Optimization of Extraction of Protein from Alfalfa Leaf (*Medicago sativa*) for Human Consumption

Milad Hadidi

Universitat de Lleida

Department of Food Technology

DOCTORAL THESIS

Optimization of Extraction of Protein from Alfalfa Leaf (*Medicago sativa*) for Human Consumption

Milad Hadidi

Dissertation presented by Milad Hadidi to fulfill the requirements of the degree of Doctor by the University of Lleida, Spain.

Doctoral program in Agricultural and Food Science and Technology

Director

Dr. Albert Ibarz Ribas

Dr. Jordi Pagan Gilabert

December 2019

Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària

University of Lleida, Spain
Department of Food Technology
Doctoral program in Agricultural and Food Science and Technology

DOCTORAL THESIS

Optimization of Extraction of Protein from Alfalfa Leaf (Medicago sativa) for Human Consumption

Dissertation presented by **Milad Hadidi** to fulfill the requirements of the degree of Doctor by the University of Lleida, Spain, under the direction of Prof. Dr. Albert Ibarz Ribas and Prof. Dr. Jordi Pagan Gilabert in the research line of Food Chemistry and Processing.

Milad Hadidi

Prof. Dr. Albert Ibarz Ribas

Prof. Dr. Jordi Pagan Gilabert

Lleida, Spain, 19 December, 2019

Abstract

The world's growing population is raising the demand for sources of cheap and more sustainable dietary plant proteins to supplement or even replace expensive and limited sources of animal protein. Alfalfa (*Medicago sativa*) is an important productive forage legume, widely cultivated around the world. Owing to its high nutritional quality and adaptability, the alfalfa leaf protein is mainly used as a feed supplement, while it only plays a minor role in the human food sector. The use of plant protein concentrates derived from alfalfa in human food is limited by their negative quality such as brown color, bitter taste, and anti-nutritional compounds.

After harvesting of the alfalfa leaves, the endogenous proteases of the leaf are degrading the protein while the polyphenol oxidase (PPO) and peroxidase (POD) enzymes with high initial activity are simultaneously causing browning in the tissue. The result of these actions is a dark and a partially degraded protein. To avoid these negative effects in the extracted protein, the authors carried out a first study on the effects of blanching by steaming the leaves of alfalfa from the beginning of the harvest. The first aim of the thesis was to optimize the inactivation of PPO, POD and plant proteases in alfalfa leaves through the steam blanching process. The effect of this process on the browning index, color, non-protein nitrogen and molecular weight was also investigated.

Some of undesirable properties of alfalfa may be due to the higher levels of saponin as a main anti-nutritional factor found in alfalfa. The second part of thesis aimed to find out the optimal conditions of ultrasound-assisted extraction for obtaining the highest yield of total saponins and their bioaccessibility for further application in the food using the RSM. Beside, UV irradiation has been employed for degradation of alfalfa saponins in different temperature and pH.

Accordingly, the extraction process of alfalfa protein as a food is extremely important for achieving a high quality protein without any safety concerns. So the ultrasonic-ultrafiltration-assisted Alkaline Isoelectric precipitation (UUAAIP) technique as a new process was developed

and employed in the alfalfa leaves' protein extraction. Finally, the composition, physiochemical and functional properties of the extracted protein were investigated and compared with the results of other extraction common methods.

By covering all the mentioned issues, this thesis tries to provide alfalfa protein isolate with high quality without any safety concerns. This research suggests that steam blanching of fresh whole alfalfa leaves under the optimum conditions was helpful for avoiding the appearance of the dark color appears and the degradation of the extracted protein. Thus, the combination of methods of extraction and purification created a new and efficient method, which is able to improve the purity, safety, quality and functional properties of alfalfa protein. UUAAIP is an appropriate technique for manufacturing alfalfa protein isolates and could resolve restrictions of human consumption of alfalfa protein.

Resum

La creixent població mundial està augmentant la demanda de fonts de proteïnes vegetals dietètiques barates i més sostenibles per a complementar o fins i tot reemplaçar fonts cares i limitades de proteïna animal. L'alfals (*Medicago sativa*) és una important lleguminosa productiva farratgera, àmpliament conreada a tot el món. A causa de la seva alta qualitat nutricional i adaptabilitat, la proteïna de la fulla d'alfals s'usa principalment com un suplement alimentós, mentre que només juga un paper menor en el sector de l'alimentació humana. L'ús de concentrats de proteïnes vegetals derivats de l'alfals en l'alimentació humana està limitat per la seva qualitat negativa, com el color marró, el sabor amarg i els compostos antinutricionals.

Després de la collita de les fulles d'alfals, les proteases endògenes de la fulla estan degradant la proteïna, mentre que els enzims polifenoloxidasa (PPO) i peroxidasa (POD) amb alta activitat inicial estan causant simultàniament l'ennegriment del teixit. El resultat d'aquestes accions és una proteïna fosca i parcialment degradada. Per a evitar aquests efectes negatius en la proteïna extreta, es va dur a terme un primer estudi sobre els efectes del blanqueig en vaporitzar les fulles d'alfals des del començament de la collita. El primer objectiu de la tesi va ser optimitzar la inactivació de PPO, POD i proteases de plantes en fulles d'alfals a través del procés de blanqueig de vapor. També es va investigar l'efecte d'aquest procés en l'índex d'enfosquiment, el color, el nitrogen no proteic i el pes molecular.

Algunes de les propietats indesitjables de l'alfals poden deure's als nivells més alts de saponina com un factor antinutricional principal que es troba en l'alfals. La segona part de la tesi va tenir com a objectiu descobrir les condicions òptimes d'extracció assistida per ultrasò per a obtenir el major rendiment de soponines totals i la seva bioaccesibilitat per a la seva posterior aplicació en els aliments utilitzant la tècnica RSM. A més, s'ha emprat la irradiació UV per a la degradació de les saponines d'alfals a diferents temperatures i pH.

En conseqüència, el procés d'extracció de la proteïna d'alfals com a aliment és extremadament important per aconseguir una proteïna d'alta qualitat sense cap problema de seguretat. Així, s'ha desenvolupat i utilitzat la tècnica de precipitació isoelèctrica alcalina assistida per ultrafiltració ultrasònica (UUAAIP) com un nou procés en l'extracció de proteïnes de les fulles d'alfals. Finalment, es van investigar la composició, les propietats fisicoquímiques i funcionals de la proteïna extreta i es van comparar amb els resultats d'altres mètodes comuns d'extracció.

En cobrir tots els problemes esmentats, aquesta tesi tracta de proporcionar un aïllat de proteïna d'alfals d'alta qualitat sense cap problema de seguretat. Aquesta recerca suggereix que el blanqueig amb vapor de fulles d'alfals senceres fresques en condicions òptimes va ser útil per a evitar l'aparició del color fosc i la degradació de la proteïna extreta. Per tant, la combinació de mètodes d'extracció i purificació va crear un mètode nou i eficient, que pot millorar la puresa, la seguretat, la qualitat i les propietats funcionals de la proteïna d'alfals. La UUAAIP és una tècnica apropiada per a fabricar aïllats de proteïna d'alfals i podria resoldre les restriccions del consum humà de proteïna d'alfals.

Resumen

La creciente población mundial está aumentando la demanda de fuentes de proteínas vegetales dietéticas baratas y más sostenibles para complementar o incluso reemplazar fuentes caras y limitadas de proteína animal. La alfalfa (*Medicago sativa*) es una importante leguminosa productiva forrajera, ampliamente cultivada en todo el mundo. Debido a su alta calidad nutricional y adaptabilidad, la proteína de la hoja de alfalfa se usa principalmente como un suplemento alimenticio, mientras que solo juega un papel menor en el sector de la alimentación humana. El uso de concentrados de proteínas vegetales derivados de la alfalfa en la alimentación humana está limitado por su calidad negativa, como el color pardo, el sabor amargo y los compuestos antinutricionales.

Después de la cosecha de las hojas de alfalfa, las proteasas endógenas de la hoja están degradando la proteína, mientras que las enzimas polifenoloxidasa (PPO) y peroxidasa (POD) con alta actividad inicial están causando simultáneamente el pardeamiento del tejido. El resultado de estas acciones es una proteína oscura y parcialmente degradada. Para evitar estos efectos negativos en la proteína extraída, se llevó a cabo un primer estudio sobre los efectos del blanqueo al vaporizar las hojas de alfalfa desde el comienzo de la cosecha. El primer objetivo de la tesis fue optimizar la inactivación de PPO, POD y proteasas de plantas en hojas de alfalfa a través del proceso de blanqueo de vapor. También se investigó el efecto de este proceso en el índice de pardeamiento, el color, el nitrógeno no proteico y la masa molecular.

Algunas de las propiedades indeseables de la alfalfa pueden deberse a los niveles más altos de saponina como un factor antinutricional principal que se encuentra en la alfalfa. La segunda parte de la tesis tuvo como objetivo hallar las condiciones óptimas de extracción asistida por ultrasonido para obtener el mayor rendimiento de saponinas totales y su bioaccesibilidad para su posterior aplicación en los alimentos utilizando la técnica RSM. Además, se ha utilizado la irradiación UV para la degradación de las saponinas de alfalfa a diferentes temperaturas y pH.

En consecuencia, el proceso de extracción de la proteína de alfalfa como alimento es extremadamente importante para lograr una proteína de alta calidad sin ningún problema de seguridad. Así, se ha desarrollado y utilizado la técnica de precipitación isoeléctrica alcalina asistida por ultrafiltración ultrasónica (UUAAIP) como un nuevo proceso en la extracción de proteínas de las hojas de alfalfa. Finalmente, se investigaron la composición, las propiedades fisicoquímicas y funcionales de la proteína extraída y se compararon con los resultados de otros métodos comunes de extracción.

Al cubrir todos los problemas mencionados, esta tesis trata de proporcionar un aislado de proteína de alfalfa de alta calidad sin ningún problema de seguridad. Esta investigación sugiere que el blanqueo con vapor de hojas de alfalfa enteras frescas en condiciones óptimas fue útil para evitar la aparición del color oscuro y la degradación de la proteína extraída. Por lo tanto, la combinación de métodos de extracción y purificación creó un método nuevo y eficiente, que puede mejorar la pureza, la seguridad, la calidad y las propiedades funcionales de la proteína de alfalfa. La UUAAIP es una técnica apropiada para obtener aislados de proteína de alfalfa y podría resolver las restricciones del consumo humano de proteína de alfalfa.

Table of contents

1. Introduction 1
1.1. Population and food demands 3
1.2. Global protein demand 5
1.2.1. Future protein demand 5
1.2.2. Distribution and availability of protein 7
1.2.3. Protein malnutrition 8
1.3. Meat reduction and transition towards plant-based protein 10
1.3.1. Meat protein 10
1.3.2. Meat replacement 13
1.3.3. Plant-based protein 16
1.4. Alfalfa protein for human consumption 19
1.4.1. Alfalfa plant 19
1.4.2. Worldwide production 20
1.4.3. Chemical composition of Alfalfa 20
1.4.4. Application of alfalfa protein 22
1.4.5. Alfalfa as a food 25
1.5. Anti-quality factors 27
1.5.1. Saponins 28
1.5.1.1. Plant materials contain saponins 30
1.5.1.2. Applications of saponins in foods 31
1.5.1.3. Alfalfa (*Medicago sativa*) saponins 33
1.5.1.4. Extraction techniques of saponins 35
1.5.1.5. Photo-degradation by UV-irradiation 41
1.5.2. Phenolic compounds 43
1.5.3. Degradation protein 45
1.6. Extraction of protein from alfalfa 48
1.6.1. Acid heating method 48
1.6.2. Isoelectric precipitate method 49
1.6.3. Salting method 50
1.6.4. Ultrafiltration method 50
2. Objectives and Working plan 51
2.1. Aims and Objectives 55
2.1.1. Main aims 55

2.1.2. Specific objectives 56
2.2. Working plan 58
3. Materials and methods 62
3.1. Materials 63
3.2. Methods 64
4. Results and discussion 86
4.1. A first approach to prevent to post-harvest undesirable characteristics of alfalfa leaves 87
4.1.1. Optimization of the steam blanching on enzymatic activity, color and protein degradation of alfalfa 88
4.1.2. Effect of the optimization of the steam blanching condition during storage 100
4.1.3. Effect of the optimization of the steam blanching condition on alfalfa leaf protein concentrate 106
4.2. Alfalfa saponins extraction by ultrasound-assisted and degradation by UV-irradiation 108
4.2.1. Optimization and kinetic study of the ultrasonic-assisted extraction of total saponins from alfalfa (*Medicago sativa*) and its bioaccessibility using the response surface methodology 108
4.2.2. Photo-degradation of alfalfa saponin by multi-wavelength irradiation 125
4.3. Producing alfalfa protein isolate for human consumption by Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique 134
4.3.1. Extraction modeling and statistical analysis 135
4.3.2. Physicochemical properties of alfalfa protein isolate 145
4.3.3. Anti-nutritional factors of alfalfa protein isolate 147
4.3.4. Functional properties of alfalfa protein isolate 149
5. Conclusion and future research 154
5.1. Conclusion 156
5.2. Recommendations for future research 160
References 163
Acknowledgements 186

List of tables

Table 4.1.1. Matrix of experimental Box-Behnken design (BBD) for the steaming time (min), particle size (mm) and harvesting to steaming time (hour) for optimization of the steam blanching conditions of alfalfa. 90

Table 4.1.2. Regression coefficients of the second-order polynomial function and the coefficients of determination (R^2). 92

Table 4.1.3. Kinetic parameters for Browning index and NPN of alfalfa during storage. 103

Table 4.1.4. Effect of blanching on optimum conditions on the color parameters of alfalfa protein concentrate. 106

Table 4.2.1. Box–Behnken design matrix with coded variables and measured and predicted values. 111

Table 4.2.2. Analysis of variance for the regression model of total saponin extraction yield and their bioaccessibility. 115

Table 4.2.3. Kinetic parameters for the yield of total saponins and their bioaccessibility during extractions. 122

Table 4.2.4. Energy emitted by lamp, mass extinction coefficients (ε_λ) of saponin and absorption coefficients of aqueous solutions of saponin for 100 $mg \cdot L^{-1}$, at different wavelengths. 132

Table 4.2.5. Parameters of pseudo first-order kinetic for photo-degradation of saponin in methanol-water solution at different pH and temperatures. 133

Table 4.3.1. Box–Behnken design matrix with coded variables and experimental and predicted values. 136

Table 4.3.2. Analysis of variance for the regression model of extraction yield and protein content. 139

Table 4.3.3. Physicochemical and anti-nutritional factors of alfalfa proteins extracted by different methods. 148

Table 4.3.4. Functional properties of alfalfa proteins extracted by different methods. 152

List of figures

Fig. 1.1. World Population Expected to Reach 9.7 Billion by 2050 (UN, 2019). 4

Fig. 1.2. Chemical structures of some common saponin present in alfalfa *(M. sativa).* 34

Fig. 1.3. Current extraction techniques employed in extraction of saponins from plant materials. 36

Fig. 1.4. The mechanism for polyphenol oxidase action on monophenols and diphenols. 45

Fig. 3.1. Scheme of Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique. 78

Fig. 4.1.1. Response surface 3D plots showing the interactive effect of blanching variables on the residual activity of PPO. 93

Fig. 4.1.2. Response surface 3D plots showing the interactive effect of blanching variables on the residual activity of POD. 95

Fig. 4.1.3. Response surface 3D plots showing the interactive effect of blanching variables on the Browning Index. 97

Fig. 4.1.4. Response surface 3D plots showing the interactive effect of blanching variables on the non-protein nitrogen. 98

Fig. 4.1.5. Effect of optimized conditions on the Browning Index of alfalfa during storage. 104

Fig. 4.1.6. Effect of optimized conditions on the non-protein nitrogen of alfalfa during storage. 104

Fig. 4.2.1. Plot of Normal probability of internally studentized residuals (Y_1). 109

Fig. 4.2.2. Plot of Normal probability of internally studentized residuals (Y_2). 109

Fig. 4.2.3. Plot of internally studentized residuals vs. predicted response (Y_1). 110

Fig. 4.2.4. Plot of internally studentized residuals vs. predicted response (Y_2). 110

Fig. 4.2.5. Response surface 3D plots showing the significant interactive effect of extraction variables on the total saponins yield and their bioaccessibility. 116
Fig. 4.2.6. Comparison between experimental (symbols) and simulated extraction kinetics (curves) of total saponins yield during extractions of ultrasound at optimum conditions and heat-reflux method. 122
Fig. 4.2.7. Comparison between experimental (symbols) and simulated extraction kinetics (curves) of their bioaccessibility during extractions of ultrasound at optimum conditions and heat-reflux method. 123
Fig. 4.2.8. Emission spectrum for HPM 12 mercury lamp. 125
Fig. 4.2.9. Evolution of saponin absorption spectrum with irradiation time 125
Fig. 4.2.10. Spectral radiant power absorbed as a function of saponin concentration 127
Fig. 4.2.11. Spectral radiant power absorbed as a function of saponin concentration from 25 to 100 $mg \cdot L^{-1}$ showing the parameters a and b fitted by linear regression. 127
Fig. 4.2.12. Incident spectral radiant power on the surface of the photo-reactor ($P(0)$) was obtained for 100 $mg \cdot L^{-1}$ saponin in methanol-water solution. 128
Fig. 4.2.13. Incident spectral radiant power at the bottom of the photo-reactor ($P(C)$) was obtained for 100 $mg \cdot L^{-1}$ saponin in methanol-water solution. 129
Fig. 4.3.1. Perturbation plots showing the effect of process factors on extraction yield. 140
Fig. 4.3.2. Perturbation plots showing the effect of process factors on protein content. 140
Fig. 4.3.3. 3D plots showing the interactive effects of process factors on extraction yield. 142
Fig. 4.3.4. 3D plots showing the interactive effects of process factors on protein content. 143
Fig. 4.3.5. Protein solubility of alfalfa proteins extracted by different methods at pH 2-10. 149

1. Introduction

Optimization of extraction of alfalfa leaf protein for human consumption

1.1.

Population and food demands

The world population growth is predicted to project by about 20% to 9.7 billion in 2050 (Fig 1.1). Compared to the preceding 50 years, population growth rates will slow down considerably. However, coming off a much bigger base, the absolute increase will still be significant, about 3 billion more humans. Nearly all of this increase in population will take place in the part of the world comprising today's developing countries. The greatest relative increase, 120 percent, is expected in today's least developed countries (UN, 2019). The sharp increases in food prices that occurred in global and national markets in recent years, and the resulting increases in the number of hungry and malnourished people, have sharpened the awareness of policy-makers and of the general public to the fragility of the global food system. This awareness must be translated into political will and effective action to render the system better prepared to respond to long-term demand growth and more resilient against various risk factors that confront world agriculture, and to ensure that the growing world population will be able to produce and have access to adequate food today and in future. There is a need to address new challenges that transcend the traditional decision making horizons of producers, consumers and policy-makers.

Furthermore, the FAO estimates that global demand of food will increase by half by 2050 (FAO, 2017).

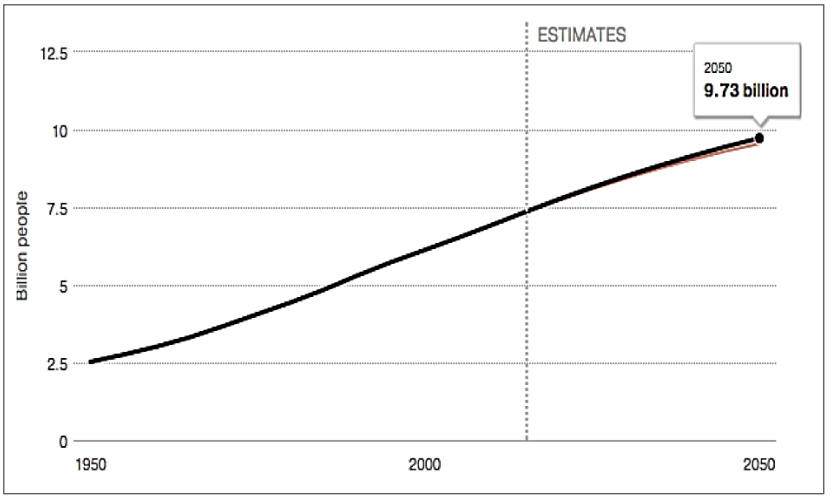

Fig 1.1. World Population Expected to Reach 9.7 Billion by 2050 (UN, 2019).

The future growth of food demand will be the combined effect of slowing population growth, continuing strong income growth and urbanization in many of the developing countries and associated shifts in diet structures, especially in the most populous ones, and gradual food saturation in many developing countries, as is already the case in developed countries. Globally the growth rate of demand will clearly be lower than during preceding decades. Nevertheless, the projected total demand increase is still significant in absolute terms, with only small differences between the main models. In addition to giving rise to an increased demand for food as a result of more mouths to feed, other changes, such as increased incomes

and urbanization, will result in changes in consumption patterns. Thus, not only will the amount of food required change, the type of foods demanded, and their relative contribution to diets, will change. How far future growth of incomes and food demand will be adequate to achieve food security will also be determined by the prospects for poverty reduction. In this context, it is encouraging to note that the secular decline of global poverty has intensified in recent decades. However progress has not been uniform and apparently it was interrupted during the current crisis. While dramatic improvement was recorded in China and several other large countries such as Indonesia, India, Pakistan, Brazil, Mexico and South Africa. Sub-Saharan Africa as a whole saw a large increase in the number of people living in absolute poverty and only a small decrease in the poverty ratio (FAO, 2011).

1.2.

Global protein demand

1.2.1. Future protein demand

In addition to increased demand arising from population growth, increased demand for protein globally is driven by socio-economic changes such as rising incomes, increased urbanization, and aging populations whereby the contribution of protein to healthy aging is increasingly recognized and recognition of the role of protein in

a healthy diet (Popkin et al, 2012). Economic development and increased urbanization is leading to major transitions in population-level dietary patterns in low and middle income countries in particular, such that most of the global increases in demand for foods of animal origin are seen in developing countries. Some forces, however, provide a countervailing force slowing demand in developed countries. Such factors include increased awareness of the impact of food production and consumption on the environment and on health. In the context of protein, the negative impact is mainly associated with animal-derived protein with reports that 12% of greenhouse gases emissions derive from livestock production and that 30% of human-induced terrestrial biodiversity loss can be attributed to animal production. Land use is also a concern; for example, in the EU two thirds of total agricultural area is used for livestock production and around 75% of protein-rich animal feed is imported from South America using large tracts of land there also. Health concerns arise with over-consumption of protein, particularly when linked with saturated fatty acids and over consumption of processed meats. Ethical issues about animal production could also stifle demand with a trend towards flexitarianism and initiatives aimed at reducing meat consumption evident in some markets. Current protein demand for the 7.3 billion inhabitants of the world is approximately 202 million tons globally. However, even accepting a 3 billion growth in population, vastly different outcomes in terms of demand for protein result depending on assumptions made about average consumption for the future (FAO, 2017). The main source of protein in the Western world diet is of animal origin, followed by dairy, and scarcely from plant sources. In the United Kingdom, according to the National Diet and Nutrition Survey, for

adults aged 1964 years, the main protein intake was from animal sources, especially meat and processed meat, as follows: from chicken and turkey 20.3%, followed by beef and veal 15.5%, other types of meat and processed meat 17%, fish (oily and white) 9.3%, and eggs 6.7%. The only plant-based protein source mentioned in the survey was baked beans at 7.0% (Henderson, Gregory, Irving, & Swan, 2003). Sans and Combris have recently published a review which encompassed worldwide meat consumption pattern over the last 50 years, observing that this rose from 23.1 kg per person per year in 1961 to 42.20 kg/person/year in 2011. The same trend was observed for proteins from dairy foods (Sans & Combris, 2015). These changes happened mostly by replacing plant-based foods from the diet; so much so that in recent times plants are not even regarded as sources of protein.

1.2.2. Distribution and availability of protein

Although protein is costly to produce, it is the greatest limitation to growth and good health (Altschul, 1965). There is a great disparity in protein usage by the various nations. The total eaten protein in the Middle East is about 50 g of protein per day, of which 39 g (78%) are of vegetable origin whereas in North America and Europe the total eaten protein is about 95 g of protein per day, of which 42 g (43%) are of vegetable origin (Kinsella, 1970). As the world population increases the problem will become more acute and will spread to larger areas. The low protein content in the diet is associated with the low level of animal protein supply and results in acute clinical symptoms, particularly among children. Moreover, even when children survive the aftermath leaves its mark on adulthood and renders the adults more susceptible to further debilitations when exposed to diseases which

normally would be tolerated by people with a history of good nourishment. Therefore, protein deficiency requires special attention for the following reasons: (a) the scientific evidence available now points to the fact that a part from calorie shortage, protein deficiency diseases are the major cause of malnutrition in low income countries, (b) protein deficiencies present special difficulties because of inadequate methods of diagnosis, (c) protein deficiencies in under developed countries are always linked with other deficiencies so that accurate clinical diagnosis and epidemiological studies may be difficult, (d) proteins are complex chemical compounds and there are wide variations in their composition based on their sources, storage, processing and cooking, (e) proteins are required in considerable amounts each day and deficiency cannot (except possibly as an emergency measure) be dealt with through provision of medicine and (f) proteins are normally among the most expensive foods and their immediate provision in many countries may entail imports which could be difficult based on general financial grounds in poor countries (Kinsella, 1970).

1.2.3. Protein malnutrition

It is not enough to present statistics which indicate that protein supplies are inadequate for large population groups. It is, thus, necessary to demonstrate that malnutrition actually occurs as a clinically identifiable disease. The most disastrous consequences occur in children where protein malnutrition manifests itself in forms of two notorious diseases: Marasmus and kwashiorkor. Kwashiorkor is a case of severe protein deficiency with the absence of serious calorie deficiency. It is a medical name for malnutrition when a child is weaned and the diet that replaces

breast milk is high in carbohydrates and deficient in protein as is common in many parts of the world where the bulk of the diet is made of starchy vegetables (Williams, 1953). The name is derived from one of the languages in coastal Ghana, translated "first-second". It means the rejected one, reflecting the development of the condition in the older child after the second one is born. As protein deprivation continues, growth failure, loss of muscle mass, swelling edema and decreased immunity occur. Other conditions include: Edema of the legs and feet, a pot-belly, light colored-thin hair, skin depigmentation, shiny skin, dermatitis, loss of teeth and enlarged liver (Cilinberto et al., 2005). Protein function in the body is to keep the blood from leaking out of the blood stream into the body tissues and cavities. When blood proteins are very low, serum seeps into the soft tissues and abdominal cavities causing defuse body swelling or edema and abdominal bloating or ascites. Aside from the direct effect of the disease, there are some secondary effects including: Lowered resistance to infection and the infection in turn reduces the capacity of the child to be nourished and enhances the malnourished condition. Kwashiorkor typically occurs at about age one after infants are weaned from breast milk to a protein deficient diet of starchy gruels or sugar water, but it can developed at any time during the formative years (WHO, 2002b; Krawinkel, 2003) Kwashiorkor patients mortality in poor countries is 18- 30% (WHO, 2002b). Mortality rate of children 1-4 years of age in countries where Kwashiorkor is rare is only 1.0-3.8 per 1000 population where mortality in countries where kwashiorkor is prevalent ranges from 12-60 per 1000 for the same age group (Badaloo et al., 2006; Pirie, 1975). Marasmus is a severe general malnutrition syndrome of both calorie and protein. It affects infants age 6-18 months old as a result of breast feeding

failure or a debilitating condition such as diarrhea. The child looks emaciated and the body may be reduced to less than 80% of normal weight for that height. The malnutrition leads to extensive tissue and muscle wasting as well as variable edema, dry skin, loose skin folds hanging over the glutei and axcille, vomiting, lethargy and impaired immunity. The afflicted child becomes fretful, irritable and voraciously hungry (Cilinberto et al., 2005). Protein malnutrition in adults is not easy to detect as in children and yet there may be far reaching effects including the prospect of permanent damage to organs in those adults who had a protein malnutrition experience when young which would influence the later habits and activities (WHO, 2002b).

1.3.

Meat reduction and transition towards plant-based protein

1.3.1. Meat protein

Based on the FAO Food Balance Sheet data, it is clear that global meat consumption has increased significantly in recent decades. Analysis of such data by Henchion et al. (2014) finds that overall meat consumption increased by almost 60% between 1990 and 2009. This trend is expected to continue, driven in particular by income growth in countries such as Asia, Latin America and the Middle East. Using 1997 as the base year, Rosegrant, Paiser, Meijer and Witcover

(2001) expect the quantity of meat demanded by consumers in developing countries to double by the year 2020. This growth is tempered somewhat by a suggestion that meat consumption per capita appears saturated in developed countries (FAO, 2017), compounded by aging populations, changing demographics and increased health and dietary awareness. While the United Nations, some governments and several NGOs are implementing campaigns to reduce the amount of meat consumed (Kanerva, 2013) global meat consumption is expected to increase by 76% by 2050. This increased demand requires significant production growth. Given constraints on land and water availability and the impact of meat production on climate change, increased efficiencies in production practices and improvements throughout the wider food chain will be critical (Gerber, et al. 2015). Meat is an important component of the human diet, and beef in particular has played a key role in food security in terms of providing energy, protein, and essential micronutrients. As highlighted by Gerber and colleagues, ruminants play a key role in converting fibrous material, which cannot be digested by humans, into protein which has a high nutritional value. Raw meat contains 20–25%. Protein depending on source and fat content, which, on loss of water due to cooking, can correspond to 28–36% in cooked meat. Protein from meat is an excellent source of essential amino acids and has high net protein utilization and digestibility (Bax, et al. 2013). In addition, meat is a key source of, often highly bioavailable, minerals (iron, zinc, and selenium) and vitamins (A, B9&12, D, and E), and has an ability, referred to as the "meat factor", to enhance iron availability from other sources (Hurrell and Egli, 2006). While meat consumption carries clear health benefits, over-consumption can lead to negative health impacts. The International Agency for Research on Cancer

(IARC) report suggested processed meat should be classified as “carcinogenic to humans” (every 50 g portion of processed meat increases risk of colorectal cancer by 18%). Red meat, on the other hand, was classified as “probably carcinogenic to humans” (every 100 g of red meat consumption leads to a 17% increased risk). While causation has not been fully elucidated, heme iron is thought to play a critical role through catalysis of N-nitroso-compounds, generation of lipid oxidation products and a possible direct cytotoxic effect. However, as discussed by De Smet and Voosen (2016) “the benefits and risks associated with red and processed meat consumption should not necessarily cause dilemmas, if these meats are consumed in moderate amounts as part of balanced diets”. In relation to cardiovascular disease (CVD), however, research seems more inconsistent and there is debate regarding the paucity of data from randomized controlled trials (RCT). A recent meta-analysis of RCT supports the idea that consumption of under 0.5 servings, or 35 g, of total red meat per day does not negatively impact on CVD risk factors. Public health concerns about livestock production are also relevant in relation to health, including zoonoses such as avian influenza, and concerns about emergence of novel diseases at animal-human-ecosystem interface. From an environmental perspective, meat production, at a global level, contributes significantly to climate change and land use change (De Smet and Voosen, 2016). High levels of greenhouse gases are produced during meat production with ruminants contributing in a significant way. Land use, water, energy and chemical inputs (e.g., fertilizers) all reflect negatively on the environmental footprint from meat production. Of course, the relative level of impact varies depending on factors such as the species under consideration, production system (e.g., grass vs. concentrate production system), requirement for

deforestation, and others. In particular, there is a case to be made for the promotion of meat produced from grass-fed ruminants (Wu, et al. 2014). In tandem to meat production, animals also produce a variety of other goods and services. Animals and cattle in particular, often serve as financial instruments and a route out of poverty in developing countries. Meat production is important for economic growth and poverty reduction in rural areas but needs to be managed carefully to ensure social effects are not negative. Plant-derived alternatives to meat are being developed with many products already on the market. Quorn is the most well-known of these. It is derived from a fungus, using a natural fermentation process and wheat-derived glucose syrup. Quorn Foods, founded in 1985, currently sells about 22,000 tons of Quorn in 16 countries, with investment underway to double production capacity in their UK plant. In vitro meat is presented as a longer-term alternative.

1.3.2. Meat replacement

It seems clear that, from both an environmental and public health perspective, it would be beneficial to reduce the quantity of meat and increase the volume of plant foods eaten by the average Westerner. This leads us to the question of how likely those in the West are to adopt such dietary change. To attempt to answer this question, we now turn to look at three factors which may impact the likelihood of any large-scale dietary change: (1) the complex nature of food choice; (2) the difficulties encountered when trying to convince a nation to change its behavior; and (3) the special status that is afforded to meat. Food choice is an extremely complex phenomenon (Sobal & Bisogni, 2009). It is influenced by both

physiological and psychosocial impulses (Sobal, Bisogni, & Jastran, 2014), is both a conscious and an unconscious process, is affected by both internal and external (ie, social) forces, and has been approached from a myriad of theoretical positions and disciplines-psychologists, behavioral economists, social scientists, public health researchers, and neuroscientists are all represented in the quest to better understand why we choose to eat what we do. With over 200 food choices made by a person each day (Wansink & Sobal, 2007), the task of unpicking and evaluating the motivations that drive these choices seems daunting. With regards to public eating patterns, most attempts to bring about change have involved measures that fall near the bottom of the intervention ladder. The most common methods applied to bring about dietary change have been public information and education campaigns (Mazzochi, Traill, & Shogren, 2009), and while these appear to increase public awareness of the campaign messages, actual behavioral change seems to be limited at best (Brambila-Macias et al., 2011; Capacci et al., 2012). However, Gordon, McDermott, Stead, and Angus (2006) argue that social marketing campaigns, whereby the techniques and tools used by marketers are adopted to promote socially beneficial behavioral change, can be a successful method in changing the eating habits of a populace, and a sustained social marketing campaign in Australia seemed to lead to an increase in fruit and vegetable consumption (Pollard et al., 2008). Any such campaigns may highlight the environmental benefits of reducing meat consumption, as consumers seem unaware, or skeptical of, the positive environmental impact of reducing meat consumption, and those who consumed most meat were also least likely to believe that reducing consumption would have a positive effect on the environment (Tobler, Visschers, & Siegrist, 2011).

With regards to decreasing meat consumption, and increasing plant-based alternatives, previous research points to a general reluctance to make such a change. Although it has been suggested that industrialized nations may be reducing their meat intake (Fresco, 2009), overall global meat consumption is expected to rise as more countries modernize and mimic Western dietary patterns (FAO, 2006; Henchion, McCarthy, Resconi, & Troy, 2014). As the GDP of a country increases, so does the demand for meat (Schroeder, Barkley, & Schroeder, 1996). Therefore if and when more nations modernize we can expect global demand for meat to increase significantly. One explanation for this may be the symbolic value of meat as a marker of prosperity (Heinz & Lee, 1998). However, this is not the only symbolic value that we associate with meat, with advertising used as a means to create and spread these symbolic meanings. These symbols have the effect of separating us from a more accurate picture of the meat we consume (ie, factory farming techniques, slaughterhouses, etc.) (Heinz & Lee, 1998). Meat is depicted as a vital part of a healthy diet (Heinz & Lee, 1998), and eating meat is portrayed as part of human nature, and the reason that humans are the dominant species on the planet (Peace, 2008). Meat advertisements also frequently employ the symbolism of meat as a masculine activity, in contrast to the feminization of men through ideas such as metrosexuality (Rogers, 2008; Buerkle, 2009). There is a dearth of research looking at the factors affecting meat consumption. In a review of previous studies, Latvala et al. (2012) found that men eat more meat than women, meat consumption decreases with age, and people with high incomes eat more meat than those on low incomes. Taste, healthiness, price, and the safety of meat were all found to be important factors in meat consumption, with animal welfare becoming more so and

environmental questions generally not asked. Clearly, more research is required to discover meat consumption patterns. There are potential opportunities to be explored for reducing meat consumption. It would be wrong to assume a meat eater/vegetarian dichotomy, with consumers instead sitting somewhere on a meat-eating to meat-avoiding continuum. Up to 40% of the UK population was found to be "meat reducers," with the primary reason for a reduction in meat consumption being concerns regarding health (Baker, Thompson, & Palmer-Barnes, 2002). Flexitarians are individuals who have reduced their meat consumption but do not identify as vegetarians (Dagevos & Voordouw, 2013). Better understanding of the motivations and circumstances of such individuals may open up pathways for more widespread meat reduction and adoption of more plant-based diets. The generally negative perception of current meat alternatives can also be used as a stimulus to develop new plant-based protein products (de Boer & Aiking, 2011). Making such products with greater similarity to meat may also tempt those who currently consume most meat (Hoek et al., 2011). This section has sought to briefly introduce some of the key problems that need to be addressed if people are to be convinced to reduce their meat intake and concurrently increase the amount of plant-foods that they eat.

1.3.3. Plant-based protein

There is an increased awareness of the association between good health and regular consumption of plant foods. Numerous epidemiological studies have demonstrated the beneficial effects in the prevention of cancer, coronary heart disease (CHD), and/or many other chronic diseases (Rizkalla, Bazzano, Tees, & Nguyen, 2008;

Rochfort & Panozzo, 2007; Bazzano et al., 2001) Human studies of epidemiology and intervention have also indicated an inverse correlation between legume consumption and the risk of CHD (Bazzano et al., 2001), type II diabetes mellitus, obesity (Rizkalla et al., 2002), and a significant decrease in low-density lipoprotein (LDL) cholesterol and triglycerides and increase in high-density lipoprotein (HDL) cholesterol (Anderson & Major, 2002; Bazzano et al., 2008; Neacsu, Fyfe, Horgan, & Johnstone, 2014). Further associations have been made between the frequency of legume consumption and CHD and cardiovascular disease (CVD); legume consumption four or more times per week compared with less than once a week has been associated with 22% and 11% lower risk of CHD and CVD, respectively (Flight & Clifton, 2006). Anderson and Major (2002), produced a meta-analysis of clinical trials looking at the emerging evidence that indicates that regular intake of pulses, as part of a heart-healthy diet, significantly decreases risk for CVD. They found that the intake of pulses decreases serum cholesterol or LDL cholesterol by 7% and serum triacylglycerols by more than 10%, however pulses do not significantly affect serum HDL cholesterol values. The hypocholesterolemic effects of legumes appear to be related to their soluble dietary fiber, vegetable protein, oligosaccharides, isoflavones, phospholipids and fatty acids, and saponins (Anderson & Major, 2002). Research data have confirmed that soy protein can lower LDL cholesterol to a small but significant degree (56%); isoflavones may play a small role in cholesterol-lowering and in lowering heart disease rates (Flight & Clifton, 2006). It is also considered that the bile acid binding and increased excretion of bile acids may play a prominent role in the hypocholesterolemic effects of pulses. Another finding of the meta-analysis conducted by Anderson and Major

was that intake of pulses, with their low glycemic index (GI) and mineral content, has favorable effects on blood pressure, glycemic regulation, and weight management (Anderson & Major, 2002). Legumes, pulses, and cereals, in addition to being an important source of macronutrients and minerals, also contain a rich variety of bioactive phytochemicals, including phytosterols and natural antioxidants (Amarowicz & Pegg, 2008; Rochfort & Panozzo, 2007), that are increasingly being recognized for their potential benefits for human health. However, plant-based foods are also associated with a series of compounds, known as antinutrients, such as protease inhibitors, α-galactosides, vicine, convicine, tannins, saponins, alkaloids, and phytates (Muzquiz, 2000). These compounds interfere with the assimilation of some nutrients and in some cases these can be toxic or cause undesirable physiological effects, for example, flatulence (Muzquiz et al., 2012). There is a balance between deleterious and beneficial effects of these plant components depending on their chemical structure, concentration, bioavailability, time of exposure, and their interaction with other dietary components. Consequently, they are classed as antinutrients or not displaying negative and/or positive effects on health (Champ, 2002; Campos-Vega, Loarca-Pina, & Oomah, 2010).

1.4.

Alfalfa protein for human consumption

1.4.1. Alfalfa plant

Alfalfa (*Medicago sativa*) is an important productive forage legume, widely cultivated around the world. Owing to its high nutritional quality and adaptability, alfalfa is also used in livestock feeding as hay and pasture (Hadidi, Ibarz, Conde, & Pagan, 2019). Leaves of alfalfa, in particular, have significant potential for human consumption due to their high protein content. Dried alfalfa leaves contain more than 250 g kg-1 protein dry basis (DB). Albumin is the main protein in alfalfa leaves, while gluten and globulin are less important. The application of alfalfa leaves' protein in human consumption is limited by their low quality and sensory attributes, especially due their brown and dark color that has important influence on acceptance by consumers and buying their decisions (Wang & Kinsella, 1976; Hadidi, et al., 2019). M. sativa is a cool season perennial legume living from three to twelve years, depending on variety and climate. The general morphology of M. sativa plant was considered by Teuber and Brick (1988), and Barnes and Sheaffer (1995). The mature M. sativa plant is characterized by a strong taproot. This taproot may eventually surpass 6 m or more in length with several to many lateral roots connected at the crown when M. sativa is grown in deep, well drained, moist soils.

The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. Tri- or multi-foliolate leaves form alternately on the stem, and secondary and tertiary stems can develop from leaf axils. A plant in a typical forage production field has between 5 and 15 stems and can reach nearly 1 m in height.

1.4.2. Worldwide production

M. sativa is the most cultivated legume in the world. Worldwide production was around 436 million tons (436000000 metric tons) in 2006. The USA is the largest M. sativa producer in the world, but considerable area is found in Argentina (primarily grazed), Australia, South Africa, and the Middle East (FAO, 2006). Within the USA, the leading M. sativa growing states are California, South Dakota, and Wisconsin. The upper Midwestern states account for about 50% of US production, the Northeastern states 10%, the Western states 40%, and the Southeastern states almost none. M. sativa has a wide range of adaptation and can be grown from very cold northern plains to high mountain valleys, from rich temperate agricultural regions to Mediterranean climates and searing hot deserts.

1.4.3. Chemical composition of Alfalfa

Alfalfa leaf proteins, green or white fraction, are characterized by an equilibrated aminogram with a composition in essential amino acids in accordance with the recommendations of the FAO (Food and Agriculture Organization) and the WHO (World Health Organization). Furthermore, alfalfa leaf proteins have great functional properties and could have many applications in the food and

pharmaceutical fields. When comparing the protein content, the leaf meal contains 20.4 g protein per 100 g (Aganga and Tshwenyane, 2003). Alfalfa is a valuable source of vitamins A and E, it contains B carotene, thiamine, riboflavin, niacin, pantothenic acid, biotin, folic acid, choline, inositol, pyridoxine, vitamin B12 and vitamin K (Aganga and Tshwenyane, 2003). It is also an outstandingly good source of vitamin C (1.78 mg/g) (Levy and Fox, 1935), but it loses 80 per cent on drying (Aganga and Tshwenyane, 2003), while vitamins B and D are present only in low concentrations. This is in agreement with Horst et al. (1984) who demonstrated the presence of vitamin D_2 and vitamin D_3. Morrison (1961) reported that alfalfa hay has a higher mineral content than grains like maize and wheat, because of the accumulation of minerals in the leaves during growth, soil washed onto the growing plants by rain, and dust settling on the roughage before it is stored. Levy and Fox (1935) examined the chemical composition and digestibility of 168 alfalfa hay samples. With respect to macro minerals, it was reported that in alfalfa hay, the sodium (Na) value varied the most, followed by chloride (Cl), manganese (Mg), potassium (K), calcium (Ca), phosphate (P) and sulphur (S). Ca and Mg concentrations in alfalfa hay are greater than for grasses at equivalent stages of maturity (Scholtz, 2008). In addition, about 8 mg of iron are present per g, or nearly double that found in spinach (Levy and Fox, 1935). Maturity of the alfalfa plant seems to have the biggest influence on the variation of chemical parameters as the quality of alfalfa hay drops as the plant matures. This drop is largely due to the increasing stem: leaf ratio and to an increase in the fibre content of the stems. During the early flowering stage, the leaves contain a greater concentration of digestible nutrients, proteins, fats, fibre, total non-structural carbohydrates and

other micronutrients than the stems. Stems have more sugars, fibre, K and Cl, therefore it is clear that the alfalfa leaves contain more nutrients than the stems (Scholtz, 2008). Alfalfa hay quality (nutritive value) varies considerably and is influenced by factors such as harvesting at specific physiological stages, climatic factors, edaphic factors such as soil conditions, leaf losses during haymaking, storage and feeding practice, disease and insects, weeds, alfalfa cultivar, moisture content during storage, water supply and fertilization (Scholtz, 2008).

1.4.4. Application of alfalfa protein

Worldwide, legumes are a unique protein source in the diet. According to Tharanathan and Mahadevamma (2003) the dietary importance of legumes is expected to grow in the years, because of the protein demand of the increasing world population and the need of reducing the health risks and high costs related to consumption of animal food sources, especially in the pattern of low-income groups and developed countries. It is estimated that over 800 million people in the world are currently malnourished (Helms, 2004; Hishamunda and Ridler, 2006). These exorbitant costs of protein from animal sources have led to a growing interest in industrial application of legume proteins to challenge the increasing demand for protein in the food and non-food market. Alfalfa protein is a good source for producing nutritious and functional food, which is extracted from alfalfa leaf (NLO, 2010). Research has been conducted on preparation procedures, application, property and nutritional value of alfalfa leaf protein. Proteins have been isolated from dry powders of six one-year old and two more than one-year-old Australian alfalfa herbages with a sequential extraction procedure, where albumin was found

to be the main protein and glutenin and globin were present in small amounts (Xie et al., 2008). Research efforts are now focusing on identification and evaluation of the potential of alfalfa with good nutritional qualities that are well adapted to adverse environmental conditions; high seed yield, pests and diseases resistant (Eromosele et al., 2008). Alfalfa biomass has potential biotechnological importance in the production of low fiber, juice-derived co-products such as particulate (chloroplastic) protein concentrates, soluble protein concentrates, carotenoids, vitamins, minerals, growth factors, pharmaceutical agents, cosmetic products, and transgenic enzymes (Sreenath et al., 2001). A study by Xie et al. (2008) even illustrated antioxidant activity of peptides isolated from alfalfa leave protein (ALP) hydrolysate. The ALPs was believed to have high nutritive value in addition to antioxidant activity. Kapel et al. (2006) characterized an antihypertensive peptide from an Alfalfa white protein hydrolysate, which was produced by a continuous enzymatic membrane reactor. Alfalfa protein concentrate (APC) has been recognized as a potential source of high quality protein (45-60%) for human consumption (D'Alvise et al., 2000). The use of plant protein concentrates derived from alfalfa in human food is limited by their negative sensory properties: dark colour due to polyphenols, granules texture, poor solubility and their grassy taste (D'Alvise et al., 2000; Xie et al., 2008) possibly because of the saponin content (Thacker and Haq, 2008). Food properties that may be influenced by these proteins include water holding capacity, emulsification, foaming, viscosity, gelation, and texture (Lamsal et al., 2007). APC (dose 10 g/day) has been tested for its nutritional value in several clinical trials carried out in countries such as Peru, India and Congo. The APC has been used since 1992 as a food supplement to combat

malnutrition in several non-EU countries in the world with no reported deleterious effects. APC (320 tons) were consumed in 20 countries throughout the world and no deleterious effects of APC were reported by the various National Government Organizations that used the product. APC is also authorized in the USA, Canada and Mexico. Products are available in forms such as food supplements, drinks and chocolate bars, but mostly in the form of food supplements (capsules, tablets, powder). A survey was conducted in 25 Members States in 2006 with feedback from five countries confirming that alfalfa is consumed in the human diet in the form of food supplements and as an ingredient in common foods (soups, salads). However, no quantitative data are available (EFSA, 2009). Petin and Luzerne (2010a) stated that in response to alfalfa's loss of competitiveness against rival products (such as oil seed cakes) and to falling subsidies, the alfalfa industry had to diversify in Europe. Leaf protein has also been recognized by the Food and Agriculture Organization (FAO) as a potential source of high quality protein for human consumption due to their abundance of source, nutritive value, and absence of animal cholesterol (Xie et al., 2008). With several years of research, supported by the European Union, the fractionation of alfalfa juice to create nutritional and functional protein ingredients for the food and non-food industry (FRALUPRO) project was established and performed. Through advanced technology (i.e. fractionation of alfalfa, extracting the protein) it could be demonstrated that alfalfa contains a protein, called Rubisco (Petin and Luzerne, 2010a, b). Rubisco (ribulose 1, 5-bisphosphate carboxylase), also called Fraction-I protein, accounts for up to 30-70/100 g of soluble alfalfa leaf proteins (SLP) (Lamsal et al., 2007). Rubisco accounts for approximately 2 per cent of the total dry matter fraction of alfalfa and

also helps plants to convert energy from the sun. It could profitably replace soya as a source of protein in food. At the moment, almost 80 per cent of the plant proteins in food come from soya, but none of them covers humans' nitrogen and amino acid requirements. By contrast, Rubisco contains all the essential amino acids which humans need and is closer to milk proteins. It also has foaming and emulsifying properties, which could be applied in foods (Petin and Luzerne, 2010a).

1.4.5. Alfalfa as a food

Alfalfa has been incorporated in recipes such as alfalfa: puree saute´, tortilla, tea, croquettes, pudding, soup, as well as raw and cooked salad (Bolton, 1962; NLO, 2010). Green alfalfa has been used as a human food in parts of Russia, China and America, whilst in South Africa it is occasionally used as a substitute for spinach. Alfalfa meal was incorporated into a special "cereal mixture" and used in the feeding of small children (Levy and Fox, 1935). Chinese farmers also treated it as a kind of vegetable (Hao et al., 2008). In a study by Hao et al. (2008) alfalfa was utilized to increase the protein, dietary fiber, mineral and vitamin content of wheat flour. Alfalfa is one of the most popular sprouts available on the European markets. They are consumed often raw or slightly cooked in salads and sandwiches or as decorative appetizers (Pen˜as et al., 2009). According to Cotto (2010) many consumers will not like the taste of alfalfa and (might also create the sense that it is burning the tongue tip) and they recommend that one should persevere as it is definitely an acquired taste. Studies done by Kalac et al. (1996) and Sen et al. (1998) indicated that saponins are responsible for the bitter taste in alfalfa. Recent sensory test trials performed with human volunteers using saponins isolated from

alfalfa aeriel parts, showed that zahnic acid tredismoside is responsible for the bitter taste (Oleszek, 2002). Alfalfa can help to reduce exhaustion and nervous agitation. Due to alfalfas' phytoestrogen (isoflavones and coumarins) content, it is reputed to regulate menstrual cycles and to stimulate milk flow in breastfeeding women. Experiments carried out by clinical nutritionists in 1982 showed that eating alfalfa helped to protect monkeys from atherosclerosis that were on a high-cholesterol diet. They also proved the effectiveness of alfalfa in decreasing blood-cholesterol levels. Alfalfa seeds can be added to the diet to help normalize serum cholesterol concentrations in patients with type II hyperlipoproteinemia (HLP) (Mo¨lgaard et al., 1987). Alfalfa may also be used as a traditional plant treatment for diabetes. The administration of alfalfa in the diet (62,5 g/kg) and drinking water (25 g/l) reduced the hyperglycaemia of streptozotocin-diabetic mice. The results demonstrated the presence of antihyperglycaemic, insulin-releasing and insulin-like activity in alfalfa. Leaves of alfalfa are used traditionally as a tea to treat diabetes in South Africa. The use in human subjects of alfalfa as an antidiabetic agent has, at least in part, been attributed to its relatively high Manganese content (Gray and Flatt, 1997).

1.5.

Anti-quality factors

Alfalfa includes some anti-nutritional factors like coumestrol, isoflavones, saponins, and L-canavanine. However, many of the anti-nutritional compounds are mostly in the stem and sprouts of alfalfa plant (EFSA, 2009). Another problem is the bitter taste as a negative sensory factor of alfalfa. A large variety of ingredients are responsible for manufacturing bitter tastes in foodstuffs such as amino acids, saponins, polyphenols and peptides. Previous studies have shown that saponins are responsible for the bitterness in alfalfa leaves (Oleszek, 2002; Kalac, Price & Fenwick, 1996). The use of plant protein concentrates or isolates derived from alfalfa in human food is limited by their negative quality and sensory properties (Wang and Kinsella, 1976), especially due its brown color, which has a strong effect on consumer acceptance and purchasing decisions (Gokmen, 2010; Medina-Meza, Barnaba, Villanim, & Barbosa-Canovas, 2015). Another major problem is protein degradation resulting in lower quality and lesser recovery (Zhang, Grimi, Jaffrin, & Ding, 2015) Postharvest protein degradation in ensiled forage crops results in the conversion of true protein into different forms of non-protein nitrogen (NPN), such as oligopeptides, free amino acids (AA) and ammonia amino acids and

peptides, resulting in a decrease in the performance of the protein extracted with human consumption and leading to economic losses in cattle feeding, which must supplement rations with other sources of true protein. Such losses are especially high in alfalfa, approaching $100 million annually in the United States alone. Furthermore, the loss of true protein in preserved forages has negative environmental impacts, as excess NPN is excreted by ruminants as urea, increasing N burdens in the environment (Sullivan & Hatfield, 2006).

1.5.1. Saponins

Saponins are second metabolites which are widely distributed in the plant kingdom. It acts as a chemical barrier or shield in the plant defense system to counter pathogens and herbivores (Augustin Kuzina, Anderson, & Bak, 2011). Therefore, it is found in plant tissues that are most vulnerable to fungal or bacterial attack or insect predation (Wina, Muetzel, & Becker, 2005). Saponins divided into two major classes which are triterpenoid and steroid glycosides which their structure characterization are varied by the numbers of sugar units attached at different positions (Hostettmann & Marston, 1995). The classification and occurrence of saponins in the plant kingdom are reviewed in detail by Vincken, Heng, de Groot, and Gruppen (2007). Saponins, which are derived from soapwort (Saponaria officinalis L.), have been widely used for centuries as household detergent (Sparg, Light, & van Staden, 2004) due to its amphiphilic nature with the presence of a lipid-soluble aglycone and water-soluble chain(s) in their structure (Güçlü-Üntündağ & Mazza, 2007). The seeds of Barringtonia asiatica Kurz (Lecythidaceae) which have known to contain saponins, have been used

traditionally by native Asian and Pacific fisherman as fish poison to enhance their catches (Sparg et al., 2004). Saponin-containing plant materials, i.e., Yucca schidigera, alfafa, were used as feed additives to increase growth, milk or wool in ruminant production (Wina et al., 2005). The molluscicidal saponins derived from soapnut (Sapindusmukorossi Gaerth) have been found having inhibitory effects against golden apple snail, which is the major pests of rice and other aquatic crops in Asian countries (Huang, Liao, Kuo, Chang, &Wu, 2003). The discovery of biological activities of saponins is not only limited to the traditional uses, but more recently, also in pharmaceutical applications (Güçlü-Üntündağ & Mazza, 2007; Sparg et al., 2004). Saponins have been found having pharmaceutical properties of hemolytic, molluscicidal, anti-inflammatory, antifungal, antibacterial or antimicrobial, antiparasitic, antitumor, and antiviral (Sparg et al., 2004). It employs as a starting point for the semi-synthesis of steroidal drugs in pharmaceutical industry. Sheng and Sun (2011) reviewed the clinical significance of triterpene saponins in prevention and treatment of metabolic and vascular disease. The pharmaceutical property discoveries, especially anticancer, have intensified the seeking of saponins from plant materials. These have driven the emergence of various new extraction technologies with the main purpose of maximizing the yield in order to accommodate the recent need. Saponins are also known possessing mineral complexes of iron, zinc, and calcium (Milgate & Roberts, 1995). The beneficial effect of saponins intake in plasma cholesterol for human is another important factor that contributes to the continuous sorting of saponins (Milgate & Roberts, 1995). Besides anticancer (Cheng et al., 2011; Man, Gao, Zhang, Huang, & Liu, 2010; Waheed et al., 2012), saponins have been discovered scientifically

having pharmaceutical properties of antioxidant (Chan, Khong, Iqbal, & Ismail, 2013; Dini, Tenore, & Dini, 2009; Li, Zu, et al., 2010).

1.5.1.1. Plant materials contain saponins

Saponins are mainly derived from various plant materials (Sparg et al., 2004; Vincken et al., 2007), but several of them are found in sea cucumber and starfish (Augustin et al., 2011; Demeyer et al., 2014). The most widely studied plantmaterial thatwas found having saponins is ginseng (Kwon, Bélanger, Pare, & Yaylayan, 2003) even though saponins derived from alfalfa have been carried out as early by Van Atta, Guggolz, and Thompson (1961). Other plant materials which have been discovered containing saponins were soymilk (Lai, Hsieh, Huang, & Chou, 2013), sugar beet (Ridout, Price, Parkin, Dijoux, & Lavaud, 1994), soy and chickpea (Serventi et al., 2013), asparagus (Vázquez-Castilla et al., 2013), marion blackberry, strawberry, and plum fruit (Yoon & Wrolstad, 1984). Saponin distribution has been found to vary in individual plant parts. For example, the roots of *Medicago truncatula* (Huhman, Berhow, & Sumner, 2005) and *Allium nigrum L.* (Mostafa et al., 2013) have been revealed containing the greatest total amount of saponins accumulation. The yam tuber cortex has been discovered to possess the highest amount of saponins of 582.53 μg/g dw which was about 2.55 times higher than tuber flesh of 227.86 μg/g dw (Lin & Yang, 2008). However, the total saponin concentration has been reported to contain the highest level in leaves from the four varieties of Swithch grass (Lee et al., 2009) and greenhouse grown Maesa lanceolata (Theunis et al., 2007).

1.5.1.2. Applications of saponins in foods

Apart from pharmaceutical applications, saponins have been used in foods as natural surfactant and serve as preservative in controlling microbial spoilage of food. More recently, due to consumer preference for natural substance, Quillaja saponin has been used as a natural small molecule surfactant in beverage emulsions in replacing synthetic surfactant of Tweens (Piorkowski & McClements, 2013). The effectiveness of the natural surfactant isolated from the bark of the Quillaja saponaria Molina tree for forming and stabilizing emulsions with a synthetic surfactant (Tween 80) has been compared by Yang, Leser, Sher, and McClements (2013). After comparing the influence of homogenization pressure, number of passes, and emulsifier concentration on the particle size produced from these two surfactants, they suggested that the natural surfactant is an effective surfactant that may be able to replace synthetic surfactants in food and beverage products. This natural surfactant has been further proven its stability and effectiveness at forming edible Vitamin E delivery systems, thus it is recommended for functional food encapsulation and beverage applications (Yang & McClements, 2013). Due to its natural foam-like characteristic, the application of saponins as a natural bio-surfactant to improve the surface properties of food is intensively studied recently. Wojciechowski, Kezwon, Lewandowska, and Marcinkowski (2014) have conducted a study to evaluate the surface activity between Quillaja bark saponin with β-casein of bovine milk protein. From their results obtained, they suggested that the Quillaja bark saponin can be used as a natural low molecular weight bio-surfactant. A recent study indicated that process which soaked with saponin, a

surfactant extracted from soapnut fruit, showed differences in the degree of modification and morphology of the cellulose fibers (Cordeiro, Faria, Abraham, & Pothan, 2013). The results clearly show that the saponin can provide a continuous path of hydrogen bonds between the fiber surfaces which will thus enhance the hydrophobic and the acid–base nature of the fiber surface. This behavior will lead to better polymer/fiber interaction during the composite preparation. Andreuccetti, Carvalho, and Grosso (2010) evaluated the incorporation of hydrophobic lasticizers in a matrix of gelatin, using the saponin extracted from *Yucca schidigera* (yucca) as emulsifier, in the production of biodegradable emulsified films using the casting technique. Their results showed that the gelatin-based films produced have good mechanical resistance, low values of water vapor permeability and reduced drying times, even though the films presented limited elongation, considerable solubility and opacity. Therefore, they suggested that the possibility of using this natural surfactant may allow for new applications of biodegradable emulsified films. The use of saponins as a natural biochemical substance in inactivation of food-borne viruses has been reviewed by Li, Baert, and Uyttendaele (2013). The saponins-extract from *Sapindus saponaria* combined with heat-treatment was recommended to inactivate *Alicyclobacillus acidoterrestris*, a spoilage-causing bacterium, in orange juice (Alberice, Funes-Huacca, Guterres, & Carrilho, 2012). Tea saponin, a tea seed derived natural surfactant, combining with *Bacillus amyloliquefaciens* (Hao, Li, Hu, Yang, & Rizwan-ul-Haq, 2011) and imazalil and prochloraz (Hao et al., 2010), have been used for postharvest treatment of Mandarin fruit and results showed that the incidence of green and blue mold and sour rot were reduced.

1.5.1.3. Alfalfa (*Medicago sativa*) saponins

Alfalfa saponins are secondary plant metabolites found in roots and aerial parts. Their presence in alfalfa has several important implications. High levels of saponins severely restrict the growth of monogastric animals (Cheeke, 1983; Heghest and Linkswiler, 1980), cause the hemolysis of red blood cells, and inhibit the growth of some fungi and insects (Assa et al., 1972; Hober, 1972), or harm the development of plants (Oleszek et al., 1992a). The alfalfa saponins have been identified as mono-, bis-, or tridesmosides of medicagenic acid, hederagenin, zanhic acid, and soyasapogenol B (Timbekovaet al., 1989; Massiot et al., 1988, 1991; Oleszek et al., 1990, 1992). Among them, medicagenic acid and hederagenin glycosides have been recognized as the "biologically active" compounds and they were determined with biological tests. The most often used tests are based on inhibition of the growth of richoderma uiride (Zimmer et al., 1967) and plant seedlings (Pedersen, 1975) or on the hemolytic activity (Jurzysta, 1979). However, the results obtained by all of these tests are strongly influenced by both the aglycon and the carbohydrate composition of the saponin molecule (Oleszek, 1990; Oleszek et al., 1990a). Thus, the determination of total saponin content with these biological procedures neither provides the content of individual compounds nor gives any information on their composition. It is highly probable that the relative quantities of particular glycosides may vary under different environmental conditions and may depend on the alfalfa variety. Observed changes in total saponin content under these circumstances (Hanson et al., 1963; Guenzi et al., 1964) may simply arise from the variation in the content of most active compounds. Moreover, glycosides of zanhic

acid and soyasapogenol B may show negligible response to biological tests, but their presence in alfalfa cannot be neglected. Therefore, more selective analytical methods are needed to obtain reliable results.

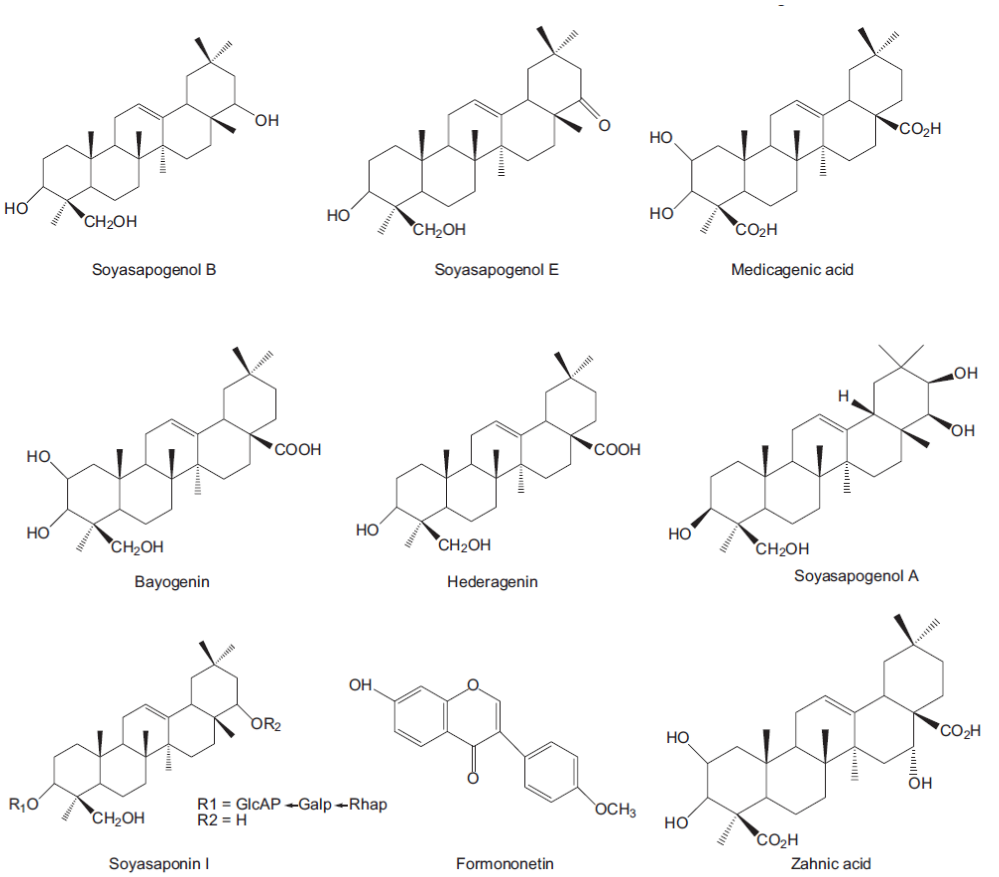

Fig 1.2. Chemical structures of some common saponin present in alfalfa *(M. sativa).*

1.5.1.4. Extraction techniques of saponins

The recent advances in extraction of bioactive compound from plant material have been intensively reviewed (Azmir et al., 2013; Wang &Weller, 2006) and this might be due to the increase in public awareness of preventative health care which could be promoted through the consumption of plant material extract. In general, the extraction techniques employed in saponin extraction can be classified into two categories, the conventional and the green technologies. The conventional extraction techniques are maceration, Soxhlet, and reflux extraction, where the green technologies are ultrasound-assisted, microwave-assisted, and accelerated solvent extraction (Heng, Tan, Yong, & Ong, 2013). The conventional extraction is relied on the solubility of solute from plant materials into solvent. Therefore, it often utilizes a large quantity of solvent to extract the desired solute, even though sometimes is aided with elevated temperature by heating, and mechanical stirring or shaking. On the other hand, the green extraction techniques involved less hazardous chemical synthesis, safer chemicals used, energy efficiency, use of renewable feedstock, and pollution prevention (Azmir et al., 2013). The design of green extraction technologies is governed under these measurements. The importance of saponins as pharmaceutical properties especially in countering cancer has provoked the invention of new extraction methods in order to obtain the maximum output to cope with the increasing demand. Therefore, a synthesis of previous literature in extraction technique selection may provide useful information in related processing industry. Fig. 1.3 clearly demonstrates that researchers are more inclined to selection of the conventional extraction techniques (70%) which include the subsequent methods, compared to the green technologies (30%), even though the

green techniques use minimal solvent. The selection of these methods usually was governed by the research focus of the studies being conducted. To further analyze the selection of extraction technique made by researchers. For isolation of new saponins and pharmaceutical property studies, 78% and 91% of the previous works were using the conventional extraction methods. However, in works focused on quantification and optimization studies, 58% and 67% of the previous works have selected green extraction technologies. It is also noteworthy that the ultrasound-assisted extraction is the most selected green extraction technologies in quantification studies which gave an implication of its capability and efficacy in obtaining significant saponin yields.

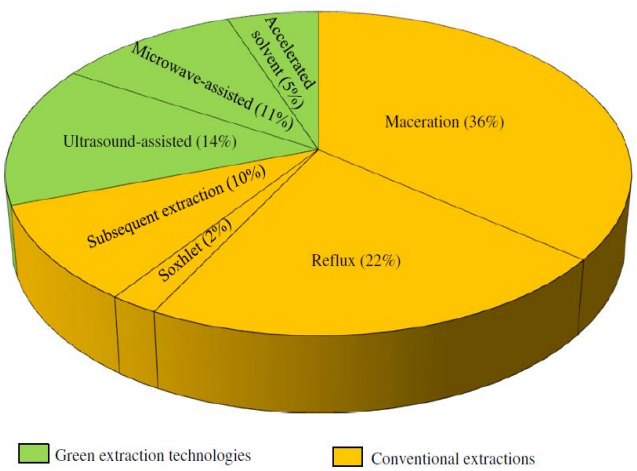

Fig 1.3. Current extraction techniques employed in extraction of saponins from plant materials.

- Maceration extraction

The maceration extraction is a solid–liquid extraction where the bioactive compound (solute) inside the plant material is extracted by soaking the plant material in a specific solvent for a period of time (Takeuchi et al., 2009). The efficacy of maceration process is determined by two main factors, solubility and effective diffusion. The solubility is governed by basic rule of "like dissolves like" which indicated that polar compounds dissolve in polar solvents, and nonpolar compounds dissolve in nonpolar solvents (Reichardt & Welton, 2011). The rate of dissolution of a solute in the extraction solvent is determined by the rate of mass transfer of a solute from the plant material to the solvent (Takeuchi et al., 2009). Due to the concentration gradient in the solid–liquid interface, the transfer of the solute inside the plant material occurs showing that an effective diffusion takes place (Takeuchi et al., 2009). No complicated utensil and equipment are needed for the set-up of a maceration extraction system that has made it a popular choice for researchers. The only paramount factor to be paid attention in enhancing extractability is the knowledge of similarity of bioactive compound interest and solvent polarity. Ethanol and methanol were the extraction solvents used to extract saponins from plant material, and ethanol preferred better probably due to environment friendly concern. The duration of extraction time is long and sometimes takes up to weeks using this method, therefore, maceration extraction often aided with mechanical shaker (Cheng et al., 2011; Huhman et al., 2005; Lee et al., 2009; Sylwia, Bogumil, & Wieslaw, 2006) or magnetic stirring (Verza et al., 2012) to shorten the extraction time.

- Reflux and Soxhlet extractions

Due to the similar working principle of Soxhlet and reflux extractions, the discussion is carried out under the same sub-title. The only difference between reflux and Soxhlet is that Soxhlet apparatus consists of a thimble to house the plant material. Reflux and Soxhlet extraction involved distillation process which is widely used in food and nonfood industrial and laboratories. The process involves heating a solution to boiling and then returning the condensed vapors to the original flask (Bart, 2011). The disadvantage of reflux and Soxhlet extractions is time consuming where it required at least one hour for an extraction. Ethanol is still the most used solvent in reflux extraction, although there are few used methanol as extraction solvent. The extraction duration of reflux extraction was varied from 1 to 4 h, while for Soxhlet was 24 to 72 h.

- Ultrasound-assisted extraction (UAE)

The phenomenon of ultrasound in creating cavitation bubbles in the solvent by acting as a microjet to denature the plant cell wall when the bubbles collapse at rare fraction resulted in a greater extraction yield of bioactive compounds. Few researchers have reviewed the ultrasound effect on the technological properties and bioactivity of food (Soria & Villamiel, 2010), and the applications of ultrasound-assisted extraction on bioactive principles from herbs (Vinatoru, 2001), food industry and processing (Mason, 1998; Vilkhu, Mawson, Simons, & Bates, 2008), Although ultrasound-assisted extraction is commonly employed in many bioactive compound extraction (Cheok, Chin, Yusof, Taib, & Law, 2013; De Koning,

Janssen, & Brinkman, 2009; Jadhav, Rekha, Gogate, & Rathod, 2009; Zhang et al., 2008).

- Microwave-assisted extraction (MAE)

Microwaves are non-ionizing electromagnetic waves with a frequency range from 0.3 to 300 GHz (Heng et al., 2013; Takeuchi et al., 2009). Recently, MAE has drawn attention in bioactive compound extraction from plant material due to short extraction time, minimal solvent usage, and its special heating mechanism (Heng et al., 2013). The recent applications of MAE of plant secondary metabolites such as flavonoids, quinones, phenylpropanoids, terpenoids, alkaloids and saponins have been reviewed (Zhang, Yang, & Wang, 2011). Microwaves are able to penetrate into biomaterials and generate heat by interacting with polar molecules such as water inside the materials. The penetration depth of microwaves into plant matrix depends on dielectric constant, moisture content, temperature, and the frequency of the electrical field (Takeuchi et al., 2009). The water contained in a plant material is responsible for the absorption of microwave energy which led to internal superheating and cell structure disruption, and consequently, facilitates the diffusion of bioactive compound from the plant matrix (Takeuchi et al., 2009). The efficacy of MAE is relied on the effect of microwave on extraction solvent and plant matrix cell structure (Takeuch et al., 2009). Although the potential application of microwave extraction for flavonoids has been reviewed thoroughly (Routray & Orsat, 2011), only few works of saponin extraction using MAE has been mentioned in previous reviews (Güçlü-Üntündağ & Mazza, 2007; Zhang et al., 2011). The superiority of MAE in saponin extraction in comparison with other extraction

methods, in terms of higher yield (Chen, Xie & Gong, 2007; Li, Zu, et al., 2010; Mandal & Mandal, 2010; Xu et al., 2012) and shorter extraction time (Chen, Xie, et al., 2007; Kwon et al., 2003; Mandal & Mandal, 2010; Xu et al., 2012), has been found in previous literature.

- Accelerated solvent extraction (ASE)

Accelerated solvent extraction has been regarded as a green technique in plant material sample preparation prior to chromatographic analysis (Azmir et al., 2013; Heng et al., 2013). This technique was introduced by Dionex Corporation in 1995. It is also known as pressurized liquid extraction, pressurized solvent extraction, and enhanced solvent extraction. Sometimes it is referred as pressurized hot water extraction, sub-critical water extraction or superheated water extraction, when water is used as solvent (Mustafa & Turner, 2011). It is an automated rapid extraction technique that uses minimal solvent at elevated temperature and pressure. The merit of using increased temperature is to enhance the solubility and mass transfer of solute to solvent, and elevated pressure keeps the solvent below its boiling point, enabling fast, safe, and efficient extraction of target analytes from plant materials into the extraction solvent (Mottaleb & Sarker, 2012). An extraction process is usually completed in 15–25 min using only 15–45 ml consumption of solvent. Therefore, it has been widely applied in the fields of environmental, food, polymer, and pharmaceutical researches.

1.5.1.5. Photo-degradation by UV-irradiation

Ultraviolet irradiation constitutes an alternative to thermal treatment that is being studied and developed to obtain a better final product sensory quality, but without neglecting microbial safety. The light radiation has to be absorbed to carry out a photochemical change; therefore, the kind of light source to be used is determined by the absorption spectrum of the reactants. To study the effect of irradiation on a compound, the specific absorption spectrum at different wavelengths has to be known. This spectrum can be obtained irradiating a solution with a well-known concentration of the compound at different wavelengths, and measuring its resulting absorbance. This absorption spectrum usually presents maximum absorbance peaks, which determine the optimum area of working wavelengths (Ibarz & Esplugas, 1989; Ibarz, Esplugas, & Graell, 1985). Ultraviolet irradiation is widely applied in industries to disinfect drinking water, air and surface disinfection and toxin degradation (Falguera, Pagán, Garza, Garvín, & Ibarz, 2011). Dong et al. (2010) reported that UV irradiation at the exclusive germicidal wavelength (254 nm) could lead to a reduction and/or elimination of patulin in apple cider and juice products without unwanted alterations of the final product. Assatarakul, Churey, Manns, and Worobo (2012) later concluded that the reaction followed a first-order kinetic model and Tikekar, Anantheswaran, and LaBorde (2013) concluded the more time needed than for the reduction of pathogens. Due to food complexity in terms of the variety of components they contain, UV radiation can affect many of these compounds. An extensive exposure of the effects of such radiation on the multitude of compounds present in food will not be made. However, some representative cases will be cited, trying to give an idea of the potential, both

positive and negative, of the photochemical treatment processes. Whited, Hammond, Chapman, and Boor (2002) studied the effect of light on the vitamin A in skimmed, semi-skimmed and whole milk, concluding that fat had a protective effect on the degradation of this vitamin. In all cases it was observed, apart from the loss of this vitamin, a loss of milk quality because in sensory analysis panelists highlighted the flavor of oxidized milk. Riboflavin, known as vitamin B12, is a yellow water-soluble vitamin, consisting of a dimenthyl isoalloxazine ring linked with ribitol, an alcohol derived from ribose. The three rings form the isoalloxazine and ribitol is the 5-carbon chain at the top. Furaya, Warthesen, and Labuza (1984) studied the photo-degradation of riboflavin in macaroni, skimmed milk powder, and buffer solutions. In liquid systems these authors found first order photo-degradation kinetics, while in solid food systems a two-step mechanism was observed. Due to potential health problems that artificial food dyes may have, some attention has been paid to the development of natural pigments to be used as colorants. Among the red pigments, betalains and anthocyanins are potential pigment sources. However, natural pigments can be degraded by various agents. Light is one of these factors affecting their stability. In this way, the effect of light on betanine extracted from beet and on cranberry anthocyanins has been studied (Attoe & von Elbe, 1981). For both kinds of pigments it was found that photochemical degradation follows first order kinetics, observing that the presence of molecular oxygen in the medium plays an important role in the degradation of these compounds.

1.5.2.Phenolic compounds

The application of alfalfa leaves' protein in human consumption is limited by their low quality and sensory attributes, especially due their brown and dark color that has important influence on acceptance by consumers and buying their decisions (Wang & Kinsella, 1976; Hadidi, et al., 2019). Especially due its brown color, which has a strong effect on consumer acceptance and purchasing decisions (Gokmen, 2010; Medina-Meza, Barnaba, Villanim, & Barbosa-Canovas, 2015). Color is an important quality attribute in the food and bioprocess industries, and it influences consumer's choice and preferences. Food color is governed by the chemical, biochemical, microbial and physical changes which occur during growth, maturation, postharvest handling and processing. Color measurement of food products has been used as an indirect measure of other quality attributes such as flavor and contents of pigments because it is simpler, faster and correlates well with other physicochemical properties. This review discusses the techniques and procedures for the measurement and analysis of color in food and other biomaterial materials. It focuses on the instrumental (objective) and visual (subjective) measurements for quantifying color attributes and highlights the range of primary and derived objective color indices used to characterize the maturity and quality of a wide range of food products and beverages. Different approaches applied to model food color are described, including reaction mechanisms, response surface methodology and others based on probabilistic and non-isothermal kinetics. Color is one of the most widely measured product quality attributes in postharvest handling and in the food processing research and industry. Polyphenol oxidase (PPO) and peroxidase (POD) have been identified as the causes of browning

reactions (Duangmal & Apenten, 1999; Tomas-Barberan & Espin, 2011). PPO catalyzes hydroxylation of monophenols to diphenols and oxidation of diphenols to quinones. The oxidation reaction is relatively rapid and the resulting quinones are colored. Subsequent reactions of the quinones lead to melanin accumulation, which is the brown or black pigment associated with browning in plants (Toivonen & Brummell, 2008). Furthermore, the synergistic activity of PPO and POD is due to the generation of hydrogen peroxide during the oxidation of phenolic compounds in PPO-catalyzed reactions (Tomas-Barberan & Espin, 2011).

The mechanism for browning involves the interaction of polyphenolic substrates with PPO in the presence of oxygen (Fig. 1.4). PPO catalyzes two reactions: (1) hydroxylation of monophenols to diphenols and (2) oxidation of diphenols to quinones. The hydroxylation reaction is relatively slow and results in colorless products, while the oxidation reaction is relatively rapid and the resultant quinones are colored. Subsequent reactions of the quinones lead to melanin accumulation, which is the brown or black pigment associated with "browning" in plant tissues. The specific reaction sequence which results in browner black-colored products depends on the specific structure of the polyphenolic substrate.

The inhibitions of this enzyme's activity and the browning have been an ongoing challenge for the vegetable food processing industry (Mayer, 2006). Residual PPO and POD endogenous enzymes may also cause quality changes during storage in dried vegetables. Both enzymes are considered to have an empirical relationship to off-flavors and especially off-colors in raw and unblanched dried vegetables (Schweiggert, Schieber, & Carle, 2005).

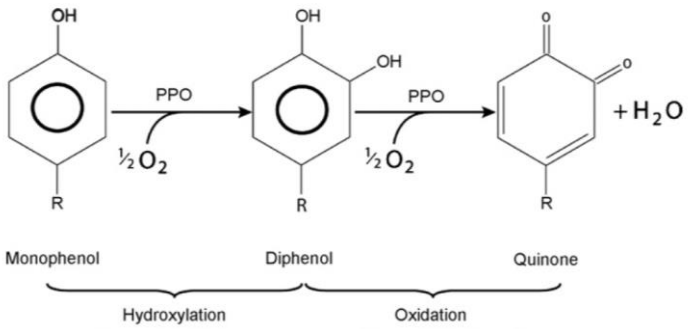

Fig 1.4. The mechanism for polyphenol oxidase action on monophenols and diphenols.

1.5.3. Protein degradation

Another major problem is protein degradation resulting in lower quality and lesser recovery (Zhang, Grimi, Jaffrin, & Ding, 2015). Proteolysis in forage results mainly from plant proteolytic enzymes. Postharvest protein degradation in ensiled forage crops results in the conversion of true protein into different forms of non-protein nitrogen (NPN), such as oligopeptides, free amino acids (AA) and ammonia amino acids and peptides, resulting in a decrease in the performance of the protein extracted with human consumption and leading to economic losses in cattle feeding, which must supplement rations with other sources of true protein. Such losses are especially high in alfalfa, approaching $100 million annually in the United States alone. Furthermore, the loss of true protein in preserved forages has negative environmental impacts, as excess NPN is excreted by ruminants as urea, increasing N burdens in the environment (Sullivan & Hatfield, 2006). The cost and logistics of many additives for inhibiting proteolysis, such as formic acid, limit their utilization in practice (Guo, Cheng, Yang, & Zhou, 2011). There is still no

satisfactory or feasible means of inhibiting proteolysis in ensiling alfalfa because the most rapid proteolysis occurs within 24–48 h of ensiling (Fairbairn & Baker, 1998; Guo, Zhou, & Zhu, 2007). Conservation of alfalfa forage as silage is an alternative to avoiding damage by weather and loss of leaf by shattering during the process of haymaking. During ensilage, however, residual proteolytic enzymatic activity results in the hydrolysis of plant proteins and the accumulation of non-protein-N (NPN), which consists principally of free amino acid-N (AA-N), peptide-N and ammonia-N (NH_3-N) (McDonald et al., 1991). The N constituents in silage are less efficiently utilized for rumen microbial-N synthesis than those in fresh or dried forages (Siddons et al., 1985; Givens and Rulquin, 2004). Reduction in proteolysis during ensilage could improve the utilization of silage-N because the efficiency of rumen microbial-N synthesis is improved by supplementing silage with protein-N rather than NPN (Rooke and Armstrong, 1989). Treatment with commercially available silage additives can effectively reduce NPN formation during ensiling, such as formic acid (Nagel and Broderick, 1992), formaldehyde (McDonald et al., 1991; Henderson, 1993) and tannins (Salawu et al., 1999; Santos et al., 2000). These studies mainly focused on the inhibitory effects of the above additives on the formation of NPN at the end of ensilage, but little attention has been paid to the effect of these additives on proteolytic actions and N distribution during the process of ensiling of forage. In addition, most of the N transformation in ensiled forage results from plant proteases (Oshima and McDonald, 1978; Winters et al., 2000). Several studies have been conducted to characterize the proteases of alfalfa forage. Protease activity in a leaf extract of alfalfa was found to be dependent on pH, temperature and time of handling (Finley et al., 1980;

McKersie, 1985; Jones et al., 1995). McKersie (1981) demonstrated the presence of at least three proteolytic enzymes in alfalfa, i.e. carboxypeptidase, aminopeptidase and acid proteinase. Each differed in their pH and temperature optima and their sensitivity to inhibitors. However, these studies on the characterization of proteolytic enzymes in ensiled alfalfa were generally restricted to fresh forages except for the study by McKersie and Buchanan-Smith (1982). Few, if any, studies have been conducted to investigate the effects of additives on alfalfa proteases during the ensilage process.

1.6.

Extraction of protein from alfalfa

Leaf protein preparation includes alfalfa juice separation, leaf protein separation and leaf protein concentration. Separation and concentration of leaf protein function as the core of preparation process. In order to recover protein in alfalfa juice, numerous protein separations and concentration technologies have been used to concentrate and produce alfalfa leaf proteins. According to different separation mechanisms, they can be divided into three categories: (1) discrepancy of solubility: salting, organic solvent fractionation, chromatography, crystallization, heating and centrifugation; (2) differences of molecular size and shape: molecular sieve chromatography and membrane; (3) charge difference: ion exchange.

1.6.1. Acid heating method

Acid heating method is a complex type of leaf protein separation method which combines acidic and heating methods. After a dual role of isoelectric precipitation and heat denaturation, the aggregation effect and sensitivity of leaf protein improve, leading to fast aggregation of leaf proteins. In comparison with traditional isoelectric precipitation method, these aggregated proteins of acid heating approaches have a more compact structure and can be separated easily, due to a

higher protein coagulation rate, ensuring high yield rate of leaf protein separation. Besides, acid heating has a clearly lower energy consumption and operation cost than those of heating method. With different pH and temperature conditions, the leaf proteins extracted by acid heating method have different characteristics. Horigome et al. (1990) analyzed chemical composition and nutritional value of leaf protein extracted from ryegrass and oats under different pH and temperature and revealed that the addition of calcium before heating could achieve a better separation. The augment of pH increased the contents of ash, calcium and magnesium content in precipitate. The precipitate extracted at pH=6 had the highest protein content and this protein was digested and absorbed most easily. However, acid heating operation is a complex process and still has a much higher energy cost than that of other methods. In addition, it also calls for expensive equipment.

1.6.2. Isoelectric precipitate method

Proteins aggregate and precipitate at the isoelectric point. Suitable pH value can reduce the decomposition activity of protein and promote the stability of carotene and lutein in juice. In acid method, pH of juice can be adjusted to 4.0~4.5 by adding HCl, then leaf proteins precipitate and produce crude proteins after centrifugation. As for alkaline method, NaOH or $NH_3 \cdot H_2O$ can adjust pH to 8.0~8.5, while flocculation efficiency improves. A pH method could reduce the activity of protease and enhance stability of carotene and lutein. But its precipitation structure is loose and imposes a negative effect upon separation performance. Besides, it also

accelerates the oxidation of unsaturated fatty acid and causes a high loss of carotene.

1.6.3. Salting method

Salting method uses macromolecular compound (polyethylene glycol) and electro-dialysis device to extract various matters at different salt concentration, then separate and purify proteins. It can be conducted at room temperature and its operation is simple and safe together with low cost. On the other hand, it can keep protein activity. But, its production quality is not good enough and the yield rate of leaf protein separation is low, because during the salting process, other impurities were produced simultaneously. Selecting a suitable temperature could improve the quality of alfalfa protein separated by salting method.

1.6.4. Ultrafiltration method

Based on penetration and UF principle, a UF membrane with good permeability and mechanical properties could effectively separate leaf proteins from juice using its size difference and other solute molecules. Leaf protein particles with large size cannot cross a UF membrane and are rejected, whereas small solute molecules including electrolytes and sugar are able to cross the membrane. According to the study of Werner Koschuh et al. (2004), the yield rate of leaf protein in UF (52%) was similar to that of thermo-coagulation method (53%). Moreover, UF operation has low energy cost, less investment and stable production efficiency. Nevertheless,

the most serious problem for restricting its further application is membrane fouling after long-term operation, which reduces the permeate flux and enhance the operation cost. In membrane process, operational temperature remains below 55°C, which can sustain the protein quality and its nutritional value. In the study of Fernández et al. (2010), protein concentrate by UF still exhibited excellent performance in water absorption property, solubility, emulsification and foam-ability, implying that the membrane process could maintain stable proteins., membrane process has higher contents of Lysine compared with other separation processes, Threonine and Valine, and lower contents of Cysteine + methionine, Isoleucine and Phenylalanine + tyrosine, indicating that leaf protein quality and its nutritional value of membrane process are similar with that of other separation processes. Membrane process can keep the leaf protein quality and its nutritional value at a high level.

2. Objectives and Working plan

Optimization of extraction of alfalfa leaf protein for human consumption

2.1.

Aims and Objectives

2.1.1. Main aims

a) Use of steam blanching process to prevent to post-harvest undesirable characteristics and to improve the quality of its edible alfalfa protein.

b) Study of the alfalfa saponins extraction by ultrasound-assisted and degradation by UV-irradiation.

c) Application of new technique for producing alfalfa protein isolate for human consumption and its composition, physiochemical and functional properties.

2.1.2. Specific objectives

a1) to optimize steam blanching on enzymatic activity, color and protein degradation of alfalfa (*Medicago sativa*).

a2) to kinetic study of the process on browning and non-protein nitrogen content of the alfalfa protein.

a3) to evaluate the effect of steam blanching on the Browning Index, color, non-nitrogen protein and molecular weight.

b1) to optimize the ultrasonic-assisted extraction of total saponins from alfalfa and its bioaccessibility

b2) to study extraction kinetics of total saponins and its bioaccessibility for ultrasound-assisted comparing to the conventional method.

b3) to photo-degrade of alfalfa saponin by multi-wavelength irradiation.

c1) to produce alfalfa protein isolate by a new Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique.

c2) to optimize and develop a new Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique for maximizing both extraction yield and protein content simultaneously.

c3) to investigate effect of UUAAIP technique on the anti-quality factors of alfalfa protein isolate.

c4) to improve physiochemical and functional properties of alfalfa protein isolate by UUAAIP method.

c5) to compare UUAAIP method with Heat-coagulation extraction (HCE) as conventional industrial process and alkaline isoelectric precipitation extraction (AIPE) as commonest extraction method.

2.2.

Working plan

- Thorough bibliographic review of all the different issues that will be addressed and determination of the specific samples and variables to be analyzed in each step.

- Analytical and statistical methods preparation and optimization.

- Pre-testing of extraction of protein from alfalfa and determination of anti-nutritional compounds and composition.

- Optimization of steam blanching on enzymatic activity of polyphenol oxidase (PPO) and peroxidase (POD), color and protein degradation of alfalfa (*Medicago sativa*) to improve the quality of its edible protein.

- Kinetic study of steam blanching on browning index and non-protein nitrogen of alfalfa protein concentrate during storage.

- Optimization of the ultrasonic-assisted extraction of total saponins from alfalfa (*Medicago sativa*) and its bioaccessibility using the response surface methodology

- Kinetic study of extraction of total saponins from alfalfa (*Medicago sativa*) and its bioaccessibility

- Photo-degradation of alfalfa saponin by multi-wavelength irradiation in different condition

- Application of Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique for producing alfalfa protein isolate for human consumption.

- Optimization of UUAAIP process by a five-factor-three-level Box-Behnken Design (BBD) for maximizing both extraction yield and protein content simultaneously.

- Improvement of physicochemical and functional properties of alfalfa protein isolate

- Study of composition, physiochemical and functional properties of the extracted protein and comparing with the results of other common methods such as heat-coagulation extraction (HCE) and alkaline isoelectric precipitation extraction (AIPE).

3. Materials and Methods

Optimization of extraction of alfalfa leaf protein for human consumption

3.1.

Materials

Fresh alfalfa was harvested from experimental alfalfa fields of the University of Lleida's Agriculture Research Group Farm in Lleida province, Spain. All experiments described in this section were carried out in duplicate.

All chemical reagents were obtained from Sigma-Aldrich- Co. Ltd. (St Louis, Mo, USA), and Panreac Química S.A. (Barcelona, Spain). All the other chemicals used were of analytical grade.

Gastric enzymes including gastric lipase from porcine pancreas (EC. 3.1.1.3) and gastric pepsin from porcine gastric mucosa (EC 3.4.23.1) were supplied from Sigma-Aldrich Co (St Louis, Mo, USA).

3.1.

Methods

Experimental procedure of steam blanching

After harvesting in the fields, leaves were separated into plastic bags and labelled with the time between harvesting and the steaming process (2 to 24 hours). Then the leaves were ground, separated by sieves into three fractions by particle size: 1 to 23 mm (non-cut). To apply blanching, a thin layer of every set of alfalfa leaves was separately exposed to steam from a laboratory steamer (Taurus SL, Lleida, Spain) at atmospheric pressure. The proportion of alfalfa leaves exposed to steam was 1/10 (w/v). After blanching, the samples were cooled in an ice bath until they reached 7 ^{0}C. The fractions were dried in a vacuum oven dryer for 4 h at 40 ^{0}C. A non-blanched sample was treated under the same conditions as the other samples. Finally, the non-blanched alfalfa and the one treated under the optimum steam blanching conditions were stored for 60 days at room temperature and then compared to verify the effects of the optimization on the color and non-protein nitrogen during the storage.

Preparation of enzyme extracts

2 g of powdered alfalfa leaves were ground with 1.2 g of polyvinylpolypyrrolidone in a mortar on ice. The PPO and POD enzymes were extracted by further grinding the polyvinylpolypyrrolidone-treated alfalfa leaf powder with 60 mL of an extraction buffer mix (50 mmol/L 2-[Nmorpholino] ethane sulfonic acid (MES), 0.2 mol^{-1} Na_2SO_4, 1 mmol L^{-1} benzamidine, 0.3% V/V Triton X-100, pH 5.5) as described by Bonnely, Davis, Lewis, & Astill (2003) The mixture was centrifuged at 4 ^{0}C and 10000 g for 20 min in a Beckman Coulter (Avanti 5-26XP, Mervue, USA) centrifuge. The supernatant obtained as a crude extract was used for the PPO and POD activity assays.

Determination PPO and POD activity

PPO and POD activity determined following the methodology described by Bonnely et al. (2003) For the PPO activity analysis, a reaction mixture consisted of 100 µL enzyme extract, 150 µL 60 mmol L^{-1} catechin as substrate, 2750 µL 100 mmol L^{-1} sodium-phosphate-citrate buffer and the pH was adjusted to 5.5 using 1 M HCl or NaOH. the enzyme activity was carried out using UV/VIS Spectrophotometer (Unico 2150, USA) at 400 nm (40 ^{0}C) in an against a blank solution.

For the POD activity analysis, 150 µL enzyme extract mixed with 150 µL 60 mmol L^{-1} guaiacol as substrate, 50 µL H_2O_2 (1.5% V/V in distilled water) and 2650 µL 100 mmol L^{-1} sodium phosphate-citrate buffer (pH 5.5). UV/VIS Spectrophotometer (Unico 2150, USA) was used to measure the enzyme activity. Absorbance was determined at 400 nm against a blank solution (30 ^{0}C).

The rate of enzyme reaction was calculated from the slope of the linear part of the curve and defined as the change of absorbance per min (ΔAbs/min). One unit of PPO and POD activity (U) was expressed as the rate of reaction per mg protein in the extracts (ΔAbs/min/mg protein). The protein concentration from the enzyme extract was determined according to the method of Bradford's method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Browning Index (BI) measurement

The color was measured by direct reading in a Konica Minolta colorimeter (Chroma Meter CR 410, Tokyo, Japan) using the three-dimensional CIE L*a*b* system. The instrument was calibrated with a white ceramic plate (L = 98, a = 0.17, b = 1.37). The Browning Index (BI), which represents the purity of brown color was calculated according to the following equations: (3.1-3.2) (Palou, Lopez-Malo, Barbosa-Canovas, Welti-Chanes, & Swanson, 1999).

$$BI = \frac{100(X-0.31)}{0.17} \qquad (3.1)$$

Where.

$$X = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \quad (3.2)$$

Determination of non-protein nitrogen (NPN)

The NPN was calculated as the difference between the total crude protein nitrogen and the value of the precipitated true protein nitrogen according to a modified

method by Licitra, Hernandez, & Van-Soest (1996). 1 gr dry ground sample was mixed with 100 ml of distilled water for 30 min. Then, 20 ml 10% (W/V) trichloroacetic acid (TCA) was added and centrifuged at 10000 g for 20 min and the supernatant was retained to calculate the NPN value by subtracting the residual nitrogen from total nitrogen obtained by the Kjeldahl method.

Optimization by the Response Surface Methodology (RSM) for steam blanching

To select the optimal experimental conditions, an optimization process was performed using response surface methodology (RSM), which is a simple and effective statistical method (Sharif et al., 2014). Box-Behnken Design (BBD) was employed to optimize the parameters of the steaming process and access the interactions between factors based on the single-factor experiment results. The independent variables for the steam blanching of alfalfa leaves, namely the steaming time (min) (X_1), particle size (mm) (X_2) and time between harvesting to steaming process (hour) (X_3), were optimized using Box-Behnken Design (BBD) of the Response Surface Methodology (RSM). The four responses of interest were the polyphenol oxidase residual activity (PPO) (Y_1), the peroxidase residual activity (POD) (Y_2), the Browning Index (BI) of the alfalfa leaves (Y_3) and the non-protein nitrogen (NPN) (Y_4). The Design Expert 10.0.3 software was used to analyze the results by keeping two variables at their central levels, 3D plots of two factors. The related data of the independent and response variables were analyzed to obtain the second-order polynomial model as shown in the following equation: (3.3)

$$Y = b_0 \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i<j=1}^{n} b_{ij} X_i X_j \quad (3.3)$$

Where Y is the response variable to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables.

Extraction of protein from alfalfa

Protein from blanched and unblanched alfalfa leaves was extracted by the method of alkali and acid precipitation following Hojilla-Evangelista et al. (2016) with some modifications. Ground alfalfa leaves were first defatted with hexane at 25 ºC. The defatted leaves were dispersed in distilled water (10% w/v) and the pH was adjusted to 10.0 with 1N NaOH. The dispersion was stirred using a magnetic stirrer for 2h at 50 ºC. The insoluble non-protein fraction was separated from the protein extracted by filtration with a cloth, then this solution was centrifugated at 10000 x g for 20 min. The collected protein extract (supernatant) was acidified with 1N HCl to pH 3.5. The protein precipitate thus obtained was collected by centrifuging at 10000 x g for 20 min. To eliminate all soluble components, the pellet was washed with distilled water and the pH was adjusted to 7.0. Finally, the protein prepared was concentrated in ultrafiltration equipment with a peristaltic pump (Prominent, Heidelberg, Germany) using a 10 kDa MW cut-off (MWCO) ceramic membrane (Tami Industries, Nyon, France). The peristaltic pump was used to supply and recirculate the feed stream through the membrane with a transmembrane flow pressure range of 350 kPa. The filter was operated at a feed pressure of 4 bar,

membrane clearance of 5mm and rotor speed of 15 rev s^{-1}. The retentate was then freeze-dried with a lyophilizer (Cryodos-50/230V-50 Hz, Telstar, Madrid, Spain). Besides, protein recovery was calculated building on the protein content of the fresh alfalfa leaves.

Ultrasonic-assisted extraction procedure and purification of alfalfa saponin

To extract and purify saponin from alfalfa leaves, we followed the method used by Oleszek (1988). For this purpose, we defatted 50 g of dried powdered alfalfa leaves with hexane in a Soxhlet equipment for 2 h. Then it was diluted using different concentrations of ethanol (60-90%) at a solvent/raw material ratio of 5-15 mL/g using a KQ-2200DB ultrasonic cleaner bath (Kunshan Instrument Co., Ltd., Jiangsu, China) in a designed ultrasonic power (50–150 w), sonication time (1-3 h) and extraction temperature (50-80 ^{o}C). Extractions were carried out at different conditions. The experimental design matrix is presented in Table 1. After filtration, the solvent was removed in a rotary evaporator at 50 ^{o}C (Hei-VAP Platinum 3, Heidolph, Germany). Then it was diluted by using methanol to a final concentration of 35% (w/v). For purification, the solution was loaded to a previously pre-conditioned C18 column with 35% methanol (6 cm X 10 cm, 55 pm, Waters Associates, 50 g). To eliminate sugars and phenolics, the column was washed first with 200 mL of 35% methanol and then with another 200 mL of methanol to elute the saponins. Saponin was purified by removing methanol in the rotary evaporator and freeze dryer (Cryodos-50/230V, Telstar, Madrid, Spain) that yielded a yellowish powder that was weighted in each experiment.

Conventional method of extraction saponin

Heat-reflux extraction as a conventional method was carried out at 75 ^{o}C for 3 h on a mixture consisting of 50 g of defatted alfalfa leaves powder and 500 ml of 80% methanol. When the extraction was complete, the cooled extract was filtered on double layers of filter paper of Whatman 41 and the solvent was eliminated in a rotary evaporator (Kwon et al, 2003). Purification of alfalfa extract was performed by C18 column according to the previous section.

Determination of Saponin Content

Saponin content was determined using the method described by Navarro del Hierro et al. (2018). The dried methanolic extracts were prepared at 2 mg/mL in methanol. Aliquots of 0.5 mL were transferred to vials, followed by 0.5 mL 0.8% (w/v) vanillin solution in ethanol and 5 mL of sulphuric acid in water (72% v/v). A control sample using methanol was also prepared. Samples were vortexed and heated at 60 °C for 10 min. Vials were cooled in ice for 5 min and absorbance was measured at 520 nm using a spectrophotometer (s2150, Uv-visible, Unico, USA) against the control sample containing methanol. The total saponins content was obtained from a standard curve of oleanolic acid ranging from 100 to 1500 μg/mL. Results were expressed as g of total saponins per 100 g of samples.

Bioaccessibility of total saponins in vitro

The method of Navarro del Hierro et al. (2018) was used to determine the total saponin bioaccessibility in vitro. Firstly, the saponins extracts (100 mg) from each run and lecithin (10 mg) were mixed with the gastric solution (14 mL). The pH was

adjusted to 2.5 by $CaCl_2$ (6 mM) and NaCl (150 mM). The samples were incubated at 37 ^oC while shaking end-over-end for 10 min. For gastric digestion, we added 3 mL of the extract gastric enzymes including gastric lipase (170 mg) and gastric pepsin (15 mg) in gastric solution. Gastric digestion was done for 50 min at 37 ^oC. The pH at the end of gastric digestion was 4.5. then A biliary secretion solution including lecithin (0.1 g), bile salts (0.25 g), of 325mM $CaCl_2$ solution (0.5 mL), 3.25M NaCl solution (1.5 mL), and Tris maleate buffer 100mM (10 mL) was prepared at pH 7.5. The mixture was stirred (Polytron, PT 3000, Littau, Swiss) at 8000 rpm for 10 min. Then this biliary secretion was added immediately, and shaken in the incubator (orbital) for 1 min at 200 rpm and 37 °C. Next, we added the extract of fresh pancreatin (0.5 g of pancreatin was mixed in 3 mL of Tris maleate buffer, stirred for 10 min and centrifuged at 2688 g for 15 min). Intestinal digestion was continued for 60 min. The final pH of the mixture was set at 7. Control digestions were performed using the same procedure without saponins. Finally, the digestion medium was centrifuged at 37 ^oC and 2688 g for 40 min in a Beckman Coulter (Avanti 526XP, Mervue, USA) centrifuge. Then an upper aqueous phase (micellar phase) and a minor precipitated phase were obtained. Aliquots of both the digestion medium (before centrifugation) and the aqueous phase were taken. The saponin content of aqueous phases of digestion medium was determined spectrophotometrically. To do so, the same procedure described previously, with the difference that standard curves of quillaja bark saponin diluted in either the control digestion medium or the control aqueous phase (ranging from 100 to 1500 μg/mL) were employed to quantify the saponin content of the digestion medium or the aqueous phase, respectively. Absorbance was determined at 520 nm

against a reagent blank containing Tris maleate buffer. At the end, the total saponins' bioaccessibility was calculated:

$$\text{Bioaccessibility (\%)} = \frac{\text{Amount of saponins in aqueous phase}}{\text{Amount of saponins in digestion medium}} \times 100$$

Optimization of ultrasound-assisted extraction of alfalfa saponin by RSM

RSM, as an effective statistical method was to determine the optimal experimental conditions, an optimization extraction process was carried out using RSM. Box-Behnken Design (BBD) was used to optimize the extraction process parameters and access their interactions according to the results of single-factor experiment (Box et al, 1978; Hadidi, Ibarz, Conde, & Pagan, 2019). The independent variables for the ultrasonic-assisted extraction of total saponins from alfalfa and their bioaccessibility were studied at three different levels of 1 (low), 0 (medium), and +1 (high), namely the solvent/raw material ratio (mL/g) (X_1), time (hour) (X_2), temperature (^oC) (X_3), ultrasonic power (w) (X_4), and ethanol concentration (%) (X_5). The response variables were the extraction yield of saponin (%) (Y_1) and their bioaccessibility (%) (Y_2). The Design Expert 10.0.3 software was employed to analyze the results through maintaining two variables at their central levels and constructing 3D plots of two factors. Data analysis of the response and independent variables was done to achieve the second-order polynomial model as shown in Equation (3.4):

$$Y = b_0 \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i<j=1}^{n} b_{ij} X_i X_j \qquad (3.4)$$

Where, Y is the predicted response, X_i and X_j shows the independent variables, b_0 is the constant coefficient, b_i is the linear coefficient, b_{ij} represent the interaction coefficient, b_{ii} is the quadratic coefficients, and n shows the number of independent variables.

UV Irradiation of alfalfa saponin samples

800 mL of the methanol-water solution (20% v/v) to be irradiated were placed in a reactor made of methacrylate with dimensions 12 × 10.5 × 10 cm. so that the surface of the sample was 22.5 cm from the lamp and the solution height was 2.4 cm. The irradiation of each solution was carried out in an installation consisting of a black chamber containing the reactor and the lamp. The reactor was mixed using a magnetic stirrer. The working temperatures (20, 40, 60 and 80 ± 1 ^{o}C) were maintained in the reactor with a refrigerant coil. A mid-pressure mercury lamp Philips HPM 12 (Philips, The Netherlands) with a 460 W nominal power was employed, emitting in a wavelength range of 250-740 nm (Fig. 1). In order to ensure a constant UV emission, the lamp was turned on 10 min before placing the samples to be irradiated. Every sample was irradiated for 80 min and taking out 2 mL of solution every 10 min in order to analyze their total saponin content and, in some cases, to obtain the absorption spectrum. (Ibarz et al., 2014).

Absorption spectra

The absorption spectra were obtained for 100 mg/L alfalfa saponin solutions in a spectrophotometer (s2150, Uv-visible, Unico, USA), using a 1-cm wide quartz cell and scanning all the wavelengths from 250 to 750 nm.

Photo-degradation of alfalfa saponin kinetics

The present research intends to obtain a kinetic model defining the variation of alfalfa saponins with irradiation time. A mechanism of degradation in three stages is proposed (Ibarz, et al., 2015; Ibarz et al., 2014). Firstly, the saponin molecule absorbs energy from a ground state (saponin) into an excited one (saponin*). The excited molecule can decline to its fundamental state or degrade to form photo-products. Hence, the mechanism of degradation pf saponin can be described as:

$$\text{Saponin} \xrightarrow{P_{abs}} \text{Saponin*}$$

$$\text{Saponin*} \xrightarrow{k_D} \text{Saponin}$$

$$\text{Saponin*} \xrightarrow{k} \text{photo-products}$$

Where P_{abs} is the total spectral radiant power absorbed by the saponin solution per volume unit, the units being Einsteins (mols of photons) per unit of volume and time. Considering a pseudo steady state for the excited intermediate saponin*, the intensive reaction rate can be expressed as:

$$C_{s*} = \frac{1}{k_D + k} P_{abs} \qquad (3.5)$$

And

$$\frac{dCs}{dt} = -P_{abs} + k_D C_{s^*} = -\frac{k}{k_D+k} P_{abs} = -K_s P_{abs} \quad (3.6)$$

$$K_s = \frac{k}{k+k_D} \quad (3.7)$$

If the irradiation process is performed in a stirred reactor of volume *V*, working on a batch process, from the combination of the mass balance for saponin, the next equation is obtained:

$$r_s = -K_s P_{abs} \quad (3.8)$$

where r_s is the intensive reaction rate for saponin and K_s global quantum yield.
In order to solve equation 3.6, the relation between P_{abs} and saponin concentration needs to be known.

If P_{abs} depends linearly on the concentration of the absorbing substance, a straight line with a non-zero origin ordinate, leading to a pseudo first-order kinetic.

$$P_{abs} = a + bC_s \quad (3.9)$$

$$\ln\left(\frac{a+b\cdot C_S^0}{a+b\cdot C_S}\right)=m_S\cdot t$$

(3.10)

$$m_S=b\cdot K_S$$

(3.11)

$$C_S=-\frac{a}{b}+\left(\frac{a}{b}+C_S^0\right)\exp\left(-bK_S\cdot t\right)$$

(3.12)

Process of alfalfa protein extraction by UUAAIP

At first, steam blanching of fresh alfalfa leaves was applied according to the method of Hadidi et al. (2019) for inactive enzymes to avoid browning color and protein degrading in alfalfa leaves. Two hours after harvesting, fresh whole leaves of alfalfa (without chopping) were steamed by a laboratory steamer (Taurus SL, Lleida, Spain) in 4.4 min. The samples were dried for 4 h at 40 ºC in a vacuum oven dryer. The dried alfalfa leaves were ground to obtain fine powder and sieved

(50 mesh size). Then they were defatted with hexane at room temperature. The scheme for the process of alfalfa protein extraction (UUAAIP) is presented in Fig. 3.1. The defatted leaves were dispersed in distilled water at different solvent/solid ratios (1:30-1:50) in ultrasonic cleaner bath (Kunshan Instrument Co., Ltd., Jiangsu, China) and the pH was adjusted to (9-11) with 1 N NaOH. The extraction temperature was set in the range of 30-50 ºC by ultrasonic temperature control. The peristaltic pump (Prominent, Heidelberg, Germany) was employed to provide and recirculate the feed stream from the ultrasonic cleaner bath as a feed tank (retentate) through a 10 kDa MW cut-off (MWCO) ceramic membrane (Tami Industries, Nyon, France). The flow rate was adjusted at 8-16 L/h that was controlled by the feed pressure. When the extraction time (60-120 min) was completed, the retentate was centrifugated at 10000 x g for 20 min. The collected protein extract (supernatant) was acidified with 1 N HCl to pH 3.5. Centrifugation was used for protein precipitation at 10000 x g during 20 min. The pellets were washed with distilled water to remove all soluble components, and the pH was set at 7.0. The retentate was then freeze-dried with a lyophilizer (Cryodos-50/230V-50 Hz, Telstar, Madrid, Spain).

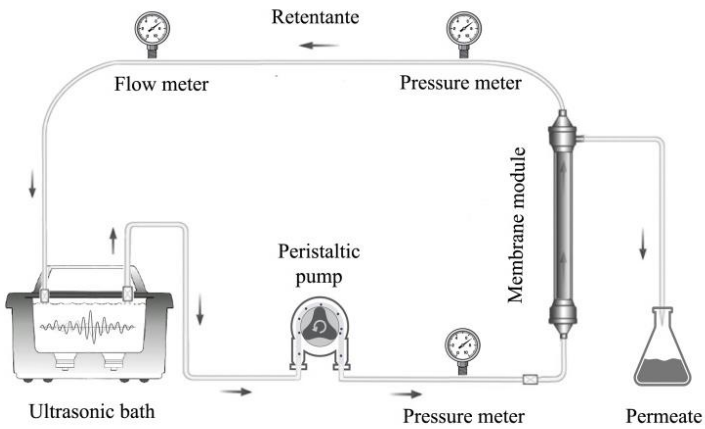

Fig. 3.1. Scheme of Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique.

Experimental design and optimization of UUAAIP process

The optimization extraction process was accomplished using RSM as an effective statistical method. Box-Behnken Design (BBD) was used to optimize the extraction process parameters and access their interactions according to the results of single-factor experiment. The independent variables for the ultrasonic-ultrafiltration-assisted alkaline extraction of alfalfa protein were studied at three different levels of 1 (low), 0 (medium), and +1 (high), namely the solvent/solid material ratio (mL/g) (X_1), pH (X_2), extraction temperature (^oC) (X_3), extraction time (min) (X_4), and flow rate (L/h) (X_5). The response variables were the extraction yield (%) (Y_1) = g extract/100 g of dried alfalfa powder, and the protein content (%) (Y_2) = g protein of extract/100 g of extract obtained from each run. The protein yield was

determined by Kjeldahl method. The Design Expert 10.0.3 software was employed to analyze the results through maintaining two variables at their central levels and constructing 3D plots of two factors. Data analysis of the response and independent variables was done to achieve the second-order polynomial model follows:

$$Y = b_0 \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i<j=1}^{n} b_{ij} X_i X_j \quad (3.13)$$

Where, Y is the predicted response, X_i and X_j show the independent variables, b_0 is the constant coefficient, b_i is the linear coefficient, b_{ij} represents the interaction coefficient, b_{ii} is the quadratic coefficient, and n shows the number of independent variables.

Conventional methods of extraction protein

Heat-coagulation extraction (HCE) was employed as conventional industrial process of alfalfa leaves' protein production. Freshly harvested alfalfa leaves were chopped and extruded. Then their juice was obtained by screw press. After preheating, the juice pH was adjusted to 7.5, a steam injection of 85 °C and a centrifugal sedimentation of 3000 rpm. Then the wet protein concentrate was dried by vacuum dryer (Zhang et al., 2017).

The second type of conventional method for alfalfa protein extraction was alkaline isoelectric precipitation extraction (AIPE). Briefly, dispersions of dried protein in water (5%, w/v) were adjusted to pH 9.0 at 25 °C, and then centrifuged (Avanti 5-26XP, Mervue, USA) at 4000 rpm for 10 min to achieve a supernatant. To deposit the protein, the extracts' pH was adjusted to 4 by adding 1 N HCl. The proteins

were obtained by centrifugation (4000 rpm, 15 min) after the supernatant was removed by decanting and freeze-dried. Finally, the protein powder was weighed and kept until next experiments (Du et al., 2018).

Total saponin content in protein samples

Total saponin content (TSC) of the protein samples was measured spectrophotometrically according to the method of Navarro del Hierro et al. (2018) and Oleszek (1988) with some modifications. For extraction of saponin, 10 g of the dired samples was diluted in ethanol (80%) at a solvent/raw material ratio of 10 mL/g using a KQ-2200DB ultrasonic cleaner bath (Kunshan Instrument Co., Ltd., Jiangsu, China) at ultrasonic power of 100 w for 2 h at 70 ^{o}C. After filtration, the solvent was removed in a rotary evaporator (Hei-VAP Platinum 3, Heidolph, Germany). Then it was diluted by using methanol to a final concentration of 35% (v/v). For purification and determining the saponin content, the solution was loaded to a previously pre-conditioned C18 column with 35 % methanol (6 cm X 10 cm, 55 pm, Waters Associates, 50 g). To eliminate sugars and phenolics, the column was washed first with 200 mL of 35% methanol and then with another 200 mL of methanol to elute the saponins. Saponins were purified by removing methanol in the rotary evaporator and lyophilizer (Cryodos-50/230V, Telstar, Madrid, Spain). Dried methanolic extracts (2 mg) were mixed in methanol (1 mL). Then 125 µL of the mixture was added to vials, followed by 125 µL of freshly prepared ethanolic vanillin (0.8%, w/v) and 1.25 mL of sulphuric acid (72% v/v). A control sample was also prepared by methanol. The samples were mixed by vortexing, and subsequently, heated at 60 °C for 10 min. The vials were cooled down in ice for 5

min, and the absorbance was determined at 545 nm by a spectrophotometer (s2150, Uv-visible, Unico, USA) against the control sample containing methanol. Total saponin content was acquired from a standard curve of oleanolic acid ranging from 100 to 1500 μg/mL. The results were reported as g of total saponins per 100 g of samples.

Total polyphenol content

Total polyphenol content (TPC) was determined using the Folin-Ciocalteu method described by Singleton, Orthofer, & Lamuela-Raventós (1999) with some modifications. Firstly, 2 g of each sample was homogenized (IKA T-18, Bonn, Germany) with 20 mL of water/methanol (1:1 v/v) for 2 min and, subsequently centrifuged for 10 min at 6700 x g at 20 °C. The supernatant was separated and used as the extract. Then 0.1 mL of the extract was mixed with 0.5 mL of Folin–Ciocalteu's reagent, 1.5 mL of 20% sodium carbonate (w/v) and 7.9 mL distilled water. The mixture was kept in the dark for 2 h at 25 °C. After incubation, the absorbance was measured at 750 nm. A control sample was also prepared. After incubation at room temperature for 1 h in darkness, the absorbance of the mixture was read at 760 nm using water/methanol mixture as blanks. The results were expressed as mg of gallic acid equivalents (GAE) per g of sample based on a calibration curve using a gallic acid standard.

Color analysis

The color of samples was determined by direct recording in a Konica Minolta colorimeter (Chroma Meter CR 410, Tokyo, Japan) using the three-dimensional CIE L*a*b* system. It was calibrated with a white ceramic tile (L* = 97.4, a* =

0.22, b* = 0.50). L* value (0 = black and 100 = white), a* value (-100 = green and +100 = red) and b* value (-100 = blue and +100 = yellow) were carried out three times for each sample.

Molecular weight distribution

An Agilent 2100 electrophoresis Bioanalyzer® (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to apparent molecular weight distribution. The protocol of protein electrophoresis was measured according to the methodology of manufacturer. An Agilent protein 230 kit (Agilent Technologies, Waldbronn, Germany) was employed as molecular weight standard. The kit includes nine standard proteins with molecular weights of 4.5; 7.0; 15.0; 28.0; 46.0; 63.0; 95.0; 150.0 and 240.0 kDa. It was used for measuring the average molecular weight of the samples considering the protein's polydispersity.

Solubility

Protein solubility was determined based on the method reported by Adebiyi & Aluko (2011). 1% (w/v) Protein solutions were prepared in phosphate buffers (0.01 M) from pH 2.0 to 10.0. The solutions were mixed for 60 min at 25 °C in an orbital mixer incubator (Ratek, VIC, Australia), the mixtures were centrifuged at 10,000g for 20 min. For sample controls, a 1% (w/v) protein solution was prepared in 0.1 N NaOH and centrifuged. The protein content (%) of the sample (P_1) and control (P_2) was determined based on the Bradford's method using bovine serum albumin (BSA) as a standard.

Protein solubility (%) = $(P_1 / P_2) \times 100$

Water-holding capacity (WHC)

WHC of the protein samples was measured based on the method described by Chandi & Sogi (2007). For this purpose, 1 g (W) of protein was mixed with 20 mL (W_1) distilled water. Next, the solution was kept still for 80 min and then centrifuged. The free water was separated, and its volume (W_2) was recorded. Finally, WHC was calculated as follows:

WHC (g water/g) = (W_1 - W_2) / W

Oil-banding capacity (OBC)

OBC of the samples was assessed as according to the modified method of Chandi & Sogi (2007). Briefly, the protein sample (1 g) (W) was dissolved in soybean oil (10 g) (W_1), and stirred evenly with static set 30 min. The mixture was then centrifuged at 4000g for 20 min after mixing every 5 min. Free oil was removed, and the quantity of free oil (W_2) was recorded. OBC was expressed as the weight of oil adsorbed per gram of sample, and measured by the following equation:

OBC (g oil/g) = (W_1 - W_2) / W

Emulsifying activity index (EAI) and emulsion stability (ES)

The emulsifying properties of protein samples were conducted based on Pearce & Kinsella's (1978) method with minor modification. 30 mL of the suspension at protein concentrations (1% w/v) was added to 10 mL of soybean oil and homogenized for 3 min at 20 °C (IKA T-18, Bonn, Germany). Then 250 µL of the emulsion fraction was mixed to 25 mL of SDS solution (0.1% w/v) immediately.

By using a spectrophotometer (s2150, Uv-visible, Unico, USA), the absorbance of the solution was recorded at 500 nm (A_0). The absorbance was determined again at 10 min after emulsion formation (A_0). EAI and ES were calculated by the following equations, respectively:

EAI (m^2/g) = $2 \times 2.303 \times A_0 / 0.25$

ES (min) = $A_0 / A_{10} - A_0 \times 10$

Foam capacity (FC) and foam stability (FS)

FC and FS of protein samples were determined as defined by Wang et al. (2010) with some modification. Ten milliliters of the protein solution at 0.5% (w/v) was homogenized using an Ultra-turrax homogenizer (IKA T-18, Bonn, Germany) to merge the air at 25 °C for 2 min. The samples were, subsequently, transmitted into a graduated cylinder instantly. After whipping, the total volume was determined immediately. The expansion of foam was defined as percentage of volume increment after homogenization at 0 min and the foam stability was measured as the volume of foam remaining after 30 min as follows:

FC (%) = $[(V_0 – V_b) / V_b] \times 100$

FS (%) = $[(V_{30} – V_0) / V_0] \times 100$

Where, V_b is the total volume before homogenization, V_0 is the volume exactly after homogenization (mL), and V_{30} is the volume after 30min keeping at room temperature

4. Results and Discussion

Optimization of extraction of alfalfa leaf protein for human consumption

4.1.

A first approach to prevent to post-harvest undesirable characteristics of alfalfa leaves

According to the different reasons that have been in the introduction the use of plant protein concentrates derived from alfalfa in human food is limited by their negative quality and sensory properties (Wang and Kinsella, 1976), especially due its brown color, which has a strong effect on consumer acceptance and purchasing decisions. Another major problem is protein degradation resulting in lower quality and lesser recovery. Postharvest protein degradation in ensiled forage crops results in the conversion of true protein into different forms of non-protein nitrogen (NPN), such as oligopeptides, free amino acids (AA) and ammonia amino acids and peptides, resulting in a decrease in the performance of the protein extracted with human consumption and leading to economic losses in cattle feeding, which must supplement rations with other sources of true protein. There is still no satisfactory or feasible means of inhibiting proteolysis in ensiling alfalfa because the most rapid proteolysis occurs within 24–48 h of ensiling.

4.1.1. Optimization of the steam blanching on enzymatic activity, color and protein degradation of alfalfa

Steam blanching is an essential operation for processing many vegetables and fruit. It is an excellent tool for inactivating enzymes. It contributes to the inactivation of polyphenol oxidase (PPO), peroxidase (POD), and also affects other quality attributes of products, especially by improving color (Xiao, et al., 2017). Due to the importance of preserving the color of the raw material before any processing, the inactivation of naturally-occurring enzymes, including PPO and POD, is the key. The proteolysis of ensiled forage and changing true protein to non-protein nitrogen also results mainly from plant proteolytic enzymes (Ohshima & McDonald, 1978). Table 4.1.1 shows the results obtained from 15 experimental runs following the BBD design. The statistical parameters, such as coefficient (R^2) and p value and lack of fit values, were measured with the analysis of variance (ANOVA). The results of the ANOVA and lack of fit tests with correlation coefficients are shown in (Table 4.1.2). The results of the correlation coefficient (R^2) values were 0.997, 0.999, 0.984 and 0.997 for Y_1, Y_2, Y_3 and Y_4 respectively. R^2 value was used to judge the adequacy of the models. The results showed that the models developed for responses were significant. There was no lack of fit in any of equations ($p<0.05$). The data normality, which was checked by using a normal plot of the residuals and the difference between the Experimental values and the regression values predicted, showed that the experimental points were normally distributed around a straight line, so the assumption of normality was satisfied. The plot of the residuals versus the predicted values showed that the residuals were scattered randomly around zero with no outliers or unexpected errors, as a result, it indicated

that all the values were within the accepted range required to validate the model (plot not shown). This means that the models are suitable for representing the relationship between the Independent and responses variable.

The results of the ANOVA indicated that the contribution of the quadratic polynomial model was significant at each of the responses analyzed. The fitted quadratic models for PPO residual activity (Y_1), POD residual activity (Y_2), Browning Index (Y_3) and non-protein nitrogen (NPN) (Y_4) in coded variables are given in Equations [4.1.1- 4.1.4], respectively.

$$Y_1 = +17.70 - 27.84X_1 - 4.54X_2 + 5.23X_3 + 3.10X_1X_2 - 2.02X_1X_3 + 1.68X_2X_3 + 10.05x_1^2 + 0.20x_2^2 + 2.78x_3^2 \quad (4.1.1)$$

$$Y_2 = +3.37 - 17.40X_1 - 4.34X_2 + 1.91X_3 + 3.70X_1X_2 - 2.25X_1X_3 - 0.32X_2X_3 + 13.05x_1^2 + 1.03x_2^2 + 1.38x_3^2 \quad (4.1.2)$$

$$Y_3 = +118.37 - 4.64X_1 - 1.36X_2 + 4.55X_3 + 0.15X_1X_2 - 0.33X_1X_3 + 0.52X_2X_3 + 13.34x_1^2 - 2.56x_2^2 + 2.13x_3^2 \quad (4.1.3)$$

$$Y_4 = +256.67 - 7.38X_1 - 5.63X_2 + 100.75X_3 + 0.25X_1X_2 - 1.00X_1X_3 - 5.00X_2X_3 - 6.71x_1^2 - 0.21x_2^2 + 15.04x_3^2 \quad (4.1.4)$$

Table 4.1.1.

Matrix of experimental Box-Behnken design (BBD) for the steaming time (min), particle size (mm) and harvesting to steaming time (hour) for optimization of the steam blanching conditions of alfalfa.

Run	Independent variable						Response variable			
	Steaming time X_1 (min)		Particle size X_2 (mm)		Harvesting to steaming X3 (hour)		Residual activity PPO Y_1 (%)	Residual activity POD Y_2 (%)	Browning Index Y_3 (BI Unit)	NPN Y_4 (g^{-1}kg TN)
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Experimental value			
1	0	4	0	10.5	0	13	17.7	3.5	118.6	256
2	1	6	1	20	0	13	0	0	123.2	239
3	0	4	0	10.5	0	13	18.2	3.4	118.1	253
4	0	4	0	1	-1	2	21.5	8.4	108.5	171
5	1	6	1	10.5	1	24	4.4	0	127.8	355
6	-1	2	-1	1	0	13	62.1	42.3	135.4	261
7	-1	2	0	10.5	-1	2	52.6	31.1	130.7	173
8	0	4	1	20	1	24	23.2	2.5	117.8	362
9	0	4	1	20	-1	2	7.4	0	106.8	164
10	0	4	0	10.5	0	13	17.2	3.2	118.4	261
11	1	6	-1	1	0	13	1.2	0.9	125.6	244
12	1	6	0	10.5	-1	2	0	0	122.3	162
13	0	4	-1	1	1	24	30.6	12.2	121.6	389
14	-1	2	0	10.5	1	24	65.1	40.1	137.5	370
15	-1	2	1	20	0	13	48.5	26.6	132.4	255

Effect of steaming on PPO inactivation

The response surface plot showing the effects of the steaming time (X_1), the particle size of the alfalfa leaves (X_2), and time between harvesting and steaming (X_3) on the residual activity of the polyphenol oxidase (PPO) are presented in Fig. 4.1.1 The percentage of the PPO residual activity was measured by the (U/U_0) ×100, where U is the enzyme activity after blanching and U_0 is the initial enzyme activity of the sample without treatment. It was observed from the ANOVA data that the model was significant ($P<0.05$) and the lack of fit was not significant (Table 4.1.2). All linear, quadratic and interaction variables were significant except the quadratic effect of particle size and interaction between particle size and harvesting to steaming time (X_2X_3) ($P<0.05$).

All factors influenced the PPO residual activity, especially the steaming time and the minimum time between harvesting and steaming and the low particle size of the alfalfa leaves. The residual activity of the polyphenol oxidase decreased sharply when the steaming time increased especially during the first minutes. PPO showed a rapid decrease from 1 min to 3 min and slowly from 3 min to 7 min in steam blanching (Ndiaye, Xu, & Wang, 2009). The effect of steaming time (X_1) and the time between harvesting and steaming (X_3) on the PPO residual were highly significant ($p<0.05$). With an increase in time between harvesting and steaming, the polyphenol oxidase residual activity decreased. As a comparison, the PPO activities in fresh tea leaves were measured at three-hour intervals from harvesting until the process of steam blanching, and the increase in the PPO activity in the tea leaves was found to be 13% and 33% after 3 h and 6 h, respectively (Ozturk et al., 2016).

Table 4.1.2.

Regression coefficients of the second-order polynomial function and the coefficients of determination (R^2).

Variable	Residual activity PPO Y_1		Residual activity POD Y_2		Browning Index (BI) Y_3		NPN Y_4	
	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
β_0	17.7	-	3.36667	-	118.367	-	256.66	-
X_1	-27.8375	< 0.0001	-17.4	< 0.0001	-4.6375	0.0009	-7.375	0.0195
X_2	-4.5375	0.0008	-4.3375	< 0.0001	-1.3625	0.0954	-5.625	0.0491
X_3	5.225	0.0004	1.9125	0.0006	4.55	0.0010	100.75	< 0.0001
X_1X_2	3.1	0.0180	3.7	0.0001	0.15	0.8793	0.25	0.9384
X_1X_3	-2.025	0.0431	-2.25	0.0014	-0.325	0.7433	-1	0.7584
X_2X_3	1.675	0.1201	-0.325	0.4018	-0.525	0.6002	-5	0.1652
X_1^2	10.05	0.0001	13.0542	< 0.0001	13.3417	< 0.001	-6.70833	0.0904
X_2^2	0.2	0.8384	1.02917	0.0386	-2.55833	0.0472	-0.20833	0.9507
X_3^2	2.775	0.0308	1.37917	0.0135	-2.13333	0.0808	15.0417	0.0054
Lack of fit	0.041		0.022		0.010		0.024	
R^2	0.9977		0.9992		0.9841		0.9970	

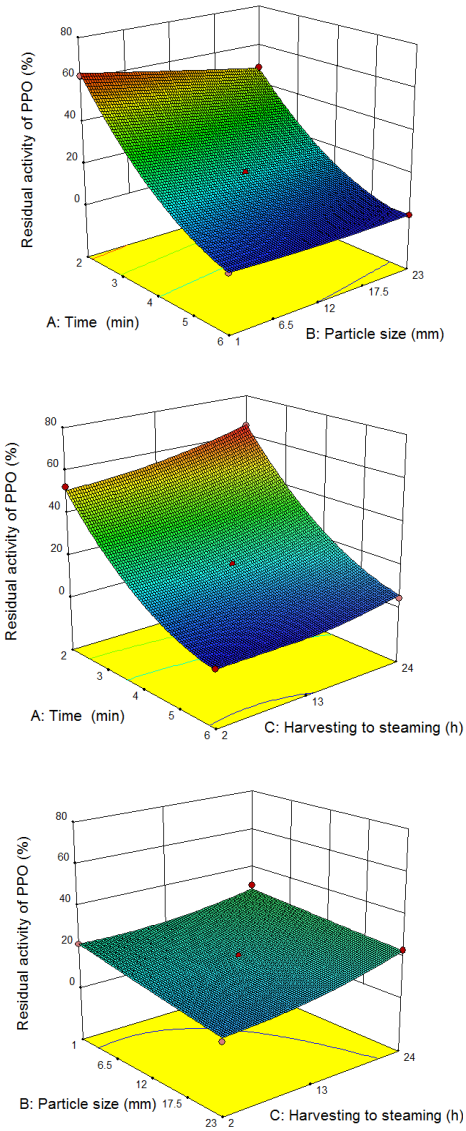

Fig. 4.1.1. Response surface 3D plots showing the interactive effect of blanching variables on the residual activity of PPO.

Effect of steaming on POD inactivation

Fig. 4.1.2 shows the effect of steaming time (X_1), the particle size of alfalfa leaves (X_2), and time between harvesting and steaming (X_3) on the residual activity of peroxidase (POD) (Y_2). The residual activity of POD was measured in a similar way to the PPO residual activity. The ANOVA data showed that the model is significant, and the lack of fit is not significant (Table 4.1.2). The high values of the coefficient (R^2= 0.99) also showed that the model is strongly significant. A lack of a fit value of 0.002 is found to be insignificant relative to the pure error. The response model for the level of the POD residual activity has a similar shape to the response model of the PPO residual activity. Except for interaction between the particle size and harvesting to steaming time (X_2X_3), all linear, quadratic and interaction variables were significant ($P < 0.05$). Steam blanching of mango slices for 5 min inactivated POD completely, while PPO activity remained at 1.71% (Ndiaye et al., 2009). Lin et al. (2012) reported thermal resistance of PPO higher than POD in blancing process of Rabdosia serra (Maxim.) leaf.

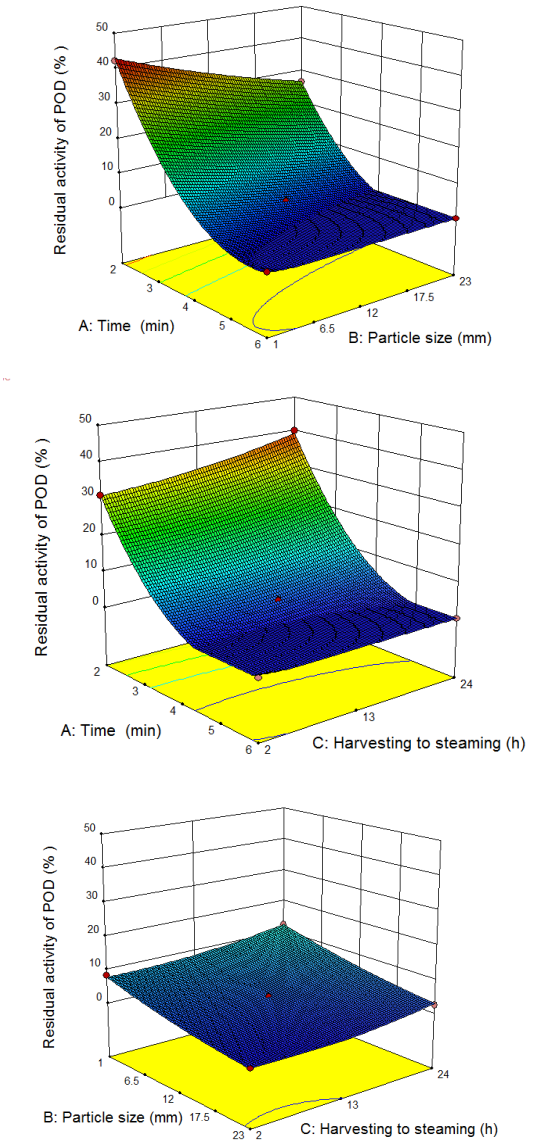

Fig. 4.1.2. Response surface 3D plots showing the interactive effect of blanching variables on the residual activity of POD.

Effect of steaming on browning

The effects of steaming time (X_1), particle size of alfalfa leaves (X_2), and time between harvesting and steaming (X_3) on the Browning Index is depicted by the response surface plots (Fig. 4.1.3). Table 4.1.2 shows that the linear and quadratic effect of the two independent variables (steaming time (X_1) and time between harvesting and steaming (X_3) on the Browning Index (Y_3) was significant ($P < 0.05$). The high value of the correlation coefficient ($R^2 = 0.98$) indicates the suitability of the model for predicting the Browning Index (Y_3) of alfalfa leaves. At first, when the steaming time increased, the Browning Index decreased to 4 minutes, and then, as the steaming time rose, the Browning Index went up again in all treatments. These effects could be due to the PPO inactivation during the initial minutes. Later, a non-enzymatic browning of Maillard appears to be caused by the protein degradation products and sugars due to the high steam temperature. Ndiaye et al. (2009) observed that the Browning Index value of mango slices showed no variation after 7 min of steam-blanching treatment, which is in strong correlation with the results found for the PPO activity by this author because at that time, PPO and POD were completely inactivated. A perfect correlation with carrot browning has also been found (Zhang, Tan, McKay, & Yan, 2005).

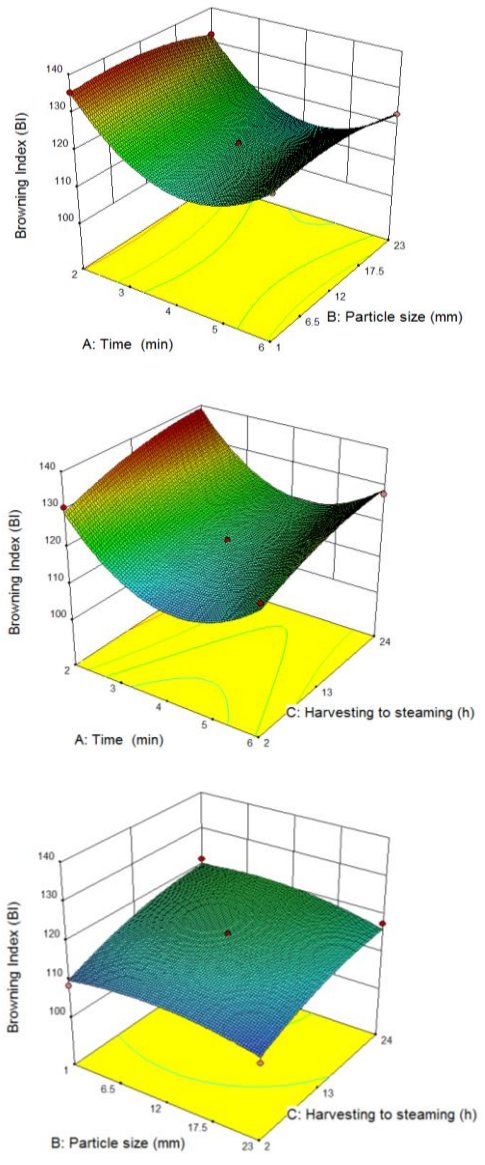

Fig. 4.1.3. Response surface 3D plots showing the interactive effect of blanching variables on the Browning Index.

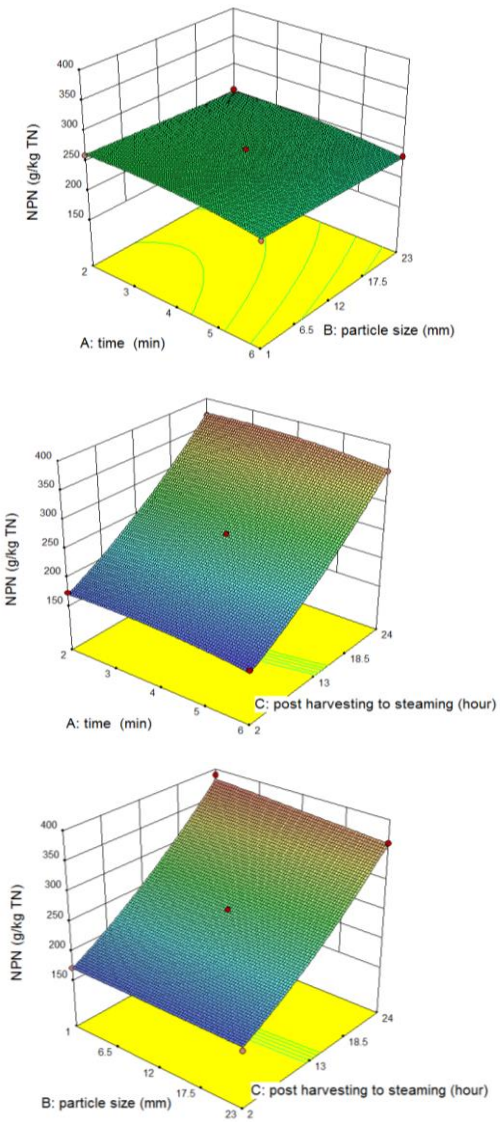

Fig. 4.1.4. Response surface 3D plots showing the interactive effect of blanching variables on the non-protein nitrogen.

Effect of steaming on non-protein nitrogen (NPN)

The non-protein nitrogen (Y_4) values obtained under different steam blanching conditions specified by the BBD matrix are presented in Table 4.1.2. The lowest Y_4 value (162 g kg^{-1} of total N) was obtained in the experimental run 12 under the following steam blanching conditions: 6 min steaming process, alfalfa leaf size of 10.5 mm and minimum time between harvesting and steaming (2 h). As shown in Fig. 4.1.4, when the time between harvesting and steaming increased from 2 to 24 h, the non-protein nitrogen increased sharply in alfalfa leaf.

4.1.2. Effect of the optimization of the steam blanching condition during storage

Effect of optimization of the steam blanching conditions on BI

Fig. 4.1.5 shows the effect on the Browning Index of 60 days of storage at the room temperature. It is observed that the BI index values significantly increased in the untreated alfalfa during storage especially in the first day after harvesting compared to the values obtained from the leaves blanched with steam.

In the process of the enzymatic browning removal by the blanching process, it is difficult to establish a reaction mechanism and to obtain a kinetic model that adequately describes the whole process. by the analysis of the data obtained of the evolution of BI over time, can be considered two steps, the first one, the color formation and the second one, the color degradation by the steam or during the storage. Both stages have been described in browning formation and degradation processes.

$$\text{Phenols} + O_2 + \text{PPO} \xrightarrow{k_0} \text{Brown pigments}$$

$$\text{Brown pigments} \xrightarrow{k_1} \text{non-colored pigments}$$

Typically, browning reactions of food color formation follow the zero-order kinetics (Labuza, 1972). First-order kinetics has been suggested for the destruction of natural plant pigments (Ibarz, Pagán, & Garza, 2000).

Thus:

$$\frac{d\,BI}{dt} = k_0 - k_1\,BI \qquad (4.1.5)$$

Where: BI is the Browning Index

k_0 is the zero-order kinetic constant

k_1 is the first-order kinetic constant

Integrating for initial condition: t =0 BI = BI_0

$$BI = K - (K - BI_0)\exp(-k_1 t) \qquad (4.1.6)$$

Where: $K = \frac{k_0}{k_1}$ and k_1 is the first-order kinetic constant

The values shown in Fig. 4.1.5 have been fitted to this equation. The values of the constants of equation 4.1.6 are also shown in Table 4.1.3. Taking into account that the kinetic constant values (K) were much higher than 1 (K>1) so, the browning formation predominates over degradation ($k_0>k_1$). It can be observed from the table a notable decrease in the value of the kinetic constant k_1 of the blanched samples in comparison with the untreated ones. Then, the reaction rate has decreased to at 5.95 % from the initial reaction rate after blanching. Therefore, treatment of the samples by blanching can be considered effective to prevent browning of the alfalfa samples during storage and therefore to extract protein with the least possible coloration.

Effect of optimization of the steam blanching conditions on NPN during storage

Fig. 4.1.6 shows the effect on the NPN concentration of 60 days of storage at the room temperature and it can be observed the decrease of these values on the untreated samples by steam blanching at optimum conditions. Numerous studies have been conducted into the contribution of microbe-originated enzymes and plant enzymes to the formation of NPN during storage. The dominant role that plant enzymes play in proteolysis during ensiling has been proven by a classical method of sterilizing forage on ensiling (Heron, 1986) or by using specific inhibitors of endo- and exopeptidases (Guo et al., 2011; Tao, Zhou, Guo, Long, & Zhu, 2011). Plant enzyme inactivation before ensiling further validated the leading role of plant enzymes in proteolysis and could effectively reduce both the extensive proteolysis and lipolysis which occur in ensiled alfalfa. The NPN content of alfalfa without any

treatment increased significantly from 167 to 605 g kg^{-1} of total N at 60 days, but only increased by about 100 g kg^{-1} of total N under optimum blanching condition after 60 days storage. Ding, Long, & Guo (2003) reported that the NPN content of fresh alfalfa went up from 155 g kg^{-1} of total N to 733 during 60-day ensiling. The alfalfa forage was autoclaved to inactivate plant enzymes so the NPN content in the autoclave-treated silage only increased by about 50 g kg^{-1} of total N.

In a similar way to that described in the process of elimination of browning by steam blanching, in the case of the elimination of the proteolytic activity of alfalfa using the same process, from the analysis of the data obtained, it is suggested that the process can also be carried out in two stages: the first the formation of NPN products from the degradation of proteins by the endogenous proteases of alfalfa and a second stage of NPN degradation mainly to amino acids by the steam at high temperature.

$$\text{Proteins + proteolytic enzymes + } H_2O \xrightarrow{k_0} \text{NPN}$$

$$\text{NPN} \xrightarrow{k_1} \text{NPN degradation products}$$

The equations proposed describing this kinetics are

$$\frac{d\,NPN}{dt} = k_0 - k_1\,NPN \qquad (4.1.7)$$

Where: k_0 is the zero-order kinetic constant, k_1 is the first-order kinetic constant

Integrating for initial condition: t =0 NPN = NPN_0

$$NPN = K - (K - NPN_0)\exp(-k_1 t) \quad (4.1.8)$$

Where: $K = \frac{k_0}{k_1}$ and k_1 is the first-order kinetic constant

The values shown in Fig. 4.1.6 have been fitted to this equation. The values of the constants of equation 4.1.8 are also shown in Table 4.1.3 and it can be observed a decrease in the value of the kinetic constant (k_1) of the blanched samples of alfalfa compared to the untreated ones. In addition, kinetic constant value (K) of degradation protein and NPN production were much higher than 1 (K>1) so, the NPN formation predominates ($k_0 > k_1$).

Blanching reduced the initial reaction rate to at 7.31%. That is why is necessary to perform the blanching treatment to prevent the protein degradation on alfalfa leaves during storage and therefore to increase efficiency in the process of protein extraction from de plant matrix.

Table 4.1.3

Kinetic parameters for Browning index and NPN of alfalfa during storage.

Factors	Variable	$K=k_0/k_1$	k_1 (h^{-1})	R^2
BI	Non-treated	163.1	0.0386	0.985
	Treated on optimum condition	123.6	0.0023	0.944
NPN	Non-treated	580.4	0.0238	0.912
	Treated on optimum condition	255.8	0.0017	0.876

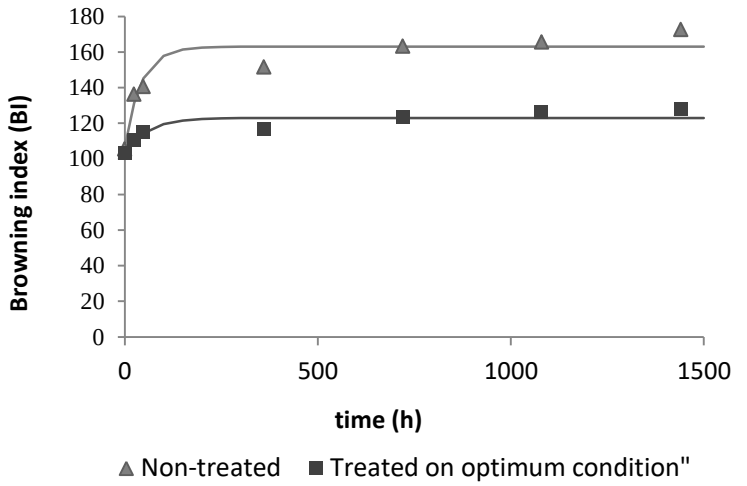

Fig. 4.1.5. Effect of optimized conditions on the Browning Index of alfalfa during storage.

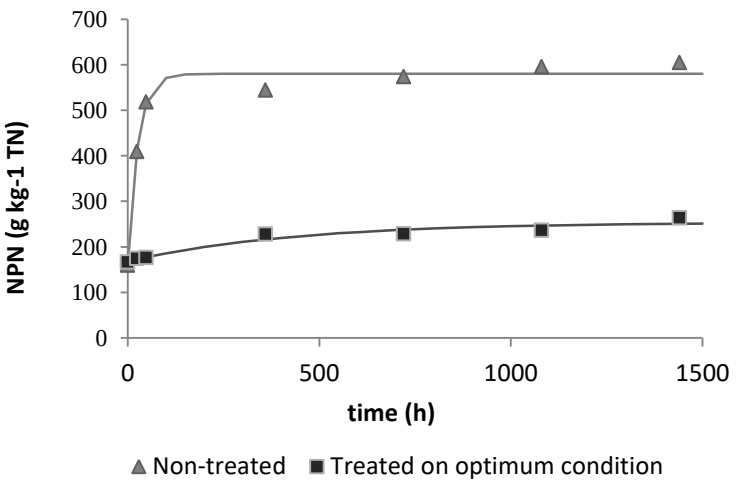

Fig. 4.1.6. Effect of optimized conditions on the non-protein nitrogen of alfalfa during storage.

4.1.3. Effect of the optimization of the steam blanching condition on alfalfa leaf protein concentrate

Table 4.1.4 shows these effects. The protein content of alfalfa leaf concentrate without the steam-blanching treatment contained 586 g kg^{-1} DB. This protein content is comparable to the one reported by Hojilla-Evangelista et al. (2016) (600 g kg^{-1} DB) and Wang & Kinsella (1976) (580–670 g kg^{-1} DB) and is lower than the amount we detected in alfalfa leaf protein concentrate treated under the optimum treatment condition (702 g/kg DB). The protein recovered from the alfalfa leaves in our extraction without any treatment and with treatment under optimum conditions was 31.3 and 39.8%, respectively. This loss of protein content caused by the degradation of peptides and free amino acids by plant enzymes during ensiling, especially in the first hours after harvesting (Guo et al., 2007; Jones, Hatfield, & Muck, 1995) is why the increase in the molecular weight of the extracted protein from 68 to 96 kDa can be observed when the leaves were blanched. The protein recovery under the best condition (39.8%) was greater than the 23–28% reported by Wang & Kinsella (1976) and 36% Hojilla-Evangelista et al. (2016).

By blanching alfalfa under optimum conditions, the lightness of the protein concentrate increased sharply compared to the untreated alfalfa leaves. On the other hand, changes in the a* and b* parameters indicate that the color turned lighter. This was due to the decrease in melanin because of the inactivation of the PPO and POD enzymes (Toivonen & Brummell, 2008).

Table 4.1.4.

Effect of blanching on optimum conditions on the color parameters of alfalfa protein concentrate.

alfalfa Samples	Protein content (g^{-1}kg DB)	Protein recovery (%)	Color			Molecular Weight (kDa)
			L*	a*	b*	
Non-treated	586±13	31.3±0.8	58.9±2.3	-2.6±0.2	27.2±1.5	68±3.2
Treated on optimum condition	702±24	39.8±1.1	63.4±1.6	-1.2±0.1	26.3±0.9	96±5.8

4.2.

Alfalfa saponins extraction by ultrasound-assisted and degradation by UV-irradiation

A major problem of alfalfa is the bitter taste as a negative sensory factor of alfalfa. A large variety of ingredients are responsible for manufacturing bitter tastes in foodstuffs such as amino acids, saponins, polyphenols and peptides. Previous studies have shown that saponins are responsible for the bitterness in alfalfa leaves. Additionally, they are known as anti-nutrients for having hemolytic and inhibitory activity and effect the muscosal cells' permeability in the small intestine.

4.2.1. Optimization and kinetic study of the ultrasonic-assisted extraction of total saponins from alfalfa (*Medicago sativa*) and its bioaccessibility using the response surface methodology

Model fitting and statistical analysis

The results of 43 experimental runs using the Box-Behnken Design along with the measured and predicted values for both responses (Y_1 and Y_2) for each trial in the experimental design are shown in Table 4.2.1. Also the ANOVA results are presented in Table 4.2.2. The coefficient of correlation (R^2) values of Y_1 and Y_2 were 0.980 and 0.905, respectively. R^2 value was used to judge the models' adequacy. It was revealed that the developed models for responses were significant ($p<0.0001$). The values of adj-R^2 (0.9626-0.8804) suggested that the total variation

of 96% for the yield of total saponins and 88% for their bioaccessibility were attributed to the independent variables. Meanwhile, low values of CV (2.48-2.64%) implies the high precision and good reliability of the actual values.

To analyze the independent variable' effect on the extraction yield and bioaccessibility of total saponins, the following second-order polynomial equation was generated:

$$Y_1 = +1.55 + 0.09X_1 + 0.17X_2 + 0.08X_3 + 0.08X_4 + 0.04X_5 + 0.08X_2X_3 - 0.14X_3X_5 - 0.16X_1^2 - 0.15X_2^2 - 0.16X_3^2 - 0.26X_4^2 - 0.33X_5^2 \quad (4.2.1)$$

$$Y_2 = +16.44 - 0.24X_1 + 0.81X_2 + 0.78X_3 + 1.40X_5 - 0.93X_1X_5 - 0.53X_2^2 + 1.07X_5^2 \quad (4.2.2)$$

It is usually necessary to check the fitted model for ensuring that it provides an adequate approximation to the real system. The residuals from the least squares are crucial for judging a model's adequacy. By constructing the normal probability plots of the residuals, a normality assumption check was done (Fig. 4.2.1, 4.2.2). The normality assumptions were satisfied as the residual plots approximated along a straight line. Figures 4.2.3 and 4.2.4 illustrate the plots of residuals vs. the predicted responses. Generally, the residuals scatter on the display randomly, implying that the variances of these figures' original observation are constant for all values of Y_1 and Y_2. The plots are satisfactory; therefore, it can be concluded that the empirical models are adequate for describing the extraction yield and bioaccessibility of total saponin by response surface.

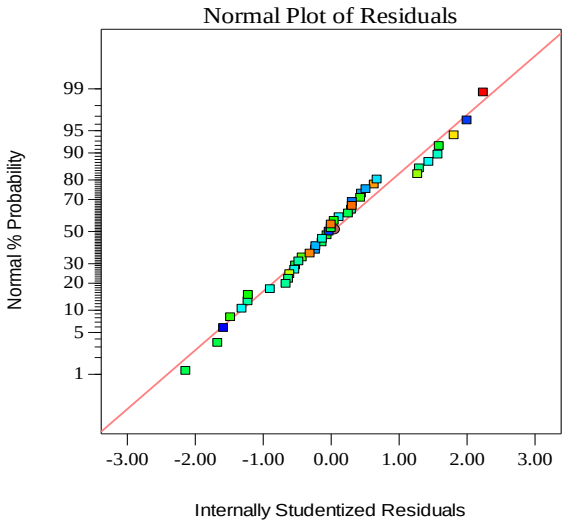

Fig. 4.2.1. Plot of Normal probability of internally studentized residuals (Y_1).

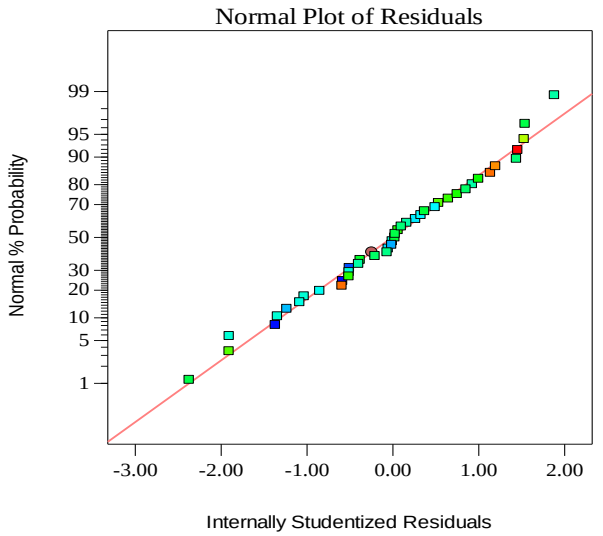

Fig. 4.2.2. Plot of Normal probability of internally studentized residuals (Y_2).

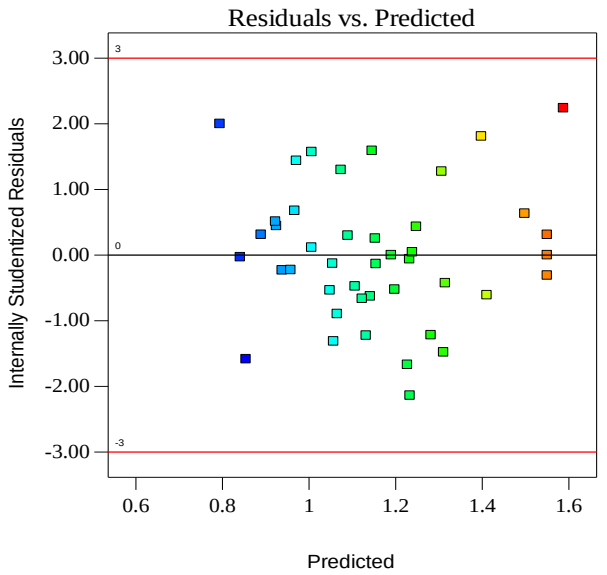

Fig. 4.2.3. Plot of internally studentized residuals vs. predicted response (Y_1).

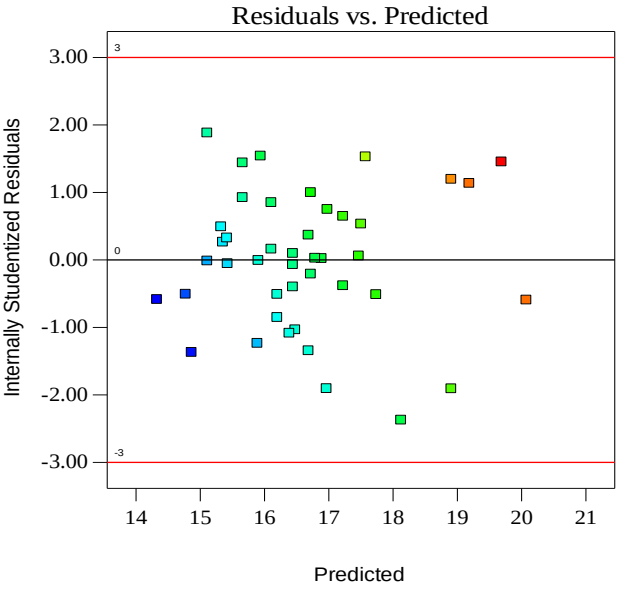

Fig. 4.2.4. Plot of internally studentized residuals vs. predicted response (Y_2).

Influence of process variables on the extraction yield of total saponin

P-value is used to check the significance of each coefficient. Smaller the p-value shows the more significance of the corresponding coefficient. Data shown in Table 4.2.2 indicate that the linear effects of all values were significant and positive with linear effect to the increase in total saponin yield (Y_1). All liner and quadratic and two interactions (time with temperature and temperature with ethanol concentration) terms affected the extracted saponin yield significantly. The response surface plots that show the effect of solvent/raw material ratio (X_1), extraction time (X_2), temperature (X_3), ultrasonic power (X_4) and ethanol concentration (X_5) on the extraction yield of total saponin (Y_1) are illustrated in Fig. 4.2.5.

Table 4.2.1.

Box–Behnken design matrix with coded variables and measured and predicted values.

Run	Factors					Responses			
	Solvent/raw material ratio X_1 (mL/g)	Time X_2 (h)	Temperature X_3 (^oC)	Power X_4 (W)	Ethanol conc X_5 (%)	Total saponins yield Y_1 (%)		Total saponins bioaccessibility Y_2 (%)	
						Measured	Predicted	Measured	Predicted
1	10	2	65	50	90	0.94	0.93	17.80	18.66
2	10	3	65	150	75	1.46	1.44	16.60	16.81
3	15	1	65	100	75	1.15	1.13	14.10	14.21
4	15	3	65	100	75	1.52	1.52	15.90	16.53
5	10	3	80	100	75	1.65	1.59	17.80	17.62
6	10	1	65	100	60	0.80	0.84	14.50	14.79
7	5	2	65	150	75	1.10	1.10	15.90	16.18
8	5	1	65	100	75	1.02	0.99	15.50	15.40
9	10	1	65	50	75	0.90	0.93	16.20	15.51

10	5	2	50	100	75	1.03	1.05	15.90	15.83
11	10	2	65	100	75	1.56	1.55	16.40	16.37
12	5	2	65	50	75	0.95	0.98	16.90	16.90
13	10	3	50	100	75	1.24	1.24	16.80	16.15
14	10	2	50	150	75	1.09	1.14	16.20	15.90
15	10	1	80	100	75	1.11	1.07	15.20	16.10
16	10	2	65	50	60	0.84	0.84	16.60	16.96
17	15	2	80	100	75	1.39	1.42	17.40	16.90
18	5	3	65	100	75	1.30	1.29	15.90	16.31
19	10	2	50	50	75	0.99	0.96	16.50	16.06
20	15	2	50	100	75	1.23	1.23	15.60	15.24
21	10	2	65	100	75	1.54	1.55	16.20	16.37
22	5	2	65	100	60	0.94	0.92	15.40	15.12
23	10	3	65	50	75	1.16	1.20	17.30	16.93
24	10	1	65	100	90	0.93	0.96	18.40	17.54
25	15	2	65	100	60	1.09	1.11	16.80	16.49
26	10	1	50	100	75	1.04	1.07	14.00	14.44
27	15	2	65	100	90	1.19	1.19	17.50	17.44
28	10	2	65	100	75	1.55	1.55	16.50	16.37
29	10	1	65	150	75	1.05	1.02	15.10	15.00
30	5	2	80	100	75	1.17	1.22	17.50	17.29
31	15	2	65	150	75	1.35	1.33	15.70	16.10
32	5	2	65	100	90	1.06	1.01	19.80	19.77
33	10	2	80	100	60	1.26	1.25	16.90	16.45
34	10	3	65	100	90	1.24	1.26	19.80	19.20
35	10	2	50	100	60	0.85	0.79	15.60	16.09
36	10	2	65	150	60	1.01	1.01	16.20	15.55
37	15	2	65	50	75	1.12	1.12	15.90	16.01
38	10	2	80	100	90	1.02	1.06	20.50	20.45
39	10	3	65	100	60	1.18	1.22	15.80	16.35
40	10	2	65	150	90	1.10	1.09	19.60	19.45
41	10	2	80	50	75	1.20	1.16	17.60	17.78
42	10	2	50	100	90	1.16	1.15	16.80	17.69
43	10	2	80	150	75	1.26	1.30	17.00	17.31

Fig 4.2.5 indicates the increasing trend of the total saponin extraction yield as the solvent/raw material ratio increases up to 11.4 and slightly decreases when the ratio goes beyond 11.4. This indicates that the solvent mainly dissolves the total alfalfa saponins when the solvent/raw material ratio reaches 11.4 mL/g. Hu et al. (2012) observed the same tendency in the extraction of saponin from *Eclipta prostrata* plant and they concluded that as the liquid/solid ratio continued to increase, more protein and polysaccharide were dissolved in solution, hinders saponin dissolution. Extraction time has effect on the surface area contact of the solid matters and solvent (Bonilla, Mayen, Merida, &, Medina, 1999). As shown in Fig. 4.2.5, extraction time positively and linearly effects on the yield of total saponin extraction until 2.8 h. In addition, there was no change obviously in the total saponin extraction yield as the time increases more. To improve efficiency and save energy, the extraction time of 2.8 h was adequate. As illustrated in Fig. 4.2.5, when the temperature increases to 75 ^{o}C, the yield increases regularly. The maximum yield is achieved at 76.8 ^{o}C. By increasing the temperature, the molecular movement is accelerated; thus, the temperature increases the solubility of saponins (Wettasinghe & Shahidi 1999; Yang et al., 2010). As the extraction temperature rises to above 77 ^{o}C, the yield of total saponins shows a little reduction. Ultrasound power can significantly affect the cost of the process. So studying its effect on the extraction of saponins is necessary. With Increase in the yield with the increase in ultrasonic power (up to 110 W), the yield also increase due to the larger amplitude of ultrasound that travels through the solvent. Hence, the higher is the power input, the more violent will be the cavity collapse, and so higher yield of extraction will be obtained. Based on these results, the optimum extraction ultrasound power is

112 W. Higher extraction yields can be due this fact that sonication stimulates a breakdown of the cell wall and facilitates the washing out of the cell content by formation of micro-jet and acoustic streaming (Vinatoru, 2001). On the other hand, ultrasound power above 110 (W) can increase the bubble numbers in the solvent during cavitation, and thus reduce the efficiency of the ultrasound energy transmitted into the medium (Filgueiras et al., 2000) and decrease the yield. When the concentration of ethanol increases from 60 to 80%, the yield of total saponin increases steadily. The yield reaches a maximum at 78% of ethanol concentration. According to the similarity and intermiscibility theory, when the polarity of solute and solvent is similar, the solute simply dissolves from the cells (Gribova, Filippenko, Nikolaevskii, Belaya, & Tsybulenko, 2008).

Table 4.2.2.

Analysis of variance for the regression model of total saponin extraction yield and their bioaccessibility.

Source	Total saponin yield			Total saponin bioaccessibility		
	Coefficient	F-Value	p-value	Coefficient	F-Value	p-value
Model	1.55	55.02	< 0.0001	16.37	10.60	< 0.0001
X_1	0.092	83.30	< 0.0001	-0.24	2.59	0.1217
X_2	0.17	291.51	< 0.0001	0.81	28.35	< 0.0001
X_3	0.089	78.82	< 0.0001	0.78	26.62	< 0.0001
X_4	0.083	67.16	< 0.0001	-0.16	1.06	0.3134
X_5	0.042	17.30	0.0004	1.40	85.47	< 0.0001
X_1X_2	0.023	1.25	0.2758	0.35	1.34	0.2602
X_1X_3	0.005	0.062	0.8062	0.050	0.027	0.8704
X_1X_4	0.020	0.99	0.3313	0.20	0.44	0.5159
X_1X_5	0.005	0.062	0.8062	-0.93	9.33	0.0058
X_2X_3	0.085	17.82	0.0004	-0.050	0.027	0.8704
X_2X_4	0.037	3.47	0.0759	0.10	0.11	0.7444
X_2X_5	-0.017	0.76	0.3941	0.025	0.068	0.9350
X_3X_4	-0.010	0.25	0.6243	-0.075	0.061	0.8067
X_3X_5	-0.14	46.64	< 0.0001	0.60	3.92	0.0602
X_4X_5	0.002	0.015	0.9023	0.55	3.30	0.0830
X_1^2	-0.16	105.57	< 0.0001	-0.26	1.18	0.2885
X_2^2	-0.15	91.05	< 0.0001	-0.49	4.25	0.0512
X_3^2	-0.16	97.14	< 0.0001	0.21	0.74	0.3983
X_4^2	-0.25	255.41	< 0.0001	0.19	0.63	0.4369
X_5^2	-0.33	430.39	< 0.0001	1.10	21.03	0.0001
C.V. %	2.48			2.64		
R^2	0.9804			0.9059		
Adj-R^2	0.9626			0.8804		

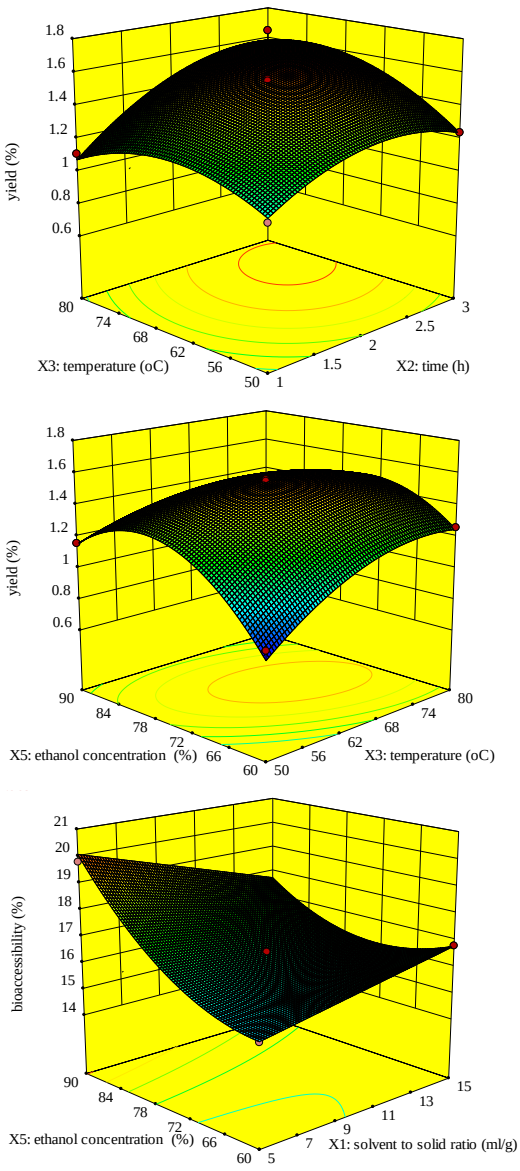

Fig. 4.2.5. Response surface 3D plots showing the significant interactive effect of extraction variables on the total saponins yield and their bioaccessibility.

Influence of process variables on the total saponins bioaccessibility

The quadratic effects of the ultrasound-assisted extraction factors X_1 (solvent/raw material ratio), X_2 (extraction time), X_3 (extraction temperature), X_4 (ultrasound power) and X_5 (ethanol concentration) were carefully analyzed for the total bioaccessibility of saponins response (Table 4.2.2). F-values and p-values were used to determine the significance of each coefficient. Higher F-values with lower p-values lead to more significant correspondence among different variables. The table shows that the linear coefficients of extraction time, temperature and ethanol concentration (X_2, X_3, and X_5), time and ethanol concentration quadratic term coefficients and cross product coefficients (X_1X_5) are significant with very small p-values ($p<0.05$). The other coefficients are not significant due to their higher p-values ($p>0.05$).

The results indicated that as the ultrasound-assisted extraction temperature and time increase, the bioaccessibility of saponins increases. This might be due to the already damaged cellular tissues which release saponins from matrix by the ultrasound cavitation and heat during the extraction process. As previously explained, there is a positive correlation between the saponin's bioaccessibility and solubility, so probably the positive effect of temperature on bioaccessibility of alfalfa saponin can be attributed to the effect of temperature on increasing the solubility of saponins. With regard to saponins, bioaccessibility is the fraction of total ingested saponins that remains solubilized before cell absorption (Navarro del Hierro et al, 2018). Based on this definition, a deeper study and understanding of bioaccessibility of saponin is necessary as many of the bioactivities of saponins are because of their residence at the gastrointestinal level due to their poor absorption.

However, reports on the saponin bioaccessibility and the effect of different process conditions on it are scarce. Sagratini et al. (2013) reported that following the gastrointestinal digestion of cooked lentils in vitro, the bioaccessibility of soyasaponin ranged 9-10%. Additionally, the bioaccessibility values were correlated with the lentils' cooking time. Moreover, the bioaccessibility of carotenoids in vitro increased between 10 and 14% with the application of ultrasound-assisted extraction for 30 min at 75 ^{o}C (Mercado-Mercado et al, 2018). Navarro del Hierro et al. (2018) reported that the type of solvents in ultrasound-assisted extraction affected on the saponins' bioaccessibility. In vitro gastrointestinal digestion of the ethanol extracts significantly increased the bioaccessibility of saponins from edible seeds comparing to the ethanol/water extracts. Total saponins bioaccessibility was found to increase with increasing the ethanol concentration from 60 to 90%

Kinetic model for total saponin extraction yield

The extraction kinetics of total saponin yield from alfalfa by ultrasound on optimum conditions and heat-reflux method were investigated (Fig. 4.2.6). The extracted quantity of saponins increases with time to reach an equilibrium concentration. As noticed, the maximum total saponin extraction yield obtained after ultrasound-assisted method on optimum treatment was 1.64% in comparison with the heat-reflux method by methanol (1.27%). Oleszek (1996) reported that total saponins content of alfalfa stems and leaves were 2.41 and 1.53% (db), respectively. Fenwick et al. (1991) reported that total saponins content of alfalfa was ranged between 0.15 and 1.71% that depends on the plant spices, plant parts

and harvesting season. Data analysis showed that the evolution of total saponin yield over time occurs in the following two steps: a) the release of saponins from the matrix, and b) the saponins' retention during the extraction:

$$\text{Saponins—matrix} \xrightarrow{k_0} \text{saponins + matrix}$$

$$\text{Saponins + matrix} \xrightarrow{k_1} \text{saponins — matrix}$$

The extraction kinetics for total saponin yield (%) from alfalfa through ultrasound-assisted extraction and conventional method were investigated according to the pseudo-first order model.

Thus:

$$\frac{dC_n}{d_t} = k_0 - k_1 C_n \qquad (4)$$

Where, C_n is the concentration of total saponins, k_0 is the kinetic constant of zero-order, and k_1 is the kinetic constant of first-order.

Integrating for initial condition: $t = 0$ $C_n = C_{n0}$

$$C_n = K - (K - C_{n0})\exp(-k_1 t) \quad (5)$$

Where, $K = \frac{k_0}{k_1}$ and k_1 is the kinetic constant of first-order.

Fig. 4.2.6 illustrates a consistency between experimental and simulated values in the ultrasound extraction (R^2 = 0.991) and heat-reflux method (R^2 = 0.995). The

constants values of Equation 5 are also given in Table 4.2.3. Taking into account that the values of kinetic constant (K) were higher than 1 (K>1), the realization of saponins from the matrix predominates over the saponins retention ($k_0>k_1$). It can be observed from Table 4.2.3 that the kinetic constant value (k_1) of the ultrasound is almost two times more than that of heat-reflux. Wu, Lin, & Chau (2001) observed that the ultrasound-assisted extraction yield of ginseng root saponins was three times faster than that of the thermal conventional method. Therefore, treatment by ultrasound at optimum conditions can be considered effective to increase the yield rate the alfalfa saponins during extraction. The extraction yield of saponins is sensitive to time. For instance, the yield of saponins from *Eclipta prostrasta* showed increase as the increasing of ultrasound-assisted extraction time was to 3 h (Hu et al., 2012).

Kinetic model for total saponin bioaccessibility

For the first time, the bioaccessibility of total saponins from alfalfa was investigated using an in vitro gastrointestinal digestion model that simulates the physiological condition of the human intestinal compartments. The extraction kinetics of total saponin bioaccessibility using an in vitro gastrointestinal digestion model that simulates the physiological condition of the human intestinal and that was adapted by Navarro del Hierro et al. (2018) to allow the measurement of saponins (Fig. 4.2.7). As indicated, application of both methods had a positive effect on the bioaccessibility of alfalfa saponins. Rate constants showed an increasing trend as the extraction time increased. The kinetics of bioaccessibility of total saponins could be divided into two phases: the fast phase that takes around 90

min, and the slow phase, for the rest of the study time. As shown in Fig. 4.2.7, the highest saponin bioaccessibility in the ultrasound-assisted and heat-reflux by methanol extraction methods reached to 20.4 and 17.8%, respectively. These results may be due to the cavitation influence on the structure of the cell wall that released saponins to enhance their bioaccessibility. Table 4.2.3 shows that the kinetic constant's value (k_1) of ultrasound is bigger than that of the heat-reflux method.

Poor bioaccessibility of alfalfa saponins can be due to a collection of factors. Navarro del Hierro et al (2018) reported the bioaccessibility of saponins from edible seeds were variable (13-100%) considering the type of saponins' source and solvent factors. Serventi et al. (2013) reported that the bioaccessibility values of type A, B, E, and DDMP saponins from soy-chickpea bread formulations were 30%, 45–65%, 86–91%, 51–61%, respectively. So, the kinetics of ultrasound-assisted and heat-reflux extractions for alfalfa saponin bioaccessibility (%) were studied according to the pseudo-first order model similar to the one described in the process of saponin extraction kinetic:

$$BA_n = K - (K - BA_{n0})\exp(-k_1 t) \quad (6)$$

Where, $K = \frac{k_0}{k_1}$ and k_1 is the kinetic constant of first-order

Table 4.2.3.

Kinetic parameters for the yield of total saponins and their bioaccessibility during extractions.

Factors	Extraction methods	$K=k_0/k_1$	k_1 (h^{-1})	R^2
Yield	Ultrasound on optimum condition	1.663±0.068	0.013±0.002	0.991
	Conventional method (Heat-reflux)	1.443±0.094	0.006±0.001	0.995
Bioaccessibility	Ultrasound on optimum condition	20.491±0.325	0.020±0.004	0.984
	Conventional method (Heat-reflux)	17.913±0.121	0.0164±0.001	0.996

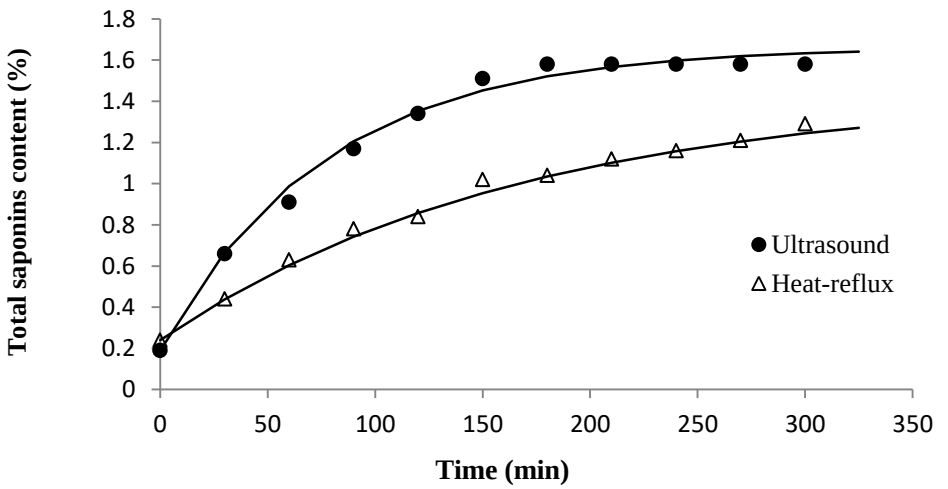

Fig. 4.2.6. Comparison between experimental (symbols) and simulated extraction kinetics (curves) of total saponins yield during extractions of ultrasound at optimum conditions and heat-reflux method.

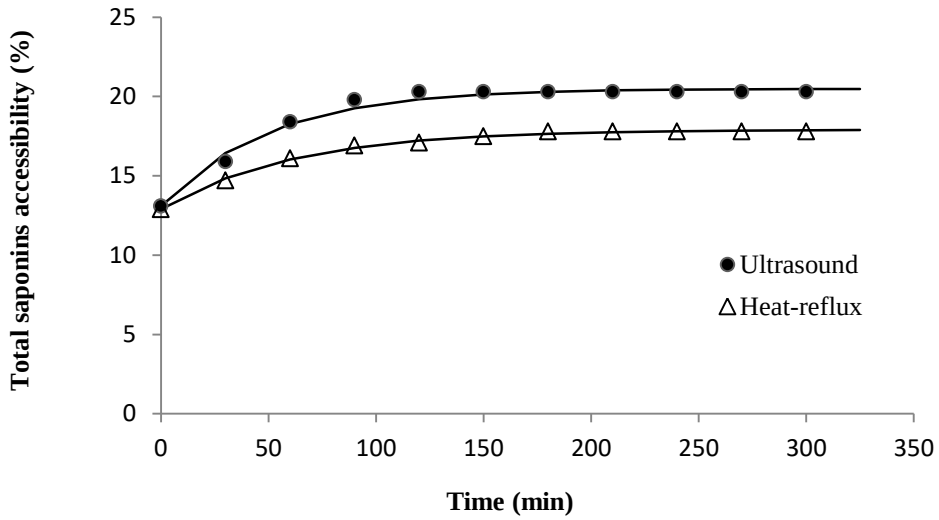

Fig. 4.2.7. Comparison between experimental (symbols) and simulated extraction kinetics (curves) of their bioaccessibility during extractions of ultrasound at optimum conditions and heat-reflux method.

Optimized conditions

The simultaneous optimization of ultrasound-assisted extraction was established for improving the extraction yield and their bioaccessibility of alfalfa saponins. Based on the experimental data, the optimal extraction conditions included: solvent/raw material ratio 11.4 mL/g; extraction time 2.84 h; extraction temperature 76.8 ^{o}C; ultrasound power 112.0 w; and ethanol concentration 78.2%. The yield of total saponins and bioaccessibility were 1.61% and 18.6%, respectively using the numerical optimizer. Additionally, individual optimization based on the extraction yield was done. The optimized condition for the highest extraction yield of saponin (1.68%) was obtained at: solvent/raw material ratio 11.6 mL/g; extraction time 2.73 h; extraction temperature 72.8 ^{o}C; ultrasound power 106.0 w; and ethanol

concentration 75.1%. To optimize extraction procedure for the maximum bioaccessibility of alfalfa saponins (19.7%), solvent/raw material ratio 9.5 mL/g; extraction time 2.90 h; extraction temperature 79.1 ^{o}C; ultrasound power 111.0 w; and ethanol concentration 88.2% were considered as the best extraction conditions based on the bioaccessibility of alfalfa saponins.

4.2.2. Photo-degradation of alfalfa saponin by multi-wavelength irradiation

Absorption spectrum

Fig. 4.2.8 shows the emission spectrum of the lamp used and Fig. 4.2.9 shows the evolution of the absorption spectra of a solution with an initial alfalfa saponin concentration of 100 mg/L. All these absorption spectra were obtained at pH 4 and 20 °C and indicated absorption in the range of wavelengths between 470 and 610 nm with a maximum absorption peak around 543 nm. In addition, similar to what Hu et al. (2012) found, the maximum absorption of eclalba-saponin was at 554 nm with a broad peak in the range of about 450-640 nm. The absorption spectra for the initial alfalfa concentration of 100 mg/L were also obtained for pH 5, 6 and 7, matching the absorption spectrum for pH 4. Therefore, the UV absorption does not depend on the pH value in the range 4–7. Comparing the emission and absorption spectra, it can be seen that within a range between 470 and 610 nm the radiation emitted by the lamp can be absorbed by alfalfa saponins. Table 4.2.4 shows the extinction coefficients (ε_λ) for each wavelength obtained from the data corresponding to the initial solution in Fig. 4.2.9 applying the Lambert–Beer equation. It also shows the absorption coefficient (μ_λ) for a 100 mg/L alfalfa

saponins methanol-water solution and the power emitted by the lamp ($P_{emit,\ \lambda}$) at each wavelength within the considered range.

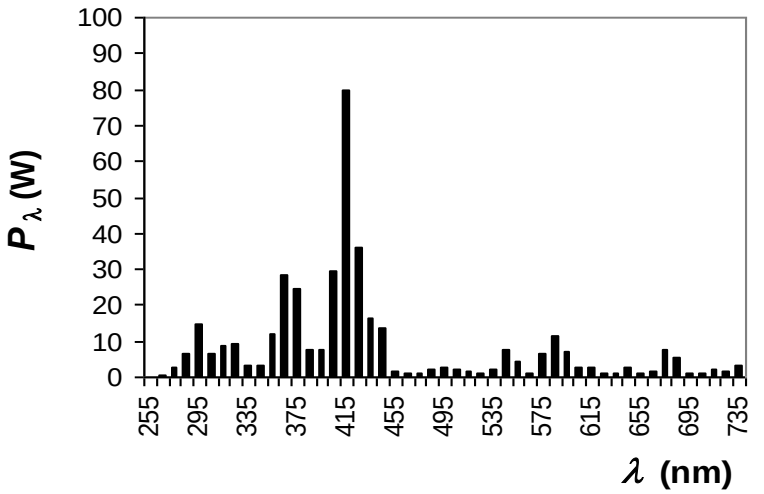

Fig. 4.2.8. Emission spectrum for HPM 12 mercury lamp.

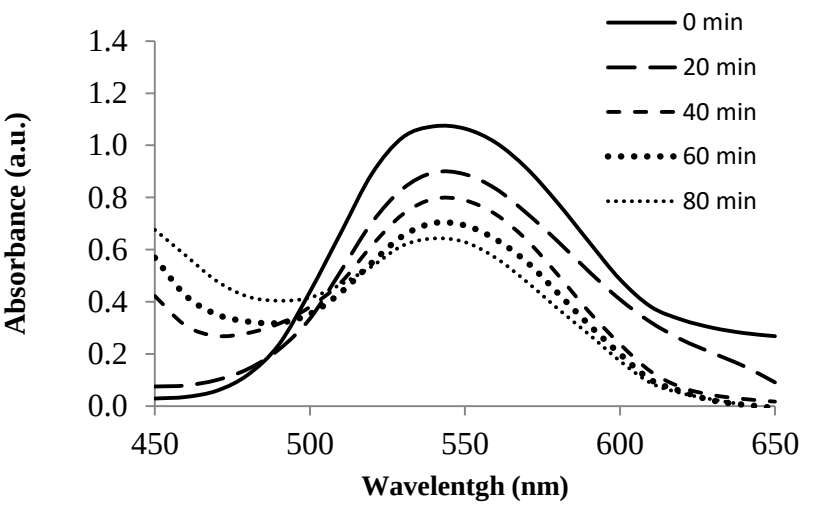

Fig. 4.2.9. Evolution of saponin absorption spectrum with irradiation time, at pH=4 and 20ºC.

Dependence of Pabs on the alfalfa saponin content

Table 4.2.4 indicates the absorption coefficients (μ_λ) for the initial alfalfa saponin concentration of 100 mg/L that was irradiated in all the cases, showing values up to 2.402 cm^{-1}. It is known that for those high values all the radiation reaching the surface of the reactor will be absorbed in the first few millimetres (Garvin et al., 2015). Figures 4.2.10 and 4.2.11 show the dependence of P_{abs} on the alfalfa saponin concentration using two different scales. Fig. 4.2.10 shows how the relation between P_{abs} and the alfalfa saponin concentration is linear for low concentrations and trends towards an asymptote for high concentrations. The asymptote coincides with the value calculated for the incident radiant power on the surface of the reactor (P(0)) (Garvin et al., 2015). Fig. 4.2.10 shows how P_{abs} could be considered a constant value for alfalfa saponin concentrations between 100 and 300 $mg \cdot L^{-1}$. P_{abs} varies from 6.82 x 10^{-6} to 7.28 x 10^{-6} $Einstein \cdot L^{-1} \cdot s^{-1}$ for 100 and 300 $mg \cdot L^{-1}$ respectively, the average being 5.57 x 10−6 $Einstein \cdot L^{-1} \cdot s^{-1}$. This figure shows fitted values for 4.2.10 and 4.2.11 these being respectively.

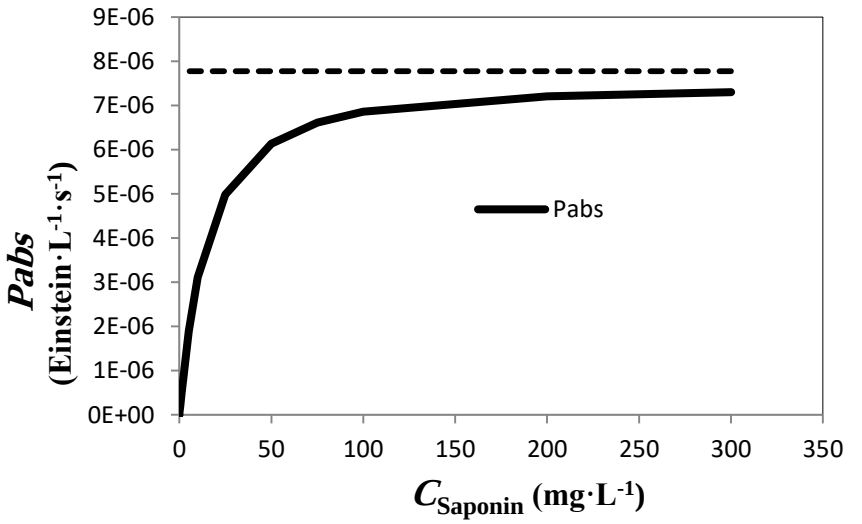

Fig. 4.2.10. Spectral radiant power absorbed as a function of saponin concentration from 0 to 350 mg·L^{-1}.

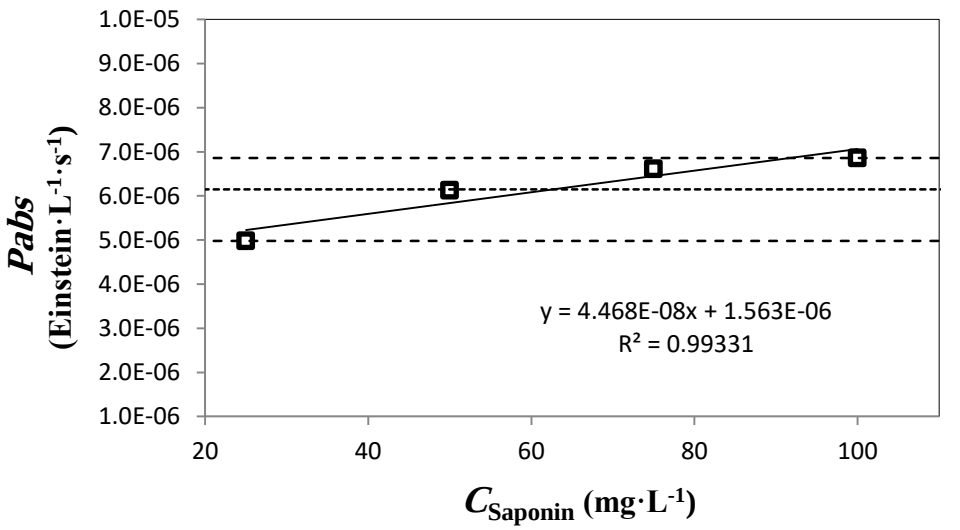

Fig. 4.2.11. Spectral radiant power absorbed as a function of saponin concentration from 25 to 100 mg·L^{-1} showing the parameters *a* and *b* fitted by linear regression.

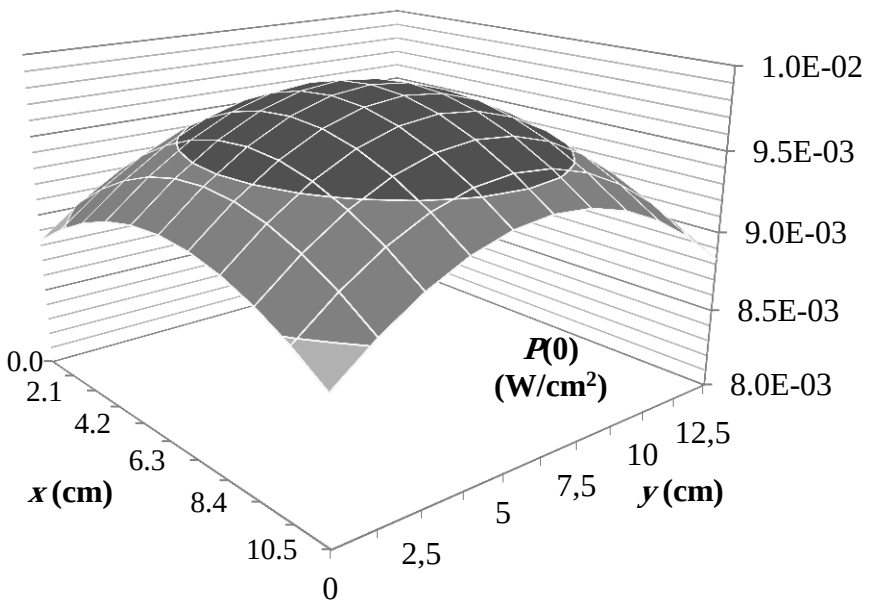

Fig. 4.2.12. Incident spectral radiant power on the surface of the photo-reactor ($P(0)$) was obtained for 100 mg·L^{-1} saponin in methanol-water solution.

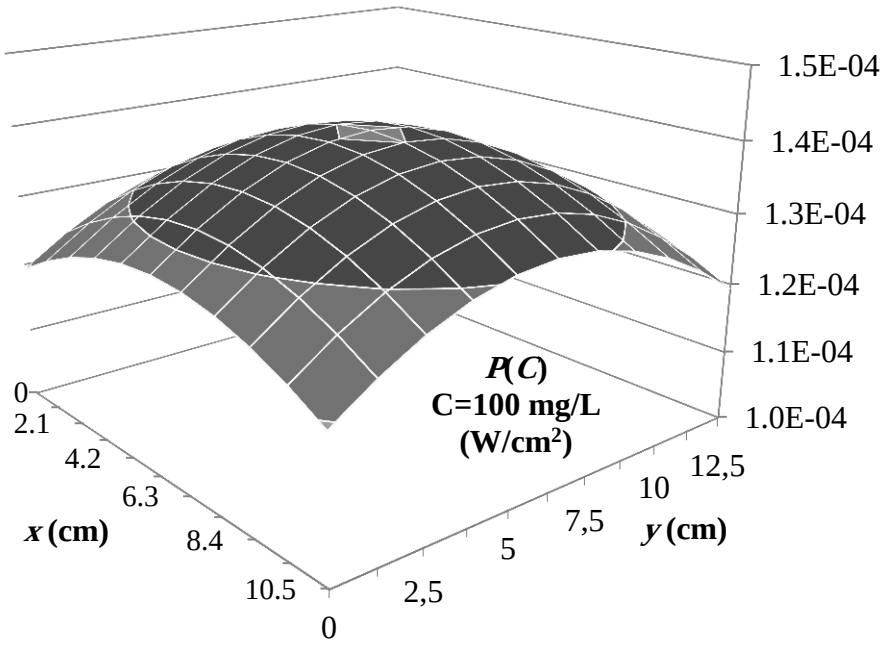

Fig. 4.2.13. Incident spectral radiant power at the bottom of the photo-reactor ($P(C)$) was obtained for 100 mg·L^{-1} saponin in methanol-water solution.

Photo-degradation of alfalfa saponin in a methanol-water solution. Effect of Temperature and pH

The evolution of the alfalfa saponin content when a methanol-water solution of 100 mg·L−1 is irradiated is illustrated in figures 4.2.14 - 4.2.15. In all the cases, alfalfa saponin is degraded during the irradiation of the solutions, showing a straight line with non-zero origin ordinate, matching a pseudo-first-order kinetics.

Fig. 4.2.14 shows the effect of temperature for solutions at pH = 4. While at temperatures of 20, 40, 60 and 80 °C, the alfalfa saponin concentration decreases about 58% after 80 min of irradiation at 20 °C, at 80 °C the degradation reaches 80%. Therefore, It can be seen that the higher the temperature, the faster the alfalfa saponin photo-degradation rate. Alfalfa saponin degraded more easily at pH = 4 than at pH = 7. It can also be observed that for a given pH, the more the temperature increased, the more easily the alfalfa saponin was degraded. For solutions of pH = 7 and temperatures of 20 and 80 °C, after a 90-minute irradiation time, the alfalfa saponin contents were about 79 and 65% of the initial value, respectively. All the values of the k_D/k ratio calculated in Tables 4.2.5 are greater than 1. As this parameter is the ratio of the rates between the stage of declining to the fundamental state and the stage of forming photo-products, it means that, in all the cases, the declining step was faster than the photo-product formation. It can also be observed that the ratio decreases when the temperature is increased, confirming that the efficiency of the photo-degradation increases with temperature. Table 4.2.5 also enable it be concluded that the higher the temperature, the faster the photo-degradation, as was also found by Aguilar et al. (2015). The m_s and Ks values show the same trend observed in Fig. 4.2.14 - 4.2.15, confirming that the optimal conditions for degrading alfalfa sapoin in the range studied are 80 °C and pH=4.

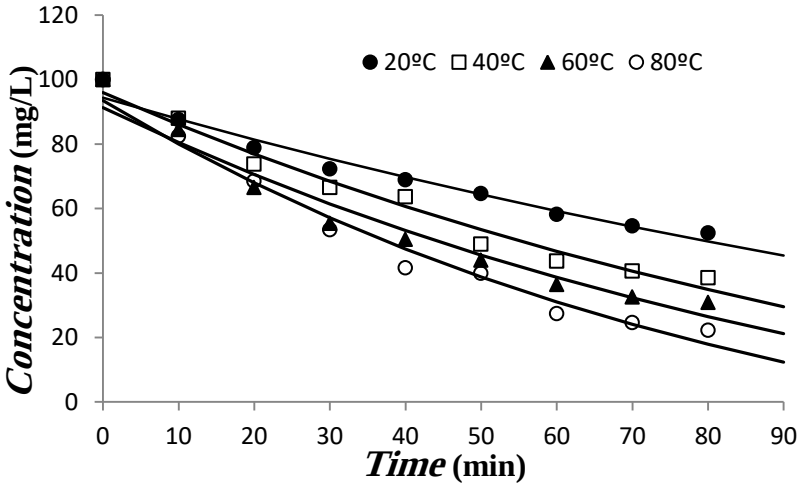

Fig 4.2.14. Evolution of saponin concentration with irradiation time at different temperatures pH = 4.

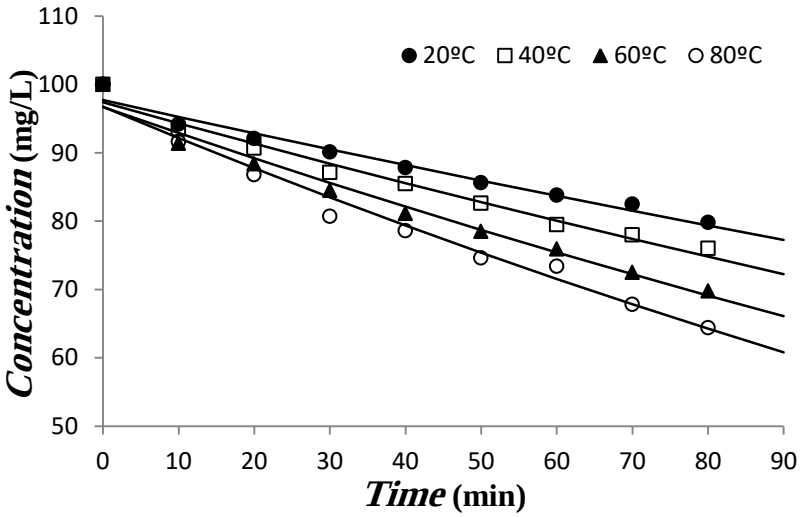

Fig 4.2.15. Evolution of saponin concentration with irradiation time at different temperatures pH = 7.

Table 4.2.4. Energy emitted by lamp, mass extinction coefficients (ε_λ) of saponin and absorption coefficients of aqueous solutions of saponin for 100 mg·L^{-1}, at different wavelengths.

λ (nm)	P_{emit} (W)	$\varepsilon_\lambda \times 10^3$ (L·mg^{-1} cm^{-1})	μ_λ (C=100 mg·L^{-1}) (cm^{-1})
255	0.1792	11.61	1.161
485	0.8946	2.07	0.207
495	2.0580	4.74	0.474
505	1.7900	9.39	0.939
515	1.4315	14.62	1.462
525	0.9842	19.83	1.983
535	1.7900	23.05	2.305
545	6.4430	24.02	2.402
555	3.4896	23.81	2.381
565	1.0738	22.57	2.257
575	5.5476	20.29	2.029
585	9.3060	17.22	1.722
595	5.9061	13.82	1.382
605	5.4963	10.50	1.050
615	2.1477	8.08	0.808
625	1.0738	6.93	0.693
635	0.9842	6.22	0.622
645	5.3170	5.76	0.576
655	1.0738	5.48	0.548
665	1.3419	5.25	0.525
675	6.4430	4.95	0.495
685	4.5634	4.44	0.444
695	0.8050	3.73	0.373
705	0.8050	2.23	0.223

Table 4.2.5. Parameters of pseudo first-order kinetic for photo-degradation of saponin in methanol-water solution at different pH and temperatures.

$$\ln\left(\frac{a+bC_S^0}{a+bC_S}\right) = m_S\, t = b \cdot K_S \cdot t$$

pH	*T* (ºC)	*Intercept*	m_S x 10^4 (s^{-1})	K_S (mol/Einstein)	k_D/k	R^2
4	20	0.043±0.036	0.88 ± 0.13	0.0039	253	0.9750
	40	0.030±0.055	1.31±0.19	0.0059	170	0.9738
	60	0.066±0.075	1.50±0,26	0.0067	148	0.9638
	80	0.049±0.077	1.85±0.27	0.0083	120	0.9741
5	20	0.032±0.031	0.79±0.11	0.0035	282	0.9770
	40	0.025±0.049	1.11±0.17	0.0050	200	0.9709
	60	0.013±0.029	1.46±0.10	0.0065	152	0.9940
	80	0.025±0.049	1.69±0.17	0.0076	131	0.9871
6	20	0.016±0.017	0.48±0.06	0.0021	464	0.9811
	40	0.022±0.024	0.64±0.09	0.0029	348	0.9776
	60	0.031±0.024	0.71±0.08	0.0032	314	0.9826
	80	0.035±0.035	0.94±0.11	0.0042	237	0.9842
7	20	0.017±0.012	0.31±0.04	0.0014	720	0.9758
	40	0.019±0.015	0.39±0.05	0.0017	572	0.9779
	60	0.025±0.018	0.49±0.06	0.0022	455	0.9808
	80	0.024±0.022	0.59±0.08	0.0026	378	0.9791

4.3.

Producing alfalfa protein isolate for human consumption

Producing alfalfa protein isolate for human consumption by Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique

The combination of methods can create a new and efficient method, which is able to improve the purity, safety, quality and functional properties of alfalfa protein for human consumption. In this section, ultrasonic-ultrafiltration-assisted Alkaline Isoelectric precipitation (UUAAIP) technique as a new process was developed and employed in the alfalfa leaves' protein extraction. Response surface methodology (RSM) was used to optimize the extraction factors (solvent/solid ratio, pH, extraction temperature, time, and flow rate) to maximize both the extraction yield and protein content of alfalfa protein. Finally, the composition, physiochemical and functional properties of the extracted protein were investigated and compared with the results of other common methods such as heat-coagulation extraction (HCE) and alkaline isoelectric precipitation extraction (AIPE).

4.3.1. Extraction modeling and statistical analysis

A total number of 43 runs were carried out using the Box-Behnken Design (BBD) in order to study and optimize the combined effect of five process factors (solvent/solid material ratio, pH, extraction temperature, extraction time, and flow

rate) on the extraction yield and protein content of alfalfa protein (Table 4.3.1). The developed model equation was evaluated using ANOVA (Table 4.3.2). The higher model F-values for both responses (49.59-52.74) and the lower p-values ($p<0.0001$) illustrated the significance of fitted models. They also demonstrate that most of the variation in the responses could be described by the regression equations. The F-values (13.43-3.98) and p-values (0.071-0.026) of lack-of-fit models implied that it was not significantly relative to the pure error. For the quadratic regression models of Y_1 and Y_2, the calculated correlation coefficients (R^2) were 0.980 and 0.972, respectively, demonstrating that 98% and 97.2% of the variations could be explained through the fitted models. Besides, the R^2-Adj values (0.973-0.964) were very close to the correlation coefficients, implying a high correlation degree between the actual and predicted values. Furthermore, the coefficient of variation (CV) of Y_1 and Y_2 were not significant at very low values (4.38 and 2.08, respectively), suggesting that the predicted values were close to the actual values. The responses of extraction yield (Y_1) and protein content (Y_2) of alfalfa protein obtained from the 43 runs were entered into a second-order polynomial equation in order to define the relationship between the process factors and responses:

$$Y_1 = +13.55 + 2.71X_1 + 0.77X_2 + 0.42X_3 + 0.53X_4 - 0.42X_5 - 0.87X_1X_4 - 0.95X_2X_4 + 0.83X_4X_5 - 1.92X_1^2 - 0.60X_2^2 - 3.78X_4^2$$

$$Y_2 = +86.90 + 4.89X_1 + 2.00X_2 + 1.64X_3 + 10.94X_4 - 0.94X_5 - 2.00X_1X_4 - 2.30X_1X_3 - 1.80X_2X_4 - 3.89X_1^2 - 3.04X_2^2 - 2.15X_3^2 - 6.96X_4^2 - 1.30X_5^2$$

Table 4.3.1.

Box–Behnken design matrix with coded variables and experimental and predicted values.

Run	Factors					Responses			
	Solvent/solid material ratio X_1 (mL/g)	pH X_2	Temp X_3 (^oC)	Time X_4 (min)	Flow rate X_5 (L/h)	Extraction yield Y_1 (g/100g)		Protein content Y_2 (g/100g)	
						Experimental	Predicted	Experimental	Predicted
1	20	10	40	90	16	8.40	8.50	73.50	75.86
2	50	10	40	120	12	10.10	10.25	92.20	90.76
3	35	11	40	120	12	9.50	9.55	87.60	88.04
4	35	10	50	90	16	12.50	13.55	85.40	84.28
5	50	10	40	90	16	14.50	13.93	85.50	85.69
6	35	9	40	90	16	11.60	11.76	82.10	81.07
7	35	9	40	60	12	7.00	6.94	63.40	62.16
8	35	10	50	120	12	10.50	10.75	88.00	90.00
9	35	10	40	90	12	14.40	13.55	86.10	86.90
10	50	10	40	90	8	14.80	14.78	87.50	87.47
11	35	11	40	90	16	13.40	13.31	81.60	82.17
12	35	10	30	90	16	13.10	12.71	81.50	80.73
13	35	10	40	120	16	10.80	10.73	89.10	88.94
14	20	9	40	90	12	8.20	7.55	70.60	71.11
15	35	9	40	90	8	12.30	12.61	81.50	80.06
16	20	10	40	60	12	4.40	3.76	60.50	59.14
17	35	10	40	120	8	10.20	9.93	88.90	90.23
18	35	9	40	120	12	9.70	9.90	87.40	87.64
19	35	9	50	90	12	12.50	12.60	80.00	81.31
20	35	10	40	60	16	7.20	8.02	66.50	66.45
21	35	11	40	90	8	13.90	14.16	86.80	86.96
22	35	10	30	60	12	9.20	8.85	64.50	64.81
23	50	10	30	90	12	13.40	13.93	85.00	86.37
24	50	11	40	90	12	14.10	14.53	84.50	84.84
25	35	11	40	60	12	10.60	10.39	70.80	69.76
26	20	10	40	90	8	8.70	9.35	75.70	77.85
27	20	11	40	90	12	9.20	9.10	79.10	79.11
28	20	10	30	90	12	7.80	8.51	71.60	72.05

29	35	10	40	90	12	14.20	13.55	87.80	86.90
30	35	10	30	120	12	9.50	9.91	87.50	87.45
31	20	10	50	90	12	8.70	9.35	81.70	79.95
32	50	10	40	60	12	10.70	10.94	71.50	71.07
33	20	10	40	120	12	7.30	6.58	85.60	83.23
34	50	10	50	90	12	14.90	14.77	85.90	85.07
35	35	10	30	90	8	13.80	13.56	84.50	82.87
36	35	9	30	90	12	11.20	11.76	77.30	78.11
37	35	10	40	60	8	9.90	10.52	67.50	68.94
38	35	10	40	90	12	14.10	13.55	86.80	86.90
39	35	11	30	90	12	13.50	13.31	82.50	82.01
40	35	11	50	90	12	14.30	14.15	85.40	85.41
41	50	9	40	90	12	13.60	12.98	84.00	84.84
42	35	10	50	90	8	14.70	14.40	87.90	85.92
43	35	10	50	60	12	10.10	9.68	66.50	68.86

Effect of process factors on extraction yield

As shown in Table 4.3.2, in the case of extraction yield, the p-values of all liner terms were less than 0.01; this indicates that X_1, X_2, X_3, X_4 and X_5 had highly significant effects. In addition, the solvent/solid material ratio, pH and time showed significant quadratic effects at $p < 0.01$. The interactions of X_1X_4, X_2X_5 and X_4X_5 were statistically significant at $p < 0.01$ (Fig. 4.3.3). Analysis of the model clearly shows that the solvent/solid material ratio was the most significant factors. It had a positive effect on the extraction yield ($X_1 = 2.71$). As observed in Fig. 4.3.1, the solvent/solid material ratio had a major effect on the extraction yield such that the extraction yield of alfalfa protein was improved with increasing the solvent/solid ratio from 20 to 45 mL/g; however, later it showed a decreasing trend. This phenomenon could be due to an increased driving force for the mass transfer of proteins, which enhances the diffusivity of the solvent into cells and improves the

protein desorption from the cells (Bedin, Netto, Bragagnolo, & Taranto, 2019). There was an increase in the extraction yield up to 90 min and then a decrease on further increase of the extraction time. Similar study has presented that the prolonged extraction time was not favorable to improve the extraction yield of protein (Eromosele et al., 2008). Fig. 4.3.1 shows that the extraction yield of alfalfa protein increases with increase in the extraction temperature. The extraction yield significantly increased along with the increase of pH from 9 to 10. Then, the yield was almost unchanged at the pH range of 10 - 10.5, though it then a decrease. Alkaline pH can loosen the texture by the disruption of hydrogen bonds leading to the hydrogen ions' separation from the sulphate and carbolic groups (Shen, Wang, Wang, Wu, & Chen, 2008). Moreover, the surface charge of proteins converts progressively negative, improving their aqueous solubility (Jarpa-Parra, et al., 2014). On the other hand, in the pH range of 10.5-11, irreversible denaturation of protein occurs and extraction yield declines. Flow rate had a negative effect (X_5 = -0.49), meaning that the extraction yield of alfalfa protein was more favorable at low flow rates.

Table 4.3.2.

Analysis of variance for the regression model of extraction yield and protein content.

Source	Extraction yield			Protein content		
	Coefficient	F-value	p-value	Coefficient	F-value	p-value
Model	14.23	49.59	< 0.0001	86.90	52.74	< 0.0001
X_1	2.71	401.87	< 0.0001	4.86	140.34	< 0.0001
X_2	0.77	32.81	< 0.0001	2.00	23.74	< 0.0001
X_3	0.42	9.58	0.0053	1.65	16.16	0.0006
X_4	0.53	15.42	0.0007	10.94	710.86	< 0.0001
X_5	-0.42	9.87	0.0047	-0.94	5.29	0.0314
X_1X_2	-0.12	0.21	0.6487	-2.00	5.94	0.0234
X_1X_3	0.15	0.31	0.5850	-2.30	7.85	0.0104
X_1X_4	-0.87	10.45	0.0038	-1.10	1.80	0.1939
X_1X_5	0.000	0.001	1.0000	0.050	0.003	0.9520
X_2X_3	-0.13	0.21	0.6487	0.050	0.003	0.9520
X_2X_4	-0.95	12.32	0.0020	-1.80	4.81	0.0392
X_2X_5	0.050	0.034	0.8551	-1.45	3.12	0.0912
X_3X_4	0.025	0.002	0.9272	-0.38	0.21	0.6523
X_3X_5	-0.37	1.92	0.1797	0.12	0.023	0.8804
X_4X_5	0.83	9.29	0.0059	0.30	0.13	0.7183
X_1^2	-2.21	106.95	< 0.0001	-3.89	35.88	< 0.0001
X_2^2	-0.90	17.53	0.0004	-3.04	21.91	0.0001
X_3^2	-0.47	4.84	0.0385	-2.15	11.02	0.0031
X_4^2	-4.05	359.10	< 0.0001	-6.96	115.09	< 0.0001
X_5^2	-0.46	4.67	0.0418	-1.30	3.99	0.0584
Lack of Fit	-	13.43	0.071	-	3.98	0.026
C.V. %	4.38			2.04		
R^2	0.980			0.972		
Adj-R^2	0.973			0.964		

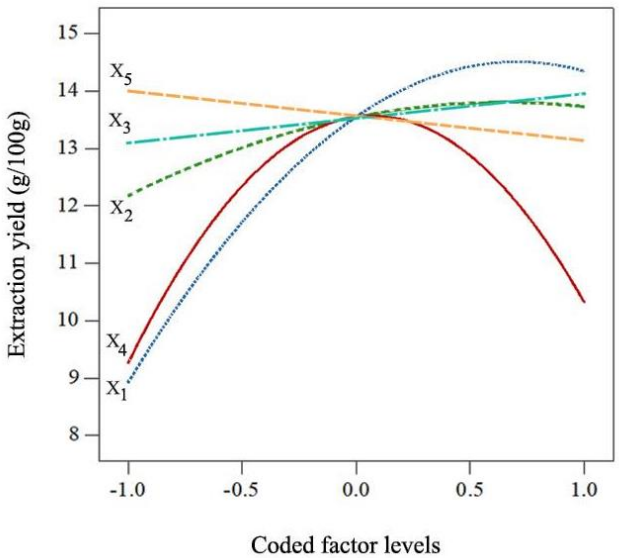

Fig. 4.3.1. Perturbation plots showing the effect of process factors on extraction yield.

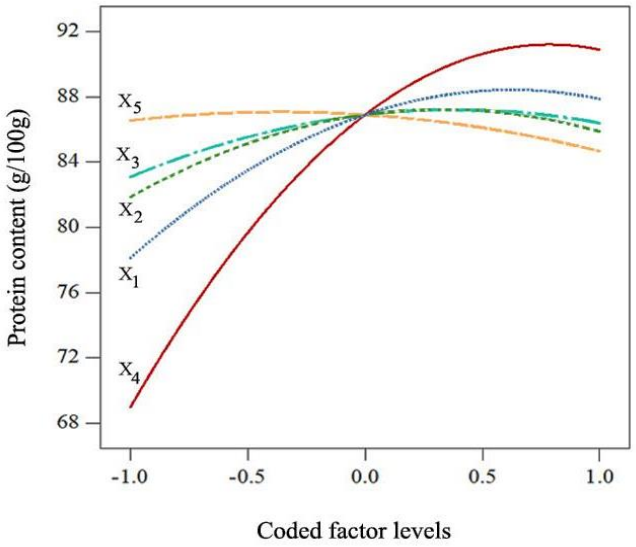

Fig. 4.3.2. Perturbation plots showing the effect of process factors on protein content.

Effect of process factors on protein content

In addition to extraction yield, protein content is the most significant characterization of protein isolates, because these two dependent variables are used as the basis in the majority of protein extraction optimization methods. In the present research, the protein content varied from 60 to 92%, depending on the process parameters. In Table 4.3.2, the results of ANOVA analysis show that the alfalfa protein content is more significantly affected by X_1, X_2, X_3 and X_4 at $p < 0.01$ than by X_5 at $p < 0.05$. It is also indicated that X_1X_2, X_1X_3 and X_2X_4 were significant terms in the model ($p < 0.05$) as shown in Fig. 4.3.4. All quadratic terms of variables were statistically significant except the flow rate factor ($p < 0.01$) as confirmed by their regression coefficient values. Extraction time was the most significant factor on alfalfa protein content (Y_2). Protein content was found to increase with increasing extraction time (Fig. 4.3.2). This might be due to the reason that the mixture crosses through the membrane filtration for more times, so more impurities are separated from protein. Arogundade & Mu (2012) found that when ultrafiltration was performed more times, the corresponding purity level of the extracted sweet potato protein increased significantly. The results showed that with an increase in temperature from 20 to 45 ^{o}C, the protein content was improved gradually. A high temperature can decrease the viscosity of liquid phase, and enhance the permeate flux until 50 °C. Then the temperature increase can cause heat denaturation of the proteins (Atra, Vatai, Bekassy-Molnar, & Balint, 2005).

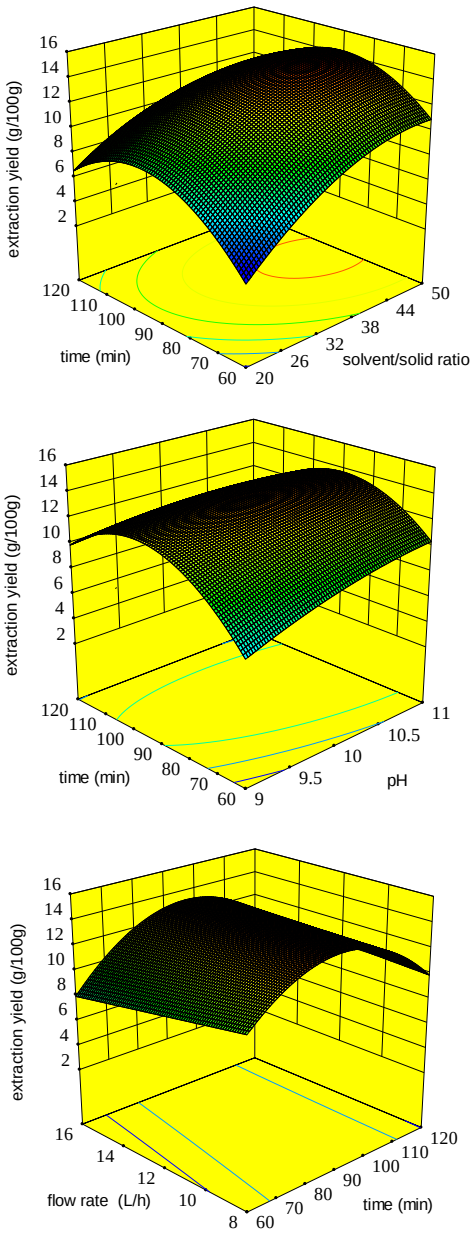

Fig. 4.3.3. 3D plots showing the interactive effects of process factors on extraction yield.

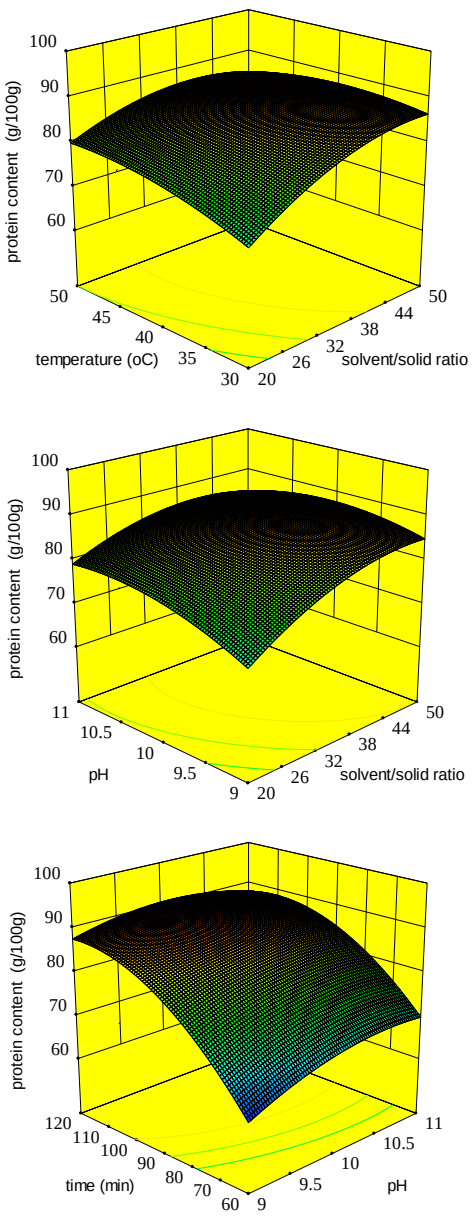

Fig 4.3.4. 3D plots showing the interactive effects of process factors on protein content.

Simultaneous optimization and verification of the model

Simultaneous optimization of the UUAAIP method was carried out for increasing the combination of protein extraction yield and protein content from alfalfa leaves. RSM was successfully applied to optimize the UUAAIP process and analyze the effects of factors and their interactions. The optimized conditions were solvent/solid material ratio of 43.3 mL/g, pH of 10.1, extraction temperature of 42.5 ^{o}C, extraction time of 102 min, and flow rate of 9.7 L/h. Under these conditions, the highest predicted extraction yield and protein content were 14.5 and 91.1 g/100g, respectively with the desirability value of 0.983. The strong correlation between the real and predicted results confirmed that the response model was adequate to reflect the expected optimization. So, BBD was regarded to be detailed and influential tool for predicting both responses using the UUAAIP technique.

4.3.2. Physicochemical properties of alfalfa protein isolate

Extraction yield and protein content

Table 4.3.3 illustrates the extraction yield and content of alfalfa protein extracted by the UUAAIP, HCE and AIPE techniques. HCE showed the highest extraction yield (16.6 g/100g) and the lowest protein content. Protein content of UUAAIP, AIPE and HCE methods was 91.1, 74.5 and 63.9 g/100g, respectively. These results indicate that HCE retained more impurities in the concentrate than AIPE and UUAAIP so the HCE protein was not purified. The addition of isoelectric precipitation step as part of AIPE and UUAAIP increased the purity of the resulting protein extract by isolating the proteins by precipitation selectively from other polymers. Dong et al. (2011) reported that ultrasonic-assisted alkaline extraction

following by ultrafiltration of rapeseed protein was significantly better than the traditional extraction. This protein content is comparable to the one presented by Hojilla-Evangelista, Selling, Hatfield, & Digman (2016) (60 g/100g) and Knuckles & Kohler (1982) (89 g/100g). Also it is higher than the amount that we found for alfalfa leaves protein concentrate in our previous study (70 g/100g) (Hadidi et al., 2019).

Color analysis

Dark color is an important limiting factor in utilization of alfalfa protein concentrate or isolate for human consumption, which has major impact on the acceptance of consumers. UUAAIP had significantly higher L* (68.1) than AIPE (56.7) and HCE (59.3) ($p < 0.05$), implying the lighter color (Table 4.3.3). The b* values of all samples was not significantly ($P > 0.05$) affected by the method of extraction. Moreover, minimum a* value was determined for AIPE (-4.5), indicting the greener color compared to the other samples. Our findings showed that the utilization of ultrafiltration during alkaline extraction in the UUAAIP method was efficient in improving the color of alfalfa isolate due to removing the phenolic compounds. The development of dark color in protein isolates can lead to the oxidation of phenolic compounds. They undergo enzymatic and non-enzymatic oxidation to form quinones under alkaline conditions, which can then react with protein, resulting in dark color in the protein solutions. It is worth noting that the color cannot be washed from the protein isolates when they are precipitated at isoelectric points (Sosulski, 1979).

Molecular weight

The average molecular weight of the extracted alfalfa protein by different methods ranged from 71.4 to 94.5 kDa. UUAAIP showed a significantly ($P < 0.05$) higher average molecular weight compared to AIPE and HCE (Table 4.3.3). Lamsal et al., (2007) also found a different number of polypeptide bands with the MW range of 20–107 kDa in non-clarified alfalfa leaf juice. Barbeau & Kinsella (1988) reported a MW range of 6–550 kDa for the protein bands detected in protein the alfalfa herbage juice. As reported previously, the extraction method significantly affected ($p < 0.05$) on the molecular weight of Barley protein concentrates (Houde, Khodaei, Benkerroum, & Karboune, 2018). This result is in agreement with the findings of a previous study that showed the average molecular weight of alfalfa protein was in the range of 68 - 96 kDa (Hadidi et al., 2019).

4.3.3. Anti-nutritional factors of alfalfa protein isolate

Total saponin content

The saponin contents of alfalfa protein obtained with different extraction methods are shown in Table 4.3.4. The saponin content of UUAAIP protein (1.56 mg/g) was significantly lower than that of HCE (8.11 mg/g) and AIPE (7.68 mg/g) proteins, implying that UUAAIP is an efficient method for saponins elimination. The molecular weight of saponins is mostly less than 2 kDa (Francis, Kerem, Makkar & Beker, 2002); hence, they can pass through the UUAAIP membrane with 10 kDa MW cut-off and separate from the protein. Alfalfa proteins containing low level of saponin content have higher nutritional value and sensory properties because they are responsible for the bitterness in alfalfa leaves beside the anti-nutritional effect

of saponins (Oleszek, 2002). Oleszek (1996) reported that the total saponin content of alfalfa stems and leaves as 2.41 and 1.53% (db), respectively. Furthermore, the concentration of saponin in the protein concentrate obtained from alfalfa green juice by heat-coagulation method was in the range of 5 - 14 mg/g (EFSA, 2009).

Total polyphenol content

Polyphenols in alfalfa leaves are usually accompanied by problems such as dark color, unfavorable taste and low digestibility, owing to the interaction of polyphenol-protein (Yu et al., 2016; D'alvise, Lesueur-Lambert, Fertin, Dhulster & Guillochon, 2000). Alfalfa leaves contain high levels of phytoestrogens, mainly isoflavones and coumestrol (EFSA, 2009). Besides their beneficial health effects, the anti-oestrogenic properties of phytoestrogens have also raised concerns since they might act as endocrine disruptors, indicating a potential to cause adverse health effects (Rietjens, Louisse, & Beekmann, 2017).

The total polyphenol content of the extracted protein was significantly ($p < 0.05$) affected by the extraction methodology. The lowest total polyphenol content was detected in UUAAIP protein (4.45 mg/g) followed by HCE (6.07 mg/g) and AIPE (8.58 mg/g) proteins, respectively. The protein extracted from rapeseed stem by heat coagulation decreased the polyphenol content significantly, which shows that the effect of heating on the reduction of polyphenols concentration (Yu et al., 2016). Color measurement confirmed that the lower content of phenolic compounds in canola protein isolates improved the color and taste of the isolates (Xu & Diosady, 2002). D'alvise et al. (2000) removed 94% of polyphenols from alfalfa white protein concentrate by ultrafiltration and adsorbent resin separations methods.

Table 4.3.3.

Physicochemical and anti-nutritional factors of alfalfa proteins extracted by different methods.

Parameters	Extraction methods		
	HCE	AIPE	UUAAIP
Extraction yield (g/100g)	16.6 ± 1.1 [a]	13.9 ± 2.3 [b]	14.5 ± 0.7 [b]
Protein content (g/100g)	63.9 ± 4.6 [c]	74.5 ± 5.1 [b]	91.1 ± 3.8 [a]
Total saponin content (mg/g)	8.11 ± 0.05 [a]	7.68 ± 0.04 [a]	1.56 ± 0.02 [b]
Total polyphenols content (mg/g)	6.07 ± 0.17 [b]	8.58 ± 0.5 [a]	4.45 ± 0.46 [c]
Average molecular weight (kDa)	71.4 ± 3.2 [b]	73.0 ± 2.5 [b]	94.5 ± 3.8 [a]
L*	59.3 ± 1.8 [b]	56.7 ± 2.6 [c]	68.1 ± 1.1 [a]
a*	-0.4 ± 0.2 [a]	-4.5 ± 0.3 [c]	-1.7 ± 0.3 [b]
b*	22.4 ± 3.7 [a]	21.9 ± 2.1 [a]	22.7± 1.5 [a]

* Different letters in each row indicate significant differences ($P < 0.05$).

4.3.4. Functional properties of alfalfa protein isolate

Solubility

The solubility of HCE, AIPE and UUAAIP in the pH range of 2.0-10.0 is shown in Fig. 4.3.5. All of the extracted alfalfa protein samples showed a similar solubility (NSI) trend as a function of pH. The results indicated that the highest solubility for HCE (92.2%) was observed at pH 9.0, and for AIPE (93.6%) and UUAAIP (94.7%) at pH 8.0. The lowest solubility was at the isoelectric point in all the samples. At the isoelectric point, the positive and negative charges on protein are balanced; as a

result, the overall charge of the protein is zero. Hydrophobic interactions among proteins are much greater than the hydrophilic and hydration repulsive forces created by charged residues (Eromosele et al., 2008). The minimum solubility of HCE was at pH 4.0-5.0, and for AIPE and UUAAIP at pH 3.0-4.0. Similarity, Du et al. (2018) observed the lowest solubility of Mung bean protein and albumin detected at pH 4-5. At neutral pH, a maximum solubility of 72.8% was shown for UUAAIP while HCE had the minimum value of 58.1%. Also the present study results showed that HCE caused more protein denaturation, so lower protein solubility was indicated compared with the AIPE and UUAAIP methods. In addition, similar to what Hojilla-Evangelista et al. (2016) found, the minimum solubility of alfalfa protein concentrate was at pH 4 and the highest solubility was at alkaline pH.

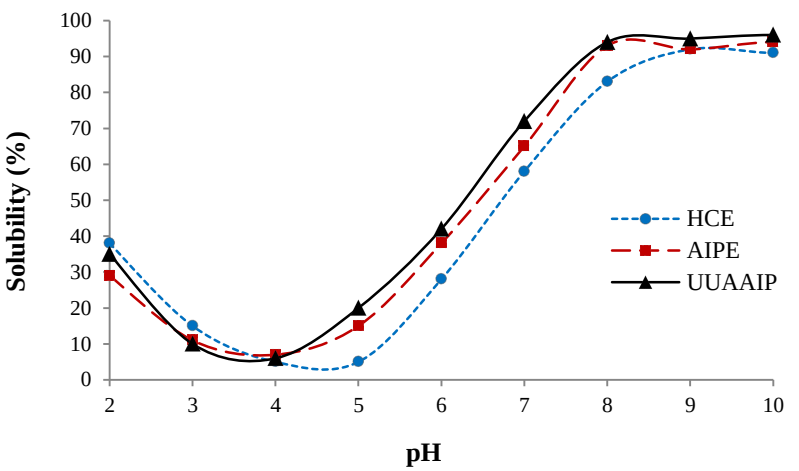

Fig. 4.3.5. Protein solubility of alfalfa proteins extracted by different methods at pH 2-10.

Water-holding capacity (WHC) and oil-banding capacity (OBC)

Water-holding and oil-banding capacities are influenced by the parameters such as the method used to process the protein, amino acid composition, the ratio of surface polarity to hydrophobicity, and particle size (Dong et al., 2011; Du et al., 2018). As shown in Table 4.3.4, the WHC and OBC of all alfalfa protein samples were significantly ($p < 0.05$) different. For example, the WHC of UUAAIP (4.35 g water/g) was significantly ($p < 0.05$) higher than that of AIPE (3.90 g water/g) and HCE (3.34 g water/g). This result is in accordance with the previously published literature (Fiorentini & Galoppini, 1981), where the WHC and OBC of alfalfa protein extracted by alkaline isoelectric precipitation method were 4.5 g water/g and 4.1 g oil/g, respectively. WHC is a great property for foods with high viscosity like soups, pastas, confectionaries, and bakery products. In present study, the OBC of all alfalfa protein samples was greater than the WHC. UUAAIP had the highest OBC with 4.88 g oil/g, which significantly higher than that of AIPE (4.27 g oil/g) and HCE (3.95 g oil/g). Furthermore, our results are in agreement with the findings of a previous study by Knuckles & Kohler (1982).

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability (ES) of the extracted alfalfa protein by different methods are illustrated in Table 4.3.4. Two main parameters for the emulsifying characteristics of protein are EAI and ES. The EAI of UUAAIP, AIPE and HCE was 142.9, 195.9 and 190.6 (m^2/g), respectively. The EAI and ES of UUAAIP were significantly ($p < 0.05$) lower than those the AIPE and HCE. Hojilla-Evangelista et al. (2016) reported similar EAI (187 m^2/g) and ES

(18.9 min) values for alfalfa concentrate at pH 7. On the other hand, this finding is in contradiction with the results of rapeseed protein study, which reported that ultrafiltration of protein improved its emulsifying capacity and stability (Dong et al., 2011; Yoshie-Stark, et al., 2008). This phenomenon could be explained by the lower saponin content in UUAAIP and the effect of ultrafiltration on the removal of alfalfa saponins. Saponins have been used as an emulsifier and gel-forming agent in foodstuffs and cosmetics (Moses, Papadopoulou, & Osbourn, 2014).

Foaming properties

Table 4.3.4 illustrates that the foam capacity and stability of all the extracted alfalfa proteins are very high, and though foam capacity of AIPE (521.4%) and HCE (492.5%) is higher than that of the UUAAIP (365.8%). The foam stability results varied in a similar manner (Table 3). The foam stability of alfalfa protein samples was ranged from 53.6 to 60.2%. The high foam capacity and stability of AIPE and HCE could be also explained by their higher amount of saponin content. In fact, Saponins are known to contribute in the process of foam formation. The combination of hydrophobic aglycone backbone and hydrophilic sugar molecules confers the foaming properties of saponins (Moses, Papadopoulou, & Osbourn, 2014). The average foam capacity and stability of the extracted alfalfa protein were comparable with those of lentil protein isolate (Jarpa-Parra, et al., 2014).

Table 4.3.4.

Functional properties of alfalfa proteins extracted by different methods.

Parameters	Extraction methods		
	HCE	AIPE	UUAAIP
Water-holding capacity (g water/g)	3.34 ± 0.31^{c}	3.90 ± 0.17^{b}	4.35 ± 0.54^{a}
Fat-binding capacity (g oil/g)	3.95 ± 0.19^{c}	4.27 ± 0.09^{b}	4.88 ± 0.24^{a}
Emulsifying activity index (m^2/g)	190.6 ± 35.1^{a}	195.9 ± 27.5^{a}	142.9 ± 20.9^{b}
Emulsifying stability (min)	17.3 ± 1.2^{a}	16.8 ± 0.5^{a}	14.5 ± 0.8^{b}
Foam capacity (%)	521.4 ± 15.2^{a}	492.5 ± 17.6^{a}	365.8 ± 20.3^{b}
Foam stability (%)	60.2 ± 3.2^{a}	59.8 ± 6.5^{a}	53.6 ± 4.9^{b}

* Different letters in each row indicate significant differences ($P < 0.05$).

5. Conclusion And Future research

Optimization of extraction of alfalfa leaf protein for human consumption

5.1.

Conclusion

a) Response surface methodology was used to determine the optimum steam blanching conditions that inactivated the PPO and POD enzymes and the minimum values of the Browning Index and non-protein nitrogen (NPN) of alfalfa leaves for producing higher quality alfalfa protein concentrates for human consumption. Based on the second-order polynomial experiments, the RSM was used to estimate and to optimize the experimental variables: the steaming time (X_1), the particle size of the alfalfa leaves (X_2), and the time between harvesting and steaming (X_3). The best conditions calculated to avoid the browning and the protein degradation in alfalfa leaves under the response surface methodology indicated that the combination of a steaming time of 4.36 min, particle size without cutting (23 mm) and minimum of time between harvesting and steaming (2 hour) gave the optimum result. Under these conditions, the experimental residual activity of polyphenol oxidase was 1.31% and peroxidase enzyme was completely inactivated (0%), the Browning Index was 108.3 (BI unit) and the non-protein nitrogen values 170.2 BI (g/kg TN) respectively, producing a protein with a lighter

color and a higher molecular weight. This optimization maintained the quality and color of alfalfa well during storage.

b) To the best of our understanding, the present research reported is the first in which ultrasound-assisted extraction has been optimized for both the extraction of saponin and their bioaccessibility. Optimization of the ultrasound-assisted extraction conditions for maximizing the yield and bioaccessibility of alfalfa saponins was done. Statistical analysis of high coefficients of correlation accepts the proposed models validity. The optimal extraction parameters were as follows: solvent/raw material ratio 11.4 mL/g; extraction time 2.84 h; extraction temperature 76.8 ^{o}C; ultrasound power 112.0 w; and the ethanol concentration 78.2%. Under the most suitable conditions, the yield and bioaccessibility of total saponins were 1.61% and 18.6%, respectively. Validation of the optimized ultrasound-assisted extraction conditions also shows small deviation as the experimental values established were well within 95% interval of prediction. The yield rate constant for the ultrasound extraction almost two times more than that of the conventional method. Ultrasonic-Assisted extraction, comparing to heat-reflux method, was found to be a good alternative method to enhance the yield and bioaccessibility of saponins.

   The degradation of alfalfa saponins by multi-wavelength UV irradiation follows pseudo first-order kinetic. Ultra-violate irradiation is a suitable method for its degradation when a UV multi-wavelength emitting lamp is used. It indicated absorption in the range of wavelengths between 470 and

610 nm with a maximum absorption peak around 543 nm. The increase in temperature and decrease in pH enhanced the photo-degradation process. It can be seen that the higher the temperature, the faster the alfalfa saponin photo-degradation rate. Alfalfa saponin degraded more easily at pH = 4 than at pH = 7. The optimal conditions for the ranges studied were 80 °C and pH = 4, reaching a reduction of 80% in the initial alfalfa saponin content after 80 min.

c) In this part, the three methods of ultrasound, ultrafiltration and alkaline extraction were employed simultaneously for producing protein isolates from alfalfa leaves. The simultaneous optimization of UUAAIP technique was established for improving the extraction yield and protein content of alfalfa isolates. RSM was successfully applied to optimize the UUAAIP process and analyze the effects of variables and their interactions. The obtained results showed that solvent/solid material ratio, pH, extraction temperature, extraction time and flow rate affected the measured responses significantly. The maximum yield (14.5 g/100g) and protein content (91.1 g/100g) were reached at the following optimized conditions: solvent/solid material ratio of 43.3 mL/g, pH of 10.1, extraction temperature of 42.5 oC, extraction time of 102 min, and flow rate of 9.7 L/h. The anti-nutritional factors and physicochemical and functional characteristics of alfalfa protein isolates were affected by the protein extraction methodology. UUAAIP technique improved the average molecular weight, color and protein content of the extracted isolates. Moreover, it was efficient in removing the

saponins and phenolic compounds compared to the conventional and industrial methods. UUAAIP protein had greater solubility, water-holding and oil-binding capacities but lower emulsifying and foaming properties than those of HCE and AIPE. Thus, UUAAIP is an appropriate method for manufacturing alfalfa protein isolates and could resolve restrictions of human consumption of alfalfa protein.

5.2.

Recommendation and Future research

On account of conclusions that have been reached within the different studied fields, some final considerations must be stated in order to define the next steps in this research line.

Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) technique should be used for producing protein for human consumption from other sources of protein.

The effect of different drying conditions and methods should be studied on some quality factors of alfalfa protein.

Alfalfa protein isolate with high quality should be replaced to meat protein in food products.

Future investigation is required at a molecular scale, in order to understand the interaction between different saponins and UV-vis irradiation that lead to saponins degradation.

References

Optimization of extraction of alfalfa leaf protein for human consumption

A

Abete, I., Parra, D., & Martinez, J. A. (2009). Legume, fish, or high-protein-based hypocaloric diets: Effects on weight loss and mitochondrial oxidation in obese men. *Journal of Medicinal Med Food*, 12, 100-108.

Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum L.*) seed protein isolate. *Food Chemistry, 128*(4), 902–908.

Aiking, H. (2011). Future protein supply. *Trends in Food Science and Technology*, 22(2), 112-120.

Aiking, H. (2014). Protein production: Planet, profit, plus people? *American Journal of Clinical Nutrition*, 100(Suppl.), 483-489.

Amarowicz, R., & Pegg, R. B. (2008). Legumes as a source of natural antioxidants. *European Journal of Lipid Science and Technology*, 110, 865-878.

Anderson, J. W. (2002). Whole-grains intake and risk for coronary heart disease. In L. Marquart, J. L. Slavin, & R. G. Fulcher (Eds.), Whole-grain foods in health and disease (pp. 187-200). St Paul, Minnesota, USA: American Association of Cereal Chemists.

Anderson, J. W. (2003). Whole grains protect against atherosclerotic cardiovascular disease. *Proceedings of the Nutrition Society*, 62, 135-142.

Anderson, J. W., & Major, A. W. (2002). Pulses and lipaemia, short-and long-term effect: Potential in the prevention of cardiovascular disease. British Journal of Nutrition, 88(S3), 263-271.

Atra, R., Vatai, G., Bekassy-Molnar, E., & Balint, A. (2005). Investigation of ultra-and nanofiltration for utilization of whey protein and lactose. *Journal of Food Engineering, 67*(3), 325–332.

Arogundade, L. A., & Mu, T. H. (2012). Influence of oxidative browning inhibitors and isolation techniques on sweet potato protein recovery and composition. *Food Chemistry, 134*(3), 1374–1384.

Augustin, J. M., Kuzina, V., Anderson, S. B., & Bak, S. (2011). Molecular activities, biosynthesis and evolution of triterpenoid saponins. *Phytochemistry, 72*, 435-457.

Aw, T. L., & Swanson, B. G. (1985). Influence of tannin on Phaseolus vulgaris protein digestibility and quality. Journal of Food Science, 50(1), 67-71.

B

Baker, S., Thompson, K. E., & Palmer-Barnes, D. (2002). Crisis in the meat industry: A values-based approach to communications strategy. *Journal of Marketing Communications*, 8(1), 19-30.

Barbeau, W. E., & Kinsella, J. E. (1988). Ribulose bisphosphate carboxylase/oxygenase (rubisco) from green leaves - Potentials as a food protein. *Food Reviews International, 4*(1), 93–127.

Barba, F.J., Mariutti, L. R. B., Bragagnolo, N., Mercadante, A. Z., Barbosa-Cánovas, G. V., & Orlien, V. (2017). Bioaccessibility of bioactive compounds from fruits and vegetables after thermal and nonthermal processing. *Trends in Food Science & Technology, 67*, 195-206.

Barnes, J., Anderson, L. A., & Phillipson, J. D. (2002). *Herbal Medicines*. London: Pharmaceutical Press. 38-41.

Bax, M.-L.; Buffière, C.; Hafnaoui, N.; Gaudichon, C.; Savary-Auzeloux, I.; Dardevet, D.; Santé-Lhoutellier, V.; Rémond, D. Effects of meat cooking, and of ingested amount, on protein digestion speed and entry of residual proteins into the colon: *A study in minipigs. PLoS ONE* 2013, 8, 612-652.

Bazzano, L. A., He, J., Ogden, L. G., Loria, C., Vupputuri, S., Myers, L., & Whelton, P. K. (2001). Legume consumption and risk of coronary heart disease in US men and women: NHANES I epidemiologic follow-up study. *Archives of Internal Medicine*, 161(21), 2573-2578.

Bazzano, L. A., Tees, M. T., & Nguyen, C. H. (2008). Effect of non-soy legume consumption on cholesterol levels: A meta-analysis of randomized controlled trials. *Circulation*, 118, 1122.

Bedin, S., Netto, F. M., Bragagnolo, N., & Taranto, O. P. (2019). Reduction of the process time in the achieve of rice bran protein through ultrasound-assisted extraction and microwave-assisted extraction, *Separation Science and Technology*. DOI: 10.1080/01496395.2019.1577449.

Brambila-Macias, J., Shankar, B., Capacci, S., Mazzocchi, M., Perez-Cueto, F. J., Verbeke, W., & Traill, W. B. (2011). Policy interventions to promote healthy eating: A review of what works, what does not, and what is promising. *Food and Nutrition Bulletin*, 32(4), 365-375.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.

Bonnely, S., Davis, A. L., Lewis, J. R., & Astill, C. (2003). A model oxidation system to study oxidized phenolic compounds present in black tea. Food Chemistry, 83(4), 483-492.

Bonilla, F., Mayen, M., Merida, J., & Medina, M. (1999). Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chemistry, 66*(2), 209-215.

Box G. E. P., Hunter W. G., Hunter, J. S. (1978). *Statistics for Experimenters*, New York: John Wiley.

Buniowska, M,. Carbonell-Capella, J. M., Frigola, A., & Esteve, M. J. (2017). Bioaccessibility of bioactive compounds after non-thermal processing of an exotic fruit juice blend sweetened with *Stevia rebaudiana. Food Chemistry, 221*, 1834-1842.

Buerkle, C. W. (2009). Metrosexuality can stuff it: Beef consumption as (heteromasculine) fortification. *Text and Performance Quarterly*, 29(1), 77-93.

C

Cam, A., & de Mejia, E. G. (2012). Role of dietary proteins and peptides in cardiovascular disease. *Molecular nutrition and food research*, 56(1), 53-66.

Campos-Vega, R., Loarca-Pina, G. F., & Oomah, B. D. (2010). Minor components of pulses and their potential impact on human health. *Food Research International*, 43, 461-482.

Capacci, S., Mazzocchi, M., Shankar, B., Macias, J. B., Verbeke, W., Pe´rez-Cueto, F. J., Traill, W. B. (2012). Policies to promote healthy eating in Europe: A structured review of policies and their effectiveness. *Nutrition Reviews*, 70(3), 188-200.

Champ, M. M. (2002). Non-nutrient bioactive substances of pulses. *The British Journal of Nutrition*, 88, 307-319.

Chang, K. C., & Satterlee, L. D. (1981). Isolation and *characterization of the major protein from Great Northern Beans (*Phaseolus vulgaris L.). *Journal of Food Science*, 46, 1368-1373.

Charalampos, P., & Michael, K. (2008). Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds. *Food Science and Technology, 41*(4), 652-659.

Chandi, G. K., & Sogi, D. (2007). Functional properties of rice bran protein concentrates. *Journal of Food Engineering, 79*(2), 592–597.

Cheok, C. Y., Salman, H. A. K., & Sulaiman, R. (2014). Extraction and quantification of saponins: A review. *Food Research International*, 59, 16–40.

D

Dagevos, H., & Voordouw, J. (2013). Sustainability and meat consumption: Is reduction realistic. *Sustainability: Science, Practice, and Policy*, 9(2), 60-69.

De Boer, J., & Aiking, H. (2011). On the merits of plant-based proteins for global food security: Marrying macro and micro perspectives. *Ecological Economics*, 70, 1259-1265.

De Smet, S.; Voosen, E. Meat: The balance between nutrition and health. *A review. Meat Sci*. 2016, 120, 145–156.

Ding, W. R., Long, R. J., & Guo, X. S. (2003). Effects of plant enzyme inactivation or sterilization on lipolysis and proteolysis in alfalfa silage. *Journal of Dairy Science*, 96(4), 2536-2543.

Duangmal, K., & Apenten, R. K. O. (1999). A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum var. Romano*). *Food Chemistry*, 64, 351–359.

D'alvise, N., Lesueur-Lambert, C., Fertin, B., Dhulster, P., & Guillochon, D. (2000). Removal of polyphenols and recovery of proteins from alfalfa white protein concentrate by ultrafiltration and adsorbent resin separations. *Separation Science and Technology, 35*(15), 2453–2472.

Dong, X. Y., Guo, L. L., Fang, W., Li, J. F., Jiang, M. L., Li, G. M., Zhao, Y. D., & Chen, H. (2011). Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. *Journal of the Science of Food and Agriculture, 91*,1488–1498.

Du, M., Xie, J., Gong, B., Xu, X., Tang, W., Li, X., Li, C., & Xie, M. (2018). Extraction, physicochemical characteristics and functional properties of Mung bean protein. *Food Hydrocolloids, 76*, 131–140.

E

Eromosele, C. O., Arogundade, L. A., Eromosele, I. C., & Ademuyiwa, O. (2008). Extractability of African yam bean (*Sphenostylis stenocarpa*) protein in acid, salt and alkaline aqueous media. *Food Hydrocolloids, 22*(8), 1622–1628.

European Food Safety Authority (EFSA). (2009). Scientific Opinion of the Panel on Dietetic Products Nutrition and Allergies on a request from the European Commission on the safety of 'Alfalfa protein concentrate' as food. *The EFSA Journal, 997*, 1–19.

European Commission - Standing Committee on Agricultural Research (SCAR) (2011). Sustainable food consumption and production in a resource constrained world. In: The 3rd SCAR foresight exercise.

F

FAO. (2017). *The future of food and agriculture – Trends and challenges*. Rome, Italy.

FAO. (2011). *How to Feed the World in 2050*. Rome, Italy.

Flight, I., & Clifton, P. (2006). Cereal grains and legumes in the prevention of coronary heart disease and stroke: A review of the literature. *European Journal of Clinical Nutrition*, 60, 1145-1159.

Fresco, L. O. (2009). Challenges for food system adaptation today and tomorrow. *Environmental Science and Policy*, 12(4), 378-385.

Fairbairn, R., Alli, I., & Baker, B. E. (1988). Proteolysis associated with the ensiling of chopped alfalfa. *Journal of Dairy Science*, 71(1), 152-158.

Firdaous, B., Fertin, L., Khelissa, O., Dhainaut, M., Nedjar, N., & Chataigné, G. (2017). Adsorptive removal of polyphenols from an alfalfa white protein concentrate: Adsorbent screening, adsorption kinetics and equilibrium study. *Separation and Purification Technology*, 178(7), 29-39.

Firdaous, L., Dhulster, P., Amiot, J., Gaudreau, A., Lecouturier, D., & Kapel, R. (2009). Concentration and selective separation of bioactive peptides from an alfalfa white protein hydrolysate by electrodialysis with ultrafiltration membranes. Journal of Membrane Science, 329(1-2), 60-67.

Fonteles, T. V., Leite, A. K. F., Silva, A. R. A., Carneiro. A. P. G., Miguel E. C., Cavada, B. S., Fernandes F. A. N., & Rodrigues, S. (2016). Ultrasound processing to enhance drying of cashew apple bagasse puree: Influence on antioxidant properties and in vitro bioaccessibility of bioactive compounds. *Ultrasonics Sonochemistry, 31*, 237-249.

Fiorentini, R., & Galoppini, C. (1981). Pilot Plant Production of an Edible Alfalfa Protein Concentrate. *Journal of Food Science, 46*(5), 1514–1517.

Firdaous, B., Fertin, L., Khelissa, O., Dhainaut, M., Nedjar, N., & Chataigné, G. (2017). Adsorptive removal of polyphenols from an alfalfa white protein concentrate: Adsorbent screening, adsorption kinetics and equilibrium study. *Separation and Purification Technology, 178*(7), 29–39.

Francis, G., Kerem, Z., Makkar, H.P.S. Beker, K. (2002). The biological action of saponins in animal systems: a review. *British Journal of Nutrition, 88*, 587–605.

Filgueiras, A. V., Capelo, J. L., Lavilla, I., Bendicho, C., & Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry. 8*(3), 303-313.

G

Gerber, P.J.; Mottet, A.; Opio, C.I.; Falcucci, A.; Teillard, F. (2015). Environmental impacts of beef production: Review of challenges and perspectives for durability. *Meat Sci*, 109, 2–12.

Ghodsvali, A., Haddad, M. H., Vosoughi. M., & Diosady, L. L. (2005). Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Research International*, *38*, 223–231.

Gribova, N. Y., Filippenko, T. A., Nikolaevskii, A. N., Belaya, N. I., & Tsybulenko, A. A. (2008). Optimization of conditions for the extraction of antioxidants from solid parts of medicinal plants. *Journal of Analytical Chemistry*, *63*(11), 1034-1037.

Gordon, R., McDermott, L., Stead, M., & Angus, K. (2006). The effectiveness of social marketing interventions for health improvement: What's the evidence? *Public Health*, 120(12), 1133-1139.

Gokmen, V. (2010). Enzymes in fruit and vegetable processing: Chemistry and engineering applications Chapter 5: selection of the indicator enzyme for blanching of vegetables. *In A. Bayindirli (Ed.)*. CRC Press Boca Raton, FL, pp. 123-144.

Güçlü-Üstündağ, Ö., & Mazza, G. (2007). Saponins: properties, applications and processing. *Critical Reviews in Food Science and Nutrition*, 47 (3), 231-258.

Gue´guen, J., & Cerletti, P. (1994). Proteins of some legume seeds, soybean, pea, fababean and lupin. In B. J. F. Hudson (Ed*.), New and developing sources of food proteins*. New York: Chapman and Hall.

Guo, X. S., Cheng, W., Yang, F. Y,. & Zhou, H. (2011). Contribution of endopeptidase to the formation of nonprotein nitrogen during the ensiling process of alfalfa. *Animal Feed Science and Technology*, 168(1-2), 42-50.

Guo, X. S., Zhou, H., & Zhu, Y. (2007). Changes in the distribution of N and plant enzymatic activity during ensilage of lucerne treated with different additives. *Grass Forage Science*, 62(1), 35-43.

H

Hadidi, M., Ibarz, A., Conde, J., & Pagan, J. (2019). Optimisation of steam blanching on enzymatic activity, color and protein degradation of alfalfa (*Medicago sativa*) to improve some quality characteristics of its edible protein. *Food Chemistry*, 276, 591–598.

Heinz, B., & Lee, R. (1998). Getting down to the meat: The symbolic construction of meat consumption. Communication Studies, 49(1), 86-99.

Henchion, M., McCarthy, M., Resconi, V. C., & Troy, D. (2014). Meat consumption: Trends and quality matters. Meat Science, 98(3), 561-568.

Henderson, L., Gregory, J., Irving, K., & Swan, G. (2003). The National Diet & Nutrition Survey: Adults aged 19 to 64 years (Vols. 1-4). Norwich: HMSO.

Heron, S. J. E., Edwards, R. A., & McDonald, P. (1986). Changes in the nitrogenous components of gamma-irradiated and inoculated ensiled ryegrass. Journal of the Science of Food and Agriculture, 37, 979-985.

Heng, M. Y., Tan, S. N., Yong, J. W. H., & Ong, E. S. (2013). Emerging green technologies for the chemical standardization of botanicals and herbal preparations. *Trends in Analytical Chemistry*, *50*, 1-10.

Heng, L., Vincken, J. P., Hoppe, K., van Koningsveld, G. A., Decroos, K., Gruppen, H., van Boekel, M. A. J. S., & Voragen, A. G. J. (2006). Stability of pea DDMP saponin and the mechanism of its decomposition. *Food Chemistry*, 99 (2), 326-334.

Hoek, A. C., Luning, P. A., Weijzen, P., Engels, W., Kok, F. J., & de Graaf, C. (2011). Replacement of meat by meat substitutes. A survey on personand product-related factors in consumer acceptance. *Appetite*, 56(3), 662-673.

Hojilla-Evangelista, M. P., Selling, G. W., Hatfield, R., & Digman, M. (2016). Extraction, composition and functional properties of dried alfalfa (*Medicago sativa L.*) leaf protein. *Journal of the Science of Food and Agriculture*, 97(3), 882-888.

Houde, M., Khodaei, N., Benkerroum, N., & Karboune, S. (2018). Barley protein concentrates: Extraction, structural and functional properties. *Food Chemistry*, *254*, 367–376.

Hu, T., Guo, Y. Y., Zhou, Q. F., Zhong, X. K., Zhu, L., Piao J. H, Chen, J., & Jiang J. G. (2012). Optimization of Ultrasonic-Assisted Extraction of Total Saponins from Eclipta prostrasta L. Using Response Surface Methodology. *Journal of Food Science*. 77(9), 975-982.

Huang, Z., Liu, Y., Cui, Z., Fang, Y., He, H. H., Liu, B. R., & Wu, G. L. (2018). Soil water storage deficit of alfalfa (Medicago sativa) grasslands along ages in arid area (China). *Field Crops Research,* 221, 1–6.

Hurrell, R.; Egli, I. (2006). Iron bioavailability and dietary reference values. *Am. J. Clin. Nutr*. 91, 1461–1467.

Hu, T., Guo, Y. Y., Zhou, Q. F., Zhong, X. K., Zhu, L., Piao J. H, Chen, J., & Jiang J. G. (2012). Optimization of Ultrasonic-Assisted Extraction of Total Saponins from *Eclipta prostrasta L*. Using Response Surface Methodology. *Journal of Food Science. 77*(9), 975-982.

I

Ibarz, A., Pagán, J., & Garza, S. (2000). Kinetic models of non-enzymatic browning in apple puree. Journal of the Science of Food and Agriculture, 80, 1162-1168.

J

Ja¨rvi, A. E., Karlstro¨m, B. E., Granfeldt, Y. E., Bjo¨rck, I. E., Asp, N. G., & Vessby, B. O. (1999). Improved glycemic control and lipid profile and normalized fibrinolytic activity on a low-glycemic index diet in type 2 diabetic patients. *Diabetes Care*, 22(1), 10-18.

Jenkins, D. J., Wolever, T., Kalmusky, J., Giudici, S., Giordano, C., Patten, R., Csima, A. (1987). Low glycemic index diet in hyperlipidemia: Use of traditional starch foods. *American Journal of Clinical Nutrition*, 46, 66-71.

Jurzysta, M. Hemolytic micro method for rapid estimation of toxic alfalfa saponins. *Acta Agrobot.* 1979, 32, 5-11.

Jones, B. A., Hatfield, R. D., & Muck, R. E. (1995). Characterization of proteolysis in alfalfa and red clover. *Crop Science,* 35(2), 537-541.

Jarpa-Parra, M., Bamdad, F., Wang, Y., Tian, Z., Temelli, F., Han, J., et al. (2014). Optimization of lentil protein extraction and the influence of process pH on protein structure and functionality. *LWT-Food Science and Technology*, *57*, 461–469.

K

Kalac, P., Price, K. R., & Fenwick, G. R. (1996). Changes in saponin content and composition during the ensilage of alfalfa (*Medicago sativa L.*). *Food Chemistry*, 56, 377–380.

Kamaljit, V., Raymond, M., Lloyd, S., & Darren, B. (2008). Application and opportunities for ultrasound assisted extraction in the food industry: a review. *Innovative Food Science and Emerging Technologies, 9*(2), 161-169.

Kanerva, M. Meat Consumption in Europe: Issues, Trends and Debates; Artec-Paper 187; Universität Bremen: Bremen, Germany, 2013; p. 58, ISSN 1613-4907.

Key, F. B., & Mathers, J. C. (1995). Digestive adaptations of rats given white bread and cooked haricot beans (*Phaseolus vulgaris*): Large-bowel fermentation and digestion of complex carbohydrates. *British Journal of Nutrition*, 74, 393-406.

Kinsella, J. E., Damodaran, S., & German, B. (1985). Physicochemical and functional properties of oilseed proteins with emphasis on soy proteins In A. M. Altschul, & H. L. Wilcke (Eds.), *New Proteins Foods* (Vol. 5, pp. 107-179). New York: Academic Press.

Kneafsey, M., Dowler, E., Lambie-Mumford, H., Inman, A., & Collier, R. (2013). Consumers and food security: Uncertain or empowered? *Journal of Rural Studies*, 29, 101-112.

Knuckles, B. E., & Kohler, G. O. (1982). Functional properties of edible protein concentrates from alfalfa. *Journal of Agricultural and Food Chemistry*, 30(4), 748-752.

Kwon, J. H., Belanger, J. M. R., Pare, J. R. J., Yaylayan, V. A. (2003). Application of the microwave-assisted process (MAP) to the fast extraction of ginseng saponins. *Food Research International 36* , 491-498.

Knuckles, B. E., & Kohler, G. O. (1982). Functional properties of edible protein concentrates from alfalfa. *Journal of Agricultural and Food Chemistry, 30*(4), 748–752.

L

Labuza, T. P. (1972). Nutrient losses during drying and storage of dehydrated foods. CRC Critical *Reviews in Food Technology*, 3(2), 217-223.

Lamsal, B. P., Koegel, R. G., & Gunasekaran, S. (2007). Some physicochemical and functional properties of alfalfa soluble leaf proteins. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology*, 40(9), 1520-1526.

Lang, V., Bellisle, F., & Oppert, J. M. (1998). Satiating effect of proteins in healthy subjects: A comparison of egg albumin, casein, gelatin, soy protein, pea protein, and wheat gluten. *American Journal of Clinical Nutrition*, 67(6), 1197-1204.

Licitra, G., Hernandez, T. M., & Van Soest, P. J. (1996). Standardization of procedures for nitrogen factionation of ruminant feeds. *Animal Feed Science and Technology*, 57(4), 347-358.

Lin, L, Lei, F, Sun, DW, Dong, Y, Yang B, Zhao, M. 2012. Thermal inactivation kinetics of Rabdosia serra (*Maxim.*) Hara leaf peroxidase and polyphenol oxidase and comparative evaluation of drying methods on leaf phenolic profile and bioactivities. *Food Chemistry*, 134(4), 2021–2029.

Lin, J., Tang, C. Y., Ye. W., Sun, S. P., Hamdan, S. H., & Volodin, A. (2015). Unraveling flux behavior of superhydrophilic loose nanofiltration membranes during textile wastewater treatment. *Journal of Membrane Science*, 493, 690-702.

M

Muzquiz, M., Varela, A., Burbano, C., Cuadrado, C., Guillamo, E., Muzquiz, M., Pedrosa, M. M. (2012). Bioactive compounds in legumes: Pronutritive and antinutritive actions. Implications for nutrition and health. *Phytochemistry Reviews*, 11(2-3), 227-244.

Mathers, J. C. (2002). Pulses and carcinogenesis: Potential for the prevention of colon, breast and other cancers. *British Journal of Nutrition*, 88(S3), 273-279.

Mazzochi, M., Traill, W. B., & Shogren, J. F. (2009). Fat economics: N*utrition, health and economic policy*. Oxford: Oxford University Press.

Muzquiz,, M. (2000). Factores antinutricionales en fuentes proteicas. In J. Vioque,, A. Clemente,, J. Bautista,, & F. Millan, (Eds.), Jornada internacional sobre proteı´nas alimentaras. Spain: Universidad de Sevilla.

Massiot, G.; Lavaud, C. L.; Guillaumen, D.; Le Men-Olivier, L.Reinvestigation of sapogenins and prosapogenins from alfalfa (Medicago sativa*). J. Agric. Food Chem*. 1988a, 36,901-909.

Massiot, G.; Lavaud, C. L.; Le Men-Olivier, L.; Binst, G.; Miller, S.; Fales, H.M. Structural elucidation of alfalfa root saponins by MS and NMR analysis*. J. Chem. Soc., Perkin Trans*. 198813,

Massiot, G.; Lavaud, C.; Besson, V.; Le Men-Olivier, L.; Binst, G. Saponins from aerial parta of alfalfa (*Medicago sativa*). *J. Agric. Food Chem.* 1991,39,78-82.

Medina-Meza, I. G., Barnaba, C., Villanim F., & Barbosa-Canovas, G. V. (2015). Effects of thermal and high-pressure treatments in color and chemical attributes of an oil based spinach sauce. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology*, 60(1), 86-94.

Miller, R. E., Fremery, D., & Kohler, G. O. (1975). Soluble protein concentrate from alfalfa by low-temperature acid precipitation. *Journal of Agricultural and Food Chemistry*, 23(6), 1177-1179.

Mayer, A. M. (2006). Polyphenol oxidases in plants and fungi: Going places? *A review. Phytochemistry,* 67(21), 2318-2331.

Martin, D., Navarro del Hierro, J., Villanueva Bermejo, D., Fernández-Ruiz, R., Fornari, T., & Reglero, G. (2016). Bioaccessibility and antioxidant activity of Calendula officinalis

supercritical extract as affected by in vitro co-digestion with olive oil. *Journal of Agricultural and Food Chemistry, 64*(46), 8828-8837.

Mercado-Mercado, G., Montalvo-González, E., González-Aguilar, G. A., Alvarez-Parrilla, E,. & Sáyago-Ayerdi, S. G. (2018). Ultrasound-assisted extraction of carotenoids from mango (*Mangifera indica L. 'Ataulfo'*) by-products on in vitro bioaccessibility. *Food Bioscience, 21*, 125-131.

Mechmeche, M., Kachouri, F., Chouabi, M., Ksontini, H., Setti. K., & Hamdi, M. (2017). Optimization of Extraction Parameters of Protein Isolate from Tomato Seed Using Response Surface Methodology. *Food Analytical Methods 10*, 809–819.

Moses, T., Papadopoulou, K. K., & Osbourn, A. (2014). Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. *Critical Reviews in Biochemistry and Molecular Biology, 49*(6), 439–462.

N

Navarro del Hierro, J., Herrera, T., Fornari, T., Reglero, G., & Martin, D. (2018a). The gastrointestinal behavior of saponins and its significance for their bioavailability and bioactivities. *Journal of Functional Foods, 40*, 484-497.

Navarro del Hierro, J., Herrera, T., García-Riscoa, M,. Fornari, T., Reglero, G., & Martin, D. (2018b). Ultrasound-assisted extraction and bioaccessibility of saponins from edible seeds: quinoa, lentil, fenugreek, soybean and lupin. *Food Research International, 109*, 440-447.

Ndiaye, C., Xu, S. Y., & Wang, Z. (2009). Steam blanching effect on poly phenol oxidase, peroxidase and color of mango (*Mangifera indica L.*) slices. *Food Chemistry*, 113(1), 92-95.

Neacsu, M., Fyfe, C., Horgan, G., & Johnstone, A. M. (2014). Appetite control and biomarkers of satiety with vegetarian (soy) and meat-based high protein diets for weight loss in obese men: A randomized crossover trial. *The American Journal of Clinical Nutrition*, 100(2), 548-558.

Nowacka, J., & Oleszek, W. (1992). High performance liquid chromatography of zanhic acid glycoside in alfalfa (*Medicago sativa*). *Phytochemistry Analysis*, 3, 227-230.

Nowacka, J., & Oleszek, W. (1992). High performance liquid chromatography of zanhic acid glycoside in alfalfa (*Medicago sativa*). *Phytochemistry Analysis, 3*, 227-230.

O

Ohshima, M,. McDonald, P., (1978). A review of the herbages in nitrogenous compounds in herbage during ensiling. *Journal of the Science of Food and Agriculture*. 29 (6), 497-505.

Oh, M. M., & Rajashekar, C. B. (2009). Antioxidant content of edible sprouts: effects of environmental shocks. *Journal of the Science of Food and Agriculture. 89*(13), 2221-2227.

Oleszek, W. A. (2002). Chromatographic determination of plant saponins. *Journal of Chromatography A*, 967, 147–162.

Oleszek, W. Solid-phase extraction-fractionation of alfalfa saponins. *J. Sci. Food Agric*. 1988, 44, 43-49.

Oleszek, W. Structural specificity of alfalfa saponin hemolysis and its impact on two hemolysis-based quantification methods. J. Sei. Food Agric. 1990, 53, 477-485.

Oleszek, W. (1991). Saponin fractions from Medicago sativa. Atlas of chromatograms. *J. Chromatogr. Sci*.,29, 128.

Oleszek, W.; Jurzysta, M. (1986). Isolation, chemical characterization and biological activity of alfalfa (*Medicago media Pers*.) root saponins. *Acta SOCB. ot. Pol.*,55,2 3-33.

Oleszek, W.; Price, K. R.; Colquhoun, I. J.; Jurzysta, M.; Ploszynski, M.; Fenwick, G.R. (1990a), Isolation and identification of alfalfa (*Medicago sativa L*.) root saponins: Their activity in relation to a fungal bioassay. *J. Agric. Food Chem*. 38, 1810-1817.

Oleszek, W.; Jurzysta, M.; Price, K. R.; Fenwick, G. R. (1990b) High performance liquid chromatography of alfalfa root saponins. *J. Chromatogr.*, 519, 109-116.

Oleszek, W.; Jurzysta, M.; Gorski, P. (1992a). Alfalfa saponins-the allelopathic agent. In Allelopathy, basic and applied aspects; Rizvi, S. 1. W., Rizvi, V., Eds.; Chapman and Halk London,

Oleszek, W.; Jurzysta, M.; Ploszyfiski, M.; Colquhoun, I. J.; Price, K. R.; Fenwick, G.R. (1992b) Zanhic acid tridesmoside and other dominant saponins from alfalfa (*Medicago sativa L.*) aerial parte. *J. Agric. Food Chem*, 40,191-196.

Oleszek, W.; Nowacka, J.; Gee, J. M.; Wortley, G. M.; Johnson, I. T. (1994). Effects of some purified alfalfa (*Medicagosatioa*) saponins on transmural potential difference in mammalian small intestine. *J. Sei. Food Agric*., in press.

Oleszek, W. (1988). Solid-phase extraction-fractionation of alfalfa saponins. *Journal of the Science of Food and Agriculture*, *44*, 43-49.

Oleszek, W. (1996). The Alfalfa Saponins: Structure, Biological Activity, and Chemotaxonomy. *Advances in Experimental Medicine and Biology, 405*,155-170.

Ozturk, B., Seyhan, F., Ozdemir, I., Karadeniz, B., Bahar, B., Ertas, E., & Ilgaz, S. (2016). Change of enzyme activity and quality during the processing of Turkish green tea. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology*, 65, 318-324.

Oleszek, W. (1988). Solid-phase extraction-fractionation of alfalfa saponins. *Journal of the Science of Food and Agriculture*, *44*, 43–49.

Oleszek, W. A. (2002). Chromatographic determination of plant saponins. *Journal of Chromatography A*, *967*, 147–162.

P

Palou, E., Lopez-Malo, A., Barbosa-Canovas, G. V., Welti-Chanes, J., & Swanson, G. B. (1999). Polyphenoloxidase activity and color of blanched and high hydrostatic pressure treated banana puree. Journal of Food Science, 64(1), 42-45.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, *26*, 716–723.

Pedersen, M. W. Relative quantity and biological activity of saponins in germinated seeds, roots, and foliage of alfalfa. Crop Sei. 1975, 15, 541-543.

Piorkowski, D. T., & McClements, D. J. (2013). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocolloids*, *42*(1), 5-41.

Popkin, B.M.; Adair, L.S.; Nq, S.W. Global Nutrition and the pandemic of obesity in developing countries. Nutr. Rev. 2012, 70, 3–21.

Pollard, C. M., Miller, M. R., Daly, A. M., Crouchley, K. E., O'Donoghue, K. J., Lang, A. J., & Binns, C. W. (2008). Increasing fruit and vegetable consumption: Success of the Western Australian Go for 2and5s campaign. Public Health Nutrition, 11(03), 314-320.

Price, K. R., Johnson, J. T., & Fenwick, G. R. (1987). The chemistry and biological significance of saponins in food and feeding stuffs. *Critical Reviews in Food Science and Nutrition, 26*, 27-135.

R

Rao, V., & Al-Weshahy, A. (2008). Plant-based diets and control of lipids and coronary heart disease risk. *Current Atherosclerosis Reports*, 10(6), 478-485.

Roy MK, Juneja LR, Isobe S, Tsushida T. (2009). Steam processed broccoli (*Brassica oleracea*) has higher antioxidant activity in chemical and cellular assay systems. *Food Chemistry*; 114:263–9.

Rostagno, M. A., Palma, M., & Barroso, C. G. (2003). Ultrasound-assisted extraction of soy isoflavones. *Journal of Chromatography A. 1012*(2), 119-128.

Rochfort, S., & Panozzo, J. (2007). Phytochemicals for health, the role of pulses. *Journal of Agricultural and Food Chemistry*, 55, 7981-7994.

Rogers, R. A. (2008). Beasts, burgers, and hummers: Meat and the crisis of masculinity in contemporary television advertisements. *Environmental Communication*, 2(3), 281-301.

Rizkalla, S. W., Bellisle, F., & Slama, G. (2002). Health benefits of low glycaemic index foods, such as pulses, in diabetic patients and healthy individuals. *British Journal of Nutrition*, 88, 255-262.

Rietjens, I. M. C. M., Louisse, J., & Beekmann, K. (2017). The potential health effects of dietary phytoestrogens. *British Journal of Pharmacology, 174*, 1263–1280.

Ruiz, R. G., Price, K. R., Arthur, A. E., Rose, M. E., Rhodes, M. J. C., & Fenwick, R. G. (1996). Effect of soaking and cooking on the saponin content and composition of chickpeas (*Cicer arietinum*) and lentils (*Lens culinaris*). *Journal of Agricultural and Food Chemistry*, 44, 1526-1530.

Ruiz, R. G., Price, K. R., Fenwick, M. J., & Rhodes, M. J. (1996). Effect of processing on saponin content and composition of chickpeas. *Biochemical Society Transactions*, 24 (3), 393–395.

S

Sabate´, J., & Soret, S. (2014). Sustainability of plant-based diets: Back to the future. The American *Journal of Clinical Nutrition*, 100, 476-482.

Sage, C. (2013). The interconnected challenges for food security from a food regimes perspective: Energy, climate and malconsumption. *Journal of Rural Studies*, 29, 71-80.

Sans, P., & Combris, P. (2015). World meat consumption patterns: An overview of the last fifty years (19612011). *Meat Science*, 109, 106-111.

Sagratini, G., Caprioli, G., Maggi, F., Font, G., Giardinà, D., Mañes, J., & Vittori, S. (2013). Determination of soyasaponins I and βg in raw and cooked legumes by solid phase extraction (SPE) coupled to liquid chromatography (LC)–mass spectrometry (MS) and assessment of their bioaccessibility by an in vitro digestion model. *Journal of Agricultural and Food Chemistry, 61*(8), 1702-1709.

Schroeder, T. C., Barkley, A. P., & Schroeder, K. C. (1996). Income growth and international meat consumption. *Journal of International Food and Agribusiness Marketing*, 7(3), 15-30.

Schweiggert, U., Schieber, A., & Carle, R. N. (2005). Inactivation of peroxidase, polyphenoloxidase, and lipoxygenase in paprika and chili powder after immediate thermal treatment of the plant material, Innovative Food Science and Emerging Technologies, 6(4), 403-411.

Shi, J., Xue, S. J., Ma, Y., Li, D., Kakuda, Y., & Lan, Y. (2009). Kinetic study of saponins B stability in navy beans under different processing conditions. *Journal of Food Engineering*, 93 (1), 59- 65.

Shi, J., Arunasalam, K., Yeung, D., Kakuda, Y., Mittal, G., & Jiang, Y. (2004). Saponins from edible legumes: Chemistry, processing, and health benefits. *Journal of Medicinal Food*, 7, 67-78.

Shen, L., Wang, X., Wang, Z., Wu, Y., & Chen, J. (2008). Studies on tea protein extraction using alkaline and enzyme methods. *Food Chemistry, 107*, 929–938.

Sharif, K. M., Rahman, M. M., Azmir, J., Mohamed, A., Jahurul, M. H.A., Sahena. F., & Zaidul, I. S. M. (2014). Experimental design of supercritical fluid extraction – a review. *Journal of Food Engineering*, 124, 105-116.

Sobal, J., & Bisogni, C. A. (2009). Constructing food choice decisions. *Annals of Behavioral Medicine*, 38(1), 37-46.

Sobal, J., Bisogni, C. A., & Jastran, M. (2014). Food choice is multifaceted, contextual, dynamic, multilevel, integrated, and diverse. *Mind, Brain, and Education*, 8(1), 6-12.

Sosulski, F. W. (1979). Organoleptic and nutritional effects of phenolic compounds on oilseed protein products: a review. *Journal of the American Oil Chemists' Society, 56*, 711–714.

Singh, B., Singh, J. P., Singh, N., & Kaur, A. (2017). Saponins in pulses and their health promoting activities: A review. *Food Chemistry, 233*, 540-549.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Sullivan, M. L,. & Hatfield, R. D. (2006). Polyphenol oxidase and o-diphenols inhibit post-harvest roteolysis in red clover and alfalfa. *Crop Science*, 46, 662-670.

T

Tao, L., Zhou, H., Guo, X. S., Long, R. J., & Zhu, Y. (2011). Contribution of exopeptidase to the formation of nonprotein nitrogen during the ensiling process of alfalfa. *Journal of Dairy Science*, 94(8), 3928-3935.

Tarade, K.M., Singhal, R.S., Jayram, R.V., Pandit, A.B., (2006). Kinetics of degradation of saponins in soybean flour (Glycine max) during food processing. *Journal of Food Engineering* 76, 440–445.

Thom, E. (2000). A randomized, double-blind, placebo-controlled trial of a new weight-reducing agent of natural origin. *Journal of International Medical Research*, 28, 229-233.

Thorne, M. J., Thompson, L. U., & Jenkins, D. J. A. (1983). Factors affecting starch digestibility and the glycemic response with special reference to legumes. *American Journal of Clinical Nutrition*, 38, 481-488.

Tobler, C., Visschers, V. H., & Siegrist, M. (2011). Eating green. Consumers' willingness to adopt ecological food consumption behaviors. *Appetite*, 57(3), 674-682.

Tome, D. (2012). Criteria and markers for protein quality assessment A review. *British Journal of Nutrition*, 108, 222-229.

Timbekova, A. M.; Larin, M. F.; Jagubaev, M. P.; Abubakirov, N. K. (1989). Triterpene glycosides of alfalfa. V. Medicoside H. Khim. Prir. Soed.,5, 673-677.

Toivonen, P. M. A., & Brummell, D. A. (2008). Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biology and Technology*, 48(1), 1-14.

Tomas-Barberan, F. A., & Espin, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, 81(9), 853-876.

U

United Nations. (2019). Department of Economic and Social Affairs, Population Division, *World Population Prospects 2019: Highlights* (ST/ESA/SER.A/423).

V

Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs, *Ultrasonics Sonochemistry, 8*, 303-313.

Volenec, J., Cunningham, S., Haagensen, D., Berg, W., Joern, B., & Wiersma, D. (2002). Physiological genetics of alfalfa improvement: past failures, future prospects. *Field Crop Research*, 75(1-2), 97-110.

W

Wang, J. C., & Kinsella, J. E. (1976). Functional properties of novel proteins: Alfalfa leaf protein. *Journal of Food Science*, 41(2), 286–292.

Wang, C., Tian, Z., Chen, L., Temelli, F., Liu, H., & Wang, Y. (2010). Functionality of barley proteins extracted and fractionated by alkaline and alcohol methods. *Cereal Chemistry, 87*, 597–606.

Wansink, B., & Sobal, J. (2007). Mindless eating the 200 daily food decisions we overlook. *Environment and Behavior*, 39(1), 106-123.

Wojciechowski, K., Kezwon, A., Lewandowska, J., & Marcinkowski, K. (2014). Effect of β-casein on surface activity of Quillaja bark saponin at fluid/fluid interfaces. *Food Hydrocolloids, 34*, 208-216.

Wu, G.Y.; Fanzo, J.; Miller, D.D.; Pingali, P.; Post, M.J.; Steiner, J.L.; Thalacker-Mercer, A.E. Production and supply of high-quality food protein for human consumption: Sustainability, challenges, and innovations. *Ann. N. Y. Acad. Sci*. 2014, 1321, 1–19.

Wu, J., Lin, L,. & Chau, F, T. (2001). Ultrasound-assisted extraction of ginseng saponins from ginseng root and cultured ginseng cell. *Ultrasonics Sonochemistry, 8*, 347-352.

X

Xiao, H. W., Pan, Z. H., Deng, L. Z., El-Mashad, H. M., Yang, X. H., & Mujumdar, A. (2017). Recent developments and trends in thermal blanching–A comprehensive review. *Information Processing in Agriculture*, 4(2), 101-127.

Xu, L., & Diosady, L. L. (2002). Removal of phenolic compounds in the production of high-quality canola protein isolates. *Food Research International*, *35*(1), 23–30.

Y

Yang, L., Cao, Y. L., Jiang, J. G., Lin, Q. S., Chen, J., & Zhu, L. (2010). Response surface optimization of ultrasound-assisted flavonoids extraction from the flower of *Citrus aurantium* L. var. amara. *Journal of Separation Science, 33*(9), 1349-1355.

Yoshie-Stark, Y., Wada, Y., & Wasche, A. (2008). Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food Chemistry, 107*, 32–39.

Yu, X., Bogaert, L., Hu. R., Bals, O., Grimi, N., & Vorobiev, E. (2016). A combined coagulation-ultrafiltration method for enhanced separation of proteins and polyphenols. *Separation Science and Technology*, *51*, 1030–1041.

Z

Zhang, W., Grimi, N., Jaffrin, M. Y., & Ding, L. (2015). Leaf protein concentration of alfalfa juice by membrane technology. *Journal of Membrane Science*, 489, 183-193.

Zhang, Q., Tan, S., McKay, A., & Yan, G. (2005). Carrot browning on stimulated market shelf and during cold storage. *Journal of the Science of Food and Agriculture*. 85(1), 16-20.

Zhang, W., Grimi, N., Jaffrin, M. Y., Ding, L., Tang, B. (2017). A short review on the research progress in alfalfa leaf protein separation technology. *Journal of Chemical Technology & Biotechnology, 92*(12), 2894–2900.

Zimmer, D. E.; Pedersen, M. W.; McGuire, C. F. A bioassay for alfalfa saponins using the fungus Trichoderma viride. *Crop Sci*. 1967, 7, 223-224.

Acknowledgements

I would like to express my special appreciation and thanks to my advisors Professor Dr. Albert Ibarz and Professor Dr. Jordi Pagan, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been priceless. I would also like to thank my committee members for serving as my committee members even at hardship. I also want to thank you for letting my defense be an enjoyable moment, and for your brilliant comments and suggestions, thanks to you. I would especially like to thank my friends at University of Lleida, Spain. All of you have been there to support me when I recruited patients and collected data for my Ph.D. thesis.

A special thanks to my family. Words cannot express how grateful I am to my parents and entire family for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far.

And to you my best, Arqavan, just thanks for… standing by me.