THERMAL PROCESSING EFFECTS ON TOTAL PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY, TRYPSIN INHIBITOR ACTIVITY AND IN VITRO PROTEIN DIGESTIBILITY OF LENTILS

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THERMAL PROCESSING EFFECTS ON TOTAL PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY, TRYPSIN INHIBITOR ACTIVITY AND IN VITRO PROTEIN DIGESTIBILITY OF LENTILS

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ABSTRACT

Heat pre-treatment of nutrient-rich lentil seeds prior to their processing into flour may enhance its use by reducing processing and preparation times in value added products. In this study, changes in trypsin inhibitor content, total phenolic content, antioxidant activity, and in-vitro protein digestibility of flours prepared from hulled red lentils and unhulled green lentils were determined subsequent to various processing methods such as oven roasting (OR), boiling and microwave heating (MH).

The increasing interest in the phenolic content of plant based food-stuffs made us to assess two different lentil cultivars processed under fixed temperature and time combination. Total phenolic content and antioxidant activity (TAC) of 70% acetone lentil extracts were assayed spectrophotometrically at 760nm using Folin-Ciocalteu and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity methods, respectively. Significant differences in phenolic content and antioxidant activity were noted between hulled red and unhulled green lentil varieties. MH (5 min) led to a significant increase ($P \leq 0.05$) in total phenolic content in unhulled green lentil flours (GLF) [8.85 mg GAE/g dry weight (DW) while raw flour comparatively showed lower phenolic content [7.5 mg GAE/g DW]. A similar increase was found after oven-roasting this material for 20 min. The TAC of untreated unhulled green lentil range was around 86%, and it was higher

than the value obtained for the flour from untreated hulled red lentils. The increase in TPC of OR samples and microwave-heated samples over untreated ones may reflect reductions in TAC. Flour samples obtained from boiled lentils showed a sharp decrease in TPC and TAC values, which may be attributable to a number of factors in the matrix.

Though lentils are rich in protein, their anti-nutritional components, the length of the time required for their preparation, as well as their unfavorable flavor, and reduced protein digestibility have limited their frequency of use for human consumption. By applying heat, anti-nutritional factors such as trypsin inhibitors can be largely removed. Our results show that MH treatment produced significant reduction ($P \leq 0.05$) in trypsin inhibitor activity when compared to OR or boiling methods. *In- vitro* protein digestibility (IVPD) was improved after processing. Longer processing times associated with OR or boiling methods improved IVPD to a greater extent than MH.

Keywords: TAC- Total antioxidant activity; GA- Gallic Acid equivalent; Lentils; TPC- Total phenolic content; IVPD- *In-Vitro* Protein digestibility; MH- Microwave heating; OR- Oven roasting; GLF- Green lentil flours; RLF- Red lentil flours

RÉSUMÉ

Le prétraitment thermique de lentilles avant de les moudre en farine peut faciliter son utilisation dans la préparation de produits à valeur ajoutée. Cette étude porte sur l'évaluation des effets des prétraitments thermiques par chauffage par torréfaction(CT), par chauffage par microondes (CM), ou par l'eau bouillante (EB), sur la teneur en composés phénoliques totaux (CPT), sur l'activité anti-oxydante totale (AAOT), sur les teneurs en inhibiteur de trypsine, et sur la digestibilité *in-vitro* des protéines (DIVP). Les essais ont été faits sur des farines obtenues à partir de lentilles rouges décortiquées et de lentilles vertes non decortiquées après l'application des prétraitment thermiques.

Les deux varieties de lentilles ont été prétraitées selon des combinaisons déterminées de températures et de durées de traitment. Les teneurs en CPT et l'AAOT ont été évaluées par spectrophotométrie à 760 nm en utilisant la méthode de Folin-Ciocalteu et la méthode DPPH (1, 1- diphényl-2-picrylhydrazyle) de piégeage des radicaux libres. Les résultats ont démontré des différences significatives entre les deux types de lentilles étudiées. Le prétraitement CM (5 min) a conduit à une augumentation significative ($P \leq 0.05$) de la teneur en CPT dans les échantillons de farine de lentilles vertes [8.85 mg GAE/g de poids sec (ps) lorsque comparé aux échantillons de farine de lentilles vertes non-traitées [7.5 JEU mg/g ps]. Une tendance similaire a été observée auprés des farines de lentilles vertes torréfiées au four pendant 20 min. L'AAOT des farines de lentilles vertes non-traitées était d'environ 86% et elle était supérieure à celle obtenue à partir de lentilles rouges non-traitées. Les teneurs en CPT observées dans les

échantillons traités soit par CT soit par CM étaient plus élevées que celles des échantillons non traitées et peuvent refléter des réductions en AAOT obetnues. Les teneurs en CPT et en AAOT des échantillons obtenues à la suite du prétraitment EB étainet nettement inférieures et peut être attributées à plusieurs facteurs dans la matrice.

Bien que les lentilles soit riches en protéines, plusiers facteurs limitent leurs utilisation pour la consommation humaine. Ces principaux facteurs sont : la présence de composants antinutritionnels, le temps nécessaire à la préparation, les saveurs désagréables, et la digestibilité réduites des protéines. On peut réduire l'impacte des facteurs antinutritionnels comme les inhibiteurs de la trypsine par des traitements thermiques. Les résultats ont demontré que l'utilisation du pretraitment CM permettait de réduire significativement ($P \leq 0.05$) l'activité des inhibiteurs de trypsine lorsque comparés aux prétraitments CT et EB. Il a aussi été démontré que l'augmentation de la dureé des prétraitments thermiques par CT ou par EB permettait d'accroitre la DIVP. Dans tous les cas étudiés, l'application d'un prétraitment thermique sur les lentilles a permis d'améliorer la DIVP.

Mots clés : Lentilles vertes, lentilles rouges, farines, traitements thermiques, composés phénoliques totaux (CPT), activité anti-oxydante totale (AAOT), digestibilité in-vitro des protéines (DIVP), équivalent en acide gallique, chauffage par torréfaction (CT), chauffage par microondes (CM), chauffage par trempage dans l'eau bouillante (EB).

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CONTRIBUTIONS OF THE AUTHORS

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The authorship of the first paper (chapter 3) includes Yamuna Sampathkumar, Ashutosh Singh, D, Dr. Valérie Orsat, Yvan Gariépy and Dr. G. S. V. Raghavan. For the second paper (chapter 4) the authorship is Yamuna Sampathkumar, Yvan Gariépy, Dr. Valérie Orsat and Dr. G. S. V. Raghavan.

Co-authors, Yvan Gariépy, Department of Bioresource Engineering were involved in the development, implementation and data analysis. Yvan Gariépy, Dr. Valérie Orsat provided guidance and support in the development of the manuscripts.

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CHAPTER I

INTRODUCTION

Lentils (*Lens Culnaris* Medik.), one of the most important protein rich pulses, botanically classified as (Adsule *et al*., 1989) are of high nutritional quality. Throughout its development, pulse seeds gather proteins, reaching levels of roughly 25% on a dry weight basis (DW) (Pulse Canada, 2004; Sotelo and Adsule, 1996). Lentils are an excellent source of carbohydrates, various micronutrients viz., vitamin A, potassium, iron, B vitamins as well as fiber (Pulse Canada, 2009; Saskatchewan Agriculture and Food, 2003). In addition to micronutrients, it also contain certain essential amino acids such as arginine and lysine (Longneck *et al*., 2002), which are deficient in cereals and grains. Therefore by including legumes in our daily diet helps in gaining all most all essential amino acids necessary for improving the human health.

Compared to meat, poultry, or eggs, lentil are a protein source with virtually no associated fat, and are considered as a very good source of dietary fibre and resistant starch (Pulse Canada, 2009; Saskatchewan Agriculture and Food, 2003). They also help in weight management through delayed gastric emptying, resulting in an earlier sense of fullness, reduced hunger and increased satiety after a meal (Dilis and Trichopoulou, 2009; Leterme, 2002). Lentils both in de-hulled and whole form can be used in the form of flours and may be used as an ingredient in fortified pasta products and many other food products in combination with cereals and grains. Lentils, an important component of vegetarian diets, are used in a wide variety of dishes like soups, stews and many Indian dishes like idlis, dosas, salty-donuts, spiced-snack mixes and deserts.

The inclusion of lentils in one's daily diet has many beneficial effects in controlling and preventing various metabolic disorders such as diabetes mellitus and coronary heart disease (Tharanathan and Mahadevamma, 2003). Unfortunately, compared with other parts of the world, the legumes like lentil is relatively low in the West. On the other hand, one of the serious problems faced by most developing countries is the scarcity of food with an adequate quality of proteins for the growing population (Bressani,

1972). Thus, the use of plant proteins as an alternative for animal protein in human nutrition is of increasing interest.

However, while among seeds those of legumes tend to contain a high percentage of protein, they also contain several anti-nutritional constituents such as trypsin inhibitors, phytic acid, tannins, α-galactosides and oligosaccharides (raffinose, stachyose and verbascose) which play a major role in limiting protein and carbohydrate utilization (Vidal-Valverde *et al*., 1994). Additionally, due to a deficiency in sulphur-containing amino acids such as methionine and cysteine in lentil proteins (Evans and Bauer, 1978; Sarwar and Peace, 1986), a beany flavor (Walker and Kochhar, 2007), and length of the time required for their preparation, pulses such as lentils do not make up a significant portion of daily diets. The factors for lower legume protein digestion in humans are due to the low molecular weight protein trypsin inhibitor binds to the endopeptidase trypsin to form an inactive protein complex, thereby inhibits trypsin's function (Chavan & Adam, 1989).

Oligosaccharides are responsible for flatulence (Fleming, 1981) and phytic acid lowers the bioavailability of minerals (Reddy *et al*., 1984). Tannins are also reported to reduce the protein digestibility of lentils by forming complexes with protein and thereby reducing amino acid availability (Adsule *et al*., 1989). In contrast, Shahidi, (1997) reported that presence of some anti-nutritional constituents might exhibit beneficial health effects.

The potential health benefits of lentils also include the presence of secondary metabolites such as phenolic compounds (Xu and Chang, 2010) which plays an important role in antioxidant activities, not only protecting the human body from the damage of reactive oxygen species but also reducing their activity by scavenging free radicals, complexing pro-oxidant metals and quenching singlet oxygen (Ranilla *et al*., 2009; Madhujith and Shahidi, 2005).

Precooking (i.e., thermal processing) seeds to produce flours which can be used as functional ingredients which improve the protein content of foods can promote the

consumption of lentils and like legumes by increasing their public recognition through an accentuation of their health benefits.

Both developed and developing countries face the global issue of addressing the energy, nutrient and dietary protein requirements of their populations by using easier and quicker preparation methods. Consequently there exists a need to develop appropriate processing methods of unhulled green and de-hulled red lentils.

Since little information are reported in the literature on oven roasting, boiling and microwave heating on trypsin inhibitor activity (TIA), *in-vitro* protein digestibility and total phenolic content and antioxidant activity of lentil flours; This study was attempted on unhulled green lentil and de-hulled red lentils which are grinded before processing and all the above mentioned quality parameters are compared which would be helpful to use the processed flours in value added products.

Hypothesis

Thermal processing will provide an optimal solution for nutritional expression and reduction in anti-nutritional factors and may help in improving *in-vitro* protein digestibility of lentils.

Objectives

1. The main objective of the study was an attempt to compare whether conventional treatments like oven roasting, hydrothermal processing and electro technologies such as microwave heating had any effect on total phenolic content and antioxidant activity of hulled red lentils and non-hulled green lentils after varied times at suitable fixed temperatures.

2. The second objective was to assess whether the effects of oven roasting, boiling, and novel method such as microwave heating contributed to a reduction in TIA and improvement in protein digestibility of lentils, and further to attempt to determine whether enzyme inhibitors alone limit protein digestibility.

Scope and Significance

Due to the increasing interest in health benefits, need for low cost protein and foods rich in antioxidants are needed; hence, inclusion of lentils like legumes in our daily diet is key to the problem. Food technologists, nutritionists have been searching grain legumes as an alternative source of food to alleviate protein calorie malnutrition (PCM) in developing countries. Studies by researchers also highlighted by extending the consumption of grain legumes in the form of flours which could be used in various food applications such as baked goods, beverages, snacks, soups, salad dressings and dips amongst others (Kon & Burtea, 1979). As legumes are a versatile source of medium quality low cost proteins, their significance in human nutrition cannot be overemphasized (Salunkhe & Kadam, 1989) and they are processed before consumption depending on cultural and taste preferences. The most commonly used domestic thermal processing includes moist heating, dry and wet heating, autoclaving, boiling and drum drying process. But most of the studies were performed by applying thermal treatment on whole seeds before grinding them into flours and varieties used were also limited.

Therefore, the present study aims to study the ability of using microwave heating on unhulled green lentil and de-hulled red lentil flours and comparing it with oven roasting and boiling. Total phenolic content, antioxidant activity, trypsin inhibitor activity and *in-vitro* protein digestibility are to be investigated in roasted and boiled lentil flours compared with raw ones.

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CHAPTER II

LITERATURE REVIEW

Abstract:

Lentils, like legume crops, are an important source of various micronutrients such as proteins, carbohydrates, dietary fiber and other bioactive phenolic compounds. At present, the consumption of protein and health-oriented foods are increasing in day to day life. Consumer interest in natural food products with increased nutritional value has increased tremendously. Therefore, the food industry is creating high quality nutritious foods which are gluten-free, but high in protein content. As the traditional processing of legumes is a time-consuming process, preparing pre-cooked ready-to-use pulse ingredients will stimulate the production of legume-bearing foods and can prove to be an effective way to increase the public recognition of healthy foods. This review summarizes the chemical composition of lentils, followed by their traditional processing such as roasting, boiling and novel method such as microwave heating. It also emphasizes their antioxidant activity and phenolic compounds, which can, in the future, serve as alternatives to synthetic antioxidants and thus play a beneficial role in controlling various diseases and improving human health. Moreover it highlights the anti-nutritional compounds and low protein digestibilities which are major reasons for the low recommended intake levels of lentils.

Keywords: Lentils, Antioxidants, Anti-nutrients, microwave heating, phenolic compounds, protein digestibility.

2.1 The Lentils

Lentils (*Lens culnaris* Medikus*)*, a cool season food legume and one of the world's oldest cultivated crops, plays an important role in the diets of many cultures. Lentils are consumed daily by many inhabitants of the Middle East and Asia (Yadav *et al*., 2007). Lentils are an important commercial crop grown in Canada, with large areas in Saskatchewan being devoted to their culture (Saskatchewan Pulse Growers, 2007). Canada's production of lentils reached 1.04 Tg yr^{-1} in 2002, and its exports accounted for 44 % of total international lentil exports (Agriculture and Agri- food Canada, 2002c). Lentil is annual bushy herb with erect, spreading and compact growth habit. Its stems are slender, angular, light green in color, and the plants are 0.15-0.75 m in height. The seeds are round, small, lens shaped and weigh between 2-8g per 100 seeds (Duke 1981, Muehlbauer *et al*., 1985).

Although the lentils consumption is relatively low in western countries, the production and consumption per capita has increased over the last few years. Lentil seeds are used in various cuisines worldwide, and lentil flour can be used in soups, stews and purees, as well as mixed with cereals to make breads, cakes and food for infants (William and Singh, 1988) which provide essential amino acids which are deficient in cereal grains. Further, lentil flour also has a potential for traditional and newer product developments with various health benefits since it contain large amount of protein, and is gluten-free and low in glycemic index (Swanson, 1990).

Lentil varieties differ on the basis of their seed coat, cotyledon colour, seed size, plant hairiness, and leaf colour (Yadhav *et al*., 2007). Two main varieties of lentils are grown in Canada, red and green. Red-colored lentils, which have brown or grey seed coats and orange to red cotyledons, are mainly consumed hulled, whereas green lentils, with light green seed coats and yellow cotyledon, are consumed whole.

2.2 Chemical Composition of Lentils:

The chemical composition of lentil-like food legumes is governed by the species, cultivar, geographic location and growth conditions. Lentil seeds' main components

include carbohydrates (Iqbal, 2006), consisting of soluble and insoluble dietary fibers as well as prebiotic oligosaccharides (Vidal Valverde *et al.,* 2002). Starch is the predominant carbohydrate in lentil seeds, ranging from 34.7 to 65% of dry weight basis (dwb). The relative proportions of the polymers amylose (soluble starch) and amylopectin (insoluble starch) in starch granules, confer the latter's unique structure, crystallinity, and specific physical properties (Hoover and Ratnayake, 2002). The lentil's carbohydrate fraction includes low levels of soluble sugars such as its most prevalent monosaccharide fructose, along with glucose; sucrose is the predominant disaccharide, and ranges from 1 to 2.5% dwb. Lentils also contain several galacto-oligosaccharides such as raffinose, ciceritol, stachyose and verbascose (2-8% dwb) and non-starch polysaccharides (~20% dwb) (Frias *et al*., 1994; Vidal-Valverde *et al*., 1992).

Resistant starch and all other undigested carbohydrates, including oligosaccharides and non-starch polysaccharides are collectively termed 'dietary fiber.' Lentils contain appreciable amounts of dietary fiber (9.7 to 24.1% dwb). Compared to other legumes, lentils are lower in cellulose (4.1 to 5.7% dwb), but higher in hemicellulose (6-15.7% dwb), which account for the majority of their fiber (Reddy, Pierson *et al*., 1984; Amir *et al*., 2007; Urbano *et al*., 2007).

The second major portions of lentil seeds are proteins (17- 30% dwb). These have varying concentrations of essential amino acids (Sathe, *et al.,* 1984) and bioactive peptides including adequate high levels of lectins (Cuadrado *et al*., 2002b). Lentil proteins are generally low in the essential amino acids methionine, cysteine and tryptophan, but rich in lysine, which is deficient in cereal proteins (Adsule *et al*., 1989). Lentils also possess significant amounts of non-protein amino acids (taurine, γ-aminobutyric acid (GABA), γ-hydroxyarginine, γ-hydroxyornithine and trigonelline) which have biological effects (Rozan *et al*., 2001; Kuo *et al*., 2004).

Compared to other plant foods such as cereal grains, fruits and vegetables, legume seeds are recognized for their high protein content. The crude protein contents of whole seed, defatted seed, cotyledon flour and defatted cotyledon flour are reported at 25.9%, 27.8%, 29.1% and 31.1%, respectively (Gupta and Dhillon, 1993). Lentil protein

values have been reported to range from 15.9-31.4 g kg^{-1} dwb (El-Nahry, Mourad *et al*., 1980; Souci *et al*., 1989). Most lentil seed proteins are stored in the cotyledons, where the majority of the protein consists of salt soluble globulins (storage proteins) comprising of legumins (11S) and vicilin (7S). The remaining seed protein belongs to the albumin fraction which includes housekeeping proteins, lectins and lipoxygenases (Bhatty and Christison, 1984; Bhatty, 1986; Wang *et al*., 2003). Albumins are water soluble proteins including protease inhibitors, amylase inhibitors and lectins. Storage proteins are soluble in salt solution, whereas enzymatic proteins tend to be less soluble in water. On the other hand, some researchers have found that legumes bread with higher glutelin content may help to improve the protein quality since it contains higher concentrations of methionine and cysteine (Singh and Jambunathan, 1982). Cooking lentils in boiling water significantly increased their crude protein content and starch content, but significantly decrease their ash content. The relative increase in crude protein and starch content in boiled lentils could be attributed to the loss of soluble solids (Wang *et al*., 2009).

Lentil is low in fat (lipids), but they do contain both essential fatty acids: linoleic and linolenic acid. The former is predominant, accounting for 41 to 57% of fat across several cultivars, while the latter accounts for 0.3 to 16% (Amir *et al*., 2007; Wang and Daun, 2004). Wang and Daun, (2004) reported that Canadian green and red lentils bore 40.97-46.14% and 42.91-45.23% linoleic acid, respectively. Lentil fats also include a small amount of saturated fatty acids, mainly oleic acid and palmitic acid.

Additionally, different genotype and environmental conditions may influence in altering mineral concentrations in lentil seeds. Lentils are a significant source of essential minerals which includes macronutrients, micronutrients and trace elements. Most of the minerals are located in the cotyledons, with the exception of Ca and Fe which are predominantly present in the seed coat (Adsule *et al*., 1989). Lentils are very good source of copper, phosphorous, magnesium and sodium with potassium (Porres, López-Jurado *et al*., 2003; Urbano *et al*., 2006). However, due to the interference of anti-nutritional compounds in lentil-based foods, lentils show low bioavailability of minerals. The presence of non-nutritional compounds not only affects mineral utilization but also other macronutrients such as proteins and their digestion.

Though lentils are devoid of vitamin B_{12} and D, lentils contain significant levels of water-soluble and fat-soluble vitamins required by humans (Kylen and McCready, 1975; Savage, 1988). Lentils are an excellent source of folate, which, if deficient in humans, can lead to neural tube defects in infants, and a higher incidence of certain types of cancer and heart disease (Selhub and Rosenburg, 1996).

2.3 Traditional Processing of Lentils

While lentils are one of the best and cheapest sources of vegetable protein and are highly nutritious, they do contain several anti-nutritional factors which could limit their consumption (Adsule *et al*., 1989; Liener and Kakade, 1980). Efficient conventional thermal processing techniques are used to bring some desirable changes to lentil seeds or pre-cooked flours by changing their composition and thus increasing or decreasing their nutritional value (Sathe *et al.,* 1984).

Conventional thermal processing applied on whole seeds or milling of pulse seeds to flours has been used for decades, for processing many food products (Bar-Yosef, 1998). Some of the drawbacks of large-scale processing include slow heat conduction, long processing times, and poor commercial stability of the product. This has led food processors to look for alternative technologies. Therefore, a comparative study of traditional processes such as roasting, boiling, and electro technologies such as microwave heating, employed with legumes, is reviewed below. Each method can provide different physiochemical and structural changes, as well as improve the nutritional quality of the final product.

2.3.1. Roasting:

Roasting is a rapid processing method that uses dry heat for short period of time. Studies have shown that after roasting, grains may exhibit improved texture, enhanced crispiness and increases in volume by puffing (Hoke *et al*., 2007). Roasting is a common heating processes applied to legumes, which generally leads to a significant reduction in insoluble dietary fiber and total dietary fiber but an increase in soluble dietary fiber (Azizah and Zainon, 1997 and Mahadevamma and Tharanathan, 2004). Gahlawat &

Sehgal, (1992) reported that roasting may also improve the digestibility, colour, flavor, shelf life and reduces the anti-nutrient factors of cereals and legumes. Commercial food processing, by applying heat to seed products, helps to improve their protein quality by destroying certain anti-nutritional compounds. Thermal treatment such as roasting of flour by applying dry heat for 6-8 min at 104- 105°C has been shown to reduce its enzyme activity and lower its trypsin inhibitor and haemagglutinin activities (Aguilera, Lusas *et al*., 1982; Smith and Circle, 1972). Therefore, roasting being a simple and cost effective processing method can be used in developing countries in order to achieve maximum nutrient utilization from legumes like lentils. Under conventional heating methods, thermal energy is transferred from the product surface towards its center 10-20 times more slowly than in a microwave-heated product. Roasting has both positive and negative impacts on legume seeds and flours according to its duration.

2.3.2 Boiling (Hydrothermal processing):

Boiling is one of the commonest methods to cook any form of comestible legumes. It can be done by two methods: (i) open pan boiling, or (ii) pressure boiling

In the traditional process, comestible legumes are cooked by open pan boiling, where violent heating may result in loss of water soluble nutrients. Consequently, turning down the heat once the water starts boiling, in order to slow down the cooking process, is recommended. Appropriate cooking times for legumes can be affected by genetic factors, physical structure, chemical composition and processing (Iyer *et al*., 1989).

Parihar *et al*., (1999) reported that proportionately more proteins were retained under both forms of boiling, than fats or carbohydrates. Preliminary studies by Xu and Chang, (2008) reported that the boiling process significantly reduced total phenolic contents, free radical scavenging capacity in cool season comestible legumes. Porres, López-Jurado *et al*., (2003) found that by pressure cooking lentils at 120°C for 30 min reduces concentrations of trypsin inhibitor activity, phytate, and tannin content by 76%, 8%, and 12% respectively. *In-vitro* protein digestibility was improved by 81% after pressure cooking compared to open pan boiling (Naveeda and Jamuna, 2006). Ur-

Rehman and Salariya, (2005) studied that by ordinary boiling of different comestible legumes improved protein digestibility by 86.0-93.3% when compared to uncooked legumes.

Ma, Z., *et al*., (2010) reported that processing by boiling of different legume flours resulted in varying compositions. Protein content was significantly lower for unhulled green and red lentils, compared to hulled ones. A significant reduction in total trypsin inhibitor after boiling has been reported for all pulse flours.

2.3.3 Microwave Heating:

Alternative technologies are needed to overcome the major limitations of thermal processing of foods, further build consumer interest in nutritionally rich, synthetic preservative-free, long shelf life products, which retain their fresh flavor and appearance (Brijesh *et al*., 2011). Ahmed, *et al.,* (2009) divided emerging technologies into two categories such as electro technologies and non-thermal technologies. Electro-technologies like microwave heating are used in thermal processing of non-conductive food materials. Two frequencies namely 915 MHz and 2450 MHz are used in microwave heating applications (Decareau, 1985). Datta, (2001) characterized the rate of heat generation per unit volume at a particular location in the food during microwave heating, Q, as:

$$Q = 2\pi f \varepsilon_0 \varepsilon'' E^2 \qquad (2.1)$$

Where,

E is the strength of the electric field

f is the frequency of the microwaves

ε_0 is the permittivity of the free space (a physical constant) and

ε'' is the dielectric loss factor.

Microwave energy provides more uniform and rapid volumetric heating when compared to other conventional heating process. It operates through the interaction between an alternating electromagnetic field and a dielectric material. The mechanism

behind microwave heating involves oscillatory migration of ions which produces heat in the food under the influence of an electric field (Brijesh *et al*., 2011). According to Meda *et al*., (2005), microwave heating exploits the dielectric behavior of the substance in such a manner as to generate heat only up to a certain depth from the surface of the product. There is an exponential decay of microwave energy as the waves penetrate into the product from the surface, depending on the dielectric properties of the substance.

Kadlec *et al*., (2001) found that the sucrose and monosaccharide content of dried germinated peas was constant, but decreased after MW treatment to 78% and 83% of original levels, respectively. Stewart and Raghavan, (2003) showed that MW drying preserved TIA and retained higher quantities of fatty acids, to produce better quality soybeans [*Glycine max* (L.) Merr.]. Microwave heating at a power density of 3 W g^{-1} using a 1:1.5 samples to water ratio retained a maximum amount of vitamin content in red lentils (Dev *et al*., 2010). Sharma and Gujaral, (2011) found a decrease in TPC and antioxidant activity in barley (*Hordeum vulgare* L.) (after sand roasting and microwave roasting). The greater loss in TPC may be due to a longer heating time in the microwave oven.

By applying microwaves processing to pulses such as chickpeas (*Cicer arietinum* L.) and common beans (*Phaseolus vulgaris* L.) (Marconi, *et al*., 2000) reported changes in the cooking quality, physiochemical characteristics, along with nutritional modifications in starch and non-starch polysaccharides. Scanning electron microscopy of microwave-heated and traditionally processed legumes shows similar effects with respect to *in-vitro* starch digestibility. Gonzalez and Perez, (2002) reported that microwave irradiation and extrusion cooking caused several changes in rheological, functional and morphological characteristics of lentil starches. Both treatments reduced the retro gradation tendency of lentil starch for the processing industry, which would be an interesting for the processing industry, since this is one of the reasons that have limited its commercial use. The *in-vitro* protein digestibility of microwave and pressure-cooked comestible legumes varies significantly, but averages about 75% and 80%, respectively (Khatoon and Prakash, 2004).

Khatoon and Prakash, (2006) investigated the effects of germination on the cooking quality and nutrient retention of microwave-cooked pulses. Germinated legumes cooked in a microwave oven showed an increased *in-vitro* protein digestibility, which, however, remained low when compared to pressure cooked legumes. Also, microwave cooking required more water and time than pressure cooking. Recently, microwave heating has been applied to a great variety of foods to significantly reduce their preparation time (cooking, thawing, blanching, drying, pasteurization, sterilization, dehydration, reheating, etc.) but rarely for legume processing (rehydration, cooking, and canning). In addition, microwave heating within closed vessels has been successfully used in the analytical field for the rapid acid hydrolysis of proteins, reducing cooking time from 110 to 11 min for chickpeas and 55 min to 9 min for common beans, when compared to conventional cooking methods (Marconi, *et al*., 2000).

2.4 Antinutritional components in lentils:

Lentils are rich sources of inherent nutritional benefits, because of their micro nutrients, vitamins and minerals. Several studies follows have reported that legume seeds contain two main classes of enzyme inhibitors: protease inhibitors and α-amylase inhibitors. Muzquiz and Wood, (2007), reported those that these protease (trypsin and chymotrypsin) inhibitors disrupt protein digestion, thereby inhibiting the utilization of proteins and amino acids from the seeds. Trypsin and chymotrypsin inhibitors in lentils range from 2.7-6.1 U mg^{-1} dwb (Vidal-Valverde, *et al*., 1994; Urbano, Lopez-Jurado *et al*., 1995). Moreover, α-galactosides and trypsin inhibitors were found in appreciable amounts in raw lentil seeds.

α-galactosides are a group of non-nutritional compounds, whose content in lentils ranges from 1.8 to 7.5 g kg^{-1} dwb (Wang and Daun, 2006; Porres *et al*., 2004). α-galactosides are mainly formed of raffinose, ciceritol, stachyose and verbascose. As they are not digested in small intestine, their metabolism occurs in the large intestine. This activity results in the emission of gases (Hydrogen, CO_2 and CH_4) which are contributors to flatus. Reddy, Pierson *et al*., (1984) reported that green lentils release less flatulence than other legumes such as navy beans, red kidney, chickpea and peas.

In lentils, the phytic acid content varies from 6.2 to 8.8 g kg^{-1} dwb, while tannin levels in raw seeds average about 4.7 g kg^{-1} dwb (Wang and Hatcher, 2008). Phytic acid, major phosphorus storage constitutes 1-3% and they occupy high content in whole grains, legumes. According to Fred-lund *et al*., (2006), phytate, phosphorus storage in plants and its salts occurs as a mineral complex, which is insoluble at the physiological pH of the intestine.

2.4.1 Trypsin Inhibitors:

In legume seeds, trypsin inhibitors (TI) are both proteinanceous and non-proteinanceous in nature (Dave Oomah *et al*., 2011). Only proteinanceous TI has been extensively studied in the literature. Trypsin inhibitors are present in variety of foods, and especially in comestible legumes. Plant proteinase inhibitors vary in size from 4 to 60 kDa molecular weight (Ryan, 1981). Bowman–Birk inhibitor (BBI) proteins are double-headed serine protease inhibitors which have a molecular mass of 7-8 kDa in dicot seeds and but vary substantially in size and inhibitory sites in monocots (Ragg, Galbusera *et al*., 2006).

Birk, (1994) reported that the mechanism of TI's anti-nutritional effects, though not clearly understood, probably occurred through a mechanism of feedback inhibition which controls the pancreatic secretions, according to the level of trypsin in the small intestine at any given time. When TI's react with the enzyme trypsin, the enzyme level falls below the critical threshold value, and the pancreas is then induced to produce more of the enzyme, thus leading to pancreatic hyperactivity, which can cause hypertrophy and, hyperplasia of the pancreas. The hormone cholecystokinin (CCK) is believed to act as a mediating agent between the pancreas and trypsin. Thus TIs interfere with protein digestibility, by binding to trypsin in the small intestine to form insoluble complexes. Therefore inactivating a TI is accompanied by an increase in nutritive value of protein.

Generally, raw legumes contain higher levels of anti-nutritional factors than processed forms, hence processing plays a major role before incorporating them into food or animal diets (Hajós and Osagie, 2004). Table 2.1 illustrates the level of trypsin inhibitor activity in different legumes (Elkowicz and Sosulski, 1982). It is well

established that high levels of trypsin and chymotrypsin inhibitor activity impairs the mammalian digestive enzyme trypsin and results in reduced growth by decreasing the availability of amino acids. The presence of protease inhibitors in the diet can cause adverse effects by decreasing protein and starch digestion and thereby reducing the nutritive value of lentil seeds, and limiting their use as food (Embaby, 2000; Wang, *et al.,* 2009; Alonso, 2000). Grant, *et al.,* (1983) reported that lentils belong to a group of pulses with a lesser toxicity and low reactivity to the erythrocyte agglutination test for lectins, when compared to other highly reactive legumes like common bean, and scarlet runner bean (*Phaseolus coccineus* L.).

Table 2.1 Trypsin Inhibitor activity of comestible legumes

Legume Flour	**Trypsin inhibitor activity (TIU mg^{-1} sample)**
Lima Bean	46.81
Soybean	41.55
Chickpea	18.80
Navy Bean	18.23
Northern Bean	18.08
Mung Bean	9.96
Field Pea	7.61
Lentil	5.12

Adapted from Elkowicz and Sosulski, (1982)

Habiba, (2002), reported that the use of microwave heating is increasing in industrial cooking processes, due to the reduction in processing time. Moreover, it was found that some anti-nutrient factors such as trypsin inhibitors, α-amylase inhibitors, and lectins are greatly reduced by applying heat. However, phytic acid is heat stable, but can be greatly reduced by treatments such as germination, soaking and fermentation. All legumes, including lentils, must be cooked, not only to soften the lentil seeds, but also to reduce the trypsin inhibitor activity which normally disrupt the nutritive value of pulses

and markedly reduces the availability of some essential amino acids (Wang and Hatcher, 2008).

2.5 Protein Overview

Legumes including lentils can be consumed around the world because of their high protein content compared to cereals according to the (Table 2.2). Legumes are an important source of protein in the human diet in many parts of the world including Latin America, Africa, and the Asian subcontinent (Jood, Bishnoi *et al*., 1998). Protein malnutrition is an important problem in most developing countries, so legumes can play a major role in providing needed protein at a low cost, compared to the high cost and limited availability of animal proteins in these countries. Therefore, the consumption of legumes, including lentils, should be increased by reducing their anti-nutritional compounds, and bringing recognition to the nutritive value of legumes by introducing new valuable products.

Table 2.2 Protein content and amino acid levels in different pulses

Pulse species	Protein content (g hg^{-1} dwb)	Amino acid content (mg N g-1)		
		Lysine	Methionine	Cysteine
Pigeon pea [*Cajanus cajan* (L.) Millsp.]	20.9	450	70	90
Chickpea (*Cicer arietinum* L.)	20.1	370	110	80
Lentil (*Lens culinaris* Medik.)	24.2	400	80	110
Mung bean (*Phaseolus aureus* Roxb.)	23.9	430	70	40
Kidney bean (*Phaseolus vulgaris* L.)	22.1	60	60	60
Pea (*Pisum sativum* L.)	22.5	460	80	80
Cow pea (*Vigna unguiculata* (L.) Walp.)	23.4	410	120	110

Adapted from www.fao.org; hg^{-1} – per hundred gram

2.5.1 Low Protein Digestibility:

Proteins are large macromolecules produced by both plants and animals. They consist of long chains of polypeptide subunits called amino acids, with an average of about 400 or 500 amino acids per protein molecule. There are 20 different amino acids in human protein, among the 20, eight are "essential" amino acids and they come from protein diet. The remaining 12 amino acids are categorized as "non-essential", because they can be synthesized by cells. To synthesize a protein, all the amino acids should be present, if one or more amino acids are missing, the organism will eventually suffer from protein deficiency. The nine essential amino acids required in adequate amounts in the humans diet are: histidine, tryptophan, phenylalanine, lysine, threonine, valine, methionine, leucine, and isoleucine (file:///C/wayne/chemid1.htm, 2011). The essential amino acid lysine is rich in legumes like lentils, but they are deficient in sulphur-containing amino acids such as methionine and cysteine. Additionally, legume seeds also contain anti-nutritional compounds which can be proteinous or non- proteinous in nature (Dave Oomah *et al*., 2011).

Lentils contain a large amount of protein; however its availability is poor due to the low protein digestibility (44.6-51.9%) of raw flours (Sulieman, Mashair, 2008). Low protein digestibility of legume proteins includes intrinsic structural factors, including primary structure and protein conformation (Carbonaro *et al*., 1992, 1997, 2000; Nielsen 1991; Chau and Cheung, 1997; Semino, *et al*., 1985). The structural constraints of legume proteins are influenced by the presence of glycoproteins and sulfur-containing amino acids (Semino, *et al*., 1985; Carbonaro, Marletta *et al*., 1992). Additionally, the structural constraints and compact structure of native phaseolin in legumes also limit accessibility to enzymes to the protein (Nielsen, 1991).

Lentils contain some anti-nutritional factors like indigestible oligosaccharides, α-galactosides, and protease inhibitors (Vidal-Valverde, Frias *et al*., 1994) which may be attributed to the poor digestibility of their proteins. The use of most legumes, including lentils, is limited by some of the major anti-nutritional factors in leguminous seeds. These

include compounds such as phytate, polyphenols, enzyme inhibitors (trypsin, chymotrypsin, and α-amylase) and haemagglutinins. Reduction of these anti-nutrients was significantly greater when these legumes cooked by autoclave at 121º C or 128ºC, compared to other traditional processes (ur-Rehman and Shah, 2005). Additionally, when various lentil cultivars' flour was digested with pepsin and pancreatin, the *in-vitro* protein digestibility was reduced from 77.1%-88.0% by the process of normal cooking (Sulieman, Mashair, 2008).

Phytic acid is considered an antinutritional compound given its ability to bind essential dietary minerals as well as proteins and starch, and to consequently reduce their bioavailability in humans (Philippi, 2003). The toxic effects of lectins are due to their ability to bind to specific receptor sites on the surface of intestinal epithelial cells, which causes an interference with the absorption of nutrients across the intestinal wall. Bound lectins also impair the transfer of amino acids, leading to a decrease in protein digestibility (Adsule *et al*., 1989).

2.5.2 Effects of thermal processing on protein digestibility

Cooking, soaking, roasting, boiling, germination and other conventional cooking methods are applied to lentils in order to inactivate the antinutritional compounds and to improve of protein digestibility.

Monsoon and Yusuf, (2002), found that non-soaked lentil seeds after dry heating or autoclave treatments which caused reduction in trypsin inhibitor activity, tannins and phytic acid and increased tannin/catechins ratio without causing any significant changes in *in-vitro* protein digestibility of lentils. On the other hand, soaking of lentil seeds followed by traditional or autoclave cooking process, has been found to significant by decrease phytic acid and tannin content, and improve *in-vitro* and *in-vivo* protein digestibility (Savage and Scott, 1989; Zia-ur-Rehman and Shah, 2005). Paraná *et al*., (2000) reported that microwave treatment caused a significant reduction in trypsin inhibitor activity and hæmaglutinins of raw and soaked lentils. All the above forms of

processing have been carried out on whole or hulled seeds prior to them being milled into flour. Therefore, processing, such as soaking, improves or reduces the *in-vitro* protein digestibility to different degrees depending upon the cultivar and duration of soaking.

The process of germinating *Lens culinaris* seeds up to 75 hours has been shown to decrease the levels of antinutritional factors such as tannins, phytic acid and lead to an improvement in protein digestibility (El-Adway *et al*., 2003). Fermentation also causes an increase in *in-vitro* protein digestibility and non-protein nitrogen and a slight increase in crude protein content (Sheik, 1994). The higher the temperature and longer the period of fermentation, the greater is the reduction in the phytic acid content. The processing such as germination and fermentation shows improvement in protein digestibility after a long period of time. In order to reduce the processing time, cooking or applying heat to the legumes seeds/flours is necessary to reduce the preparation time and increase in nutritional value in a short period of time. Table 2.3 illustrates protein digestibility of various pulses reported in several studies.

Table 2.3 Protein Digestibility of Various Pulse Seeds

Source	Protein content (g hg^{-1} dwb)	Protein digestibility (g hg^{-1} dwb)	Reference
Casein	—	98.34	Monsoor and Yusuf (2002)
Soybean [*Glycine max* (L.) Merr.]	—	71.80	Han et al., (2007)
Chickpea (*Cicer arietinum* L.)	20.4	89.01	Monsoor and Yusuf (2002)
Lentil (*Lens culinaris* Medik.)	23.2	95.19	Monsoor and Yusuf (2002)
	—	79.1-79.4	Han et al., (2007)
Moth bean [*Vigna aconitifolia* (Jacq.) Marechal]	—	58.69-62.06	Khokhar and Chauhan (1986)
Yellow Pea (*Pisum sativum* L.)	—	82.0	Han et al., (2007)
Green Pea (*Pisum sativum* L.)	—	82.6	Han et al., (2007)

(Adopted from Boye and Zare, 2010), - not reported; hg^{-1} – per hundred gram

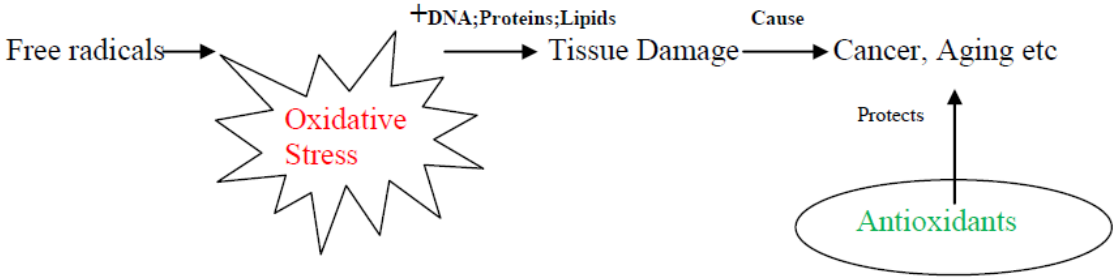
Cooking and thermal processing induces protein denaturation, enhances accessibility of susceptible sites of proteolysis and thereby improves the digestibility of proteins (Carbonaro, Cappelloni *et al*., 1997). Jood, Bishnoi *et al*., (1998) studied the protein digestibility of legume globulins and found them to be resistant in the native state, but a good source of nutrition when denatured.

2.6 Free radicals and antioxidants in human health:

Several researchers have defined free radicals as molecular fragments which bear at least one or more unpaired electrons in atomic and molecular orbitals (Gilbert, 2000; Halliwell & Gutteridge, 1999). Highly reactive, free radicals are very unstable and hence short lived ($t_{½}$ ranges from nano to milli seconds). Oxygen free radicals for (*e.g*., hydroxyl, peroxyl (RO_2•), alkoxyl (RO•), and hydroperoxyl (HO2•) radicals and nitrogen free radicals such as nitric oxide and nitrogen dioxide can be converted to other non-radical reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These free radicals cause deleterious oxidative effects at pathologic levels, but may also provide beneficial effects to both humans and animals at physiological levels (Freidovich, 1999).

According to Valko *et al*., (2006), when ROS are in high concentrations, they cause damage to cell structures, nucleic acids, lipids, and proteins. The high nucleophilic potential of deoxyribonucleic acid (DNA), lipids, and proteins lead these to react with free radicals to form stable bonds, which cause structural changes and oxidative damage. Therefore, in order to regulate the process, both enzymatic and non-enzymatic antioxidants from various natural sources play a major role in protection against various diseases (*e.g*., several forms of cancer, cardiovascular (CVD), neurological and pulmonary diseases) induced by free-radical-generated oxidative stress (Halliwell, 1994). Fig 2.1 illustrates the consequences of oxidative stress generated by free radicals and the role of antioxidants in protection against human diseases.

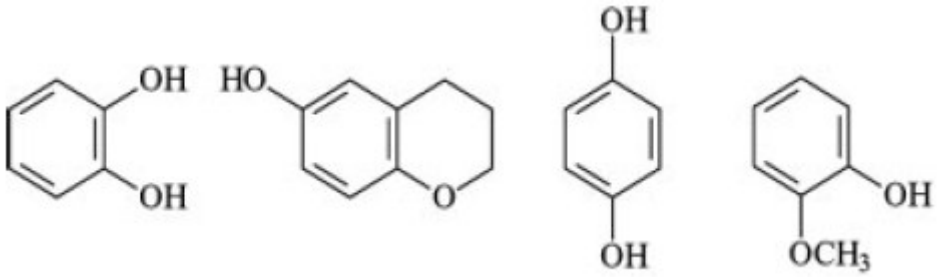
Figure 2.1: Effects of oxidative stress and antioxidants role in protein against human diseases



2.6.1 Sources of Natural Antioxidants:

Antioxidants are defined as any substance that delays oxidation of a substrate when present in lower concentration than that of the oxidizable substrate (Valenzuela *et al*., 1996). Natural antioxidants are synthesized by various organisms such as microorganisms, fungi, even animals and more often by plants. Changes in consumer preferences and food safety legislation have led the food industry and researchers to concentrate on natural antioxidant sources, their safety in the human diet and further health concerns. Natural antioxidants tend to display less toxicity when compared to synthetic ones, due to the presence of the pyrocatechol group, (Figure 2.2) *i.e.*, ortho-disubsituted phenolic compounds (Pokorny, 2007).

Figure 2.2: Basic chemical structure of an antioxidant (Pokorny, 2007)



Natural antioxidants have many potential health benefits, such as preventing many degenerative and chronic diseases such as cancer, Alzheimer's and Parkinson's disease (Chu *et al*., 2002; Chung *et al*., 1999). Natural sources rich in phenolic antioxidants are generally recommended direct for human consumption, rather than as antioxidant additives in prepared foods. Interestingly, legumes contain more phenolic antioxidant activity

than fruits and vegetables. Table 2.4 lists the major sources of tocopherols, phospholipids, ascorbic acid and their esters, rosemary resins, sage resins, and tea leaf extracts (Pokorný, 2007).

Table 2.4: Major sources of antioxidants in the human diet (Adapted from Pokorný, 2007)

Sources	Examples
Cereals	Whole wheat products, oat, rice, bran
Vegetables	Leaf vegetables, potatoes
Fruits	Apples, bananas, berries, olives
Oilseeds	Sesame seeds, hazelnuts, almonds
Legumes	Beans, peanuts, soybeans
Cocoa products	Chocolate
Beverages	Tea, coffee, red wine, beer, fruit juices
Herbs and spices	Labiatae plants (rosemary, sage, oregano, savory)

Though researchers have debated the relative advantages and safety of synthetic *vs.* natural antioxidants, both of them depend strictly on their chemical structure and polarity for their action. Adding natural antioxidants at a higher concentration (over 20%) to the diet of prematurely-aging mice improved leukocyte function and reduced oxidative stress (Alvarado, *et al*., 2006). Other sources such as garlic (*Allium sativum* L.), green tea [*Camellia sinensis* (L.) Kuntze] catechins are found to be beneficial against cardiovascular and other diseases such as cancer and the aging of skin (Rutter *et al*., 2003).

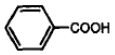
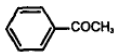
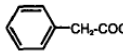
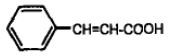
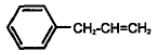
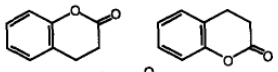
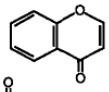
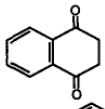
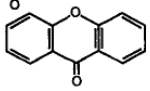
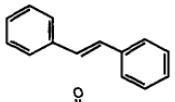
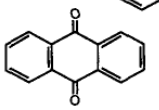
2.6.2 Antioxidant Activity and Phenolic Compounds:

As many as 25,000 deaths occur annually due to cardiovascular disease, cancer and diabetes in Canada that are related to diet. As a result, dietary modification is a practical strategy for the prevention of chronic diseases (Katzmarzyk *et al*., 2000). The World Health Organization (WHO) estimates that healthy life expectancy can be increased by avoiding the top preventable health risks: four diet-related prominent factors

(blood cholesterol, blood pressure, overweight, and low fruit and vegetable intake), as well as physical inactivity and smoking. Aggressive oxygen species (*e.g.,* the hydroxyl radical, superoxide anion and singlet oxygen) have been associated with carcinogenesis and arterial injury by ischemia, followed by reoxygenation, arteriosclerosis, ionizing radiation, etc.

Growing interest in antioxidant activity has arisen because this activity reduces oxidative damage associated with many chronic diseases, including cardiovascular diseases, cancer, arthrosclerosis, diabetes, immune deficiency diseases, and aging (Ames *et al*., 1993; Wang *et al*., 1996). Therefore, in order to prevent these diseases, consumption of foods with high levels of compounds with antioxidant activity may be necessary for day-to-day life. The potential health benefits of pulses are in large part due to the presence of secondary metabolites such as phenolic compounds and their antioxidant properties (Cardador-Martinez *et al*., (2002); Azevedo *et al*., (2000); Lazze *et al*., (2003). Polyphenols constitute one of the widely distributed products of secondary metabolism in plants, with more than 8000 phenolic structures currently known (Harborne, 1993). The major sub groups of phenolic compounds are phenolic acids, flavonoids and tannins. Kim, *et al.,* (2003) and Robbins, (2003) studied those phenolic compounds that range from simple molecules, such as phenolic acids to complex polymerized structures such as tannins that further include of an aromatic benzene ring substituted with hydroxyl groups. Polyphenols can be divided into 10 different classes based on their chemical structure (Table 2.5) (Harborne, 1989; Bravo, 1998).

Table 2.5 Main classes of polyphenolic compounds (Adapted from Bravo, 1998).

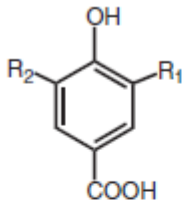
Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

Phenolics, including 'phenolic acids' — phenols with carboxyl groups — are low molecular weight compounds, with a C_6-C_1 structure (Table 2.4). These are commonly present in plants and include phenolic acids (gallic, vanillic, syringic, *p*-hydroxybenzoic) and aldehydes. Ferulic acid is one of the most abundant phenolic acids in common pulses and it occupies an intermediate level of phenolic acids between *p*-coumaric acid and sinapic acid (Lutharia and Pastor-Corrales, 2006).

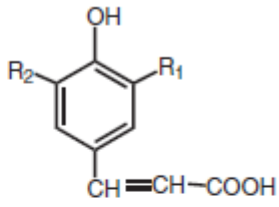
There are two subgroups of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids (Fig. 2.3) (Balasundaram *et al.,* 2006). Gallic acid, *p*-

hydroxybenzoic acid, protocatechuic acid, syringic acid and vanillic acids, which have a C_6-C_1 structure, fall under the former category, while caffeic, ferulic, *p*-coumaric acid, and sinapic acids and aromatic compounds, which have a C_6-C_3 structure, fall under the latter (Balasundram *et al*., 2006; Moyer *et al*., 2002).

Fig 2.3: Chemical Structure of a) Hydroxybenzoic acids b) Hydroxycinnamic acids (Balasundaram *et al*.,2006)



Benzoic Acid Derivatives



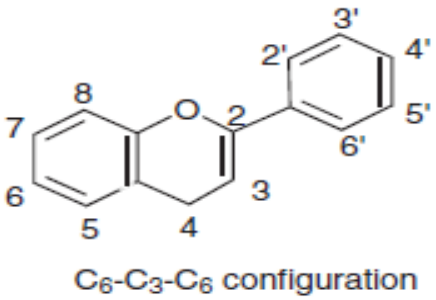
Cinnamic Acid Derivatives

Compound	R_1	R_2	Compound	R_1	R_2
p-Hydroxy benzoic acid	H	H	*p*-Coumaric acid	H	H
Vanillic acid	H	OCH_3	Ferulic acid	H	OCH_3
Syringic acid	OCH_3	OCH_3	Sinapic acid	OCH_3	OCH_3
Dihydroxybenzoic acid	OH	H	Caffeic acid	OH	H
Gallic acid	OH	OH			

Another group of phenolic compounds includes flavonoids, a largest group of plant phenolics which are further subdivided into anthocyanidins, flavonols, flavones, and flavanones. Flavonoids are low molecular weight compounds which usually occur as glycoside derivatives in plants (Kim *et al*., 2003, King and Young, 1999). The common structure of flavonoids consists of fifteen carbon atoms arranged in an oxygenated heterocycle arranged in C_6 –C_3 –C_6 configuration (diphenylpropane structure). The flavonoid structure consists of two benzene rings (A and B). The (A) ring is usually synthesized through the acetate pathway, whereas ring (B) derives from the shikimate pathway, and is connected to a heterocyclic ring (C) by a 3-carbon bridge (Fig 2.4). Isoflavones (e.g., genistein, daidzein), especially common in legumes, have a (B) ring (B) of the flavone molecule attached to the heterocyclic third carbon (Bravo, 1998; Balasundaram *et al*., 2006). According to a survey of isoflavone content (USDA, 2002),

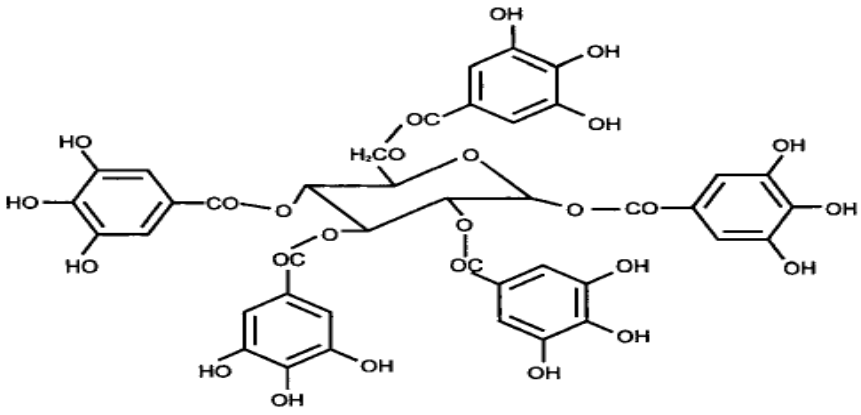
lentils do not contain any traces of isoflavone content. Therefore, simple phenols and flavonoids are mostly low molecular weight compounds which can be solubilized in alkaline conditions or be retained in the fiber matrix, based on their ester linkages (Bravo, 1998).

Figure 2.4 Basic structure and numbering system of flavonoids (Adapted from Bravo, 1998)



Another group of basic phenolics, tannins, contain at least two phenol rings. Tannins are highly hydroxylated compounds of high molecular weight, which form insoluble complexes with proteins and carbohydrates. They are divided into two groups: condensed and hydrolysable (Bravo, 1998; Kim *et al*., 2003). Tannic acid, one of the best known hydrolysable tannins (Fig 2.5) consists of a pentagalloyl glucose molecule that can be further esterified into five Gallic acid subunits (Bravo, 1998). Additionally, phenolic compounds act as radical scavengers and chelators of metals ions (Lopes *et al*., 1999, Bravo, 1998).

Figure 2.5: Basic Structure of tannic acid (Adapted from Bravo, 1998)

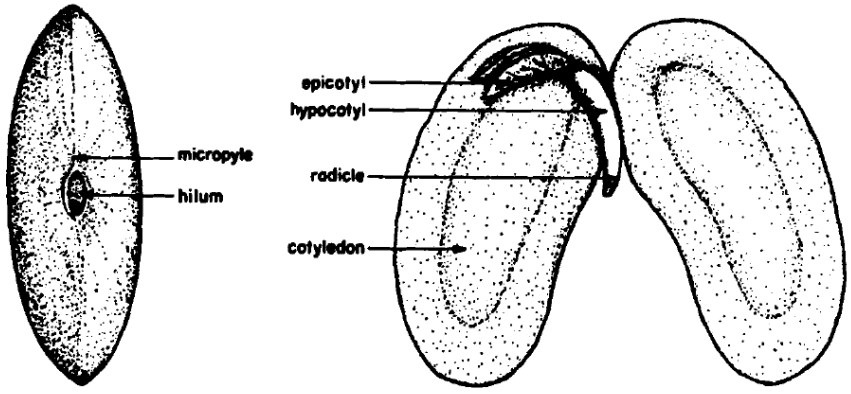


2.6.2 Antioxidant Activity and phenolic compounds in lentils:

Apart from macronutrients such as proteins and carbohydrates, legume seeds or flours provide micronutrients such as vitamins, carotenoids (Adsule *et al*., 1989) and phenolic compounds, which are considered to be bioactive compounds (De Pascual *et al*., 2000; Dueñas *et al*., 2002, 2004). Recently, phenolic substances (e.g., flavonoids, phenolic acids, and lignans) from legumes which play an important role as potential antioxidants have been extensively studied. Grain legumes/pulses play an important role in traditional diets in many parts of the world. Sauces prepared from the cooking liquors of coloured lentils, which are consumed with cooked rice and other cereals, contain dietary tannins or non-tannin phenolics, including phenolic acids. These derive mainly from seed coat pigments. The consumption of such legumes compounds has been linked to a reduced risk of diabetes and obesity and also serves a role in reducing coronary heart disease (Bazzano *et al*., 2001).

The typical legume seed is comprised of three different parts: the cotyledons, seed coat, and embryonic axis (Fig. 2.6); (Dueñas *et al*., 2004), which account for an average of 89%, 10% and 1% of total seed weight, respectively. The seed coat has the highest concentration of phenolic compounds and serves as a protective barrier for the cotyledons, which mainly house reserve substances such as proteins and carbohydrates.

Figure 2.6: Structure of typical legume seed; Left- External view, Right- internal view (Source: Northern, 1958)



According to Dueñas *et al*., (2002), lentils phenolic compounds are mainly distributed in seed coat and cotyledons. Flavonoids, such as the glycosides of flavones

and flavonols, together with trans-resveratrol-3-O-glucoside, as well as higher concentrations of proanthocyanidins are found in the seed coat, whereas non-flavonoid phenolics compounds are mainly located in the cotyledons (Dueñas *et al*., 2002).

Ames *et al*., (1983) reported that the majority of antioxidants from lentils were phenolics and polyphenols. In lentil seeds, most phenolics, both in terms of quantity and variety are located in the seed coat. Therefore, the seed coat of lentil-like legumes, which represents only a small percentage (8-11% dwb) of their total seed weight could be used as a replacement for synthetic antioxidants (Ronzio *et al*., 1998). Lentils have the highest phenolic, flavonoid and tannin contents (6.56 mg GAE g^{-1}; 1.30 and 5.97 catechin equivalents g^{-1}, respectively) compared to other common beans and pulses (Xu and Chang, 2007). An optimum antioxidant activity (95%) was obtained when the radical scavenging activity of a lentil extract was evaluated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Halvorsen and Holte, 2002). The phenolic antioxidant activities of legumes are related to the compounds' structure, for example, for the primary antioxidants flavonoids (Decker, 1997), their antioxidant activity is associated with the position and degree of hydroxylation (Rice-Evans *et al*., 1996). According to Xu and Chang (2007), lentils and red beans are rich sources of tannins. They found the highest amounts of tannins in lentils, where they ranged in content from 0.12–8.78 mg catechins equivalents g^{-1}. According to Fernandez-Orozco *et al*., (2003), the content of α- tocopherols in lentils ranges from 3.84–8.69 mg g^{-1} dwb.

2.7 Conclusion:

Consumer interest and health concerns for natural and ready-to-cook pulse ingredients have recently come into demand. Though lentil-like legumes are rich in proteins, antioxidant activity, some of their nutritional components reduced their usefulness as human food with respect to the recommended intake of certain nutrient levels. Therefore, the use of traditional processing methods such as boiling and roasting, along with newer electro-technologies such as microwave heating, were applied to the processing of lentil flours. These methods' affect with regards to improving the

nutritional value and *in-vitro* protein digestibility of lentils by reducing their levels of anti-nutritional compounds and the status of this result was investigated. Therefore, the chemical composition, antioxidant activity and phenolic compounds of lentils and reasons behind the low digestibility of untreated lentil seed protein have been reviewed. The production of lentils is comparatively low compared to that of other legumes, but the presence of high levels of phenolics and proteins in lentils encourages the improvement of their production and processing, so as to derive maximum benefits for human health.

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CONNECTING TEXT

The present study deals with the comparison of thermal processing such as (oven roasting, boiling and microwave heating) effects on total phenolic content and total antioxidant activity of unhulled green and hulled red lentils.

CHAPTER 3

THERMAL PROCESSING EFFECTS ON TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF LENTILS

Abstract

Microwave heating was applied to lentil flours and compared with traditional processing methods. Legumes like lentils are abundant in phenolic compounds, which are potential antioxidants essential for humans in fighting many diseases. Pre-cooking the flours of hulled red and whole unhulled green lentil cultivars has the potential to develop a product rich in antioxidants and phenolic compounds. Total phenolic content of whole unhulled green lentils was 7.5 mg Gallic acid equivalents (GAE) per g on a dry weight basis (dwb), compared to 2.65 mg GAE g^{-1} dwb for hulled red lentils. Total antioxidant activity (TAC) determined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay was greatest in unhulled green lentils at (86%) compared to 12% in hulled red lentils. In general, processed lentil flours exhibited a decrease in total phenolic content (TPC) compared to unprocessed flours (UPF), and these decreases were greater in magnitude with boiling than microwave or oven roasting. Two exceptions were significant ($P \leq 0.05$), a 17% increase in TPC after 5 min of microwave heating (MH) or 20 mins of oven roasting (OR), but these were followed by a steady decline thereafter in green lentil flours (GLF). Boiling (BW) resulted in a significant ($P \leq 0.05$) decrease in TPC and TAC values for both lentil cultivars, which progressed with increasing time. As a result of TAC of phenolic content, MH on GLF shows a significant increase ($P \leq 0.05$) with increasing time when compared to UPF 86% and the hulled red lentils shows an initial decrease with a slight increase as heating time increases. Overall, MH shows an increase in TPC at lesser time and retains maximum antioxidant activity of both lentil cultivars.

Keywords: TPC- Total phenolic content; TAC- Total antioxidant activity; OR- oven roasting; BW-Boiling; MH- Microwave heating; GLF- Green lentil flours; RLF- Red lentil flours; UPF- Unprocessed flours

3.1. Introduction:

Lentil (*Lens culinaris* Medik.*)* food components include bioactive phenolic compounds. In developed countries, the nutritional value of legumes is gaining considerable interest because of the demand for healthy and nutritional food products. However, certain limitations restrict the use of lentils in the human diet. These are in part related to traditional eating practices, lack of consumer understanding, poor processing techniques and diversified food products. Therefore, to improve lentils' nutritional value and palatability, heat processing is the answer. Well-established methods exist to obtain new, value added legume-based products, potentially helping to increase legume consumption in the West (Aguilera *et al*., 2010). Another, more effective way is to promote the health benefits of the population by encouraging consumption of legumes like lentils.

In addition to conventional methods, a rapid solution or method is necessary to allow the inclusion of legumes in our daily diet. Consequently, the pre-cooking of legume flours by traditional methods such as roasting and boiling, or by novel methods such as microwave heating, was investigated. Microwaves use wavelengths between radio and infrared waves, which penetrate deeply and heat rapidly (Schlegel, 1992; Giese, 1992). Microwave energy required 76% less energy when compared to conventional heating methods, and generates higher quality products in terms of taste, texture and nutritional content (Quenzer and Burns, 1981). Moderate microwave heating conveys no risk of toxicity or adverse effects on diets containing meat and legumes (Alhekail, 2001).

Antioxidants are recognized as reducing oxidative damage. Consuming legumes has several potential health benefits: reduced risk of cardiovascular disease, lower LDL cholesterol and higher HDL cholesterol, and a lower risk of type II diabetes mellitus (Villegas *et al*., 2008). Several researchers have reported that inclusion of antioxidant-rich lentils in the daily diet provides secondary metabolites such as phenolic compounds (Xu and Chang, 2010). Finally, lentil phenolic compounds have been found to exhibit antioxidant properties that protect the human body from several disorders by scavenging free radicals and protecting it from the potential damage from reactive oxygen species

(Madhujith and Shahidi, 2005; Ranilla *et al*., 2009). These compounds reduce these reactive species' activity by complexing pro-oxidant metals and quenching singlet oxygen. Phenolic compounds are abundant in legumes, and may represent significant sources of antioxidant activity in food. Phenolic content ranges from 27-298 mg/100 g dm(dry matter) in apples, 4-490 mg/100g dm in berries, and 6-180 mg/100g dm in some vegetables, while in legumes it can range from 78-1710 mg/100g dm (Bravo, 1998).

Lentils contain low molecular weight secondary plant metabolites such as hydroxybenzoics, hydroxycinnamics, catechins, and procyanidins, flavonols, flavones, dihydroflavonols and flavonones, which are phenolic compounds. Catechin and proanthocyanidin compounds make up the highest percentage (≈ 69%) of total identified phenolics (74.48 μg g^{-1}) in raw lentils, while flavonols and dihydroflavonols make up to about 17%, and hydroxybenzoics accounts for further 5% (Aguilera *et al*., 2010).

While a great deal of information exists regarding lentil polyphenols and their properties, information regarding the effect of various thermal processing methods on phenolic compounds in lentil flours is sparse. Therefore, the present investigation aimed to compare the results of microwave heating to traditional processes such as oven roasting and boiling, as applied to flour made from green and red lentil cultivars, with respect to their effects on the total phenolic content and their antioxidant activity. The effects of these processing methods on flour color and change in mass balance were also investigated.

3.2 Materials and Methods:

3.2.1 General Setup

Two lentil cultivars (*Lens culinaris)* hulled red and unhulled green lentil variety were purchased from a local retail market. Lentils were ground with a cyclone mill to pass through a 0.84 mm screen. Moisture content was determined by drying the ground samples in an oven at 110°C until a constant weight was obtained (about 12 hrs). The moisture of untreated hull-on green lentils flour was 9.37 ± 0.20 g dwb while that made

from hulled red lentils was 8.90 ± 0.00 g dwb. All calculations for quantification of total phenolics and determination of antioxidant activities were done on a dry weight basis (dwb). All reagents and solvents used were of HPLC grade (Fisher Scientific, Ottawa, ON, Canada).

For each processing treatment (oven roasting, microwave roasting, boiling) × processing duration × lentil type combination (completely randomized design), triplicate samples were independently processed. The mass balance of green and red lentil flours was determined before and after processing. Pre- and post-processing lentil flour extracts' moisture content, total phenolic content, free radical scavenging activity, and colour (L*, a*, b*) were determined.

3.2.2 Processing

3.2.2.1 Oven Roasting

For oven heating, lentil flours were evenly spread [depth approximately 1mm× 5mm] on aluminum dishes, and roasted for 1, 3, 5, 10, or 20 min in an oven preheated to 80°C. After cooling to room temperature, the flours were stored in opaque, air-tight plastic containers at 4°C until further analysis.

3.2.2.2 Boiling (Hydrothermal Processing)

For processing by boiling (hydrothermal processing), the lentil flours were dispersed by stirring in distilled water [10:100 (w/v) flour: water] for 1 h at 20°C, followed by heating in a water bath at 90 °C for 15, 20, 25, or 30 min. Triplicate samples were collected at each processing time, stored overnight in a freezer at -40°C, then freeze-dried in a laboratory freeze-dryer (Thermo Savant Modulyod-115, NY, USA). The dried samples were ground in a domestic coffee grinder and then stored at 4°C in opaque air-tight containers until further analyses.

3.2.2.3 Microwave Heating

For microwave heating, lentil flours were exposed to microwaves at a power density of 3 W g^{-1} for 1, 3, 5, 10 and 20 min in a microwave oven. Heated samples were cooled and stored until analysis in the same manner as the oven-heated samples.

3.2.3 Color measurements

Triplicate color measurements were made using a tristimulus colorimeter with D65 (day light condition) as the measurement light source. A white tile was used to calibrate the instrument, where Y = 93.32; x = .3152; y = .3233 values were compared with the standard values provided. The measured values are expressed as L*, a* and b*, where L* represents for lightness to darkness (0 = dark & 100= light), a* indicate redness (+ve) to greenness (-ve), and b* values indicate yellowness (+ve) to blueness (-ve).

3.2.4 Extraction and quantification of total phenolics.

The procedure followed that of Xu and Chang (2007) with minor modifications. Samples (1 g, in triplicate) of raw and processed lentil flours were placed in 20 ml of acetone: water: acetic acid (70: 29.5: 0.5 v/v/v), and vortexed for maximum extraction of phenolics. The flour-extract slurry was centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and used in the measurement of total phenolics and free radical scavenging activity assays.

Total soluble phenolics were determined using Folin-Ciocalteu reagent (Slinked and Singleton, 1977; Jagadeesh *et al*., 2009), including modifications suggested by Singh, (2010). The lentil flour extract supernatant solution (1 mL) was mixed with 7.5 mL of double distilled water and 0.5 mL of Folin-Ciocalteu reagent, followed by 1 mL of 7.5% (w/v) freshly prepared Na_2CO_3 solution. The mixture was incubated at room temperature for 90 min and its absorbance measured at 765 nm using a spectrophotometer (Ultrospec 2100pro, Biochrom Ltd., and Cambridge, England). A gallic acid standard curve was developed and

the total phenolic content was expressed in terms of gallic acid equivalents (GAE) in mg g^{-1} dwb.

3.2.5 Determination of total phenolic scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DDPH) radicals

The free radical scavenging activity of flour extracts was measured by way of the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay (Nair *et al*., 2007), with minor modifications. An aliquot of lentil flour phenolic extract (50 µl) was added to 1.5 ml of DPPH solution (3.94 mg DPPH per 100 ml methanol), and the decolorisation reaction allowed to proceed for 20 min. Absorption was then measured spectrophotometrically at 517 nm (Ultrospec 2100pro, Biochrom Ltd., Cambridge, England). Free radical scavenging activity (FRSA) on DPPH radicals was expressed as percent (%) inhibition:

$$FRSA = 100 \times \left(\frac{Abs_{blank}^{517nm} - Abs_{t=20\min}^{517nm}}{Abs_{blank}^{517nm}} \right) \quad (3.1)$$

where,

Abs_{blank}^{517nm} is the absorbance of a water blank (control) at 517 nm, and

$Abs_{t=20\min}^{517nm}$ is absorbance of the sample at 517 nm after 20 min incubation.

3.2.6 Statistical Analysis

Data underwent analysis of variance (ANOVA) as a completely randomized design (CRD), not as repeated measures in time, because different samples were measured at different times (not the same sample measured repeatedly). Data split up and analysed in 3 ways.

(i) Boiling treatments only, treatment factors lentil type and processing duration (2 × 5)

(ii) Single time, 20 mins, treatment factors lentil type and type of processing (2 × 3)

(iii) No boiling, treatment factors lentil type, type of processing, and processing duration (2 × 2 × 5)

Prior to an initial ANOVA with all factors and interaction (e.g., for (i) L, P, L×P) data were tested for homogeneity of variance, a precondition for ANOVA. Both a strict (Bartlett's test) and less stringent test (D'Agostino-Pearson) were used. In cases where data failed the test for homogeneity of variance ($P \leq 0.05$), TAC and TPC values for boiling process was log transformed prior to analysis (see Appendix I).

The initial ANOVA showed all 2 and 3 factor interactions to be significant in the case of almost all parameters measured. Consequently the ANOVA analysis was repeated with treatment combinations treated as individual treatments (e.g. for (i) the 2 × 5 two factor analysis became a 10 treatment single combined factor analysis) Following ANOVA, means were separated using Tukey's HSD, using CoStat (Version 6.2). A significance threshold of $P \leq 0.05$ was used throughout.

3.3 Results and Discussion

3.3.1 Color measurements, total phenolic content and its antioxidant activity changes after boiling

This portion of the experiment looked only at the effect of lentil type and duration of boiling (and not other processing methods).

Among the raw flours, the hulled red and unhulled green lentil flour showed no significant difference in L* value (Table 3.1). At all processing times, boiled unhulled green lentil flour (GLF) had a lower L* value than the equivalent hulled red lentil flour (RLF). Unhulled GLF L* values declined by 25% after 15 min, remained unchanged at 20 min and 25 min, and declined again (by 15%) at 30 min. Comparatively, hulled RLF showed no decrease in L* value at 15 min, and only 7 and 11% declines after 20 min and 25 min, with no further significant decline at 30 min.

Table 3.1 Effect of boiling on quality parameters of lentil flours boiled for different times.

Quality parameter	Lentil type / Duration of boiling (min)									
	Green, unhulled					Red, hulled				
	0	15	20	25	30	0	15	20	25	30
L*	68.25 a	51.42 d	49.51 d	51.86 d	43.73 e	70.45 a	69.88 a	64.84 b	57.52 c	55.94 c
a*	-1.31 c	0.35 b	0.43 b	0.52 b	0.56 b	3.16 a	-0.76 c	-2.02 d	-2.60 d	-5.00 e
b*	15.43 a	9.85 cde	11.36 bc	12.96 ab	9.07 cde	10.35 bcd	9.08 cde	7.32 e	7.80 de	7.88 de
Anti-ox ($\%\Delta Abs_{517}$)	85.87 a	43.93 b	42.59 b	40.83 b	24.41 c	11.81 d	3.77 e	3.25 e	2.57 e	1.47 e
Tot. Phenol (mg GAE g^{-1} DW)	7.50 a	3.24 b	2.85 c	2.83 c	2.82 c	2.65 c	1.24 d	1.19 de	1.14 ef	1.08 f

NOTE: Tot Phenol- Total phenolic content; Anti-ox- Antioxidant activity

For each quality parameter, common letters row-wise across both lentil types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD. Due to lack of homogeneity of variance (D'Agostino-Pearson test) the quality parameter 'Total phenolics' was log transformed prior to analysis.

A negative a* value was obtained for raw green lentil with hull (-1.31), but a positive a* value (3.16) for raw hulled red lentils indicating their natural green and red hues (Table 3.1). After boiling green lentil a* values were greater than those of red lentils at all processing times. After boiling 15 min, green lentil flour a* values changed significantly and became slightly positive; however, further boiling showed an increasing but non-significant trend. Comparatively, after boiling 15 min red lentil a* values changed significantly and became slightly negative, and showed a decreasing trend thereafter.

The b* values were higher for raw unhulled GLF than raw hulled RLF, showing the yellower hue of the former. For GLF boiled for 5, 20 or 30 min (but not 25 min) had lower b* values than raw flour; while for hulled red lentil boiled flour only had lower b* values after 20 min (but not 15, 25, or 30 min). After 15 min or 30 min boiling both the lentil types had similar b* values, however at 20 min and 25 min the b* values of hulled red lentil were lower than those of unhulled green lentils. Prinyawiwatkul *et al*., (1996)

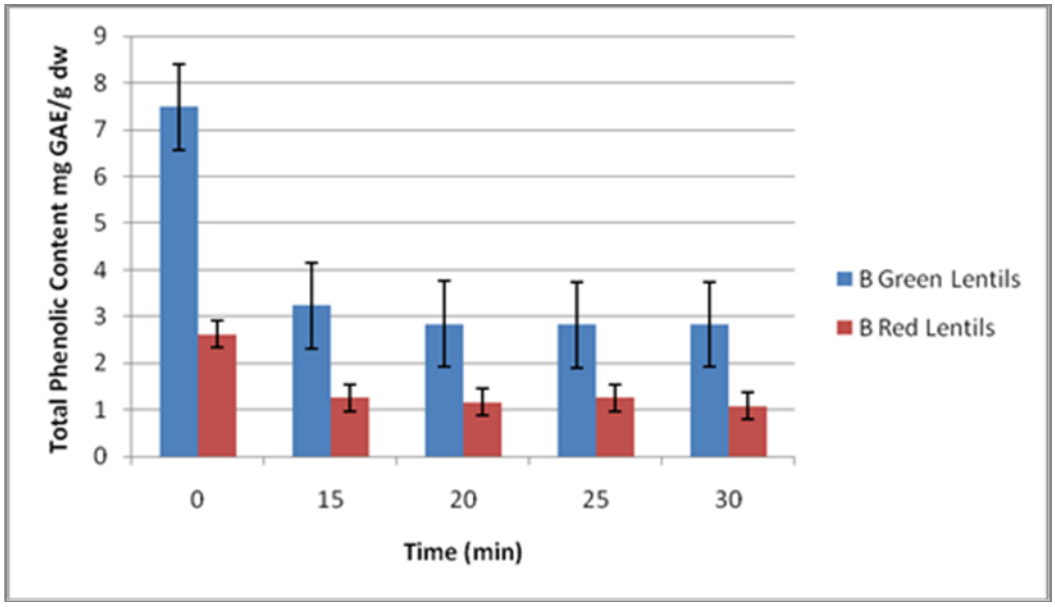
reported that it is advantageous to incorporate thermally-treated pulse flours into certain foods such as extruded snacks, cookies etc., because the alteration in color of pulse flours is desirable. It was also reported that by soaking, boiling and fermentation processes, whole cow pea seeds gave greener and blue hues, with increased lightness (higher L*) and lower a* and b* values.

Total phenolic content (TPC) of lentil was 7.5 mg GAE g^{-1} dwb for raw unhulled GLF, but significantly less (2.65 mg GAE g^{-1} dwb) for raw hulled RLF. This concurs with the findings of Xu and Chang, (2007), who reported that lentils contain 7.53 mg GAE g^{-1} dry matter suing acidified aqueous acetone. It is also in good agreement with Amarowicz and Pegg, (2008) who found approximately the same values. Amarowicz, *et al.,* (1995) reported that acetone/water system extracted maximum TPC from lentils. Boiling for any period reduced the TPC of unhulled green and hulled red lentil flour compared to unprocessed flour. Boiled hulled RLF had less TPC than similarly treated GLF, at all processing times, which is likely a result of the green lentil's greater initial TPC. Decreasing TPC value during processing are the result of phenolic compounds, primarily located in the seed coat and cotyledons being leached out or thermally or oxidatively degraded during soaking and boiling, as reported in peas, chickpeas and lentils (Xu and Chang, 2008). The levels of TPC in boiled lentils are in good agreement with those of Xu and Chang, (2007) and Barroga, *et al.,* (1985).

For quantification of free radical scavenging activity (FRSA), the DPPH method has been widely used worldwide given its simplicity, convenience, and independence from sample polarity (Koleva *et al*., 2002; Marxen *et al*., 2007). The FRSA (Table 3.1) was significantly higher in raw unhulled GLF (86%) than raw hulled RLF (12%), which when hulled has a much lesser antioxidant activity. It was stated that since the flavonoid structure of phenolic compound contributes greatly to their antioxidant activity, and since flavonol and flavone concentration are relatively high in the seed coat, whereas the cotyledon carries less potent antioxidants in the form of non-flavonoid compounds such as hydroxybenzoics and hydroxycinnamics (Fuhrman *et al*., 2002; Jovanovic *et al*., 1998), it is no surprise that the FRSA of the unhulled GLF was greater than that of the hulled

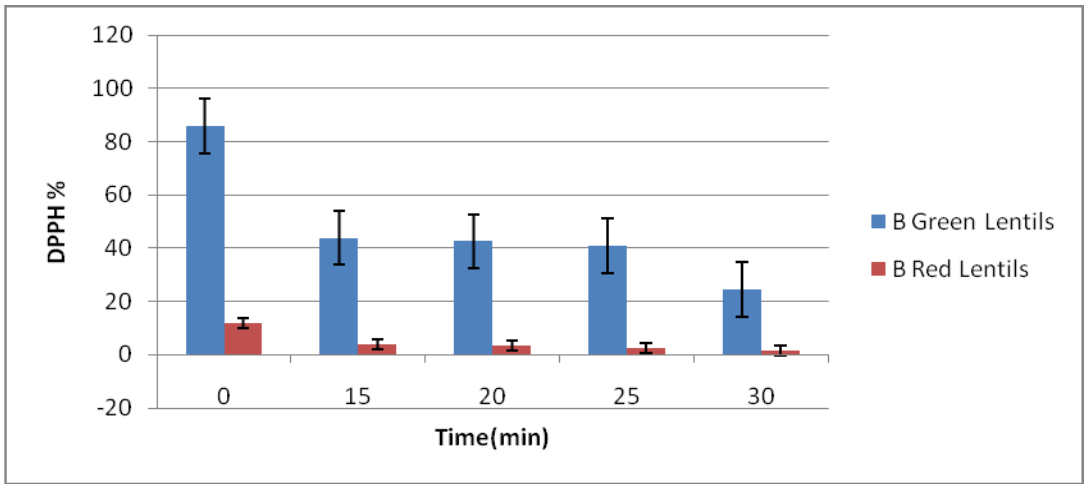
RLF. After 15 min boiling the FRSA of GLF declined by roughly half, while that of RLF decreased by two thirds (Table 3.1), which concurs with the work of Xu and Chang (2008). For the green lentils, from 15 to 25 min the FRSA remained unchanged, but declined by 40% between 25 and 30 min. In red lentils a non-significant declining trend was seen in FRSA as boiling time increased.

Figure 3.1: Comparison of total phenolic content of boiled lentil cultivars



Note: B: Boiled

Figure 3.2: Comparison of free scavenging activity of boiled lentil cultivars



Note: B: Boiled

The trend after thermal processing (such as boiling) and the standard errors in the graph for both the lentil cultivars on total phenolic content and antioxidant activity are illustrated in the Figures 3.1 and 3.2.

3.3.2 Color measurements, total phenolic content and its antioxidant activity changes after thermal processing at 20 min

This experiment compared the effects of the three processing methods (oven roasting, microwave heating and boiling) for the two lentil types, at a single processing time (20 mins) on quality parameters are illustrated in the (Table 3.2).

Keeping in mind the preprocessing values of L*, a*, b* for the green (68.25, -1.31, and 15.43) and red (70.45, 3.16, and 10.35) lentils (Table 3.1), it is clear that, in general, boiling for 20 mins alters these values to a much greater extent than either oven roasting (OR) or microwave treatment for the same period of time, and that the latter two treatments have similar and very minor effects on these parameters. This concurs with the work of Ma, Z., *et al*., (2010) who found an increase in L* values after thermal processing.

Table 3.2 Effect of oven roasting, microwave heating and boiling for 20 minutes on quality parameters of lentil flours boiled.

Quality parameter	Lentil type Processing method					
	Green, unhulled			Red, hulled		
	Oven	MW	Boiling	Oven	MW	Boiling
L*	69.82 bc	68.92 cd	49.51 e	74.41 ab	75.60 a	64.85 d
a*	-1.74 d	-2.08 d	0.43 c	5.35 b	6.48 a	-2.02 d
b*	11.08 b	17.40 a	11.36 b	17.48 a	17.24 a	7.31 c
Anti-ox (%ΔAbs_{517})	79.80 a	75.67 b	42.59 c	9.15 e	18.99 d	3.25 f
Tot. Phenol (mg GAE g^{-1} DW)	8.78 a	7.39 b	2.84 c	1.68 d	1.58 d	1.19 e

NOTE: Tot Phenol- Total phenolic content; Anti-ox- Antioxidant activity; Oven- Oven roasting; MW- microwave heating.

For each quality parameter, common letters row-wise across both lentil types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD.

The TPC after 20 min of thermal processing, was slightly (19%) higher for the OR than the microwave heated (MH) unhulled GLF, and a similar but non-significant trend was observed with the hulled RLF. The TPC levels were similar to those of the equivalent raw flours (Table 3.1). However, in both cases the TPC of the boiled flour was much lower than that of either of the other two processing methods (or of initial preprocessing levels). Thus boiling is a poorer processing method in terms of maintaining phenolic content. This concurs with Dueñas *et al*., (2006) who found that the cotyledon has lower concentrations of phenolics compounds, which consisted mainly of non-flavonoid compounds, such as hydroxybenzoics and hydroxycinnamics. Siddhuraja and Becker, (2007) reported that roasted kidney and pinto beans showed an increase in total phenolics.

The free radical scavenging activity (FRSA) of the OR and MH flours were similar for the unhulled green lentils, and greater for the MH than the OR for the hulled red lentils. However, in both cases, the FRSA of the boiled flour was much lower than either of the other processed flours. These FRSA levels are well in agreement with Dueñas *et al*., (2005). These results show that MH helps to retain maximum antioxidant activity of lentil flours, particularly when compared to boiling.

3.3.3 Oven roasting and microwave heating changes on color measurements, total phenolic content and antioxidant activity of lentil flours

Table 3.3 illustrates the color changes resulting from OR and MH for different period of time. The L* value of hulled RLF increase after 1 min of MH and 3 min of OR, to stay unchanged thereafter. For unhulled GLF only at 1 and 3 min of OR were L* values higher than in the raw flour, while only after 5 min of MH was hulled RLF L* values lower than the raw flour.

Neither OR nor MH had any significant effect on the a* value of unhulled green lentil. In red lentil both OR and MH increase the a* value compared to raw flour, but the value did not change significantly with the duration of processing. This concurs with the

work of Sharma and Gujaral, (2011) who found that barley showed an increase in a* value upon sand roasting and microwave cooking.

The b* value of hulled RLF oven roasted or MH for 1 min was greater than that of similar raw flour, and then remained essentially unchanged. For OR unhulled GLF the b* value increases, but not significantly, compared to the raw flour, after one min and there after decline, dropping significantly below that of raw flour only after 20 min. For unhulled GLF and MH, the b* value increased up to 3 min, compared to the raw flour, declining thereafter, and only returning to initial raw values after 20 min. For the unhulled GLF the MH flour generally showed higher b* values than the OR flour; however, no significant difference was seen with hulled RLF, and if anything the opposite trend was apparent. Ma, Z., *et al*., (2010) also found an increase in b* values in pulse flours after roasting. Kaur and Singh, (2005) studied the values of the flours made from Indian chickpea cultivars such as 'Kabuli' and 'Desi' chickpeas, and found values of 81.64 to 85.41 for L*, ¯0.72 to ¯1.10 for a*, and 14.1 to 20.7 for b*.

The TPC of OR and MH hulled RLF was significantly lower than that of raw hulled RLF, but did not vary significantly with processing time (Table 3.4). However, while the TPC of unhulled GLF OR for 1 min dropped below the level in raw GLF, it increased thereafter, reaching a level above that in the original raw flour after 20 min. For the same flour heated with microwave the TPC initially (1 min) decreased, then varied up and down, returning to similar levels as the raw flour after 20 min. Overall there is a significant increase in total phenolic content resulting from applying heat to legumes bearing a seed coat for a sufficiently long period of time. These results concur with those of Ee *et al*., (2011) who reported that total phenolics in wattle (a species of *Acacia*) seed flour were increased almost 10-fold greater after roasting for 30 min than in raw flour. Randhir *et al*., (2008) reported that a decrease in TPC with heating could be due to heat susceptible free phenolic compounds undergoing thermal degradation. The increase in total phenolics may be due to the partial destruction of the cell structure of the seeds, whereby roasting helps to release the bound phenolics in the cell wall or leads to the formation of heat-induced, extractable phenolics (Manzocco *et al*., 1998). Other factors

which contribute to an increase in total phenolic content may be the formation of other compounds as a result of Maillard reactions induced by roasting (Manzocco *et al*., 2001). Our results concur closely with those of Siddhuraju, (2006), Turkmen et al., (2005), and Boateng *et al*., (2007), who reported that total phenolics of dry heated samples were higher than in raw samples.

Table 3.3 Effect of oven roasting and microwave heating for different times on L*, a*, b* color-space variables of two different lentil flours.

Processing duration (min)	Color parameter/Lentil type/Processing Method											
	L*				a*				b*			
	Green, unhulled		Red, hulled		Green, unhulled		Red, hulled		Green, unhulled		Red, hulled	
	Oven	MW	Oven	MW	Oven	MW	Oven	MW	Oven	MW	Oven	MW
0	68.25de		70.45c		-1.31e		3.16d		15.43de		10.35f	
1	70.31c	69.25cd	73.46c	75.01a	-1.47e	-1.76e	6.79a	5.18bc	18.24bcd	19.43bc	18.39bcd	16.90cde
3	70.12c	66.90e	74.18ab	74.86a	-1.63e	-1.23e	5.97abc	4.89c	17.30cde	23.91a	17.86bcd	16.70cde
5	69.69cd	63.90f	73.64b	74.41ab	-1.67e	-1.34e	6.20abc	5.63abc	15.71de	20.92ab	18.15bcd	16.83cde
10	69.28cd	67.96de	73.92ab	74.40ab	-1.71e	-1.77e	5.94abc	5.72abc	14.46e	19.46bc	18.09bcd	17.12cde
20	69.81cd	68.92cd	74.14ab	75.60a	-1.74e	-2.08e	5.35abc	6.48ab	11.08f	17.40cde	17.48cde	17.24cde

NOTE: Oven: Oven roasting; MW- Microwave heating

For each quality parameter, common letters row- (time) and column-wise (lentil type and processing method) indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD.

Table 3.4 Effect of oven roasting and microwave heating for different times on antioxidant levels and total phenolics of two different lentil flours.

Processing duration (min)	Lentil type Processing Method							
	Anti-ox (%ΔAbs_{517})				Tot. Phenolics (mg GAE g^{-1} DW)			
	Green, unhulled		Red, hulled		Green, unhulled		Red, hulled	
	Oven	MW	Oven	MW	Oven	MW	Oven	MW
0	85.77a		11.70i		7.49b		2.65g	
1	74.69def	73.43ef	9.21i	9.59i	6.51ef	6.66de	1.66hi	1.57hi
3	69.43fg	66.03g	10.7i	9.93i	6.76d	6.44f	1.72hi	1.54hi
5	65.33g	80.23bc	10.4i	11.65i	7.27bc	8.77a	1.76h	1.62hi
10	77.77cde	75.30cde	11.13i	18.29h	7.38bc	7.15c	1.63hi	1.53i
20	78.80cd	75.67cde	9.15i	18.99h	8.78a	7.39b	1.68hi	1.58hi

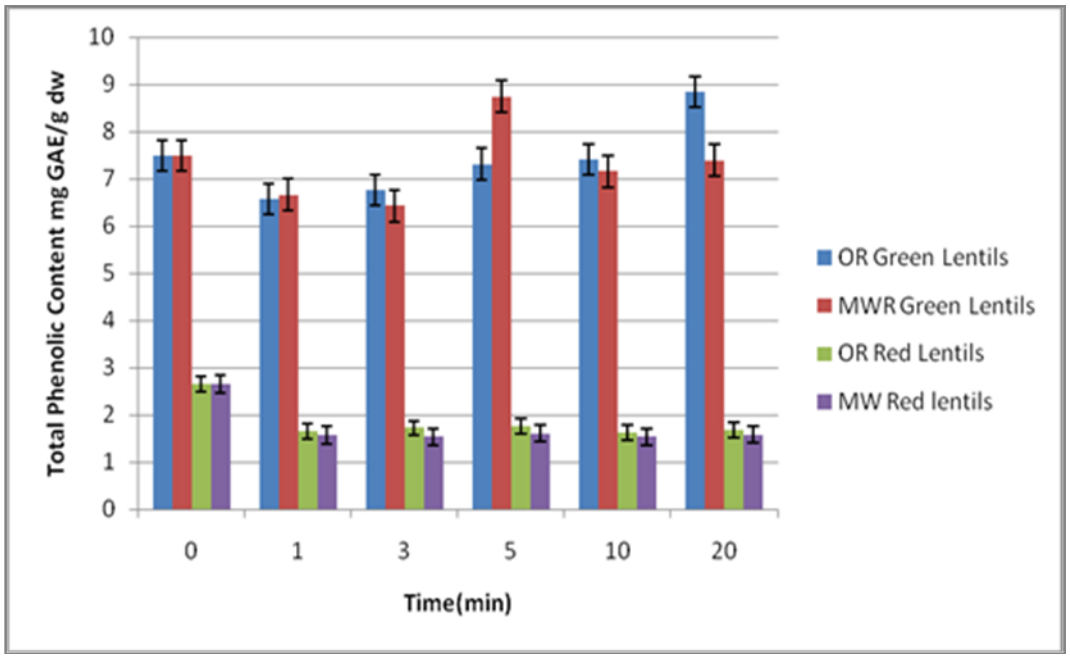
NOTE: Tot Phenol- Total phenolic content; Anti-ox- Antioxidant activity %; Oven- Oven roasting; MW- microwave heating.

For each quality parameter, common letters row- (time) and column-wise (lentil type and processing method) indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD.

The raw unhulled GLF exhibited significantly greater (P<0.05) antioxidant activity than the red hulled variety (Table 3.4). OR and MH for 1 to 20 min, led to a significant decrease in antioxidant activity of unhulled GLF, and except at 5 min, values for the two processing methods were not significantly different. For hulled RLF OR had no effect on antioxidant activity, and only after 10 min of MH did antioxidant activity increase. These results coincide with those of Xu and Chang (2008) for dry heated pulses. Furthermore, roasting is likely to exhibit more antioxidant activity due to the formation of other compounds as a result of Maillard reactions, as reported by Manzocco *et al*., (2001). However, our results show a decrease in antioxidant activity and total phenolic content of processed lentil flours. Sharma and Gujaral, (2011) found that the roasting and microwave cooking of barley, led to a decrease in the TPC inversely related to the total antioxidant capacity (TAC), which was highly dependent on individual cultivars and variety.

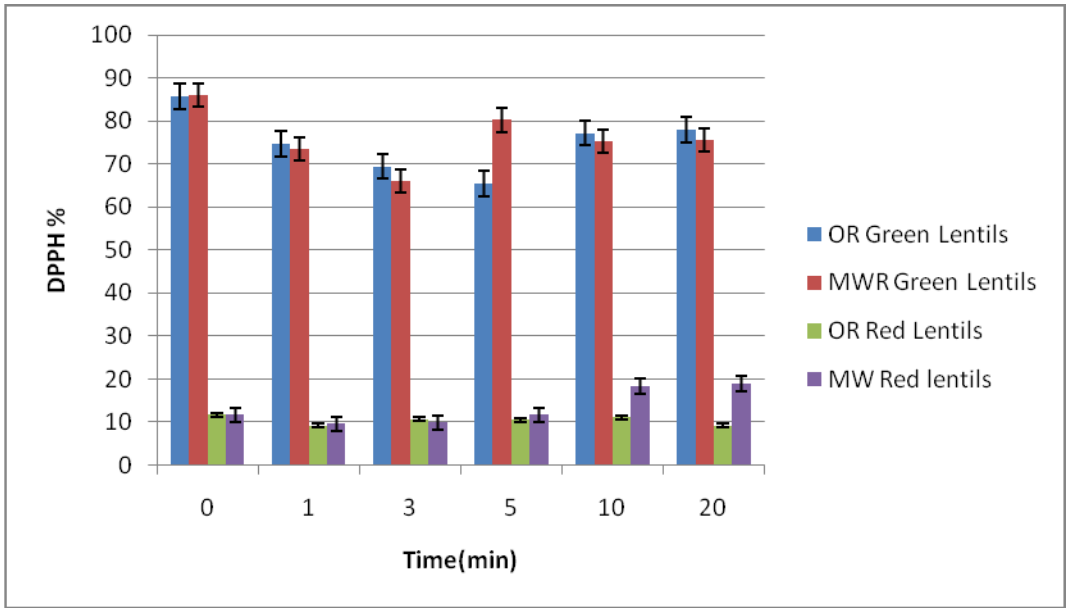
By the end of roasting process, changes induced in the flour are related to loss of water, development of flavor, color and texture. According to Açar and Gökmen, (2009), increasing roasting time tends to increase TAC values, until a maximum reached at the end of 60 min. At this point the flour would have reached steady state moisture content. At higher temperatures, the total antioxidant activity showed an increase due to the formation of non-enzymatic browning products, especially melanoids, as reported by (Woffenden *et al*., 2002).

Figure 3.3: Comparison of total phenolic content (mg GAE/g dw) for oven roasted & microwave heated lentil flour cultivars



Note: OR: Oven roasted; MW: Microwave heated

Figure 3.4 Comparison of free scavenging activity of oven roasted and microwave heated lentil flours.



Note: OR: Oven Roasted ; MW: Microwave heated

The comparative trend of both unprocessed and thermal processed (such as oven roasting and microwave heating) and the standard errors of the graphs for both the lentil cultivars on total phenolic content and antioxidant activity are illustrated in the Figures 3.3 and 3.4.

3.3.4. Comparison of microwave heating with conventional process:

Depending on the duration, MH and OR help to retain a maximum antioxidant activity, but all thermal processing results in a decrease in total antioxidant activity resulting from a decline in their phenolic content when compared to the raw flours. However, MH of unhulled GLF results in a higher percentage of TAC value then raw flour, particularly with increasing processing duration. This increase is evident from in the hulled red lentil variety after 10 min of heating. MH helps in reducing the moisture content of the flours with a shorter heating time when compared to traditional treatments; in addition it helps in increasing the total phenolic content after 5 min of heating.

3.4 Conclusions

According to Açar and Gökmen, (2009), roasting is a process applied to pulses in order to enhance their flavour and crunchy texture and it has both negative and positive impact in terms of TAC. MH leads to an increase in phenolic content after 5 min and helps in retaining maximum total antioxidant activity. Comparatively, oven roasting shows an increase in total phenolics after 20 min. Meanwhile, boiling shows a much greater decrease in TAC in both the lentil varieties with increasing time of heating. Therefore, the consumption of such MH lentil flours would not only reduce the time for preparation, but also provides necessary nutraceuticals to improve human health.

3.5 Acknowledgements

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CONNECTING TEXT

Microwave heating (MH) of antioxidant activity and total phenolic content from both the lentil cultivars maintains maximum at shorter period of heating when compared to conventional methods. Further, the effects of thermal processing are investigated to compare the changes in the additional parameters such as trypsin inhibitor activity and *in-vitro* protein digestibility of lentils.

CHAPTER 4

THERMAL PROCESSING EFFECTS ON ANTINUTRIENTS AND IN-VITRO PROTEIN DIGESTIBILITY OF LENTILS

Abstract:

Lentil-like legumes represent an important part of the human diet in many countries. Many studies report that the presence of heat labile anti-nutrient components like protease inhibitors, tannins, and phytates may interrupt the digestive enzymes which help in digesting the protein in our body. The raw samples of whole unhulled green lentil flour contained 3.18 mg g^{-1} of trypsin inhibitor activity (TIA) and hulled red lentil flour 2.25mg g^{-1}. Boiled unhulled green lentil flours exhibited a 53-82% reduction in TIA compared to unprocessed lentil flour, while hulled red lentil showed a 45.3-70.5% reduction. Microwave heating (MH) led to a significant reduction ($P \leq 0.05$) in TIA level in both cultivars after 20 min of processing, whereas, oven roasting (OR) exhibited 83% and 81% reduction for unhulled green and hulled red lentil flours, respectively. The *in-vitro* protein digestibility (IVPD) of boiled flour showed a significant increase ($P \leq 0.05$) in percentage of digestion, of about 90-95% digestion after 30 min of hydrothermal processing compared to 0.77–0.84% with OR of whole unhulled green lentil flour, but a 4.6-6.6% increase in red hulled lentil flour after 10 and 20 min of roasting. When compared to other traditional processes, MH shows a slight increase in IVPD at a shorter period of heating. This may be due to greater heating durations causing proteins in the microwave samples to undergo desirable changes.

Keywords: TIA: Trypsin inhibitor activity; IVPD: In Vitro Protein Digestibility; unhulled green lentil flours; hulled red lentil flours; MH- Microwave heating; OR- oven roasting

4.1 Introduction

Lentils like most food legumes provide a readily available and important source of dietary proteins for a large portion of the world's population. Current research suggests that consumption of legumes provides energy, dietary fiber, protein, minerals and vitamins required for human health. However, lentils-like legumes lack in amino acids such as methionine and cysteine and their proteins are less digestible than those of milk or eggs (Evans and Bauer, 1978; Sarwar and Peace, 1986). Additionally, several studies have reported that the consumption of legumes like lentils may have inherent health benefits, including reducing the risk of cardiovascular disease, cancer, diabetes, osteoporosis, hypertension, gastrointestinal disorders, adrenal disease, as well as reduction of LDL cholesterol (Hu, 2003; Jacobs & Gallaher, 2004; Pihlanto & Korhonen, 2003; Tharanathan & Mahadevamma, 2003). Such positive correlations have led to a steady rise in the use of pulse ingredients, such as flours, in the development of new food products, especially in developed countries (Ma, Z, *et al*., 2010).

In 1997 the Food and Agriculture Organisation of the United Nations (FAO), reported that world protein requirements continued to be a global issue, and over 800 million people in the developing world suffered with heightened concerns for food security and protein malnutrition (Blandford and Viatte, 1997). About half of the children in central Asia and eastern Africa, suffer with growth retardation due to inadequate supplies of protein and other macro and micro nutrients, which results in serious problem such as protein-energy-malnutrition (PEM).[1] Since most of the undernourished people depend wholly on a mono-carbohydrate diet (eg. rice, maize), they lack the necessary protein, fat, vitamin A, iodine, zinc and iron required for good human health. Therefore, there is tremendous interest in trying to meet some of these people's protein requirements by blending pulse flours such as those of lentils, peas, chickpeas and beans with other locally grown grains (Ma, Z, *et al*., 2011)

[1] http://emedicine.medscape.com/article/1104623-overview

The major proteins found in legumes are globulins and albumins. Globulins are salt solution soluble and albumins are water-soluble and include enzymes such as protease inhibitors, amylase inhibitors and lectins. The major globulins of legumin (11 S) and vicillin (7S) usually include polymorphic subunits encoded by multi-gene families (Schwenke, 2001). Roughly 90% of lentil proteins are located in the cotyledons where they range in concentration of 22 to 34.6% (g hg^{-1}) on a dry weight basis (dwb) (Adsule *et al*., 1989). According to Young and Pellet, (1994), the composition of amino-acids and protein digestibility plays an important role in the nutritional value of legume protein.

In lentils, the presence of protease inhibitors (trypsin and chymotrypsin) can interfere with the digestive utilization of protein by binding to the protease and thereby reducing its activity. This results in an incomplete digestion of dietary protein, and a higher fecal excretion of endogenous nitrogen (Green and Lyman, 1972). Lentils with higher tannin/catechins ratio also interact with proteases and reduce their activity. Furthermore, a higher degree of tannin polymerization results in a lower digestibility of proteins (Yoneda and Nakatsubo, 1998). Therefore, to increase the nutritive value and digestibility of the legume proteins, processing must be carried out to inactivate the anti-nutritional factors.

By applying heat to legume protein, the protein gets denatured; this eventually results in loss of solubility due to aggregation of unfolded molecules. Kinsella *et al.,* (1989) reported that thermal denaturation of lentil protein involves an initial stepwise dissociation of subunits in proteins and subsequent partial re-association of unfolded molecules partially, with the formation of either soluble or insoluble complexes. Monteiro *et al*., (1982) reported that denaturation phenomena are facilitated by the presence of folding of polypeptide chains in the legume's protein and subunits structure. Denatured proteins also results in change in shape and molecular size.

Monsoon and Yusuf (2002) found that dry heating of non-soaked lentil seeds caused a reduction in trypsin inhibitor activity (TIA), tannins and phytic acid and increased the utilization of tannin ratio, thereby not affecting the in-vitro protein

digestibility. The digestibility was not affected due to the benefits of eliminating the heat-labile non-nutritional components such as TIA, and reducing tannins. Dry heating of lentil seeds caused a significant reduction in TIA and increase in *in-vitro* protein digestibility (Savage and Scott, 1989; Zia-ur-Rehman and Shah, 2005). Germination causes a decrease in TIA, tannins and phytic acid and improves the protein digestibility (Vidal-Valverde et al., 1994; Urbano, Porres et al., 1995; El- Adawy et al., 2003). Sheik, (1994) reported that a significant improvement in *in-vitro* protein digestibility was found in fermented legume products.

In this study, the effects of roasting, boiling, and microwave heating are investigated in order to compare the changes in *in-vitro* protein digestibility of lentil flours along with treatment effects on trypsin inhibitor activity. The study also investigates the reduction in trypsin inhibitor activity leading to improve protein digestibility.

4.2 Materials and Methods

Two lentil cultivars (*Lens culinaris* Medik.) hulled red and seed-coat-on green (unhulled), were purchased at a local retail market. Lentils were ground with a cyclone mill to pass a 0.84 mm screen. Moisture content was determined by drying the ground samples in an air oven at 110 °C until a constant weight was obtained. All reagents and solvents used were of HPLC grade (Fisher Scientific, Ottawa, ON, Canada).

4.2.1 Processing Methods

Methods used in processing lentil flours are summarized in Fig. 4.1.

4.2.1.1 Oven Roasting

For oven heating, lentil flours were evenly spread [1mm × 5mm] on aluminum dishes, and roasted for 1, 3, 5, 10, or 20 min in an oven preheated to 80°C. After cooling

to room temperature the flours were stored in opaque, air-tight plastic containers at 4°C until further analysis.

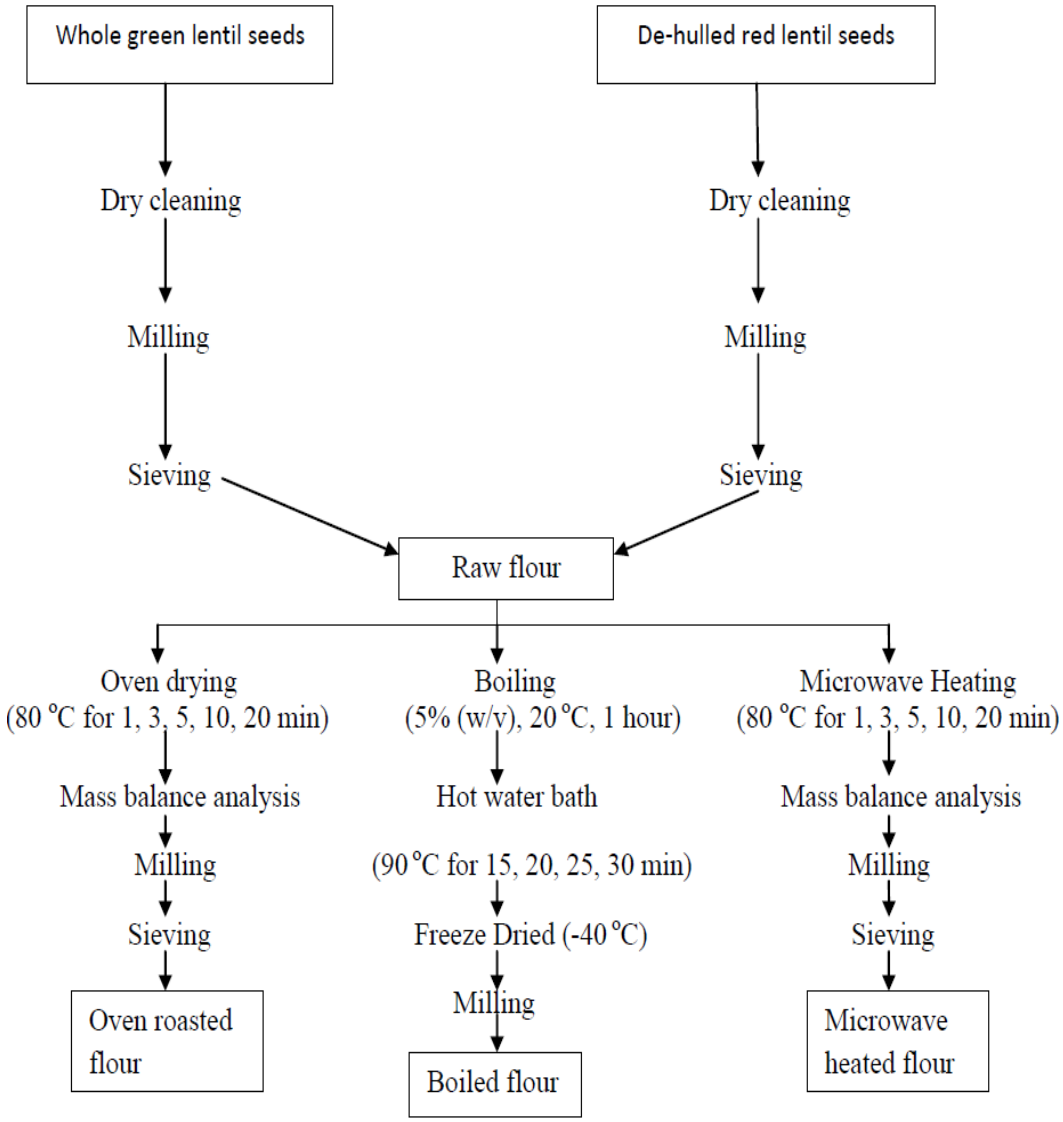
4.2.1.2 Boiling (Hydrothermal Processing)

For processing by boiling (hydrothermal processing), the lentil flours were dispersed by stirring in distilled water 1:10 (w/v) flour : water for 1 h at 20°C, followed by heating in a water bath at 90 °C for 15, 20, 25, or 30 min. Triplicate samples were collected at each processing time, stored overnight in a freezer at ⁻40°C, then freeze dried in a laboratory freeze-dryer (Thermo Savant Modulyod-115, NY, and USA). The dried samples were ground in a domestic coffee grinder and then stored at 4°C in opaque air-tight containers until further analyses.

4.2.1.3. Microwave Heating

For microwave heating, lentil flours were placed in 50 ml tubes and exposed to microwaves at a power density of 3 W g^{-1} for 1, 3, 5, 10 and 20 min in a convectional microwave oven. Heated samples were cooled and stored until analysis in the same manner as the oven-roasted samples.

Fig 4.1 Flow chart of the whole and de-hulled lentil flour samples



4.2.2. Protein Extraction

The alkaline extraction technique used for extraction of proteins from legumes took advantage of proteins' heightened solubility at alkaline pH and low solubility at the isoelectric point, pH (~ 4-5). The method of El-Adawy, (2000) was employed, with a slight modification in the centrifugation time, was used to extract maximum protein from lentil flours. Lentil flours (1:20 w/v) in water were adjusted to pH 9 with 1 N NaOH at room temperature (~30 °C), shaken for one hour, and the resultant slurry then centrifuged for 20 min at 10,000 rpm which helps to remove insoluble materials. After centrifugation,

the supernatant was collected and the extraction procedures were repeated on the residue to obtain a maximum protein yield. The supernatant extracts were combined and acidified with 1N HCl to a pH of 4.5 (isoelectric point), to precipitate the protein. The precipitate was recovered by centrifugation at 10,000 rpm for 20 min. the pellet was washed with double distilled water and re-dispersed in distilled water (El-Adawy, 2000). The protein curd was filtered with filter paper and washed three times using distilled water. The resulting protein was lyophilized over a 48 hrs time period. The weight of the protein in the lyophilized tube before and after was noted, in order to calculate the decrease in the moisture content.

4.2.3. Protein Content

The lentil flours prior to heat treatment were analyzed to determine their protein content using a BCA protein assay kit (Thermo Scientific Pierce). To determine the protein concentration, BSA (Bovine Serum Albumin) was used as a standard. Bovine serum albumin (2 mg mL^{-1}) was diluted to various concentrations (20 – 2000 μg mL^{-1}) in order to create a standard curve. The standard protocol of the BCA protein assay was followed to determine the unknown protein concentration of both red and green lentil flours. Working reagent (2 mL; made up from 50:1, Reagent A: Reagent B) was added to 0.1 ml of either standard protein (BSA) or unknown sample. After incubating at 37°C for 30 minutes, the samples were cooled to room temperature and absorbance at 562 nm was measured within 10 minutes. The resulting protein concentration was expressed in mg mL^{-1}.

4.2.4 Trypsin Inhibitor Activity (TIA)

The trypsin inhibitor activity was determined based on the methods of Kakade *et al.,* (1974) with some modifications suggested by Embaby, (2010), using BAPA (N benzoyl- DL-arginine-P-nitroanilide hydrochloride) and type III trypsin from bovine pancreas. The dilution factors used were based on 1 ml of the sample solution or aliquots producing trypsin inhibition rates between 40% and 60%.

Both the raw and processed lentil flours of both cultivars were evaluated for trypsin inhibitor activity. A sample of raw or processed lentil flour (1 g) was mixed with 50 ml of 0.01N NaOH. The mixture was shaken at ambient temperature for 2 hours. The solution was then centrifuged at 10,000 rpm for 20 min. The resulting clear supernatant was used for trypsin inhibition estimation.

Trypsin (type 1X from bovine pancreas, Sigma Chemical Co) was used as a standard. Diluted lentil flour suspension (2 ml) was pipetted into triplicate sets of test tubes containing 2 mL of trypsin in solution (Ma, Boye, *et al*., 2010). The tubes were placed in the water bath at 37°C and, after 10 min, 5 mL of BAPA solution, pre-warmed to 37°C, were added to each tube. Later after 5 min, the reaction was stopped by adding 1 ml of 30% acetic acid to each test tube. After thorough mixing, absorbance at 410 nm was measured against the reagent blank. The inhibitor activity of trypsin was calculated from the differential absorbance readings and reported as mg of trypsin inhibitor per g of sample by using the equation derived by Hamerstand et al., (1981).

4.2.5. *In-Vitro* Protein Digestibility (Multi Enzyme method)

The *in-vitro* protein digestibility (IVPD) of legume protein was determined using the three enzyme method (Hsu *et al*., 1977, Bodwell *et al*., 1980). Sufficient raw or processed lentil flour of either type (green or red) to yield 312.5 mg of protein was dissolved in 50 ml of distilled water to obtain the working protein suspension. The multi-enzyme mixture, containing 1.6 mg mL^{-1} trypsin, 3.6 mg mL^{-1} chymotrypsin, and 1.3 mg mL^{-1} peptidase was adjusted to pH 8.0 with 0.1 N NaOH and kept in an ice bath and continuously stirred. Enzyme solution (5 mL) was added to 50 mL of protein suspension, and the whole maintained at 37°C in a water bath with constant stirring. After 10 min the pH was measured, and *in vitro* protein digestibility was calculated as (Hsu *et al*., 1977):

$$IVPD = 210.46 - (18.10 \times pH_{t=10\ min}) \quad (4.1)$$

4.2.6. Statistical Analysis

Data underwent analysis of variance (ANOVA) as a completely randomized design (CRD), not as repeated measures in time, because different samples were measured at different times (not the same sample measured repeatedly). Data was split up and analyzed in 3 ways.

(i) Boiling treatments only, treatment factors lentil type and processing duration (2 × 5)

(ii) Single time, 20 mins, treatment factors lentil type and type of processing (2 × 3)

(iii) No boiling, treatment factors lentil type, type of processing, and processing duration (2 × 2 × 5)

Prior to an initial ANOVA with all factors and interaction (e.g., for (i) L, P, L×P) data were tested for homogeneity of variance, a precondition for ANOVA. Both a strict (Bartlett's test) and less stringent test (D'Agostino-Pearson) were used. Due to the lack of homogeneity of variance (D'Agostino-Pearson test), the protein content and in-vitro protein digestibility (IVPD) of lentil flours were log transformed prior to analysis (see Appendix I).

The initial ANOVA showed all 2 and 3 factor interactions to be significant in the case of almost all parameters measured. Consequently the ANOVA analysis was repeated with treatment combinations treated as individual treatments (e.g. for (i) the 2 × 5 two factor analysis became a 10 treatment single combined factor analysis) Following ANOVA, means were separated using Tukey's HSD, using CoStat (Version 6.2). A significance threshold of $P \leq 0.05$ was used throughout.

4.3. Results and Discussion

4.3.1. Effect of boiling on quality parameters of lentil flours

The effect of boiling for different periods of time on the protein quality parameters of lentil flours is summarized in Table 4.1. The protein content of both unprocessed green and red lentil flours were about 22%, similar to some measured values

(Ma, Z., *et al*., 2011), but lower than in some studies (Gueguen, 1983; Zia-ur-Rehman and Salariya, 2005). After 15 min of boiling, the whole unhulled green lentil flours showed a 57% and 66% decreases in protein content. As boiling continued protein content continued to decline in green lentil flour (a further 50% by 30 min), but remained constant in the hulled red lentil flour. This concurs with other studies (Lyimo *et al*., 1992; Clawson & Taylor, 1993) which reported losses in protein content due to the partial removal of certain amino acids and other nitrogenous compounds upon heating.

Table 4.1 Effect of boiling on quality parameter changes of lentil flours boiled for different times

Quality parameter	Lentil type Duration of boiling (min)									
	Green lentil with hull					Red, de-hulled				
	0	15	20	25	30	0	15	20	25	30
Protein content (%)	22.13 a	9.45 b	7.10 bcd	5.82 de	4.70 e	22.30 a	7.48 bcd	8.10 bc	7.82 bc	6.92 cd
Protein digestibility (%)	78.89 f	82.83 e	84.46 de	86.50 d	90.83 c	80.15 f	90.54 c	91.34 bc	94.05 ab	95.03 a
Trypsin inhibitor (mg g^{-1})	3.18 a	1.12 cd	0.92 de	0.80 efg	0.71 fg	2.25 b	1.24 c	0.93 de	0.85 ef	0.67 g

For each quality parameter, common letters row-wise across both lentil types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD. Due to lack of homogeneity of variance (D'Agostino-Pearson test) all quality parameters were log transformed prior to analysis.

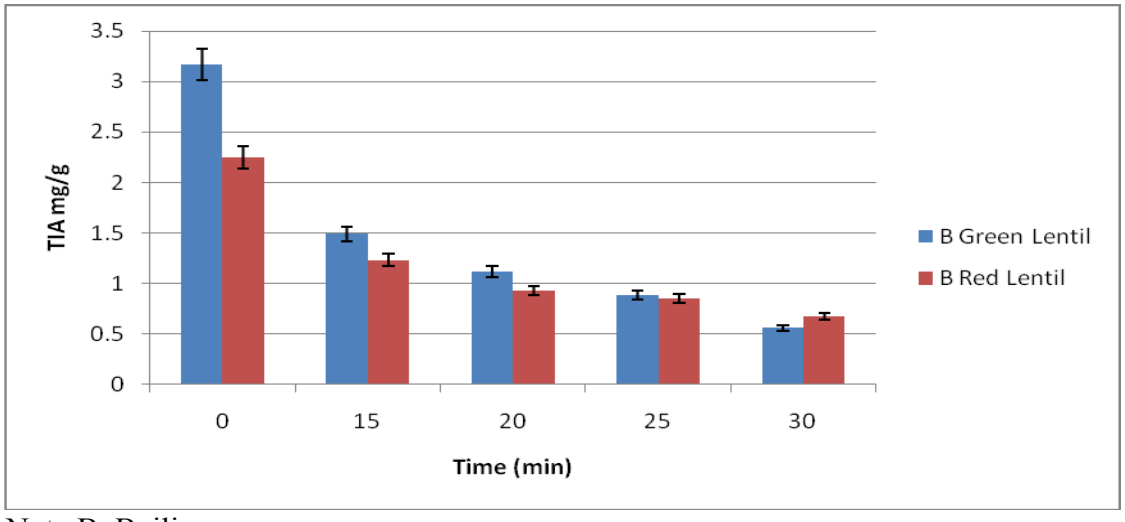
The protein digestibilities of raw whole unhulled green and red de-hulled lentil flours showed no significant difference (Table 4.1). Unhulled green and hulled red lentils flour boiled for 15 min showed significant 5% and 13% (relative) increases in protein digestibility, respectively, compared to the raw flour, and these increases continued through to 30 min (15% and 19% increases, respectively, over raw flour). The protein digestibility of hulled red lentil flour remained higher than that of unhulled green lentil flour at any given boiling duration.

These results concur with those of Melito and Tovar (1995) who measured 77-89% digestibility in boiled lentil flours, which was higher than in cooked or sonicated

flours. The absence of cell wall structures which acts as a chemical barrier may reduce the IVPD of legume flours.

Lentils are rich in proteins, but their consumption is limited due to heat-labile anti-nutritional factors such as protease inhibitors which affect trypsin and chymotrypsin. The raw unhulled green lentil flour contained a higher level of trypsin inhibition (3.18 mg g^{-1} compared to hulled red lentil flour (2.25 mg g^{-1}) of the sample (Table 4.1). These results concur with those of Ma, Z., *et al*., (2011), who found that trypsin inhibitor activity (TIA) of lentil flours was significantly higher for unhulled green lentil than hulled red lentils, but did not differ irrespective of whether the lentils were hulled or not. According to Khattab *et al*., (2009), the seeds of individual lentil cultivars differ significantly in trypsin inhibitor activity. In the present study boiled unhulled green lentil flours show a decrease of 53-82.3% in TIA according to the duration of boiling; this decrease ranged from 45.3-70.5% in hulled red lentil flour. These results concur with those of Udensi *et al*., (2007), who reported that boiling cowpea seeds in water for 15 to 45 min reduced TIA by 32.4 to 51.9%. Similarly, Wang *et al*., (2009) reported that by boiling and soaking resulted in reductions in TIA values ranging between 61.2 % and 82.6% in flours made from different lentil varieties. Wang et al. (2003) also showed that yellow field peas showed an average reduction in TIA of 84.3% after cooking.

Fig 4.2 Effects of hydro-thermal processing on trypsin inhibitor activity of lentils



Note:B: Boiling

The trend after thermal processing (such as boiling) and the standard errors of the graphs for both the lentil cultivars on trypsin inhibitor activity are illustrated in the Fig 4.2.

4.3.2 Effect of oven roasting, microwave heating and boiling for 20 minutes on quality parameters of lentil flours

The effects of 20 min of any of three thermal treatments on protein content, protein digestibility and trypsin inhibitor activity are shown in Table 4.2.

Table 4.2 Effect of oven roasting, microwave heating and boiling for 20 minutes on quality parameters of lentils.

Quality parameter	Lentil type Processing method					
	Green, unhulled			Red, de-hulled		
	Oven	MW	Boiling	Oven	MW	Boiling
Protein content (%)	26.27 a	21.38 b	7.10 d	21.85 b	20.07 c	8.10 d
Protein digestibility (%)	79.55 c	78.92 c	84.46 b	85.41 b	79.81 c	91.34 a
Trypsin inhibitor (mg g^{-1})	0.56 b	0.15 c	0.92 a	0.44 b	0.04 c	0.93 a

For each quality parameter, common letters row-wise across both lentil types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD.

After 20 min oven roasting (OR) unhulled green lentil flour showed greater protein content (26.27%) than the flour of the red hulled lentils (21.85%), and a similar pattern, if at lower protein contents (21.38 *vs.* 20.07, green *vs.* red) occurred with microwave heating (MH). In contrast, protein contents of unhulled green and hulled red lentil flours after 20 min boiling were not significantly different from each other and almost 3-fold lower than either OR or MH flours. Aguilera *et al*., (2010) reported that protein content in edible legumes may vary markedly according to cultivation conditions, maturity of the grain, and cultivar.

For both lentil types individually, protein digestibility of lentil flours was greater after boiling than either OR, MH (Table 4.2) or raw flour (Table 4.1). While the protein digestibility is greater with boiling, the protein content is, however, much reduced. Booth

et al., (2001), showed that hulling significantly increased the protein digestibility of field peas, faba beans and vetch (7.0%, 6.0% and 17% in absolute units, respectively). Nergiz and Gokgoz, (2007) found that the variation in protein digestibility between unhulled and hulled lentil flours varied slightly due to variety.

For both lentil flour types, thermal treatments led to substantial and similar magnitudes of decrease in flour TIA, compared to raw flours (Tables 4.1 and 4.2). These reductions in TIA, were of 80-82%, 95-98% and 59-71% for OR, MH and boiled lentil flours, respectively. Rajko and Szabo, (1997) discussed that MH helped to decrease the TIA in soybean by its quick and even heating, and only required simple equipment. Kadlec *et al*., (2001) concluded that microwave treatment using hot air (≈80°C) can be recommended as a method of decreasing the high anti-nutritional factor content of peas and thus improving their nutritional quality. Khattab *et al*., (2009) concluded that there can be a complete removal of trypsin inhibitor activity for pea seeds through roasting, boiling or MH, but it depends on individual cultivars.

4.3.3 Effect of oven roasting and microwave heating for different times on on quality parameters of lentil flours

Table 4.3 illustrates the changes in protein content, *in-vitro* protein digestibility and trypsin inhibitor activity of both processed (OR and MH) and raw lentil flours. The protein content of both unprocessed unhulled green and hulled red lentil flours was about 22%. With the exception of OR of green lentil flour for 20 min which increased protein content by 19% compared to raw flour, no other processing method × time combination had any significant effect on lentil flour protein content. With the exception noted above, the effect of OR and MH on protein content was identical. Aguilera *et al*., (2010) remarked that processed flours provide high energy levels due to high levels of protein and carbohydrates.

The protein digestibility of raw, unprocessed green and red lentil flours showed no significant difference. For unhulled green lentil flour neither OR nor MH (other than for 5 min) had a significant effect on protein digestibility, when compared to raw flour.

For unhulled green lentil flour, protein levels after any given processing time were the same for both processing methods. For hulled red lentils protein digestibility was generally higher for MH (*vs.* OR) lentil flour at 1 and 3 min of processing, the converse at 10 and 20 min, and the same at 5 min. For OR, digestibility went up after more than 5 min processing time, whereas for MH, digestibility went up immediately (1 and 3 min), but dropped below that of raw flour levels at 10 and 20 min processing time.

Zia-ur-Rehman and Salariya, (2005) and Van der Poel, (1990) observed that applying heat to the legumes resulted in an increase in protein digestibility that might be attributable to an increase in accessibility of proteins to enzymatic attack and by inactivation of proteinanceous anti-nutritional factors. They further noted that there was a lack in improvement in IVPD with increasing processing time: Digestive utilization of protein and the percentage of digestible protein remained little affected by duration of processing. Similar results were cited by Carbonaro *et al*., (1997), where they attributed the lack of improvement in IVPD of lentils and faba bean to denatured protein aggregation. Our results concurred with those of Khatoon and Prakash, (2006) who found the protein digestibility of raw samples to be significantly lower than that of processed legumes. They also reported that legumes processed by microwave cooking had lower in-vitro protein digestibility than those treated by pressure cooking. Preet and Purina, (2000) reported protein digestibility of cowpea varieties to range between 75.5-78.3%. Embaby, (2010) reported that microwave cooking of bitter and sweet lupin seeds improved protein digestibility by 2.53% and 1.52 %, respectively, which is in agreement with our study.

Monsoor and Yusuf, (2002) reported that dry heating of non-soaked lentil seeds led to a reduction in trypsin inhibitor activity (TIA), tannins and phytic acid, however, without significantly affecting the digestive utilization of protein. As a result, MH leads to an increase IVPD after a shorter period of heating, but the anti-nutritional compounds needed greater heating time to remove them completely. Even though there is a maximum reduction in the heat labile anti-nutrients' level when compared to the raw flours, MH causes an initial increase with decreasing tendency of IVPD. This may be due

to the prolonged heating period causing changes in protein quality and lowering the protein digestibility (Swaisgood & Catignani, 1991).

The TIA of unprocessed green lentil flour with hull (3.18 mg g^{-1}) was greater than that of unprocessed hulled red lentil flour (2.25 mg g^{-1} for RDLF); however, there was generally no difference between lentil types at a given processing time for similarly processed flours (Table 4.3). After 1 min of processing TIA of unhulled green lentil flour dropped 60% for OR and 64.7% for MH, while for hulled red lentils these reductions were 47% and 55%, respectively. When comparing the OR unhulled green lentil flours and hulled red lentil flours, after 3-20 min of processing, there is notable reduction in TIA which ranges to about 65-83% and 55%-80%. Under both processing methods the TIA continued to decrease with time, though this tended to occur significantly earlier and faster with the microwave treatment .

Udensi *et al*., (2007), found that roasting cow pea seeds from 30 to 120 min resulted in a 22.28-72.62% reduction in trypsin inhibition, and concluded that increasing the time of treatment generally increased the percentage reduction in TIA. The decrease in TIA with boiling, autoclaving or roasting is due to the heat liable nature of the trypsin inhibitor (Khokar and Chauchan, 1986). Khattab *et al*., (2009) found that a complete removal of TIA from pea seeds could be achieved by roasting, boiling or MH, but that required duration of processing varied according to the cultivar. Embaby (2010) also reported partial reductions in trypsin inhibitor level (15-62%). Clemente *et al*., (2000) concluded that increasing the heating time by 30 minutes and temperature to about 100ºC inactivated TIA by more than 50% compared to the original activity, but this was not necessarily the case for all pulse grains. Since, microwaves penetrate deep inside the product they more rapidly reduces the moisture content of the legumes. Therefore, microwave treated green and red lentil flours show a greater reduction in moisture when compared to other processing treatments. Embaby, (2010) found that MH of lupine seeds led to a greater reduction in TIA than traditional processing. Dev *et al*., (2010) concluded that microwave-boiled and autoclaved red lentils showed a range of TIA values that was too low to detect. These effects may be due to the denaturation of enzyme inhibitors

under extreme processing conditions. From the results, it is inferred that microwaves will be a promising treatment to reduce the trypsin inhibitor level over a shorter time when compared to other treatments.

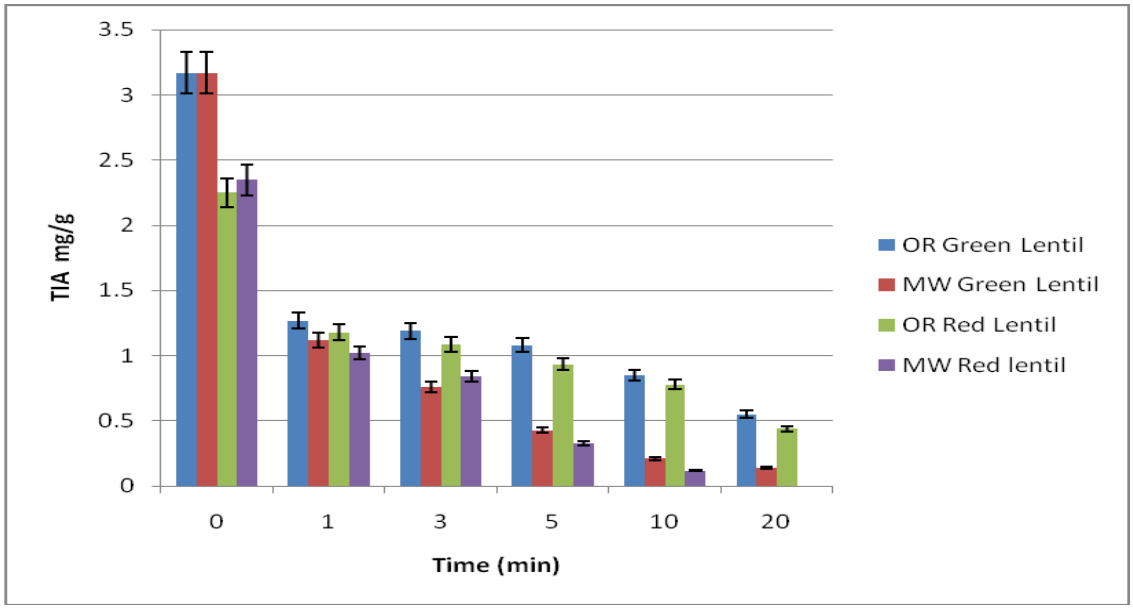
The trend after thermal processing (such as OR and MH) and the standard errors of the graphs for both the lentil cultivars on trypsin inhibitor activity are illustrated in the Figure 4.3.

Table 4.3 Effect of oven roasting and microwave heating for different times on protein content, in-vitro protein digestibility and trypsin inhibitor activity of two different lentil flours.

Processing duration (min)	Protein characteristics/Lentil type/Processing Method											
	Protein content (%)				Protein digestibility (%)				Trypsin inhibition (mg g^{-1})			
	Green, hull-on		Red, hulled		Green, hull-on		Red, hulled		Green, hull-on		Red, hulled	
	Oven	MW	Oven	MW	Oven	MW	Oven	MW	Oven	MW	Oven	MW
0	22.13 bcdefg		22.30 bcdefg		78.89 fgh		80.15 def		3.18 a		2.25 b	
1	23.37 abcdef	20.95 defg	18.72 g	21.28 defg	75.30 gh	79.81 def	78.78 fgh	88.80 a	1.27 c	1.13 cde	1.18 cd	1.02 cdefg
3	22.70 bcdef	21.08 defg	24.23 abcd	23.62 abcde	78.45 fgh	80.41 cdef	78.70 fgh	88.92 a	1.20 cd	0.77 g	1.09 cdef	0.85 fg
5	23.75 abcde	20.58 efg	25.4 ab	23.15 abcdef	78.57 fgh	74.82 h	83.87 bcde	84.17 abcd	1.09 cdef	0.43 hi	0.94 defg	0.34 i
10	23.95 abcde	20.13 fg	25.23 abc	21.88 cdefg	79.49 efg	78.38 fgh	84.79 abc	79.87 def	0.85 efg	0.21 j	0.78 g	0.12 k
20	26.27 a	21.38 defg	21.85 cdefg	20.07 fg	79.55 efg	78.92 fgh	85.41 ab	79.81 def	0.56 h	0.15 k	0.44 hi	0.04 k

For each quality parameter, common letters row- (time) and column-wise (lentil type and processing method) indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD.

Fig 4.3. Effects of thermal processing(OR and MH) on trypsin inhibitor activity of lentils



Note: OR: Oven Roasted: Boiling; MW: Microwave heated

4.4 Conclusions:

Protein malnutrition continues to be the major issue in pockets of the developed countries such as India, Africa. As shown in the study, by inactivating the anti-nutritional compounds, the protein content and digestibility has been improved. It should be noted that factors such as processing treatments, time that best suits for individual legume cultivars have to be studied in detail in order to develop an alternative protein products from pulses which food manufacturers as well as customers are looking for. Further, we can improve their nutritive value and identify novel food uses from whole pulses to develop protein rich ingredients and products that can be used in complementary with other cereals and grains which can act as a best alternative to meet the food crisis and malnutrition demands for the growing population. MH processes act as a best for TIA reduction when compared with other conventional processing methods. Within, shorter period of heating, MH improves IVPD, maintains maximum nutritive value of protein and also reduces maximum TIA. All the thermal treatment used in this study affect the composition of the protein, anti-nutritional factors such as TIA to the maximum, and improved the IVPD of both the lentil cultivars. Comparatively, boiling caused a reduction

in protein content but shows higher IVPD than other thermal treatments. It is quite clear that microwave heating shows maximum reduction in TIA and it not only saves time but slightly maintains the nutritive value.

4.5. Acknowledgements:

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CHAPTER 5

SUMMARY AND CONCLUSIONS

For most of the world's growing population, dietary protein from plants provides a major energy and nutritional source. However, protein malnutrition continues to be a global issue, particularly in developing countries in south central Asia and eastern Africa. Due to the interest of consumers and food manufacturers in achieving healthy plant-based alternative diets, the popularity of using legumes has come to light. Their consumption contributes various positive nutritional impacts, which include antioxidants, along with proteins which act as a perfect cheap protein alternative for the growing population. There has been a steady rise in development of various ready-to-use pulse ingredients and novel food products derived from pulse based flours/seeds. In order to utilize a maximum amount of protein from legumes, a number of anti-nutritional factors need to be inactivated. Natural phenolic antioxidants are abundant in the seed coats of legumes. Therefore, conventional techniques and many novel techniques have been investigated to maximally inactivate anti-nutritional compounds, improve protein digestibility and alter the content of phenolics and their antioxidant activity. In our study, unhulled lentil seeds (green) and hulled lentil (red) cultivars were used. Lentils can be consumed as seeds or flours, and are one of the most widely used legumes in various cuisines.

The first objective of the research was to study the changes in total phenolic content and its antioxidant activity in both lentil cultivars. Parameters included solvent extraction method [e.g., 70:29.5:0.5 Acetone: Water: Acetic acid], as well as different processing methods (oven roasting (OR), boiling, microwave heating (MH)) of lentil flours at a fixed temperature but varying duration for each process. After 20 min of oven roasting a maximum value of total phenolics of 8.9 mg GAE g^{-1} dwb was achieved, while same level of total phenolics was reached after only 5 min of microwave heating. MH and OR maintains maximum antioxidant activity by using 100% methanol concentration but lower when compared to the unprocessed flour inhibition. Due to lack of homogeneity, the parameters are log transformed prior to analysis for boiling processing which results lower phenolic content and antioxidant values. Both the processing except boiling well suited for maximum extraction of phenolic content and to maintain antioxidant activity for better h

The second objective of the research was to study the reduction in trypsin inhibitor activity (TIA) and any improvement in *in-vitro* protein digestibility (IVPD) of lentil flours by varying processing duration (1, 3, 5, 10, or 20 min) at a fixed temperature (80 °C) for oven roasting and microwave heating, as well as boiling for 15, 20, 25, or 30 min at a temperature of 90°C. Microwave heating led to a significant reduction (~95- 100%) in TIA levels in both the cultivars after 20 min of processing, followed by oven roasting and boiling processes. However, the protein digestibility of lentil flours showed an interesting trend, with higher digestibility for the boiling process. Microwave heating show an improvement in IVPD of about 1.18% and 10.92% for unhulled green and hulled red lentil flours, respectively, after the shorter heating times. This was followed by oven roasting which gave only a slight improvement (<1%) for both cultivars after 20 min of roasting. These results show that each processing method requires a particular heating temperature and time period for further improvement in IVPD in lentil flours. This growing trend of developing new alternative food products from pulse ingredients through easier processing techniques can serve to use pulse proteins for maximum effect in human health. The interactions among the factors (lentil type, processing treatments, and time) are well significant for IVPD and TIA of lentil flours (see Appendix III). Though there is a significant reduction in TIA, IVPD fails to improve higher in oven roasting and microwave heating process when compared to boiling.

Recommendations for further research

By using simple processing methods, and various time combinations, we studied the maximum achievement in the reduction of anti-nutritional compounds and greater percentage in protein digestion, phenolic content and antioxidant activity in lentils.

Further study can be investigated by varying heating time beyond, various solvent concentrations and different cultivar combinations could be further explored to know the maximum increase in antioxidant activity of any processed legumes. Additionally, more work is required on lentil protein and an interaction of higher content of phenolics with protein digestibility is required to explain the trends obtained during the experiments after processing.

6.1 APPENDIX I

Preliminary ANOVA and test for homogeneity of variance on the effects of boiling on all quality parameters of lentils

Experiment 1 – BOILING	Factors: lentil type (L), time (T)							
Statistic	**L***	**a***	**b***	**DPPH**	**Tot Phenolic mg GAE/g**	**Protein content %**	**IVPD %**	**Trypsin mg/g**
Test of homogeneity of variance								
Bartlett	0.4889	0.0448	0.0775	<0.0001	0.0002	0.0098	0.7297	0.7294
D'Agostino-Pearson	0.3118	0.5541	0.271	0.0534	0.0001	0.0112	0.2859	0.0009
D'Agostino-Pearson log transform				0.2258	0.4096	0.0727	0.2719	0.0909
Preliminary ANOVA (no transform)								
L	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0294	<0.0001	<0.0001
T	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
L*T	<0.0001	<0.0001	0.0006	<0.0001	<0.0001	0.0013	<0.0001	<0.0001
Preliminary ANOVA (log transform)								
L				<0.0001	<0.0001	0.0024	<0.0001	
T				<0.0001	<0.0001	<0.0001	<0.0001	
L*T				<0.0001	0.0004	0.0002	<0.0001	

6.2 APPENDIX II

Preliminary ANOVA and test for homogeneity of variance on the effects of all processes for the fixed time of 20 min on quality parameters of lentils

Experiment 2 - Fixed Time = 20 mins, all processes	Factors: lentil type (L), time (T)							
Statistic	**L***	**a***	**b***	**DPPH**	**Tot Phenolic mg GAE/g**	**Protein content %**	**IVPD %**	**Trypsin mg/g**
Test of homogeneity of variance								
Bartlett	0.5417	0.0403	0.2363	0.0434	0.1095	0.5622	0.3336	0.123
D'Agostino-Pearson	0.0644	0.1733	0.2927	0.2548	0.1484	0.1224	0.363	0.362
Preliminary ANOVA (no transform)								
L	<0.0001	<0.0001	0.1562	<0.0001	<0.0001	0.4017	<0.0001	0.0215
T	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
L*T	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0078	0.1454

6.3 APPENDIX III

Preliminary ANOVA and test for homogeneity of variance on the effects of boiling, oven roasting and microwave heating on all quality parameters of lentils

Experiment 3 - BOILING EXCLUDED	Factors: lentil type (L), time (T); Processing type (P)							
Statistic	**L***	**a***	**b***	**DPPH**	**Tot Phenolic mg GAE/g**	**Protein content %**	**IVPD %**	**Trypsin mg/g**
Test of homogeneity of variance								
Bartlett	0.3176	<0.0001	0.0308	<0.0001	<0.0001	0.0001	0.3187	0.062
D'Agostino-Pearson	0.3027	0.0567	0.1132	0.0536	0.0615	0.339	0.143	0.0001
D'Agostino-Pearson log transform							0.039	0.1383
Preliminary ANOVA (no transform)								
L	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	0.8286	<0.0001	<0.0001
P	0.0409	0.0994	<0.0001	<0.0001	0.0001	<0.0001	0.0115	<0.0001
T	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0304	<0.0001
L *P	<0.0001	0.0872	<0.0001	0.0065	0.1034	0.0015	0.0266	0.2447
L*T	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001
P*T	0.0172	0.0425	0.0011	<0.0001	<0.0001	0.0001	<0.0001	<0.0001
L*P*T	0.0242	0.0055	0.0003	<0.0001	<0.0001	0.0623	<0.0001	0.3984