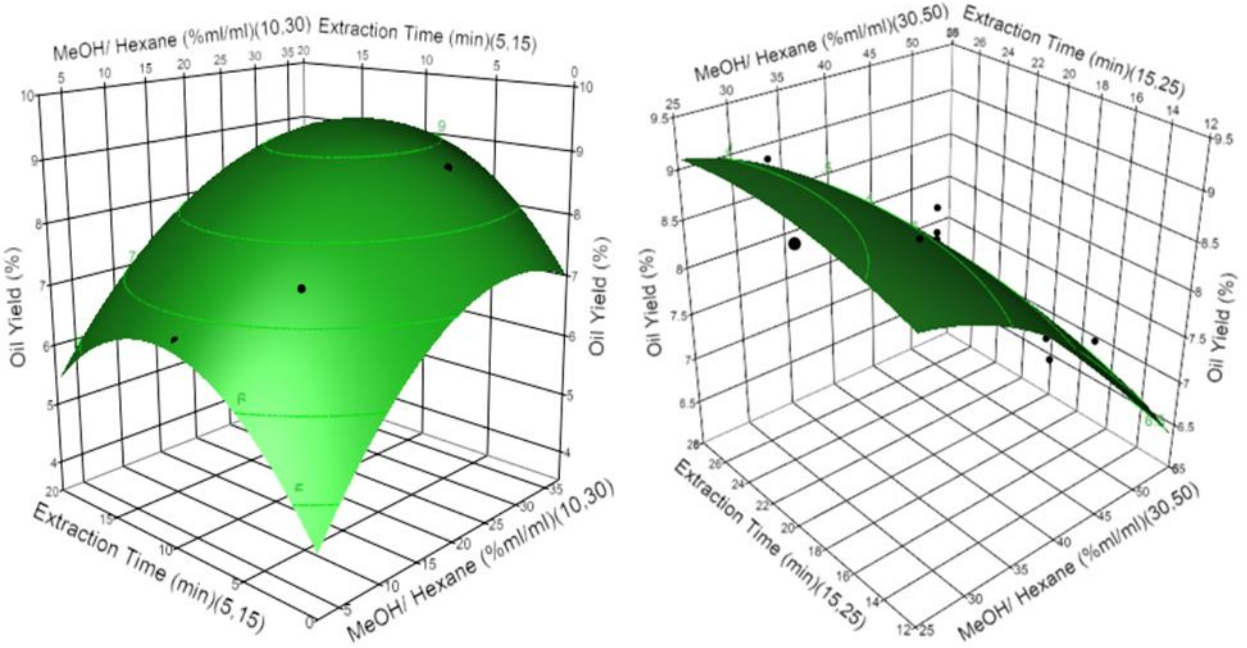



**Figure 4.3: MAE process factors effects on oil yield: CCRD - I (left) and CCRD - II (right)**

The three dimensional response of CCRD - II shown in **Figure 4.3**, clearly points out to a linearly reducing oil yield resulting from the increase in methanol concentration in hexane from 30% to 50%, whereas, the CCRD - I responses looked unclear about the yield behaviour in the event that the MeOH in Hexane concentration was increased beyond 30%.

The fact drawn from CCRD - II, is that oil yield remained constant when period of microwave exposure was increased from 15 min to 25 min while CCRD - I showed a decrease in oil yield if the extraction process was prolonged after 15 min.

The three dimensional response for squalene yield is illustrated in **Figure 4.4**, presenting the interaction between MeOH/Hexane and extraction time at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml.

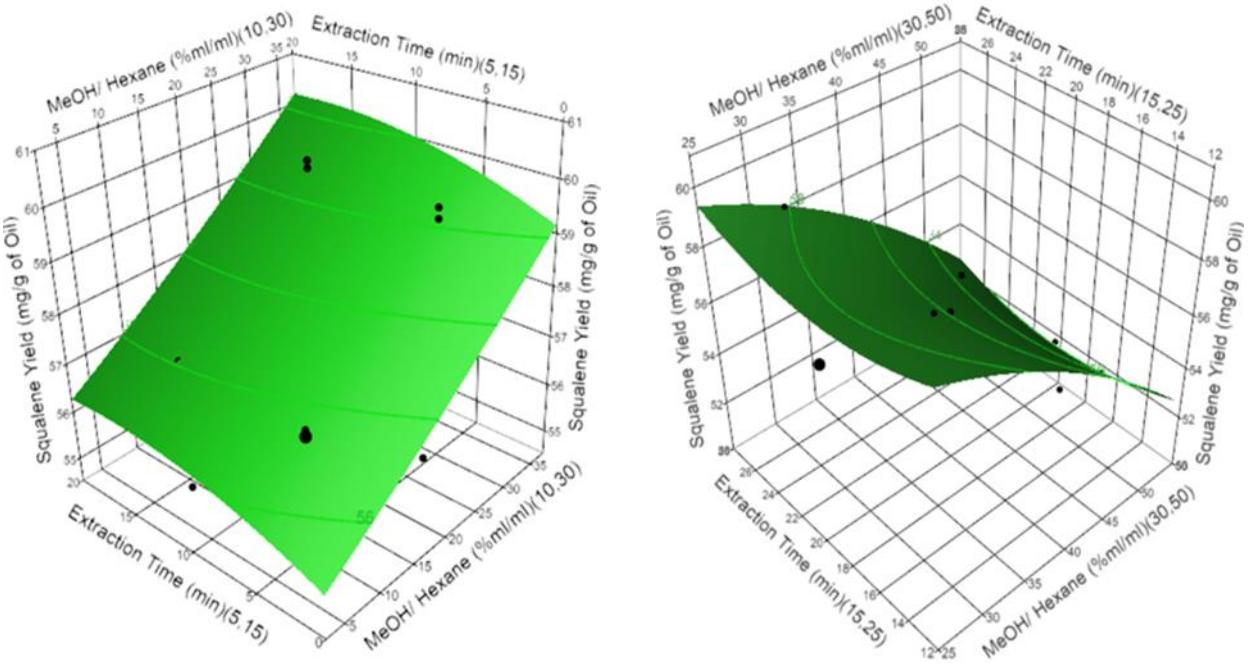



**Figure 4.4: MAE process factors effects on squalene yield: CCRD - I (left) and CCRD - II (right)**

CCRD - I predicted a linear increase in squalene yield with an increase in MeOH/Hexane and extraction time beyond 30% and 15 min respectively, in contrary the CCRD - II proved a linear decrease in squalene yield with an increase in MeOH/Hexane beyond 30% and a constant squalene yield when microwave extraction time was increased from 15 min to 25 min.

The three dimensional response for stigmasterol yield is illustrated in **Figure 4.5**, presenting the interaction between MeOH/Hexane and extraction time at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml.

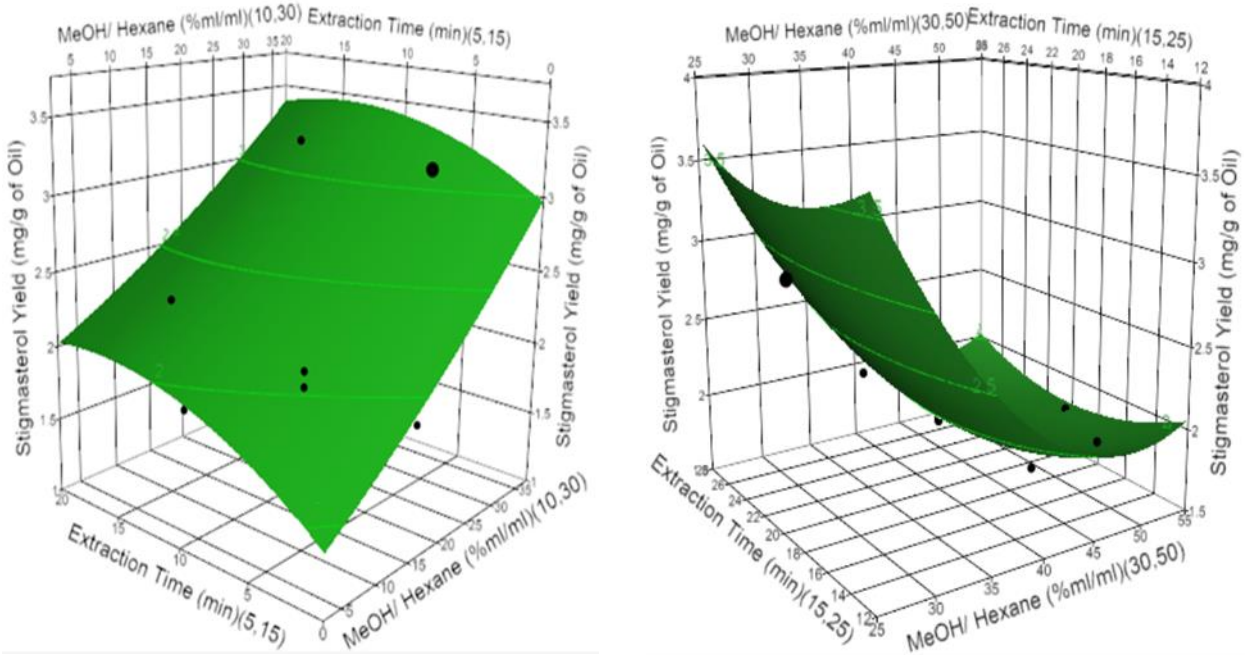



**Figure 4.5: MAE process factors effects on stigmasterol yield: CCRD - I (left) and CCRD - II (right)**

CCRD - I exhibited a linear positive impact of MeOH/Hexane and extraction time on stigmasterol yield beyond 30% and 15 min respectively. Whereas, CCRD - II demonstrated a linear negative impact of MeOH/Hexane on stigmasterol until it reached 40%, beyond that it remained constant. Furthermore, CCRD - II showed a slight parabolic pattern of stigmasterol yield caused by an increase in extraction time from 15 min to 25 min, this might be due to small variations in the biological material which can be considered as outliers. But if the focus of attention is drawn towards the stigmasterol yield obtained following 15 min and 25 min of extraction, the yield remained almost the same. This proved that there is almost no benefit of increased extraction time on stigmasterol yield beyond 15 min.

Significant lack of fit has been considered as a major aberration for predictability of any model. In statistical analysis the validity of a model is measured based on the value of error of fit (residual error) and pure error. If the model error (residual error) is in the range with pure error, the lack of fit is non-significant, while if there is large difference between model error and pure error, the lack of fit is significant. This indicates that the model error is significantly larger than the pure error. There are many criterions that could lead to a lack of fit. Nevertheless, the reason behind could be simply a very low value of pure error that tends to zero. Reproducibility can be measured by comparing the variation of the response under the same conditions, with the total

variation of the responses. The pure error is based on this variation of the responses under the same conditions. If the value of pure error tends to zero, this implies that the reproducibility factor is almost unity under the same conditions; which means the values of the response are identical (Lamberta, 1990; Ermer and Miller, 2005). As an experimental point of view, if the responses of the study are insensitive to small variations within the levels of experimental factors, it could lead to low value of pure error and hence significant lack of fit.

This can be explained further with the results obtained in this study. During the course of the optimisation process of MAE of lipids from grain amaranth, factors that greatly (negatively) affected the yields of squalene and stigmasterol were eliminated during pre-optimisation studies (**Chapter 3**). The optimisation process was operationalized using the remaining factors to which squalene and stigmasterol were minimally sensitive. Hence the difference in their yields for each combination was very little and over that even standard deviations for the replicates of each combination were also very little. This caused the pure error to be almost equal to zero. Hence the proposed models for squalene and stigmasterol yields obtained a significant lack of fit.

The main conclusion drawn from the above models predictions is that when the MAE of lipids from grain amaranth is operationalized under these independent variables: AGF (g)/50 (ml) Solvent (5 - 15 g); MeOH/Hexane (10 - 30 % ml/ml), Extraction Time (5 - 15 min), maximum amount of the squalene and stigmasterol can be extracted along with the oil. However, best extraction yields of oil and its contituents squalene and stigmasterol by MAE can be achieved with the conditions of operation at AGF (g)/50 (ml) Solvent = 5g/50 ml; MeOH/Hexane = 30/70 % ml/ml, Extraction Time: 15 min.

### 4.4.2 SOXHLET EXTRACTION OPTIMISATION DESIGN

Conventional Soxhlet extraction of amaranth grain oil was studied by CCRD for determining the best possible factor combination to have unbiased comparison with MAE. The Soxhlet CCRD layout along with mean (triplicate) values of responses are presented in **Table 4.12**.

**Table 4.12: Soxhlet CCRD with observed response for oil, squalene & stigmasterol**

| Design Points | AGF(g)/ 200 (ml) Solvent | MeOH/Hexane (% ml/ml) | Extraction Time (min) | Oil Yield (%) | Squalene Yield (mg/g of oil) | Stigmasterol Yield (mg/g of oil) |
|---|---|---|---|---|---|---|
| 1 | 20 | 10 | 60 | 8.68 | 55.72 | 2.11 |
| 2 | 20 | 10 | 120 | 8.71 | 55.78 | 2.17 |
| 3 | 20 | 30 | 60 | 8.5 | 49.38 | 1.54 |
| 4 | 20 | 30 | 120 | 8.76 | 50.1 | 1.62 |
| 5 | 30 | 10 | 60 | 8.07 | 55.59 | 2.04 |
| 6 | 30 | 10 | 120 | 8.12 | 55.67 | 2.09 |
| 7 | 30 | 30 | 60 | 7.93 | 49.21 | 1.43 |
| 8 | 30 | 30 | 120 | 8.25 | 49.03 | 1.25 |
| 9 | 16.59 | 20 | 90 | 8.88 | 58.78 | 3.06 |
| 10 | 33.41 | 20 | 90 | 8 | 57.92 | 2.87 |
| 11 | 25 | 3.18 | 90 | 8.37 | 53.81 | 1.97 |
| 12 | 25 | 36.82 | 90 | 8.45 | 48.32 | 1.16 |
| 13 | 25 | 20 | 39.55 | 8.02 | 57.12 | 2.28 |
| 14 | 25 | 20 | 140.45 | 8.43 | 57.03 | 2.17 |
| 15 | 25 | 20 | 90 | 8.38 | 57.38 | 2.26 |
| 16 | 25 | 20 | 90 | 8.39 | 57.63 | 2.48 |
| 17 | 25 | 20 | 90 | 8.41 | 57.41 | 2.34 |
| 18 | 25 | 20 | 90 | 8.36 | 57.28 | 2.49 |
| 19 | 25 | 20 | 90 | 8.51 | 57.55 | 2.23 |
| 20 | 25 | 20 | 90 | 8.47 | 57.65 | 2.45 |

#### 4.4.2.1 SOXHLET CCRD OIL YIELD (%)

The ANOVA revealed that the model was highly significant for oil yield at P value < 0.0001 with a $R^2$ value of 0.9759, which was in reasonable agreement with its adjusted $R^2$ of 0.9542 accompanied with non-significant lack of fit. This implied that the model was fitted well and could be used to predict the value of oil yield from grain amaranth by Soxhlet. The ANOVA results are presented in **Table 4.13**.

**Table 4.13: Soxhlet CCRD - ANOVA for oil yield (%)**

| Oil Yield (%) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.9759; $R^2$ Adjusted = 0.9542 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 200 (ml) Solvent (20, 30) | 1 | 1.0352 | 1.0352 | 330.9784 | < 0.0001 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 0.0000 | 0.0000 | 0.0007 | 0.9795 |
| Extraction Time (min) (60, 120) | 1 | 0.1334 | 0.1334 | 42.6381 | < 0.0001 |
| AGF(g)/ 200 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.0018 | 0.0018 | 0.5755 | 0.4656 |
| AGF(g)/ 200 (ml) Solvent x Extraction Time (min) | 1 | 0.0008 | 0.0008 | 0.2558 | 0.6240 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0313 | 0.0313 | 9.9915 | 0.0101 |
| AGF(g)/ 200 (ml) Solvent x AGF(g)/ 200 (ml) Solvent | 1 | 0.0020 | 0.0020 | 0.6531 | 0.4378 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 0.0000 | 0.0000 | 0.0078 | 0.9315 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.0592 | 0.0592 | 18.9374 | 0.0014 |
| Model | 9 | 1.2670 | 0.1408 | 45.0112 | < 0.0001 |
| Lack of Fit | 5 | 0.0145 | 0.0029 | 0.8617 | 0.5629 |
| Error | 10 | 0.0313 | 0.0031 | | |
| Total | 19 | 1.2982 | 0.0683 | | |

The linear variables such as AGF (g)/ 200 (ml) Solvent, Extraction Time (min); bilinear variable MeOH/Hexane (% ml/ml) x Extraction Time (min) and quadratic variable [Extraction Time (min)]$^2$ were found to be significant. The predicted, second – order, polynomial model for oil yield of AGF by Soxhlet extraction, in terms of coded factors is provided in **Equation 4.10**.

*Oil yield (%) = 8.4193 – 0.2753 [AGF (g)/ 200 (ml) Solvent] - 0.0004 [MeOH/Hexane (%ml/ml)] + 0.0988 [Extraction Time (min)] + 0.015 [AGF (g)/ 200 (ml) Solvent × MeOH/Hexane (% ml/ml)] + 0.01 [AGF (g)/ 200 (ml) Solvent × Extraction Time (min)] + 0.0625 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] + 0.0119 [AGF(g)/ 200 (ml) Solvent × AGF(g)/ 200 (ml) Solvent] 0.0013 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (% ml/ml)] – 0.0641 [Extraction Time (min) × Extraction Time (min)]* **(4.10)**

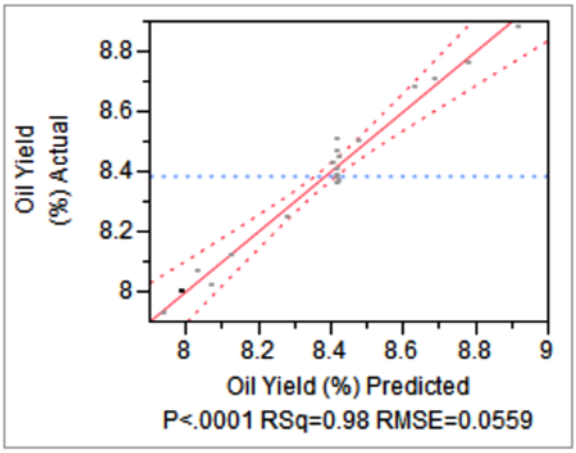



**Figure 4.6: Soxhlet Predicted vs actual plot for oil yield (%)**

The $R^2$ value for predicted vs actual plot (**Figure 4.6)** was 0.98 at P value < 0.0001. Hence this model can be used to predict oil yield (%) under different experimental conditions during Soxhlet extraction.

### 4.2.2.2 SOXHLET CCRD SQUALENE AND STIGMASTEROL YIELD (mg/g of oil)

The ANOVA for squalene as well as stigmasterol revealed that the models were significant (P < 0.05) with $R^2$ values of 0.8629 and 0.8329 respectively. The ANOVA results for squalene and stigmasterol are presented in **Tables 4.14** and **4.15** respectively.

**Table 4.14: Soxhlet CCRD - ANOVA for squalene yield (mg/g of oil)**

| Squalene Yield (mg/g of Oil ) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.8629; $R^2$ Adjusted = 0.7397 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 200 (ml) Solvent (20, 30) | 1 | 0.6271 | 0.6271 | 0.1898 | < 0.6723 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 86.0111 | 86.0111 | 26.0343 | 0.0005 |
| Extraction Time (min) (60, 120) | 1 | 0.0205 | 0.0205 | 0.0062 | 0.9388 |
| AGF(g)/ 200 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.1250 | 0.1250 | 0.0378 | 0.8497 |
| AGF(g)/ 200 (ml) Solvent x Extraction Time (min) | 1 | 0.0968 | 0.0968 | 0.0293 | 0.8675 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0200 | 0.0200 | 0.0061 | 0.9395 |
| AGF(g)/ 200 (ml) Solvent x AGF(g)/ 200 (ml) Solvent | 1 | 1.1942 | 1.1942 | 0.3615 | 0.5611 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 118.1673 | 118.1673 | 35.7675 | 0.0001 |
| Extraction Time (min) x Extraction Time (min) | 1 | 7.8627 | 7.8627 | 2.3799 | 0.1539 |
| Model | 9 | 208.0925 | 23.1214 | 6.9985 | < 0.0027 |
| Lack of Fit | 5 | 32.9265 | 6.5853 | 296.2792 | < 0.0001 |
| Error | 10 | 33.0376 | 3.3038 | | |
| Total | 19 | 241.1301 | 12.6911 | | |

The predicted, second – order, polynomial model for squalene yield of AGF by Soxhlet extraction, in terms of coded factors is provided in **Equation 4.11**.

*Squalene yield (mg/g of oil) = 57.5743 – 0.2143 [AGF (g)/ 200 (ml) Solvent] - 2.5096 [MeOH/Hexane (%ml/ml)] + 0.0387 [Extraction Time (min)] – 0.125 [AGF (g)/ 200 (ml) Solvent × MeOH/Hexane (% ml/ml)] - 0.11 [AGF (g)/ 200 (ml) Solvent × Extraction Time (min)] + 0.05 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] - 0.2879 [AGF (g)/ 200 (ml) Solvent × AGF(g)/ 200 (ml) Solvent] - 2.8635 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (% ml/ml)] – 0.7386 [Extraction Time (min) × Extraction Time (min)]*

**(4.11)**

**Table 4.15: Soxhlet CCRD - ANOVA for stigmasterol yield (mg/g of oil)**

| Stigmasterol Yield (mg/ g of Oil ) | | | | | |
|---|---|---|---|---|---|
| $R^2$ = 0.8329; $R^2$ Adjusted = 0.6826 | | | | | |
| Source of Variation | *df* | Sum of Squares | Mean Square | F Ratio | Prob > F |
| AGF(g)/ 200 (ml) Solvent (20, 30) | 1 | 0.0660 | 0.0660 | 0.8415 | 0.3805 |
| MeOH/Hexane (% ml/ml) (10, 30) | 1 | 1.1322 | 1.1322 | 14.4322 | 0.0035 |
| Extraction Time (min) (60, 120) | 1 | 0.0022 | 0.0022 | 0.0286 | 0.8691 |
| AGF(g)/ 200 (ml) Solvent x MeOH/Hexane (% ml/ml) | 1 | 0.0136 | 0.0136 | 0.1735 | 0.6858 |
| AGF(g)/ 200 (ml) Solvent x Extraction Time (min) | 1 | 0.0091 | 0.0091 | 0.1162 | 0.7403 |
| MeOH/Hexane (% ml/ml) x Extraction Time (min) | 1 | 0.0055 | 0.0055 | 0.0703 | 0.7963 |
| AGF(g)/ 200 (ml) Solvent x AGF(g)/ 200 (ml) Solvent | 1 | 0.1773 | 0.1773 | 2.2595 | 0.1637 |
| MeOH/Hexane (% ml/ml) x MeOH/Hexane (% ml/ml) | 1 | 2.1258 | 2.1258 | 27.0971 | 0.0004 |
| Extraction Time (min) x Extraction Time (min) | 1 | 0.3274 | 0.3274 | 4.1732 | 0.0683 |
| Model | 9 | 3.9109 | 0.4346 | 5.5392 | < 0.0066 |
| Lack of Fit | 5 | 0.7192 | 0.1438 | 11.0047 | < 0.0099 |
| Error | 10 | 0.7845 | 0.0785 | | |
| Total | 19 | 4.6955 | 0.2471 | | |

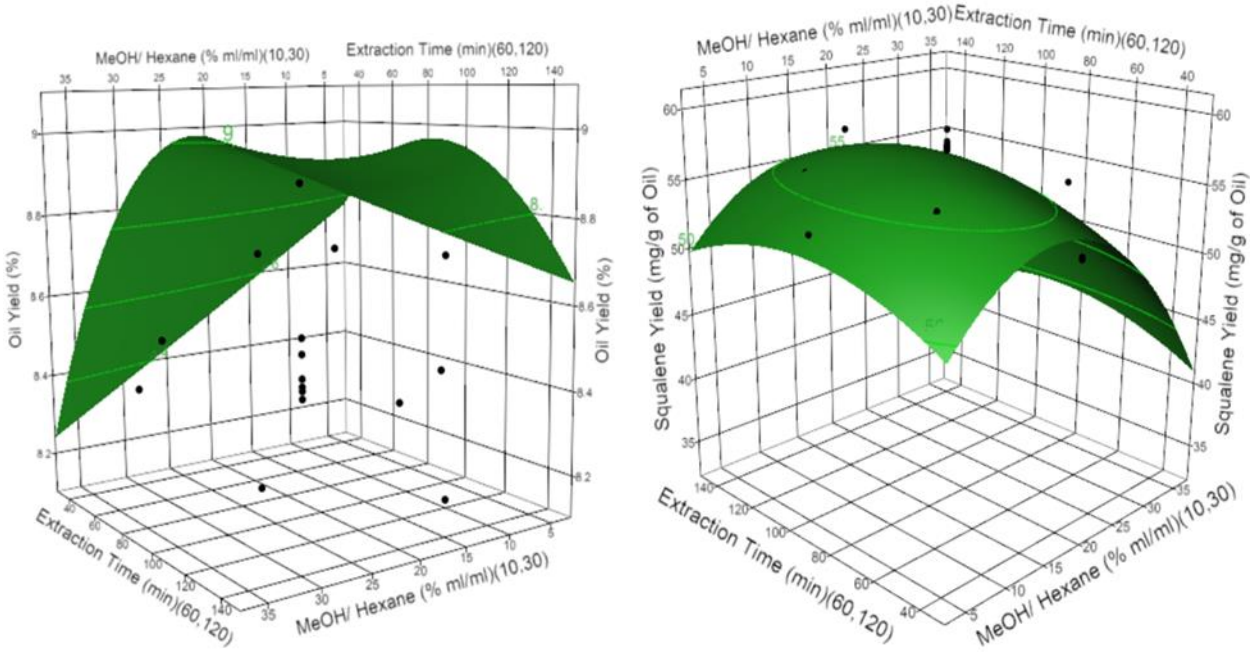
The predicted, second – order, polynomial model for stigmasterol yield of AGF by Soxhlet extraction, in terms of coded factors is provided in **Equation 4.12**.

*Stigmasterol yield (%) = 2.3899 – 0.0695 [AGF (g)/ 200 (ml) Solvent] - 0.2879 [MeOH/Hexane (%ml/ml)] - 0.0128 [Extraction Time (min)] - 0.0413 [AGF(g)/ 200 (ml) Solvent × MeOH/Hexane (% ml/ml)] - 0.0338 [AGF (g)/ 200 (ml) Solvent × Extraction Time (min)] - 0.0263 [MeOH/Hexane (% ml/ml) × Extraction Time (min)] + 0.1109 [AGF(g)/ 200 (ml) Solvent × AGF(g)/ 200 (ml) Solvent] - 0.3840 [MeOH/Hexane (% ml/ml) × MeOH/Hexane (% ml/ml)] – 0.1507 [Extraction Time (min) × Extraction Time (min)]* **(4.12)**

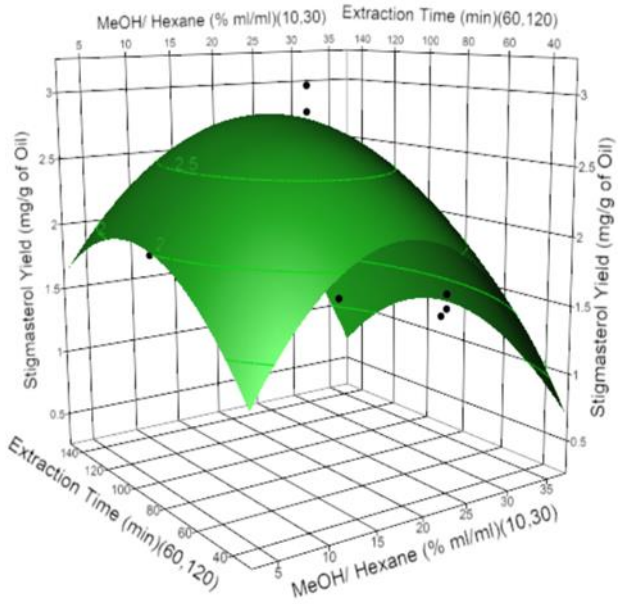
The models of squalene and stigmasterol indicated significant influence of linear variable MeOH/Hexane (% ml/ml) and quadratic variable [MeOH/Hexane (% ml/ml)]$^2$. However, the lack of fit was highly significant for both squalene and stigmasterol yields so it might not be appropriate to use these models for prediction.

### 4.4.3 INTERPRETATION OF SOXHLET EXTRACTION AND ITS COMPARISON WITH MAE

The response surface plots obtained (**Figure 4.7**) as a result of operationalizing CCRD for Soxhlet extraction, well conveyed the relationship between independent and dependent process variables and their impact on responses.




**(a)** **(b)**




**(c)**

**Figure 4.7: Soxhlet extraction process factors effects on oil (a), squalene (b) and stigmasterol (c) yields.**

It is distinctly evident from the Soxhlet model predictions that yields of oil, squalene and stigmasterol were improved by reducing the AGF to solvent ratio from 30 g/200 ml to 20 g/200 ml, moreover, responses were found to be maximised at a much lower sample to solvent ratio of 16 g/200 ml. This trend does not coincide with the trend showed by MAE, as MAE was showed to have a limit to its positive impact, with a decrease in AGF to solvent ratio.

At a constant AGF to solvent ratio, Soxhlet showed a rise in the recovery of squalene and stigmasterol with an increase in MeOH/Hexane from 10% to 20% and further increase in methanol concentration caused negative impact on their respective yields. Additionally, Soxhlet showed a negative pattern in oil yield with the rise in MeOH/Hexane. Furthermore, MAE exhibited linear increase in all the responses until it reached 30% MeOH/Hexane.

After 90 min of Soxhlet extraction operating at a constant AGF to solvent ratio, the oil yield appeared to have positive linear trend with extraction time until it reached its maximum limit, similar growth orientation was adopted by squalene and stigmasterol, however oil and squalene yield remained stable after reaching their respective maximum values whereas, stigmasterol started showing diminishing effect in its yield when the Soxhlet extraction was extended beyond 90 min. In case of MAE, extraction time and responses continued their positive linear

relationship until 15 min of process and then after reaching the maximum limit, null effect was noticed on responses with further increase in extraction time.

Soxhlet's predicted models for squalene and stigmasterol also reported significant lack of fit, as reported by MAE.

The optimised MAE method achieved the highest extraction yield of 9.1% amaranth grain oil containing 59.53 mg/g of squalene and 3.17 mg/g of stigmasterol, operating within the process factors: AGF (g)/50 (ml) solvent = 5 g/50 ml; MeOH/ Hexane (% ml/ml) = 30/70 (% ml/ml); Extraction Time = 15 min, whereas, optimised Soxhlet extraction achieved the highest extraction yield of 8.8% amaranth oil containing 58.78 mg/g of squalene and 3.06 mg/g of stigmasterol operating within the process factors: AGF (g)/200 (ml) solvent = 16.59 g/200 ml; MeOH/ Hexane (% ml/ml) = 30/70 (% ml/ml); Extraction Time = 90 min.

The experiments were carried out again using MAE and Soxhlet at their respective best factor combinations to investigate the effect of AGF's moisture content on the responses, using non freeze dried AGF (moisture content: 12.39% wet basis, SD = 0.23; moisture content of freeze dried AGF: 5.1% wet basis, SD = 0.06). The MAE study indicated a slight rise in oil yield from 9.1% to 9.38% and no significant changes in the yields of squalene and stigmasterol whereas; Soxhlet extraction was not affected by moisture content.

### 4.4.4 TOTAL POLYPHENOL AND α- TOCOPHEROL ESTIMATION

Total polyphenol and α- Tocopherol content of amaranth grain oil was obtained from MAE and Soxhlet extraction at their respective best factors combination (**Figure 4.8**).

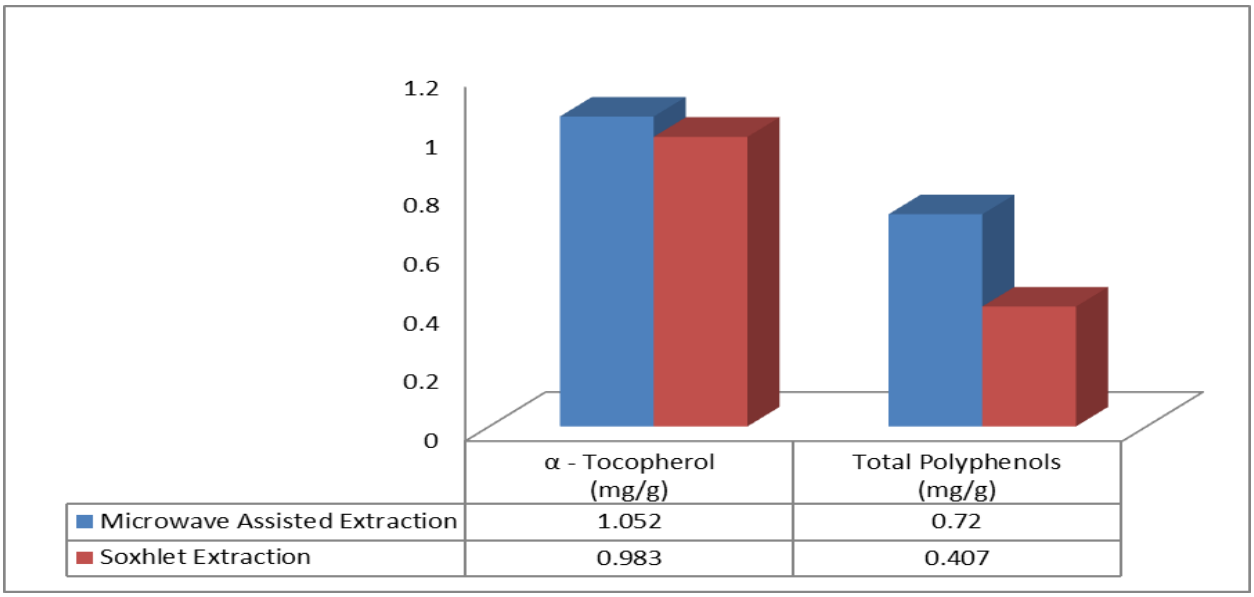



**Figure 4.8: Comparison between MAE & Soxhlet in terms of α – tocopherol and total polyphenol content**

MAE showed an increase in yield of thermo-liable constituents in oil like total polyphenols & α – tocopherol by 43.90% & 6.51% respectively when compared to Soxhlet.

### 4.4.5 ANTIOXIDANT SCAVENGING ACTIVITY

The impact of MAE and Soxhlet on the oil's antioxidant scavenging activities can be clearly viewed in Figure **4.9**

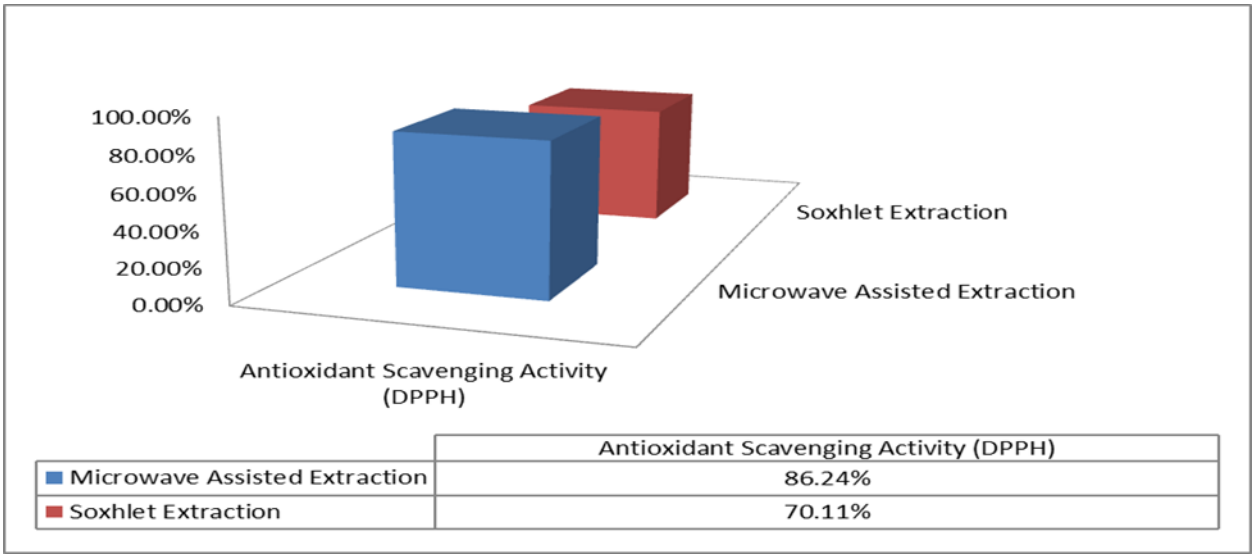



**Figure 4.9: Comparison between MAE & Soxhlet in terms of their oil's antioxidant scavenging activities.**

The antioxidant scavenging activity of the oil obtained from MAE was 16.13% greater than oil extracted by Soxhlet, which can be attributed to its higher polyphenolic and α-tocopherol content.

## 4.5 CONCLUSION

This study has established MAE as a more industrially viable methodology for extracting amaranth grain oil than standard Soxhlet extraction methodology not just limited to higher oil yield with reduced extraction time and solvent consumption but also in preserving the best quality of its high value industrially applicable thermo stable (squalene & stigmasterol) as well as the thermolabile (α – tocopherol and total tolyphenol) constituents of oil, maintaining higher antioxidant scavenging activity.

## 4.6 ACKNOWLEDGEMENT

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## PREFACE TO CHAPTER 5

**Chapter 3** and **Chapter 4** of the thesis presented the detailed process of designing and optimising the methodology for eluting lipophilic compounds from grain amaranth and compared its efficiency with standard Soxhlet method in terms of oil yield and its quality.

**Chapter 5,** concludes this thesis by summarising the main results of the presented research, highlights the intended and possible applications of the developed methodology and states the ways that the generated results contribute to the scientific knowledge.

# CHAPTER 5: **GENERAL SUMMARY AND CONCLUSION**

Amaranth based lipophilic compounds find their application in nutraceutical as well as cosmetic industries. Extraction is one of the key processing steps in recovering and purifying lipophilic ingredients contained in plant-based materials. Classical extraction technologies are very time and solvent consuming and also result in incomplete extraction from the sample matrix. Therefore, there has been an increase in the exploration of novel extraction technologies. Microwave assisted extraction (MAE) is a potential alternative to classical extraction methodologies as it takes shorter extraction time, consumes less solvent, coupled with higher extraction rates with better product quality. For efficient MAE, the operating factors of the extraction process must be first optimized.

The experimental factors which determine the performance of MAE processes using a mono-mode open microwave system include the sample matrix characteristics & size, solvent nature & volume, extraction time, microwave power and temperature.

The MAE optimisation of lipophilic content of amaranth was built upon the outcomes of a pre-optimisation study. Pre-optimisation consisted of a parameters screening study followed by a full factorial screening design. The parameters screening study indicated the significance of using amaranth grain flour (AGF) over whole amaranth grain and conducting MAE using solvent mixtures composed of a strong and a weak microwave absorbing solvent operating above the minimum microwave input power of 80 W. Based on the conclusions drawn from the parameters screening study, a full factorial screening design was conceptualised using microwave input power with four levels - 80, 120, 160 and 200 W and extraction solvent with three mixture concentrations – A, B and C (A–Methanol/Hexane = 20/80% ml/ml; B–Ethanol/Hexane = 20/80% ml/ml; C–100% Hexane) at constant AGF to solvent ratio (10 g/50 ml) and extraction time (10 min). The MAE responses were found to be maximized when operating at 120 W using solvent A. However, this full factorial screening design could not predict how the responses would be affected if values of AGF to solvent ratio (g/ml), concentrations of methanol in hexane (% ml/ml) and extraction times (min) were varied, hence at that moment it was necessary to determine whether responses could be further enhanced using these factors to create a central

composite rotatable design (CCRD) operating at a microwave input power of 120 W. However, before carrying out a response surface optimisation study, using above mentioned factors, it was necessary to decide on the levels for these factors; hence the tested levels of AGF to solvent ratio (10 g/50 ml of solvent) and extraction time (10 min) were considered as central points for a proposed central composite rotatable (CCRD) design. This would enable the study to determine best levels for maximising responses as it broadens the scope of the factor levels. Moreover, before selecting the concentration ranges for methanol in hexane that could be varied for further optimisation process, it was necessary to study how different concentrations of methanol in hexane affect the microwave absorbing capacity of the whole solvent. Therefore, a bivariate cubic polynomial mathematical model was proposed which predicted the microwave output power for MAE by varying the concentration of methanol in hexane (% ml/ml) at a fixed microwave input power of 120 W. This study proved that the microwave absorbing capacity of the methanol-hexane mixture was increased linearly until concentration of methanol reached 30% initially, hence this strongly supported to test 20% methanol in hexane (% v/v) as the recommended central point for a proposed central composite rotatable (CCRD) design experiment.

The optimisation procedures for MAE started with a CCRD (CCRD - I) framed using the following factors, that is, AGF/solvent (5g - 15g/50 ml); MeOH/ Hexane (10 - 30% ml/ml) and Extraction Time (5 - 15 min) operating at constant microwave input power of 120 W. CCRD - I observed a maximum oil yield of 9.1% at a factor combination obtained using AGF (g)/50 (ml) solvent of 5 g/50 ml, MeOH/Hexane ratio of 30/70 (% ml/ml) and an extraction time of 15 minutes. Oil yield was found to be negatively affected at AGF (g)/50 (ml) solvent of 1.59 g/50 ml, even though there was an increase in oil yield with the decrease in the AGF (g)/50 (ml) solvent. This implied that there is a limit to the positive impact on oil yield even if there is a decrease in AGF (g)/50 (ml) solvent. However, the experimental data couldn't present a limit to the linear increase in oil, squalene and stigmasterol yields with an increase in MeOH/Hexane and extraction time at a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml i.e. the linear increase should be followed either by a decrease or constant values after achieving its maximum limit. Hence it was hypothesised that the models obtained using these experimental data may not be able to predict the effects of these independent variables correctly if levels of MeOH/Hexane and extraction

time cross 30/70 (% ml/ml) and 15 min limits respectively. Additional matter of concern was the significant lack of fit of the squalene and stigmasterol models, it was assumed, if further experiments were carried out, that we might come across the values of squalene and stigmasterol that would enable us to fit data in a model with non-significant lack of fit so that we would have reliable predictability.

As a result, CCRD - II was structured using a fixed AGF (g)/50 (ml) solvent of 5 g/50 ml by varying following factors MeOH/Hexane (30 - 50% ml/ml) and Extraction Time (15 - 25 min) operating at constant microwave input power of 120 W. When the outcomes of CCRD - I & CCRD - II were amalgamated, it actualised the optimisation process. The dramatic pattern of responses revealed determining effects in the yields of oil and its constituents' squalene and stigmasterol with an increase in MeOH/Hexane beyond 30%, on the other side, null effects were noticed with an increase in extraction beyond 15 min. The main conclusion drawn from the above models predictions is that when the MAE of lipids from grain amaranth is operationalized under these independent variables: AGF (g)/50 (ml) Solvent (5 - 15 g); MeOH/Hexane (10 - 30 % ml/ml), Extraction Time (5 - 15 min), maximum amount of squalene and stigmasterol can be extracted along with the oil. However, best extraction yields of oil, squalene and stigmasterol by MAE can be achieved with the conditions of operation at AGF (g)/50 (ml) Solvent = 5g/50 ml; MeOH/Hexane = 30/70 % ml/ml, Extraction Time: 15 min.

For the unbiased comparison, Soxhlet extraction was also optimised, within the factors as follows: AGF (g)/200 (ml) Solvent (20 - 30 g); MeOH/Hexane (10 - 30 % ml/ml), Extraction Time 60 - 120 min). The optimised Soxhlet extraction achieved the highest extraction yield of 8.8% amaranth oil containing 58.78 mg/g of squalene and 3.06 mg/g of stigmasterol operating within the process factors: AGF(g)/200 (ml) solvent = 16.59 g/200 ml; MeOH/Hexane (% ml/ml) = 30/70 (% ml/ml); Extraction Time = 90 min. This result proved MAE slightly better than Soxhlet in their respective responses at a lower sample to solvent ratio and extraction time.

However, experiments were carried out again using MAE and Soxhlet at their respective best factor combinations to investigate the effect of AGF's moisture content on the responses, using non freeze-dried AGF (moisture content: 12.39% wet basis, SD = 0.23; moisture content of

freeze dried AGF: 5.1% wet basis, SD = 0.06). The MAE study indicated a rise in oil yield from 9.1% to 9.38% and there were no significant changes in the yields of squalene and stigmasterol whereas, Soxhlet extraction was not affected by moisture content.

Furthermore, MAE also proved to be superior over Soxhlet in better preserving the thermo labile constituents like polyphenols and α - tocopherol coupled with higher free radical-scavenging activity when compared at their respective best factor combinations.

The present study confirms MAE as a more industrially viable methodology for extracting lipids from amaranth grain over standard Soxhlet extraction methodology not just limited to higher oil yield with reduced extraction time and solvent consumption but also for preserving the best quality of its high value industrially applicable thermostable compounds, squalene & stigmasterol as well as the thermolabile compounds α - tocopherol and total polyphenol, maintaining higher antioxidant scavenging activity.

# REFERENCES


Aguilera, J.M. (2003). Solid liquid extraction. In Tazia, C., Liadakis, G. (Eds). Extraction optimisation in Food Engineering. Marcel Dekker Inc., New York, p: 35.

Aioi, A., Shimizu, T., Kuriyama, K. (1995). Effect of squalene on superoxide anion generation induced by a skin irritant, lauroylsarcosine. International Journal of Pharmaceutics, 113 (2), p: 159 - 164.

Alfaro, M.J., Bélanger, J.M.R., Padilla, F.C., Paré, J.R.R (2003). Influence of solvent, matrix dielectric properties and applied power on the liquid-phase microwave assisted extraction of ginger (*Zingiber officinale*). Food Research International, 36 (5), p: 499 - 504.

Amaranth Institute (2001). "Legacy" The Amaranth Institute newsletter, 14 (1). http://amaranthinstitute.org/sites/default/files/docs/14_1.pdf

Amarowicz, R. (2009). Editorial, Squalene: A natural antioxidant? European Journal of Lipid Science and Technology, 111, p: 411 - 412.

Anonymous (1998). Policy paper: Nutraceuticals / functional foods and health claims on foods. Therapeutic Products Programme and the Food Directorate from the Health Protection Branch, Health Canada, p: 3. http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/labeletiquet/nutra-funct_foods-nutra-fonct_aliment-eng.pdf

Baghurst, D.R., Mingos, D.M.P. (1992). Superheating Effects Associated with Microwave Dielectric Heating. Journal of the Chemical Society, Chemical Communications, 9, p: 674 - 677.

Bale, J.R., Kaufmann, C.S. (1992). Special issue on grain amaranth. New potential for an old crop. Food Reviews International, 8 (1), p: 5 - 49.

Baker, H., Frank, O. (1968). Clinical vitaminology methods and interpretation. Interscience Publishers Inc., New York, NY, USA, p: 172 - 175.

Baker, L. A., Rayas-Duarte, P. (1998A). Freeze – thaw stability of amaranth starch and the effects of salt and sugars. Cereal Chem, 75 (3), p: 301 - 307.

Baker, L. A., Rayas-Duarte, P. (1998B). Retrogradation of amaranth starch at different storage temperatures and the effects of salt and sugars. Cereal Chemistry, 75 (3), p: 308 - 314.

Barbero, G.F., Palma, M., Barroso, C.G. (2006). Determination of capsacinoids in peppers by microwave assisted extraction high performance liquid chromatography with fluorescence detection. Analytica Chimica Acta. 578 (2), p: 227 - 233.

Bartsch, H., Nair, J., Owen, R.W. (1999). Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis, 20 (12), p: 2209 - 2218.

Becker, R. (1994). Amaranth oil: composition, processing and nutritional qualities. In Paredes-Lopez, O. (Eds). Amaranth – biology, chemistry and technology. CRC Press, London, p: 133 - 142.

Becker, R., Wheeler, E. L., Lorenz, K. (1981). A compositional study of amaranth grain. Journal of Food Science, 46 (4), p: 1175 - 1180.

Bejosano, F.P., Corke, H. (1998). Effect of Amaranthus and buckwheat proteins on wheat dough properties and noodle quality. Cereal Chem, 75 (2), p: 171 - 176.

Bello-Pérez, L. A., de Léon, Y.P., Agama-Acevedo, E., Paredes-López, O. (1998B). Isolation and characterization of amaranth and banana starches. Starch/Stärke 50 (10), p: 409 - 413.

Belter, A., Skupinska, M., Giel-Pietranszuk, M., Grabarkiewicz, T., Rychlewski, L., Barciszewski, J. (2011). Squalene monooxygenase, a target for hypercholesterolemic therapy. The Journal of Biological Chemistry, 392 (12), p: 1053 - 1075.

Bergera, A., Monnardb, I., Dionisib, F., Gumyc, D., Hayesd, K.C., Lambeletb, P. (2003). Cholesterol-lowering properties of amaranth flakes, crude and refined oils in hamsters. Food Chemistry, 81 (1), p: 119 - 124.

Betschart, A.A., Wood, I.D., Shepherd, A.D., Saunders, R.M. (1981). Amaranthus cruentus: milling characteristics, distribution of nutrients within seed components and the effects of temperature on nutritional quality. Journal of Food Science, 46 (4), p: 1181 - 1187.

Bhagya, S., Srinivas, H. (1992). Extraction of soybean (Glycine max.) with hexane-acetic acid: Effect on oil quality. Food Chemistry, 44 (2), p: 123 - 125.

Blasco, L., Duracher, L., Forestier, J.P. (2006). Skin constituents as cosmetic ingredients: part I: a study of bio-mimetic monoglycerides behavior at the squalene-water interface by the "pendant drop" method in a static mode. Journal of Dispersion Science and Technology. 27 (6), p: 799 - 810.

Bligh, E.G., Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37 (8), p: 911 - 917.

Breene, W.M. (1991). Food uses of grain amaranth. Cereal Foods World, 36 (5), p: 426 - 430.

Brenner, D. Hauptli, H. (1990). Seed shattering control with indehiscent utricles in grain amaranth. Legacy 3:2-3. American Amaranth Institute, Bricelyn, MN.

Bressani, R. (1993). Amaranth. In Macrae, R., Robinson, R.K., Sadler, M.J. (Eds). Encyclopaedia of Food Science, Food Technology and Nutrition. Academic Press, London, 1, p: 135 – 140.

Bressani, R. (1994). Composition and nutritional properties of amaranth. In Paredes-Lopez, O. (Eds). Amaranth--Biology, Chemistry, and Technology. London, CRC Press, p: 185 - 206.

Bressani, R., Elías, L.G., González, J.M., Gómez – Brenes R., (1987A). The chemical composition and protein quality of amaranth grain germ plasm in Guatemala. Archivos Latinoamericanos de Nutrición, 37 (2), p: 364 - 377.

Bressani, R., Gonzalez, J.M., Zuniga, J., Breuner, M., Elias, L.G. (1987B). Yield, selected chemical composition and nutritive value of 14 selections of amaranth grain representing four species. Journal of the Science of Food and Agriculture, 38 (4), p: 347 - 356.

Bressani, R., Ligorria, L.E. (1994). Effect of lime cooking of grain amaranth on selected chemical components and on its protein quality. Journal of Agricultural and Food Chemistry, 42 (9), p: 1998 - 2001.

Bressani, R. (1989). The proteins of grain amaranth. Food Reviews International, 5(1), p: 13 - 38.

Bressani, R., Velásquez, L., Acevedo, E. (1990). Dietary fiber content in various grain amaranth species and effect of processing. Amaranth Newsletter, 1, p: 5 - 8.

Bruni, R., Medici, A., Guerrini, A., Scalia, S., Poli, F., Muzzoli, M. (2001). Wild Amaranthus caudatus seed oil, a nutraceutical resource from Ecuadorian flora. Journal of Agricultural and Food Chemistry, 49 (11), p: 5455 - 5460.

Budin, J.T., Breene, W.M., Putnam, D.H. (1996). Some compositional properties of seeds and oils of eight amaranthus species. Journal of the American Oil Chemists' Society, 73 (4), p: 475 - 481.

Bunzel, M., Ralph, J., Steinhart, H. (2005). Association of non-starch polysaccharides and ferulic acid in grain amaranth (*Amaranthus caudatus L.*) dietary fiber. Molecular Nutrition & Food Research. 49 (6), p: 551 - 559.

Cai, Y.Z., Corke, H., Wu, H.X., (2004). Amaranth. Encyclopedia of Grain Science, p: 1 - 10.

Cai, Y.Z., Sun, M., Wu, H., Huang R., Corke, H. (1998). Characterisation and quantification of betacyanin pigments from diverse amaranthus species. Journal of Agricultural and Food Chemistry, 46 (6), p: 2063 - 2070.

Capriles, V.D., Coelho, K.D., Guerra – Matias, A.C., Areas, J.A.G. (2008). Effects of processing methods on amaranth starch digestibility and predicted glycemic index. Journal of Food Science, 73 (7), H, p: 160 – 164.

Carlson, B.C., Jansson, A.M., Larsson, A., Bucht, A., Lorentzen, J.C., (2000). The endogenous adjuvant squalene can induce a chronic T-cell-mediated arthritis in rats. The American Journal of Physiology 156 (6), p: 2057 - 2065.

Caselato-Sousa, V.M., Amaya-Farfán, J. (2012). State of Knowledge on Amaranth Grain: A Comprehensive Review, Journal of Food Science, 77(4), p: R93 - R104.

Chana, C.H., Yusoffa, R., Ngoha, G.C., Kung, F.W.L. (2011). Microwave-assisted extractions of active ingredients from plants. Journal of Chromatography A, 1218 (37), p: 6213 - 6225.

Chauhan, G. S., Eskin, N. A. M., and Tkachuk, R. (1992). Nutritients and antinutrients in quinoa seed. Cereal Chemistry, 69 (1), p: 85 - 88.

Chemat, F., Abert-Vian, M., Zill-e-Huma, Y-J. (2009). Microwave assisted separations: green chemistry in action. In Pearlman J.T. (Eds). Green Chemistry Research Trends. Nova Science Publishers, New York, p: 33 - 62.

Chemat, S., Ait-Amar, H., Lagha, A., Esveld, D.C. (2005). Microwave-assisted extraction kinetics of terpenes from caraway seeds. Chemical Engineering and Processing, 44 (12), p: 1320 - 1326.

Cheng, W.M., Raghavan, G.S.V., Ngadi, M., Wang, N. (2006). Microwave power control strategies on the drying process I. Development and evaluation of new microwave drying system. Journal of Food Engineering, 76 (2), p: 188 - 194.

Chen, S.S., Spiro, M. (1994). Study of microwave extraction of essential oil constituents from plant materials. Journal of the Microwave Power and Electromagnetic Energy, 29 (4), p: 231 - 241.

Choi, H., Kim, W., Shin, M. (2004). Properties of Korean amaranth starch compared to waxy millet and waxy sorghum starches. Starch/Stärke, 56 (10), p: 469 - 477.

Constantinides, P.P., Han, J., Davis, S.S. (2006). Advances in the use of tocols as drug delivery vehicles. Pharmaceutical Research, 23 (2), p: 243 - 255.

Costa, L.M., Silva, F.V., Gouveia, S.T., Nogueira, A.R.A., Nóbrega, J.A. (2001). Focused microwave-assisted acid digestion of oils: an evaluation of the residual carbon content. Spectrochimica Acta Part B: Atomic Spectroscopy 56 (10), p: 1981 - 1985.

Cuadrado, C., Ayet, G., Burbano, C., Muzquiz, M., Camacho, L., Cavieres, E., Lovon, M., Osagie, A., Price, K.R. (1995). Occurrence of saponins and sapogenols in Andean crops. Journal of the Science of Food and Agriculture, 67 (2), p: 169 - 172.

Czaplicki, S., Ogrodowska, D., Derewiaka, D., Tańska, M., Zadernowski, R. (2011). European Journal of Lipid Science and Technology, 113 (12), p: 1456 - 1464.

Czerwiński J, Bartnikowska E, Leontowicz H, Lange E, Leontowicz M, Katrich E, Trakhtenberg S, Gorinstein S. (2004). Oat (*Avena sativa L*.) and amaranth (*Amaranthus hypochondriacus*) meals positively affect plasma lipid profile in rats fed cholesterol-containing diets. The Journal of Nutritional Biochemistry, 15 (10), p: 622 - 629.

De Ancos, B., Sgroppo, S., Plaza, L., Cano, M. P. (2002). Possible nutritional and health-related value promotion in orange juice preserved by high-pressure treatment. Journal of the Science of Food and Agriculture, 82 (8), p: 790 - 796.

Delazar, A., Nahar, L., Hamedeyazdan, S., Sarker, S.D. (2012). Microwave-assisted extraction in natural products isolation. Methods in Molecular Biology, 864, p: 89 - 115.

Desai, K.N., Wei, H., Lamartiniere, C.A. (1996). The preventive and therapeutic potential of the squalene-containing compound, Roidex, on tumor promotion and regression. Cancer Letters, 101 (1), p: 93 - 96.

Dhellot, J.R., Matouba, E., Maloumbi, M.G., Nzikou, J.M., Safou Ngoma, D.G., Linder, M., Desobry, S., Parmentier, M. (2006). Extraction, chemical composition and nutrional characterization of vegetable oils: Case of *Amaranthus hybridus* (var 1 and 2) of Congo Brazzaville. African Journal of Biotechnology, 5 (11), p: 1095 - 1101.

Dickinson, E. (1993). Towards more natural emulsifiers. Trends in Food Science & Technology, 4 (10), p: 330 - 334.

Dixit, B. S., Srivasatava, S. N., Pal, M. (1991). Pigments of Amaranthus, Celosia, Beta vulgaris and their utilization. Indian Journal of Natural Products, 7 (2), p: 12 - 14.

Dobos, G. (1992). Koerneramaranth als neue Kulturpflanze in Oesterreich. Introduktion und zuechterische Aspekte. PhD thesis, University of Natural Resources and Applied Life Sciences, Vienna, Austria.

Dodok, L., Modhir, A. A., Halasova, G., Polacek, I., Hozova, B. (1994). Importance and utilisation of amaranth in food industry Part 1. Characteristic of grain and average chemical constitution of whole amaranth flour. Food / Nahrung, 38 (4), p: 378 - 381.

Dominguez, H., Nunez, M.J., Lema, J.M. (1995). Enzyme-assisted hexane extraction of soya bean oil. Food Chemistry 54 (2), p: 223 - 231.

Dreher, M.L., Dreher, C.J., Berry, J.W., Fleming, S.E. (1984). Starch digestibility of foods: a nutritional perspective. C R C Critical Reviews in Food Science and Nutrition, 20 (1), p: 47 - 71.

Emmerie, A., Engel, C. (1938). Colorimetric determination of α - tocopherol (Vitamin E). Recueil des Travaux Chimiques des Pays-Bas, 57 (12), p: 1351 - 1355.

Escrich, E., Solanas, M., Moral, R., Escrich, R. (2011). Modulatory effects and molecular mechanisms of olive oil and other dietary lipids in breast cancer. Current Pharmaceutical Design, 17 (8), p: 817 - 830.

Escudero, N. L., de Arellano, M. L., Luco, J. M., Giménez, M. S., & Mucciarelli, S. I. (2004). Comparison of the chemical composition and nutritional value of *Amaranthus cruentus* flour and its protein concentrate. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum), 59 (1), p: 15 - 21.

Escudero, N. L., Zirulnik, F., Gomez, N. N., Mucciarelli, S. I., & Gimenez, M. S. (2006). Influence of a protein concentrate from *Amaranthus cruentus* seeds on lipid metabolism. Experimental Biology and Medicine, 231(1), p: 50 - 59.

FAO/WHO (1973). Energy and protein requirements: report of a joint FAO/WHO ad hoc expert committee. FAO Nutrition Meetings Report Series, WHO Technical Report Series, Rome and Geneva, 52 (522).

Folch, J., Lees, M., Sloane-Stanley, G.H. (1957). A Simple method for the isolation and purification of total Lipides from animal tissues. The Journal of Biological Chemistry, 226 (1), p: 497 - 509.

Fox, C.B. (2009). Review: Squalene emulsions for parenteral vaccine and drug delivery. Molecules, 14, p: 3286 - 3312.

Franke, W. (1989). Nutzpflanzenkunde: Nutzbare Gewächse der gemäßigten Breiten, Subtropen und Tropen. Thieme – Verlag, Stuttgart and New York.

Gamel, T.H., Mesallam, A.S., Damir, A.A., Shekib, L.A., Linssen, J.P. (2007). Characterization of amaranth seed oils. Journal of Food Lipids, 14 (3), p: 323 - 334.

Gamel, T. H., Linssen, J. P., Mesallem, A. S., Damir, A. A., and Shekib, L. A. (2005). Effect of seed treatments on the chemical composition and properties of two amaranth species: starch and protein. Journal of the Science of Food and Agriculture, 85 (2), p: 319 - 327.

Gamel, T. H., Linssen, J. P., Mesallem, A. S., Damir, A. A., and Shekib, L. A. (2006). Effect of seed treatments on the chemical composition of two amaranth species: oil, sugars, fibres, minerals and vitamins. Journal of the Science of Food and Agriculture, 86 (1), p: 82 - 89.

Garcia, L.A., Alfaro, M.A., Bressani, R. (1987). Disgestability and protein quality of raw and heat processed defatted and non-defatted flours prepared with three amaranth species. Journal of Agricultural and Food Chemistry, 35 (4), p: 604 – 607.

Gerber, M. (1994). Olive oil and cancer. In Hill, M. J., Giacosa, A., Caygill, C. P. J. Epidemiology of diet and cancer, p: 263 - 275.

Gins, M. S., Gins, V. K., Kononkov, P. F. (2002). Change in the biochemical composition of Amaranth leaves during selection for increased amaranthine content. Applied Biochemistry and Microbiology. 38, p: 474 - 479.

Gonor, K.V., Pogozheva, A.V., Derbeneva, S.A., Mal'tsev, G.I., Trushina, E.N., Mustafina, O.K. (2006a). The influence of a diet with including amaranth oil on antioxidant and immune status in patients with ischemic heart disease and hyperlipoproteidemia. Pherobase - Vopr Pitan - The Pherobase, 75(6), p: 30 - 33.

Gonor, K.V., Pogozheva, A.V., Kulakova, S.N., Medvedev, F.A., Miroshnichenko, L.A. (2006b). The influence of diet with including amaranth oil on lipid metabolism in patients with ischemic heart disease and hyperlipoproteidemia. Pherobase - Vopr Pitan - The Pherobase, 75(3), p: 17 - 21.

González, R., Carrara, C., Tosi, E., Anon, M. C., and Pilosof, A. (2007). Amaranth starch-rich fraction properties modified by extrusion and fluidized bed heating. Journal of Food Science and Technology, 40 (1), p: 136 - 143.

Gorinstein, S., Delgado-Licon, E., Pawelzik, E., Permady, H. H., Weisz, M., Trakhtenberg, S. (2001). Characterisation of soluble amaranth and soybean proteins based on fluorescence, hydrophobicity, electrophoresis, amino acid analysis, circular dichroism, and differential scanning calorimetry measurements. Journal of Agricultural and Food Chemistry, 49 (11), p: 5595 - 5601.

Gorinstein, S., Pawelzik, E., Delgado Licon, E., Yamamoto, K., Kobayashi, S., Taniguchi, H., Haruenkit, R., Yong Seo, P. Soon Teck, J., Drzewiecki, J., Trakhtenberg, S. (2004). Use of scanning electron microscopy to indicate the similarities and differences in pseudocereal and cereal proteins. Journal of Food Science and Technology, 39 (2), p: 183 - 189.

Grobelnik Mlakar, S., Turinek, M., Jakop, M., Bavec, M., Bavec, F. (2009). Nutrition value and use of grain amaranth: potential future application in bread making. Agricultura, 6 (2), p: 43 - 53.

Grobelnik Mlakar, S., Turinek, M., Jakop, M., Bavec, M., Bavec, F (2010). Grain amaranth as an alternative and perspective crop in temperate climate. Journal for Geography, 5 - 1, p: 135 - 145.

Hao, J., Han, W., Huang, S., Xue, D., Deng, X. (2002). Microwave assisted extraction of artemisin from *Artemisia annua L*. Separation and Purification Technology, 28 (3), p: 191 - 196.

Hayes, B.L., (2002). Microwave synthesis: chemistry at the speed of light, CEM Publishing, Matthews, NC.

He, H.P., Cai, Y.Z., Sun, M., Corke, H. (2002). Extraction and purification of squalene from Amaranthus grain. Journal of Agricultural and Food Chemistry, 50 (2), p: 368 - 372.

He, H.P., Corke, H. (2003). Oil and Squalene in Amaranthus Grain and Leaf. Journal of Agricultural and Food Chemistry, 51 (27), p: 7913 - 7920.

Hemwimon, S., Pavasant, P., Shotiprule, A. (2007). Microwave assisted extraction of antioxidative anthraquinones from roots of *Morinda citrifolia*. Separation and Purification Technology. 54 (1), p: 44 - 50.

Herrero, M.A., Kremsner, J.M., Kappe, C.O. (2008). Nonthermal microwave effects revisited: on the importance of internal temperature monitoring and agitation in microwave chemistry. The Journal of Organic Chemistry, 73 (1), p: 36 - 47.

Herrero, M., Mendiola, J.A., Cifuentes, A., Ibáñez, E. (2010). Supercritical fluid extraction: recent advances and applications. Journal of Chromatography A, 1217 (16), p: 2495 - 2511.

Hoover, R., Sinnott, A. W., Perera, C. (1998). Physicochemical characterization of starches from Amaranthus cruentus grains. Starch/Stärke, 50 (11- 12), p: 456 - 463.

Hosseini, M., Stiasni, N., Barbieri, V., Kappe, C.O. (2007). Microwave-assisted asymmetric organocatalysis. A probe for nonthermal microwave effects and the concept of simultaneous cooling. The Journal of Organic Chemistry, 72 (4), p: 1417 - 1424.

Hu, Z., Cai, M., Liang, H.H. (2008). Desirability function approach for the optimization of microwave-assisted extraction of saikosaponins from Radix Bupleuri. Separation and Purification Technology, 61(3), p: 266 - 275.

Imeri, A., Gonzalez, J.M., Flores, R., Elías, L.G., Bressani, R., (1987). Genetic variability and correlation of yield, grain size, chemical composition and protein quality of 25 varieties of amaranth (*Amaranthus caudatus*). Instituto de Nutrición de Centro América y Panamá, 37 (1), p: 132 - 146.

Irving, D.W., Betschart, A.A., Saunders, R.M. (1981). Morphological studies on *Amaranthus cruentus*. Journal of Food Science, 46 (4), p: 1170 - 1174.

Jadhav, D., Rekha B.N., Gogate, P.R., Rathod, V.K. (2009). Extraction of vanillin from vanilla pods: a comparison study of conventional soxhlet and ultrasound assisted extraction. Journal of Food Engineering, 93 (4), p: 421 - 426.

Jahaniaval, F., Kakuda, Y., Marcone, M. F. (2000). Fatty acid and triacylglycerol compositions of seed oils of five Amaranthus accessions and their comparison to other oils. Journal of the American Oil Chemists' Society, 77 (8), p: 847 - 852.

Jobling, S. (2004). Improving starch for food and industrial applications. Current Opinion in Plant Biology, 7 (2), p: 210 - 218.

Kigel, J. (1994). Development and ecophysiology of amaranths. In: Peredes-Lopez, O. (ed), Amaranth biology, chemistry and technology. CRC Press, p: 39 - 73.

Kim, H.K., Kim, M.J., Cho, H.Y., Kim, E.K., Shin, D.H. (2006a). Antioxidative and anti-diabetic effects of amaranth (*Amaranthus esculantus*) in streptozotocin-induced diabetic rats. Cell Biochemistry and Function, 24(3), p:195 - 199.

Kim, H.K., Kim, M.J., Shin, D.H. (2006b). Improvement of lipid profile by amaranth (*Amaranthus esculantus*) supplementation in streptozotocin-induced diabetic rats. Annals of Nutrition and Metabolism, 50(3), p: 277 - 281.

Kim, I.H., Yoon, S.H. (1990). Effect of extraction solvents on oxidative stability of crude soybean oil. Journal of the American Oil Chemists' Society, 67(3), p: 165 - 167.

Kingston, H.M., Haswell, S.J. (Eds) (1997). Microwave-Enhanced Chemistry. Fundamentals, Sample Preparation and Applications. American Chemical Society, Washington, DC.

Kingston, H.M., Jassie, L.B. (1986). Microwave energy for acid decomposition at elevated temperatures and pressures using biological and botanical samples. Analytical chemistry, 58 (12), p: 2534 - 2541.

Kingston, H.M., Jassie, L. B. (1988). Introduction to microwave sample preparation: theory and practice. American Chemical Society, Washington, DC.

Kiss, G.A.C., Forgacs, E., Serati, T.C., Mota, T., Morais H., Ramos. A. (2000). Optimisation of the microwave assisted extraction of pigments from paprika (Capsicum annum L.) powders. Journal of Chromatography A, 889, p: 41 - 49.

Klimczak, I., Malecka, M., Pacholek, B. (2002). Antioxidant activity of ethanolic extracts of amaranth seeds. Food/Nahrung, 46 (3), p: 184 - 186.

Kleinschmidt, G. (2005). Performance parameters, calculations and tests. In Ermer, J., Miller, J.H.M. (Eds). Method validation in pharmaceutical analysis: a guide to best practice. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, p: 120 - 169.

Kohno, Y., Egawa, Y., Itoh, S., Nagaoka, S., Takahashi, M., Mukai, K. (1995). Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol. Biochimica et Biophysica Acta, 1256 (1), p: 52 - 56.

Kong, X., Bao, J., Corke, H. (2009). Physical properties of Amaranthus starch. Food Chemistry, 113 (2), p: 371 - 376.

Kong, X., Bertoft, E., Bao, J., Corke, H. (2008). Molecular structure of amylopectin from amaranth starch and its effect on physicochemical properties. International Journal of Biological Macromolecules, 43 (4), p: 377 - 382.

Konishi, Y., Fumita, Y., Ikeda, K., Okuno, K., and Fuwa, H. (1985). Isolation and characterization of globulin from seeds of Amaranthus hypochondriacus, L. Agricultural and Biological Chemistry, 49 (5), p:1453 - 1459

Köseolğua, S.S., Engelgau, D.E. (1990). Membrane applications and research in the edible oil industry: an assessment. Journal of the American Oil Chemists' Society, 67 (4), p: 239 - 249,

Kremsner, J.M., Kappe, C.O. (2006). Silicon carbide passive heating elements in microwave-assisted organic synthesis. The Journal of Organic Chemistry, 71 (12), p: 4651 - 4658.

Krishnaswamy, K., Orsat, V., Gariépy, Y., Thangavel, K. (2012). Optimization of microwave-assisted extraction of phenolic antioxidants from grape seeds (Vitis vinifera). Food and Bioprocess Technology, 6 (2), p: 441 - 455.

Kubra, I.R., Kumar, D., Rao, L.J.M. (2013). Effect of microwave-assisted extraction on the release of polyphenols from ginger (*Zingiber officinale*). International Journal of Food Science and Technology, p: 1 - 6.

Kulakova, S.N., Pozdniakov, A.L., Korf II, Karagodina, Z.V., Medvedev, F.A., Viktorova, E.V., Gonor, K.V., Kamysheva, I.M., Gadzhieva, Z.M. (2006). Amaranths oil: peculiarities of chemical composition and influence on lipid metabolism by rats. Vopr Pitan, 75 (3), p: 36 - 42.

Lambert, D. (1990). Statistical methods for quality improvement. Technometrics: Journal of the American Society for Quality and the American Statistical Association, 32 (3), p: 339 - 340.

Latha, C. (2006). Microwave assisted extraction of embelin from Embelia ribes. Biotechnology Letters, 29 (2), p: 319 - 322.

La Vecchia C., Negri, E. (1997). Fats in seasoning and the relationship to pancreatic cancer. European Journal of Cancer Prevention, 6(4), p: 370 - 373.

La Vecchia, C., Negri, E., Franceschi, S., Decarli, A., Giacosa, A., Lipworth, L. (1995). Olive oil, other dietary fats, and the risk of breast cancer (Italy). Cancer Causes & Control, 6 (6), p: 545 – 550.

Lawson, H., (1995). Food oils and fats: technology, utilization, and nutrition., Chapman Jz Hall, New York, NY.

Leadbeater, N.E., Pillsbury, S.J., Shanahan, E., Williams V.A. (2005). An assessment of the technique of simultaneous cooling in conjunction with microwave heating for organic synthesis. Tetrahedron, 61, p: 3565 - 3585.

Lebovka, F., Vorobiev, N., Chemat, E. (2011). Enhancing Extraction Processes in the Food Industry. CRC Press, p: 89.

León-Camacho, M., García-González, D.L., Aparicio, R. (2001). A detailed and comprehensive study of amaranth (*Amaranthus cruentus L.*) oil fatty profile. European Food Research and Technology, 213 (4-5), p: 349 - 355.

Letellier, M., Budzinski, H., Charrier, L., Capes, S., Dorthe, A.M. (1999). Optimization by factorial design of focused microwave assisted extraction of polycyclic aromatic hydrocarbons from marine sediment. Fresenius' Journal of Analytical Chemistry, 364 (3), p: 228 - 237.

Letellier, M., Budzinski. H. (1999). Microwave assisted extraction of organic compounds. Analusis, 27 (3), p: 259 - 271.

Lew, A., Krutzik, P.O., Hart, M.E., Chamberlin, A.R., (2002). Increasing rate of reaction: microwave assisted organic synthesis for combinatorial chemistry. Journal of Combinatorial Chemistry, 4 (2), p: 95 - 105.

Lidström, L., Tierney, J., Wathey, B., Westman, J. (2001). Microwave assisted organic synthesis - a review. Tetrahedron, 57 (45), p: 9225 - 9283

Liener, I.E. (1980). Heat labile antinutritional factors. In Summerfield, R.J., Bunting, A.H. Advances in legume science. Kew Publishing, Royal Botanic Gardens, Kew, p: 157 - 170.

Linsberger-Martin, G., Lukasch, B., Berghofer, E. (2012). Effects of high hydrostatic pressure on the RS content of amaranth, quinoa and wheat starch. Starch/Stärke, 64 (2), p: 157 - 165.

Lopez-Avila, V., Young, R., Beckert, W.F. (1994). Microwave assisted extraction of organic compounds from standard reference soils and sediments. Analytical Chemistry, 66 (7), p : 1097 - 1106.

Lopez, M. G., Bello-Pérez, L. A., Paredes-Lopez, O. (1994). Amaranth carbohydrates. In: Paredes-Lopez, O (ed), Amaranth: biology, chemistry and technology. CRC Press: Boca Raton, FL, p: 107 - 131.

Lou-Bonafonte, J.M., Arnal, C., Navarro, M.A., Osada, J. (2012). Efficacy of bioactive compounds from extra virgin olive oil to modulate atherosclerosis development, Molecular Nutrition & Food Research, 56 (7), p: 1043 - 1057.

Lucchesi, M.E., Chemat, F., Smalja, J. (2004). Solvent free microwave extraction of essential oil from aromatic herbs: comparison with conventional hydrodistillation. Journal of Chromatography A, 1043 (2), p: 323 - 327.

Luque de Castro, M. D., Garcia-Ayuso, L. E. (1998). Soxhlet extraction of solid materials: An outdated technique with a promising innovative future. Analytica Chimica Acta, 369 (1 - 2), p: 1 - 10.

Luque de Castro, M.D., Jimenez-Carmona, M.M., Fernandez, V. (1999). Towards more rational techniques for the isolation of valuable essential oils from plants. Trends in Analytical Chemistry, 18 (11), p: 708 - 716.

Luque-García, J.L., Luque de Castro, M.D. (2003). Where is microwave-based analytical equipment for solid sample pre-treatment going? Trends in Analytical Chemistry, 22 (2), p: 90 – 98.

Lyon, C. K., Becker, R. (1987). Extraction and refining of oil from amaranth seed. Journal of the American Oil Chemists' Society 64 (2), p: 233 - 236.

Malecka, M. (1991). The effect of squalene on the heat stability of rapeseed oil and model lipids. Die Nahrung, 35 (5), p: 541 - 542

Mandal, V., Mohan, Y., Hemalatha, S. (2007). Microwave assisted extraction – An innovative and promising extraction tool for medicinal plant research. Pharmacognosy Reviews, 1(1), 7 - 18.

Marchini, G., Lindow, S., Brismar, H., Ståbi, B., Berggren, V., Ulfgren, A.K., Lonne-Rahm, S., Agerberth, B., Gudmundsson, G.H. (2002). The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa. British Journal of Dermatology, 147 (6), p: 1127 - 1134

Marcone, M. F., Beniac, D. R., Harauz, G., Yada, R. Y. (1994). Quaternary structure and model for the oligomeric seed globulin from Amaranthus hypochondriacus K343. Journal of Agricultural and Food Chemistry. 42 (12), p: 2675 - 2678.

Marcone, M. F. (1999). Evidence confirming the existence of a 7S globulin-like storage protein in A. hypochondriacus seed. Food Chemistry. 65 (4), p: 533 - 554.

Marcone, M.F. (2001). Starch properties of Amaranthus pumilus (seabeach amaranth): a threatened plant species with potential benefits for the breeding/amelioration of present Amaranthus cultivars. Food Chemistry, 73 (1), p: 61 - 66.

Martine Champ, M., Langkilde, A.M., Brouns, F., Kettlitz, B., Le Bail-Collet, Y. (2003). Advances in dietary fibre characterisation. 2. Consumption, chemistry, physiology and measurement of resistant starch; implications for health and food labelling. Nutrition Research Reviews, 16 (2), p: 143 - 161.

Martinez, E. N., Castellani, O. F., Anon, M. C. (1997). Common molecular features among amaranth storage proteins. Journal of Agricultural and Food Chemistry. 45 (10), p: 3832 - 3839.

Martin-Moreno, J.M., Willett, W.C., Gorgojo, L., Banegas, J.R., Rodriguez-Artalejo, F., Fernandez-Rodriguez, J.C., Maisonneuve, P., Boyle, P. (1994). Dietary fat, olive oil intake and breast cancer risk. International Journal of Cancer, 58(6), p: 774 - 780.

Martirosyan, D.M., Miroshnichenko, L.A., Kulakova, S.N., Pogojeva, A.V., Zoloedov, VI (2007). Amaranth oil application for coronary heart disease and hypertension. Lipids in Health and Disease, 6 (1), p: 1 - 12.

Meda, V., Orsat, V., Raghavan, G. S. V. (2005). Microwave heating and the dielectric properties of foods. In Schubert, H., Regier, M. (Eds.), The microwave processing of foods. Woodhead Publishing Ltd., Cambridge, p: 61 - 75.

Moreaua, R.A., Whitakerb, B.D., Hicks, K.B. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research, 41(6), p: 457 - 500

Mburu, M.W., Gikonyo, N.K., Kenji, G.M., Mwasaru, A.M. (2011). Properties of a Complementary Food based on Amaranth Grain (Amaranthus cruentus) Grown in Kenya. Journal of Agriculture and Food Technology, 1 (9), p: 153 - 178.

Mikulikova, D. and Kraic, J. (2006). Natural sources of health-promoting starch. Journal of Food and Nutrition Research, 45 (2), p: 69 - 76.

Monteros, C.J., Nieto, C.C., Caicedo, C.V., Rivera, M.M., Vimos, C.N. (1998). Iniap alegria primera variedade mejorada de amaranto para la sierra ecuatoriana. In Produccion y procesamiento de quinua en Ecuador. Retrieved from: http://www.idrc.ca

Murakoshi, M., Nishino, H., Tokuda, H., Iwashima, A., Okuzumi, J., Kitano, H., Iwasaki, R. (1992). Inhibition by squalene of the tumor-promoting activity of 12-O-Tetradecanoylphorbol-13-acetate in mouse-skin carcinogenesis. International Journal of Cancer, 52(6), p: 950 - 952.

Myers, R. H., Montgomery, D. C. (2002A). Two-level factorial designs. In Myers, R. H., Montgomery, D. C. (Eds.), Response surface methodology - process and product optimization using designed experiments, New York: John Wiley & Sons, p: 85 - 154.

Myers, R. H., Montgomery, D. C. (2002B). Two-level fractional factorial designs. In Myers, R. H., Montgomery, D. C. (Eds.), Response surface methodology - process and product optimization using designed experiments. New York: John Wiley & Sons, p: 155 - 196.

Naziri, E., Mantzouridou, F., Tsimidou, M.Z. (2011). Squalene resources and uses point to the potential of biotechnology. Lipid Technology, 23 (12), p: 270 - 273.

Nemes, S.M., Orsat, V. (2010). Screening the experimental domain for the microwave-assisted extraction of secoisolariciresinol diglucoside from flaxseed prior to optimization procedures. Food and Bioprocess Technology, 3 (2), p: 300 - 307.

Nemes, S.M. (2012). Practical Methods for Lignans Quantification. PhD Thesis. Bioresource Engineering Department, McGill University, Montreal, Canada

Newmark, H.L. (1997). Squalene, olive Oil, and cancer risk: a review and hypothesis. Cancer Epidemiology, Blornarkers and Prevention, 6, p: 1101 - 1103.

Nuchter, M., Ondruschka, B., Weiß, D., Bonrath, W., Gum, A. (2005). Contribution to the qualification of technical microwave systems and to the validation of microwave-assisted reactions and processes. Chemical Engineering & Technology, 28 (8), p: 871 - 881.

Oleszek, W., Junkuszew, M., Stochmal, A. (1999). Determination and toxicity of saponins from Amaranthus cruentus seeds. Journal of Agricultural and Food Chemistry, 47 (9), p: 3685 - 3687.

Ondruschka, B., Asghari, J. (2006). Microwave assisted extraction a state of the art overview of varieties. Chimia, 60 (6), p: 321 - 325.

Opute, F.I. (1979). Seed lipids of the grain amaranths. The Journal of Experimental Botany, 30 (116), p: 601 - 606.

Orsat, V., Raghavan, G. S. V., Meda, V. (2005). Microwave heating and the dielectric properties of foods. In Schubert, H., Regier, M. (Eds.), The microwave processing of foods. Woodhead Publishing Ltd., Cambridge, p: 105 - 118.

Pan, X., Niu, G., Liu, H. (2002). Comparison of microwave-assisted extraction and conventional extraction techniques for the extraction of tanshinones from *Salvia miltiorrhiza bunge*. Biochemical Engineering Journal, 12 (1), p: 71 - 77.

Paredes – López, O., Bello – Pérez, L.A., López, M.G. (1994). Amylopectin: structural, gelatinization and retrogradation studies. Food Chemistry, 50 (4), p: 411 - 417.

Pederson, B., Kalinowski, L.S., Eggum, B.O. (1987). The nutritive value of amaranth grain (Amaranthus caudatus). Protein and minerals of raw and processed grain. Plant Foods Human Nutrition, 36 (4), p: 309 - 324.

Périno-Issartier, S., Zill-e-Huma, Y-J., Abert-Vian, M., Chemat, F. (2011). Solvent free microwave-assisted extraction of antioxidants from sea buckthorn (*Hippophae rhamnoides* ) food by-products. Food Bioprocess and Technology, 4 (6), p: 1020 - 1028.

Peterson, E.R. (1993). Quality enhancement using microwaves. In 28th Annual Symposium Proceedings, Montreal, Canada, p: 89 - 101.

Peyre, F., Datta, A., Seyler, C., 1997. Influence of the dielectric property on microwave heating patterns: application to food materials. The Journal of microwave power and electromagnetic energy, 32 (1), p: 3 - 14

Pricea, K.R., Johnsonb, I. T., Fenwicka, G. R. Malinowcd, M. R. (1987). The chemistry and biological significance of saponins in foods and feedingstuffs. C R C Critical Reviews in Food Science and Nutrition, 26 (1), p: 27 - 135.

Proctor, A., Bowen, D.J. (1996). Ambient-temperature extraction of rice bran oil with hexane and isopropanol. Journal of the American Oil Chemists' Society, 73 (6), p: 811 - 813.

Qian, J.Y., Kuhn, M. (1999). Characterization of Amaranthus cruentus and chenopodium quinoa starch. WILEY-VCH Verlag GmbH, D-69451 Weinheim, Starch/Stärke 51 (4), S, p: 116 - 120.

Qureshi, A.A., Lehmann, J.W., Peterson, D.M. (1996). Amaranth and its oil inhibit cholesterol biosynthesis in 6 weeks – old female chickens. Journal of Nutrition, 126 (8), p: 1972 - 1978.

Radosavljevic, M., Jane, J., Johnson, L.A. (1998). Isolation of amaranth starch by diluted alkaline-protease treatment. Cereal Chem, 75 (2), p: 212 - 216.

Raghavan, G. S. V., Orsat, V., Meda, V. (2005). Microwave processing of foods. Stewart Postharvest Review, 1 (3), p: 1 - 8.

Rao, C.V., Newmark, H.L., Reddy, B.S. Chemopreventive effect of squalene on colon cancer. Carcinogenesis, 19 (2), p: 287 - 290.

Rastogi, A., Shukla, S. (2012). Amaranth: A new millennium crop of nutraceutical values. Critical Reviews in Food Science and Nutrition, 53 (2), p: 109 - 125.

Raynie, D.E. (2000). Extraction. In Wilson, I.D., Adlard, E.R., Cooke, M., Pooliem, C.F. (Eds). Encyclopedia of separation science. Academic Press, San Diego

Rosenberg, H.R. (1945). Chemistry and physiology of vitamins. Interscience Publishers Inc., New York, NY, USA, p: 452 - 453.

Routray, W., Orsat, V. (2011). Microwave-Assisted Extraction of Flavonoids: A Review. Food and Bioprocess Technology, 5, p: 409 - 424.

Reddy, B.S. (1992). Dietary fat and colon cancer: animal model studies. Lipids, 27 (10), p: 807 - 813.

Repo-Carrasco-Valencia, R., Hellstrom, J. K., Pihlava, J. M., Mattila P.H. (2010). Flavonoids and other phenolic compounds in Andean indigenous grains: Quinoa (*Chenopodium quinoa*), kaniwa (Chenopodium pallidicaule) and kiwicha (*Amaranthus caudatus*). Food Chemistry. 120 (1), p: 128 - 133.

Rissmann, R., Oudshoorn, M.H., Kocks, E., Hennink, W.E., Ponec, M., Bouwstra, J.A. (2008). Lanolin derived lipid mixtures mimic closely the lipid composition and organization of vernix caseosa lipids. Biochimica et Biophysica Acta, 1778 (10), p: 2350 - 2360.

RCS, (2007). Classic Kit: Soxhlet extractor. Chemistry World, Royal Society of Chemistry. http://www.rsc.org/chemistryworld/Issues/2007/September/ClassicKitSoxhletExtractor.asp

Saillard, R., Poux, M., Berlan, E.J. (1995). Microwave heating of organic solvents : thermal effects and field modelling, Tetrahedron, 51(14), p: 4033 - 4042.

Saint-Leger, D., Bague, A., Cohen, E., Chivot, M. (1986). A possible role for squalene in the pathogenesis of acne. I. In vitro study of squalene oxidation. British Journal of Dermatology, 114 (5), p: 535 - 542.

Sauer, J.D. (1967). The grain amaranths and their relatives: a revised taxonomic and geographical survey. Annals of Missouri Botanical Gardens, 54, p: 103 - 137.

Saunders, R.M., Becker, R. (1984). Amaranthus: a potential food and feed resource. In: Pomeranz, Y. (Eds). Advances in cereal science and technology. American Association of Cereal Chemists, 6, p: 357 - 396.

Segura-Nieto, M., Barba de la Rosa, A.P., Paredes-Lo´pez, O. (1994). Biochemistry of amaranth proteins. In Paredes-Lo´pez O (Eds) Amaranth: Biology, Chemistry, and Technology, Boca Raton, FL: CRC Press, p: 75 - 106.

Senft, J. P. (1979). Protein quality of amaranth grain. In: Proceeding of second amaranth conference Rodale Press Inc., Emmaus, Pennsylvania, p: 43 - 47.

Senthilkumara, S., Devakia, T., Manoharb, B.M., Babua, M.S. (2006). Effect of squalene on cyclophosphamide-induced toxicity. Clinica Chimica Acta, 364 (1–2), p: 335 - 342.

Shin, D.H., Heo, H.J., Lee, Y.J., Kim, H.K. (2004). Amaranth squalene reduces serum and liver lipid levels in rats fed a cholesterol diet. British Journal of Biomedical Science, 61 (1), p: 11 - 14.

Sihvonen, M., Jarvenpää, E., Hietaniemi, V., Huopalahti, R. (1999). Advances in supercritical carbon dioxide technologies. Trends in Food Science and Technology, 10 (6-7), p: 217 - 222.

Sims, R.J., Fioriti, J.A., Kanuk, M.J. (1972). Sterols additives as polymerisation inhibitors for frying oils. Journal of the American Oil Chemists' Society. 49 (5), p: 298 - 301.

Singhal, R.S, Kulkarni, P.R. (1988). Review: Amaranths - An underutilized resource. International Journal of Food Science & Technology. 23 (2), p: 125 - 140.

Singhal, R.S., Kulkarni, P.R. (1990). Effect of puffing on oil characteristics of Amaranthus (Rajgeera) seeds. Journal of the American Oil Chemists' Society, 67 (12), p: 952 - 954.

Singh, A., Sabally, K., Kubow, S., Donnelly, D.J., Gariepy, Y., Orsat, V., Raghavan, G.S.V. (2011). Microwave-assisted extraction of phenolic antioxidants from potato peels. Molecules, 16 (3), p: 2218 - 2232.

Slinkard, K., Singleton, V. L. (1977). Total phenol analysis: automation and comparison with manual methods. American Journal of Enology and Viticulture, 28, 49 – 55.

Smith, R.D. (1984). Microwave power in industry. Final report. Electric Power Research Institute, 3465, A - 8.

Smith, T.J. (2000). Squalene: potential chemopreventive agent. Expert Opinion on. Investigational Drugs, 9 (8), p: 1841 - 1848.

Smith, T.J., Yang, G.Y., Seril, D.N., Liao, J., Kim, S. (1998). Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanoneinduced lung tumorigenesis by dietary olive oil and squalene. Carcinogenesis, 19 (4), p: 703 - 706.

Souci, S.W., Fachmann W., Kraut, H. (1994). Food Composition and Nutrition Tables. Wissenschaft Verlag, Stuttgart.

Sparr Eskilsson, C., Björklund, C. (2000). Review: analytical-scale microwave-assisted extraction, Journal of Chromatography A, 902 (1), p: 227 - 250.

Stintzing, F. C., Kammerer, D., Schieber, A., Adama, H., Nacoulma, O. G., Carle, R. (2004). Betacyanins and phenolic compounds from *Amaranthus spinosus L.* and *Boerhavia erecta L.* Journal of Biosciences, 59 (1 – 2), p: 1 - 8.

Srinivas, H., Swamylingappa, B., Chand, N. (1992). Secondary extraction of soybeans using hexane-acetic acid: effect on beany flavor removal and physiochemical properties. Journal of Agricultural and Food Chemistry, 40 (2), p: 276 - 279.

Swain, M.J., Ferron, S., Coelho, A.I.P., Swain, M.V.L. (2006). Effect of continuous (intermittent) use on the power output of domestic microwave ovens. International Journal of Food Science & Technology, 41 (6), p: 652 - 656.

Talebi, M., Ghassempour, A., Talebpour, Z., Rassouli, A., Dolatyari, L. (2004). Optimization of the extraction of paclitaxel from *Taxus baccata L.* by the use of microwave energy. Journal of Separation Science, 27 (13), p: 1130 - 1136.

Tanongkankit, Y., Sablani, S.S., Chiewchan, N., Devahastin, S. (2013). Journal of Food Engineering, 117 (1), p: 151 - 157.

Tang, H.J., Mitsunaga, T.H., Kawamura, Y. (2006). Molecular arrangement in blocklets and starch granule architecture. Carbohydrate Polymers. 63 (4), p: 555 - 560.

Tatke, P., Jaiswal, Y. (2011). An Overview of Microwave Assisted Extraction and its Applications in Herbal Drug Research. Research Journal of Medicinal Plant, 5 (1), p: 21 - 31.

Teutonico, R.A., Knorr, D. (1985). Amaranth: composition, properties, applications of a rediscovered food crop. Food Technology, 39 (4), p: 49 - 60.

Thompson, L.U. (1993). Potential health benefits and problems associated with antinutrients in foods. Food Research International, 26 (2), p: 131 - 149.

Tikekar, R.V., Ludescher, R.D., Karwe, M.V. (2008). Processing stability of squalene in amaranth and antioxidant potential of amaranth extract. Journal of Agricultural and Food Chemistry. 56 (22), 10675 - 10678.

Trautwein, E.A., Van Leeuwen, A., Ebersdobler, H.F. (1997). Plant sterol profiles and squalene concentrations in common unrefined and refined vegetable oils. Bioactive inositol phosphates and phytosterols in foods. Proceedings of the 2nd workshop, COST 916, Gothenberg, Sweden, p: 79 - 81.

Trichopoulou, A., Katsouyanni, K., Stuver, S., Tzala, L., Gnardellis, C., Rimm, E., Trichopoulos, D. (1995). Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. Journal of the National Cancer Institute, 87 (2), p: 110 - 116.

Ulbricht, C., Abrams, T., Conquer, J., Costa, D., Grims-Serrano, J.M., Taylor, S., Varguese, M. (2009). An evidence-based systematic review of amaranth (*Amaranthus* spp.) by the natural standard research collaboration. Journal of Dietary Supplements, 6 (4), p: 390 - 417.

Uriyapongson, J., Rayas-Duarte, P. (1994). Comparison of yield and properties of amaranth starches using wet and dry-wet milling process. Cereal Chemistry, 71 (6), p: 571 - 577.

U.S. Department of Agriculture, Agricultural Research Service. (2012). USDA National Nutrient Database for Standard Reference, Release 25. Nutrient Data Laboratory. http://www.ars.usda.gov/ba/bhnrc/ndl

Venkatesh, M.S., Raghavan, G.S.V. (2004). An overview of microwave processing and dielectric properties of agri-food materials. Biosystem Engineering, 88(1), p: 1 - 18.

Wanasundara, J.P.D., Shahidi, F. (1994). Alkanol-ammonia-water/hexane extraction of flaxseed. Food Chemistry, 49 (1), p: 39 - 44.

Wang, L, Weller, C.L. (2006). Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology, 17(6), p: 300 - 312.

Wang, Z., Wang, L., Li, T., Zhon, X., Ding, L., Yu, Y., Yu, A., Zhang, H. (2006). Rapid analysis of the essential oils from dried *Illicium verum* Hook. f. and *Zingiber officinale Rosc*. by improved solvent-free microwave extraction with three types of microwave-absorption medium. Analytical and Bioanalytical Chemistry, 386 (6), p: 1863 - 1868.

Wang, L. J., Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. Trends in Food Science and Technology, 17 (6), p: 300 - 312.

Walkowski, A., Fornal, J., Lewandowicz G., Sadowska, J. (1997). Structure, physico – chemical properties and potential uses of amaranth starch. Polish Journal of Food and Nutrition Sciences, 6 (47), p: 11 - 22.

Williams, J.T., Brenner, D. (1995). Grain amaranth (Amaranthus species). In Williams, J.T. (Ed), Cereals and Pseudocereals. Chapman and Hall, London.

Wu, H., Corke, H. (1999). Genetic diversity in physical properties of starch from a world collection of amaranthus. Cereal Chemistry, 76 (6), p: 877 - 883.

Wu, H. X., Yue, S. X., Sun, H. L., Corke, H. (1995). Physical properties of starch from two genotypes of Amaranthus cruentus of agricultural significance in China. Starch/ Stärke, 47 (8), p: 295 - 297.

Xiao, W., Han, L., Shi, B. (2008). Optimization of microwave-assisted extraction of flavonoid from Radix Astragali using response surface methodology. Separation science and Technology, 43, p: 671 - 681.

Yamaguchi, T., Nakagawa, M., Hidaka, K., Yoshida, T., Sasaki, T., Akiyama, S., Kuwano, M. (1985). Potentiation by squalene of antitumor effect of 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-(2-chloroethyl)-nitrosourea in a murine tumor system. Japanese Journal of Cancer Research, 76 (10), p: 1021 - 1026.

Yanez, E., Zacarias, I., Granger, D., Vasquez, M., and Estevez, A. M. (1994). Chemical and nutritional characterisation of amaranthus (*Amaranthus cruentus*). Archivos Latinoamericanos de Nutrición, 44 (1), p: 57 - 62.

Yanez, G.A., Messinger, J.K., Walker, C.E. (1986). Amaranthus hypochondriacus: starch isolation and partial characterization. Cereal Chemistry, 63 (3), p: 273 - 276.

Zhao, J., Whistler R.L. (1994). Isolation and characterization of starch from amaranth flour. Cereal Chemistry, 71 (4), p: 392 - 393.

Zhou, H., Liu, C. (2006). Microwave assisted extraction of solanesol from tobacco leaves. Journal of Chromatography A, 1129 (1), p: 135 - 139.

Zuloaga, O., Etxebarria, N., Fernandez, L.A., Madariaga, J.M. (1999). Optimization and comparison of microwave assisted extraction and Soxhlet extraction for the determination of polychlorinated biphenyls in soil samples using an experimental design approach. Talanta, 50 (2), p: 345 - 357.