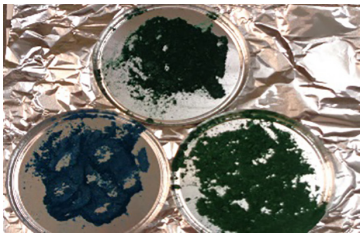
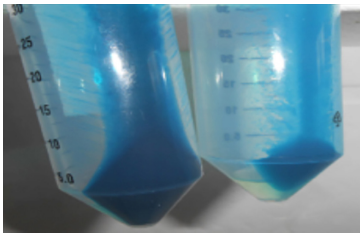
LIÈGE université
Gembloux
Agro-Bio Tech

Interfacial behavior of colored protein fractions from *Spirulina platensis*

Barka Abakoura





Promotor: Prof. Christophe Blecker

LIÈGE université
Gembloux
Agro-Bio Tech

Interfacial behavior of colored protein fractions from *Spirulina platensis*

BARKA ABAKOURA

Thesis submitted in fulfilment of the requirements for the award of PhD in Agronomic Sciences and Biological Engineering.

Promotor (supervisor): Professor Christophe BLECKER

October 2018

Copy right

ABSTRACT

This study is carried out to evaluate the interfacial activity of different fractions of *Spirulina* soluble proteins. Before the experimental steps, a broad literature review is highlighted to point out the global microalgae potential, with particular emphasis on *Spirulina* sp. The protein content of different microalgae species is presented, together with the nutritional quality of these proteins. Out of this investigation, it clearly appeared that micro algae present a higher productivity per unit area when compared to high plants. They are able to thrive and survive in drastic climatic conditions. *Spirulina* platensis particularly presents high protein content with good nutritional characteristics.

The first experimental assessment is the extraction of the colored *Spirulina* soluble protein fractions from dry *Spirulina* powder and evaluation of their physicochemical properties. Three fractions, including the blue soluble (BSSP), the green soluble (GSSP), and the total soluble (TSSP) *Spirulina* protein fractions are obtained. Investigations on their behavior at air/water interface are carried out, using dynamic methods of drop volume (TVT1), automated drop (tracker), and bubble pressure (BP100) tensiometers. Evaluation of their monolayer films mechanical behavior is done via compression isotherms using Langmuir film balance. The protein contents of the fractions are 82.76%; 82.29%; 74.53% for the blue, the green and the total fractions, respectively. Surface tension decay increases with increasing concentration for all the fractions. The tension decay is less important at pH 3 for all the fractions. The total fraction and the blue fraction appeared to form more elastic films than the green fraction. The blue soluble fraction also presented the highest collapse pressure and initial area.

The second experimental evaluation is focused on the performances of colored *Spirulina* soluble protein fractions as surfactants at water/n-dodecane interface. Evaluation of their interfacial activities is carried out using different methods as for the air/water interface. Different concentrations (0.1%; 0.3% and 0.5% (w/w)) and pH levels (3, 5, and native pH) are tested. Results show that, the interfacial tension decay increased with increased protein concentration. At 0.3% (weight/weight) colored protein concentration in the aqueous phase, the surface tension decay is greater at pH 5 compared to pH 3 and native pH. The interfacial elastic moduli of the fractions suspensions decrease with concentration unlike viscosity moduli.

Investigations on the emulsifying and foaming properties make the third experimental task. Emulsions at pH 3 are very susceptible to destabilization phenomena such as coalescence, and creaming. The emulsifying properties of the three fractions follow the same trends. However, the BSSP fraction shows a better emulsifying effect. Fractions present higher foaming capacities at their native pH, but foams are more stable at pH 3. The foaming behavior at pH 5 is close to that at the native pH.

Despite the undoubted link between *Spirulina* protein surface activity and foaming and emulsifying properties, it should be beard in mind that the best properties are not always only obtained by the conditions of greater ability of the protein to reduce surface or interfacial tension but also to its solubility. Nevertheless the surface activity of the proteins remains the prerequisite to foaming or emulsifying properties.

It should also be emphasized that the fractionation method developped in this work, unlike the methods so far available in the literature, is industrially feasible and could

allow for large production of the *Spirulina* protein fractions. One of the obtained fractions (BSSP) presents better emulsifying ptoperties. All the three fractions present interfacial activities, and can provide foaming and emulsifying properties that would allow for their industrial use as emulsifiers in lieu and place of conventional proteins. Their color may be an asset for their use in some specific applications.

RESUME

Cette étude est réalisée dans le but d'évaluer l'activité interfaciale de différentes fractions de protéines solubles de spiruline. Avant les étapes expérimentales, une vaste revue de la littérature a permis de mettre en évidence le potentiel des micro-algues, avec un accent particulier sur *Spirulina sp*. La teneur en protéines de différentes espèces de micro-algues est présentée, ainsi que la qualité nutritionnelle de ces protéines. De cette étude, il est clairement apparu que les micro-algues présentent une productivité par unité de surface supérieure à celle des hautes plantes. Elles sont capables de prospérer et de survivre sous des conditions climatiques drastiques. *Spirulina platensis* présente en particulier une teneur élevée en protéines avec de bonnes caractéristiques nutritionnelles.

La première étape expérimentale est l'extraction des fractions protéiques solubles de spiruline colorées à partir d'une poudre de spiruline commerciale et l'évaluation de leurs propriétés physicochimiques. En plus de la fraction totale (TSSP), deux fractions, la bleue (BSSP) et la verte (GSSP) sont obtenues. Les teneurs en protéines de ces fractions sont respectivement de 74,53% (TSSP), 82,76% (BSSP) et 82,29% (GSSP). Des études sur le comportement de ces différents extraits protéiques à l'interface air/eau sont effectuées, en caractérisant les cinétiques d'adsorption à l'aide de méthodes dynamiques (le tracker, les tensiomètres à goutte tombante et à pression de bulle) et les propriétés mécaniques de monocouches grâce à une balance à film de Langmuir. D'un point de vue cinétique, la réduction de la tension superficielle au cours du temps est d'autant plus marquée que la concentration est importante, et ce, pour toutes les fractions. L'étude de l'influence du pH a montré que la diminution de tension est moins conséquente à pH 3 pour toutes les fractions. En ce qui concerne les monocouches, la fraction totale et la fraction bleue semblent former des films plus élastiques que la fraction verte. La fraction bleue présente également la pression de rupture la plus élevée et une plus grande surface initiale.

La seconde évaluation expérimentale est axée sur les performances des mêmes fractions protéiques en tant que tensioactifs à l'interface liquide/liquide modèle, eau/n-dodécane, en étudiant tout comme pour l'interface air/eau l'influence de la concentration et du pH. L'évolution de la cinétique d'adsorption à cette interface présente les mêmes tendances avec une performance d'adsorption à pH 5 nettement meilleure qu'à pH 3 et pH natif.

Les investigations des propriétés technofonctionnelles gouvernées par les propriétés interfaciales (propriétés émulsifiantes et moussantes) ont constitué la troisième partie expérimentale. Les propriétés émulsifiantes des trois fractions suivent les mêmes tendances. Cependant, la fraction BSSP montre un meilleur effet émulsifiant. Les émulsions préparées à pH 3 sont très sensibles aux phénomènes de déstabilisation tels que la coalescence et le crémage. Les fractions présentent des capacités de moussage plus élevées à leur pH natif, mais les mousses sont plus stables à pH 3. Le comportement moussant à pH 5 est proche de celui du pH natif. Malgré le lien incontestable entre l'activité interfaciale des protéines de la spiruline et les propriétés moussantes et émulsifiantes, il faut garder à l'esprit que les meilleures propriétés ne sont pas toujours obtenues par les conditions de plus grande capacité de la protéine à réduire la tension

superficielle ou interfaciale. Néanmoins, l'activité de surface des protéines reste la condition préalable aux propriétés moussantes ou émulsifiantes.

Il convient de souligner que la méthode de fractionnement mise en œuvre dans ce travail, contrairement à celle jusqu'à lors disponible dans la littérature est industrialisable et pourrait permettre une grande production de ces fractions. L'une des fractions obtenues de couleur bleue (BSSP) présente de meilleures propriétés émulsifiantes. Toutes les trois fractions montrent des activités interfaciales, et peuvent fournir des propriétés moussantes et émulsifiantes qui permettraient leur utilisation industrielle en tant qu'émulsifiants en lieu et place des protéines conventionnelles. Leur couleur peut être un atout pour leur utilisation dans certaines applications spécifiques.

Acknowledgement

My deepest gratitude goes to my supervisor, Professor Christophe BLECKER who diligently supervises this work. Thanks to my thesis committee members: Professor Sabine DANTHINE, Professor ANGELIQUE Léonard and Professor Frank DELVIGNE for their time devoted and essential contribution to the realisation of this work.

I would like to thank the members of the jury Professor Romdhane KAROUI, Dr Gaoussou KARAMOKO and the president of the Jury professor Marie-Laure Fauconnier for the time devoted to assess and improve the quality of the work.

I am grateful to the government of Tchad Republic and particularly to the "*Consortium des Instutitions supérieures du Tchad-Université de Liège"* for the grant provided for realisation of this work.

Thanks to Mr Mahamat Barka, Mme Isabelle Halleux, and Mme Boutheina Ben Lamine who made it possible for me to carry out my PhD thesis in *Université de Liège*.

My gratitude to all the members of the technical and administrative staff of the research unit *Science des Aliments et Formulation Gembloux Agro Bio Tech.* for the friendly work environment they provided.

I am grateful to my sisters and brothers for their tremendous moral support that allows me to reach this level of training.

Thanks to my wife for her assistance and endurance in taking care of our children.

Last but not the least, thanks to my friends and colleagues with whom I share many things during my stay in Gembloux.

BARKA ABAKOURA

Table of contents

List of Figures

List of Tables

Abbreviations and symbols

- ∞: minus infinity
$\partial A_{p,T}$: change in area at constant pressure and temperature
∂G: change in free energy
°C: degree centigrade
0.1M: 0.1 molar
A_0: initial area
A_{280}: absorbance at 280 nanometre
A_{620}: absorbance at 620 nanometre
A_c: critical area
AFDW: ash free dry weight
AOACI: Association of Official Analytical Chemists International
ASPI: algal soluble protein isolated
ASW: Artificial sea water
A_t: transition point area
ATCC: American culture collection
BSSP: blue soluble *Spirulina* protein
BV: biological value,
$CaCl_2$: Calcium chloride
C_m: Compressibility coefficient
C-PC: C-phycocyanin
DC: protein digestibility coefficient
DD: drum dried
DSC: differential scanning calorimetry
Ɛ: Elasticity
F: force
f: correction factor
F: resulting force
FC: Foam capacity
g: gravitational constant
G: free energy
g: free energy of a molecule within the liquid
Gs: free energy at the interface
GSSP: green soluble *Spirulina* protein
ha^{-1}: per hectare
HCl: hydrochloric acid
kDa: kilodalton
l: length
m^{-2}: per meter square
$MgSO_4$: Magnesium sulfate
mg/L.d: milligram per liter per day
mN/m: milli newton per meter

n: number of molecules
NaCl: Sodium chloride
$NaHCO_3$: Sodium bicarbonate
NaOH: Sodium hydroxide
NAS-NRC: National Academy of Sciences-National Research Council
NF : norme française
nm: nanometer
NPU : net protein utilization
O/W: oil in water
O_2: Oxygen
P: pressure
PER: protein efficiency ratio
pHi: isoelectric potential
r_{cap} : capillary radius
R-PC: R-phycocyanin.
$s.\mu L^{-1}$: second per microliter
SCP: single cell protein
SD: sun dried
SDS–PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
T: temperature
TSSP: total soluble *Spirulina* protein
UV: ultraviolet
v: critical drop volume
W/O: water in oil
γ: surface tension
γ_0: initial surface tension
ΔP: pressure change
$\Delta\rho$: density difference between the two adjacent phases
ζ potential : zeta potential
π: surface pressure
π_c: critical pressure
π_t: transition point pressure

DEDICATION

This work is dedicated

to my

father Alhadji Gada

and

mother Hadja Ammakous

Strategy and objective

Strategy and objective

In order to fill the protein gap, the use of *Spirulina* soluble proteins to supplement conventional proteins is to be encouraged. To promote their use as emulsifiers or foam stabilizers in food colloids, these proteins need some investigations on their physicochemical properties, specifically on their interfacial properties. Indeed, the lack of knowledge on these properties is among the factors hindering the use of proteins from new sources for their proper applications in processed foods.

The raw material used in this study is a commercial *Spirulina* powder, from which the protein fractions are separated. The choice of this *Spirulina* powder from an industrial source as raw material is done to minimize contamination problems, and strain variabilities that can occur in naturally grown *Spirulina*.The colored soluble protein fractions of the microalgae are targeted, leaving the rest of the biomass including insoluble proteins (under the extraction conditions used) as co-products. Investigation on these co-products does not fall within the scope of the present study.

This thesis aims at evaluating the interfacial activity of the colored *Spirulina* soluble protein fractions together with some other physicochemical and sensory characteristics. Knowledge of these properties will allow for a proper use of the fractions. The incidence of the interfacial characteristics on the functional properties such foam and emulsion properties is also evaluated.

Specifically the present study is focused on:

- ✓ the extraction and characterisation of different *Spirulina* protein fractions based on an industrially feasible method: total soluble *Spirulina* protein (TSSP), green soluble (GSSP) and blue soluble (BSSP) *Spirulina* proteins;
- ✓ surface and interfacial properties of the fractions;
- ✓ foaming and emulsifying properties of the protein fractions.

The study is structured into chapters after the General introduction. The first chapter is an overview of the microalgae as potential sources of proteins, and a glance on surface and interfacial properties of their proteins. The second chapter presents the materials and methods used in the study. The protein extraction methods and some physicochemical characterisation of the fractions obtained are discussed in the third chapter. In the fourth chapter, the surface (air/water) and the interfacial (water/n-dodecane) behaviours of the protein fractions are presented. The foaming and emulsifying properties, solubility and oil absorption of the fractions are discussed in the fifth chapter followed by the overall discussion in chapter six. An overall conclusion to the whole work is presented at the end of the thesis to point out the important findings, their applicability and eventual perspectives for a future study.

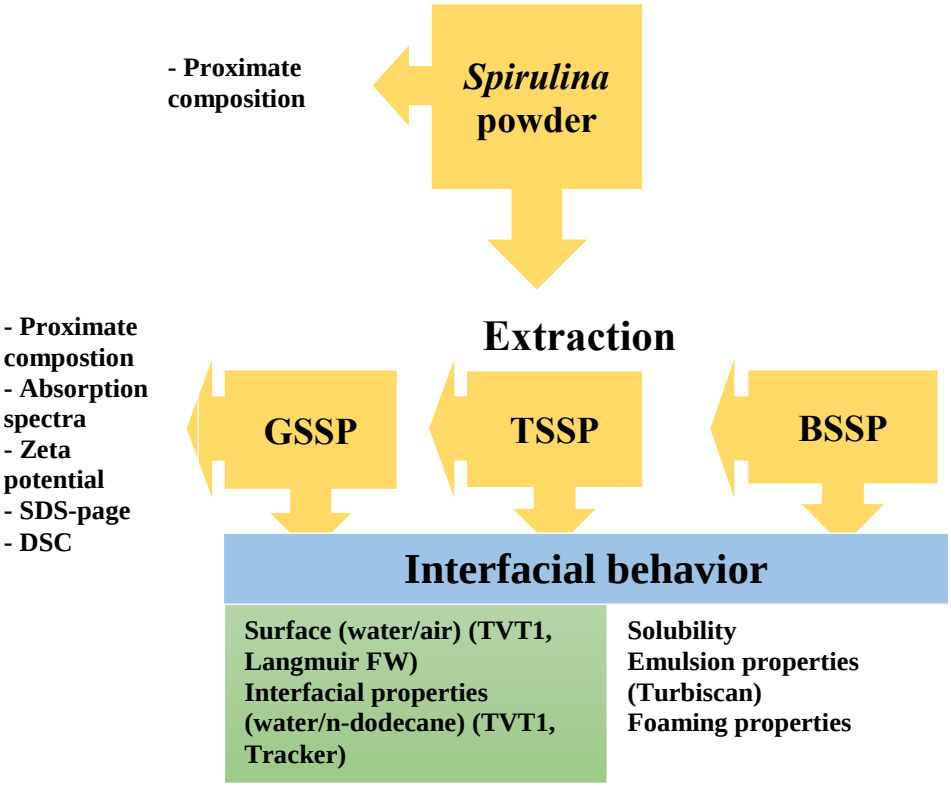


Figure 1: Overall working scheme

General Introduction

Introduction

The challenge to feed the increasing global population by implementing food production systems based on a sustainable development, and to create desirable living conditions, has brought about new research paths during the present century. A sharp protein shortage was foreseen since the early 1970s by the United Nations Advisory Committee on International Action, and recommended non-conventional crops such as autotrophic microorganisms as a source of feed or food for the expanding world population (Cohen, 1999). Microalgae could be considered as one of the reliable natural sources of nutrients and other valuable substances that can fulfil the growing needs in food and energy (Becker, 2007).

While some microalgae such as *Chlorella sp*, *Botryococcus sp*, *Chaetoceros sp*, *Ellipsoidion sp*...(Malcata, 2011) are targeted for their high lipid content that could be transesterified as biofuel (renewable energy), others such as *Spirulina* sp are attractive for their high protein content in addition to vitamins, essential fatty acids and pigments (Becker, 2007). Microalgae have higher productivity than traditional crops and can be grown under climatic conditions, such as desert and coastal areas (Christaki *et al*., 2011). Their production yield is not only high but also friendly to the preservation of the environment. Marshall (2007) cited by Christaki *et al.* (2012) reported that they are the most productive plants in the world.

Microalgae are autotrophic organisms that grow naturally on land or in aquatic media where they constitute, together with other higher photosynthetic plants the starting point of food chain in their ecosystems. They have been intensively used in aquaculture, and their use in human nutrition also experienced an historical background (De Pauw *et al.*, 1984).

Spirulina (*Arthrospira*) *sp*, a blue green microalga is among the most ancient microalgae used in human nutrition (Qian Hu, 2004). It is nowadays known as a source of a number of valuable functional substances such as vitamins, beta carotene, sulphated polysaccharides, polyunsaturated fatty acids (PUFA) and proteins. *Spirulina* proteins content is high and exhibits various functional properties. It can readily compete with animal protein and contains all the essential amino acids (Becker, 2007). Phycocyanin, the *Spirulina* blue coloured protein is one of its attractive substances exhibiting nutraceutical and technological applications (Berenice *et al.* , 2014).Thus, its uses start from a simple nutrient as any other protein, to medicinal, esthetical and technological substance. A lot of scientific publications on phycocyanins extraction and purification are available in the literature (Devendra *et al.*, 2014; Patel *et al*., 2005).

So far, few investigations on the use of *Spirulina* biomass in food formulations for malnourished children or HIV patients have been carried out mostly under clinical evaluations (Yamani *et al*., 2009; Simpore *et al*., 2005; Simpore *et al.*, 2006). Also, a lot of literature on phycocyanin extraction methods and its clinical evaluations on laboratory animals is available (Yan-Jiao *et al*., 2016). Less research had been focused on the evaluation of the physicochemical and technofunctional properties of *Spirulina* biomass or its extracts. Especially the protein fraction who's proper and controlled industrial utilization in food matrixes needs the knowledge of its physicochemical and

technological properties such as the interfacial tension, emulsion capacity and stability, foaming properties among others.

Proteins in food processing industry can be used as food ingredients, food additives, food supplement, colorant, emulsifier…They are the limited macronutrient compared to carbohydrates and lipids in food or feed. Their proper integration in a processed food in order to perform a given role, needs investigations. As a nutrient or supplement in a processed food, nutritional characteristics such as digestibility, types of amino acids, allergy, safety, etc., should be tested. When designed for its functional (technofunctional) properties, protein or protein concentrates would be conveniently used only if their physicochemical properties are well known to the food processor. These include those properties that can affect the texture and other organoleptic properties of the processed food.

Presently, there are no enough investigations on surface and interfacial behavior of colored *Spirulina* proteins. The few studies so far available on *Spirulina* protein functional behavior are focused on the total soluble *Spirulina* proteins. Chronakis (2001) evaluated the visible absorption, differential scanning calorimetry, viscosimetry and dynamic oscillatory rheological properties of the soluble *Spirulina* proteins. Some functional properties of soluble *Spirulina* proteins including oil and water absorption capacities, foaming and emulsifying properties, surface hydrophobicity and nitrogen solubility were evaluated by Bashir *et al.* (2016). Benelhadj *et al.* (2016) investigated also on nitrogen solubility, oil and water absorption capacities, and emulsifying properties of the soluble *Spirulina* proteins. To the best of our knowledge, the only study on interfacial behavior of colored fractions is that of Chronakis *et al*. (2000), who investigated on the colored fractions obtained by ultracentrifugation. According to their study, the fractions were tested for behavior at air/water, using static method with a Wilhelmy plate tensiometer. Useful information such as the knowledge of the fractions ability to present characteristics quite comparable to conventional proteins were reported. But the separation method of the colored fractions used under this study is an analytical method that could not be industrially feasible for large quantity food processing due to the high cost and energy requirements of ultracentrifugation. Also, other testing methods (dynamic methods of measuring adsorption kinetics) closer to processes of foam and emulsions preparation may provide additional informations. This will allow for a better appreciation of the behavior of *Spirulina* colored proteins within colloidal systems.

The present study is focused on some physicochemical and technofunctional characterisations of different fractions of the soluble *Spirulina* proteins i.e. the total soluble (TSSP), the green soluble (GSSP) and the blue soluble (BSSP) *Spirulina* protein fractions separated from *Spirulina* powder. Literature on the technofunctional properties of the BSSP fractions is seriously lacking, most studies on this fractions were oriented on the extraction methods and its nutraceutical uses. Nevertheless the use of this attractive natural blue coloured protein needs more investigations not only in looking for simple extraction method but also its physicochemical and technofunctional properties as a protein and a colorant. A simple industrially feasible method comparable to those currently used to prepare protein ingredients is developed under the present study, and

allows to obtain the BSSP and the GSSP fractions. These fractions are then evaluated together with the TSSP fraction.

Chapter1

Literature review

Part of this chapter was published as cited below:
Barka A and Blecker C, 2016. Microalgae as a potential source of single-cell proteins. A review. *Biotech. Agr., Soc. and Envir.*, 20 (3) 427-4

1. Introduction

This chapter is aiming at highlighting the state of art of the study in order to know what is yet to be investigated. Although the study is focused on only one microalga specie (*Spirulina platensis*), we found it vital to point out the global microalgae potentials as a whole, to show the diversity of protein and other nutrients sources that could be exploited to feel protein shortages due to the growing global populations (NAS-NRC, 1963).The new protein sources represented by the microalgae species are described with their relative protein contents presented. Their global production potential is also pointed out in order to show feasibility of their industrial mass production and the advantages they possess in terms of production yields and global nutrients supply potential.

The rest of the study was limited to microalgae proteins, and specifically those obtained from S*pirulina platensis* as the starting point of a broad research that could be extended to other nutrients from *Spirulina* or other microalgal sources. As such, knowledge on the extraction methods and the physicochemical characterisation of *Spirulina* soluble proteins which are the targeted nutrients under this study are the focus of the study. For a proper contribution to this field of study, an investigation on the background scientific knowledge concerning these aspects is obvious. The second part of this literature review includes the presentation of the key physicochemical parameters investigated in coming chapters. These include surface and interfacial properties, foaming and emulsifying properties of the protein fractions.

2. Microalgae as potential sources of Single cell Protein

The search for new protein sources to supplement the existing conventional sources in order to fill the so called « protein gap » has been the research route inspiration for many scientists. Single-cell protein (SCP) refers to protein extracted from pure or mixed cultures of algae, yeast, fungi or bacteria used as substitute to the conventional protein sources for human and animal consumption. Proteins bear a complex chemical structure, and this complexity is the basis of their multiple physiological, morphological, and technological implications. Proteins can be used as sole protein concentrates or integrated in processed foods. In the latter case, every constituent of the processed food product plays a specific role, be it nutritional, technological or functional. The knowledge of the technofunctional and nutritional properties of proteins is therefore a prerequisite for their proper utilization in food industries.

Microalgae have been identified as one of the reliable sources of protein and have attracted the attention of most actors in agricultural and food domains during the second half of the twenty first century. Some microalgal sources present protein content higher than conventional animal or plant sources e.g. *Spirulina paltensis*, protein content is 65%, higher than that of dried skimmed milk (36%), soy flour (37%), chicken (24%), fish (24%), beef (22%) and peanuts (26%) (Kelly and Bob, 2011). Despite the tremendous virtuous nutraceutical uses of substances from algal biomass reported

(Simpore *et al.*, 2005; Simpore *et al.*, 2006; Yamani *et al.*, 2009) in addition to its high protein content, there is still much to be done in term of algacultural implementation in order to make it available at an affordable cost.

Out of the more than 30.000 microalgae species existing, less than 10 are commercially produced. Yet, some of these microalgae species have been historically used as food and their industrial production is not up to the expected level.

In this review emphasis will be given to some of the microalgae species that are industrially produced and/or those that present relatively high protein content. The global microalgal potential, the protein content of some microalgae species, the technofunctional and nutritional properties of these algal proteins will be presented as reported in the literature.

2-1 Global microalgae production potential

Microalgae (unicellular organisms) and macroalgae (multicellular organisms) belong to the large algae group made up of photosynthetic microorganisms. They are known to have appeared on Earth since 3.5 billion years and are considered to be the first form of life (Margulis, 1981). They are autotrophic organisms as terrestrial plants but lack stems, leaves, flowers and are rootless. Among the microalgae, while some are eukaryotic and commonly identified as algae, others are devoid of membrane-bound nucleus (prokaryotic, cyanobacteria) and bear an intermediate structure between bacteria and plants. They have higher productivity than traditional crops and can be grown in climatic conditions, such as desert and coastal areas (Christaki *et al.*, 2011). Their production yield is not only high but also friendly to the preservation of the environment. Marshall (2007) reported that they are the most productive plants in the world. More than 30,000 species of microalgae exist but only few are being cultivated and have their chemical composition analysed (Gouveia *et al.*, 2008). Microalgae are gaining interest due to their ability to concentrate essential nutrients and functional substances.

Microalgae production has first mistakenly focused its marketing efforts on health foods which was a small market and cannot instigate a large demand for microalgae (Richmond, 2004). This orientation is found to be responsible for the limited development of the industrial mass production of microalgae. Yet, many microalgae (such as *Spirulina*, *Chlorella*, *Dunaliella*, *Scenedesmus*) when correctly processed have an attractive taste and could be thus well incorporated into many types of human foods, and will greatly increase the demand for microalgae (Richmond, 2004). The daily production rate of protein-rich microalgae cell mass presents an annual yield of some 250 t ha^{-1}, i.e. several times that of any agricultural commodity (Richmond, 2004).

Microalage production can be carried out outdoor or in bioreactors under optimal conditions. They give an opportunity to exploit the underutilised arable lands and oceans without reducing the agricultural production surface that should be supplemented to feed the growing global population. The use of microalgae in waste water treatment, for biofuel production and for atmospheric carbon dioxide sequestration (owing to their photosynthetic activities) is also boosting the demand for microalgae mass production.

Chlorella is among the most ancient microalgae species used in human diet and its commercial utilisation was introduced since 1961 by Nihon Chlorella Inc. in Japan (Iwamoto, 2000). It was cultivated for its use as health food (it contains β-1, 3-glucan, an immunoactive substance) and then in mariculture. The total amount produced in 1990s was 2000t/year (Iwamoto, 2000). Its mass production mode is either heterotrophic or mixotrophic. Production yield is higher in the latter production mode. In mixotrophic production, acetic acid can be added to the medium as an organic carbon source in addition to the carbon dioxide. In the heterotrophic mode, carbon is supplied by the sole organic carbon source. However, Chlorella biomass obtained by heterotrophic mass production mode exhibit superior quality for health food, it is rich in valuable phytochemicals and does not present contaminants (Iwamoto, 2000). A study carried out by Praveenkumar *et al.* (2014) on *Chlorella* sp modes of mass production (autotrophic and mixotrophic) revealed that the mixotrophic mode fed-batch feedings of glucose and the supply of air in dark cycles showed the highest biomass (561 mg/L.d) and fatty-acid methyl-ester (168 mg/L d) productivities.

Arthrospira (Spirulina) *sp* in human consumption exhibits also an historical background. It was recorded that *Spirulina maxima* was used in human consumption since 1521, made into dried cake in Tenochtitlan (Mexico City today) (Qian Hu, 2004). Its first commercial mass production began in 1970s in Lake Texcoco. The use of *Spirulina* in human diet has also a long history among the Kanembou tribes leaving around the Lake Chad in Chad Republic but remained unnoticed until 1960s. *Spirulina sp* is considered to be an obligatory alkalophile with the maximal growth rate being obtained at pH 9.5-9.8 (Qian Hu, 2004). Its ability to thrive in high pH environment limits the development of other microorganisms and favours its large-scale outdoor mass production. Annual production worldwide was 2000 tonnes in the year 2000 (Qian Hu, 2004). Mixotrophic mass production presents higher yields (Chen and Zhang, 1997). Today, *Arthrospira sp* mass production is effective all over the World with most of the production facilities located in Asia-Pacific region (Lee, 1997).

Dunaliella sp is the most halotelerant Eukariotic photosynthetic organism known to have a remarkable ability of adaptation to different salt concentrations from as low as 0.1M to salt saturation (4M) (Ben-Amotz, 2004). It is the most suitable organism for outdoor mass cultivation in open ponds. Autotrophic mass production mode is adapted for large-scale *Dunaliella* production in media containing inorganic nutrients with carbon dioxide as the sole carbon source (Ben-Amotz, 2004). Attempts for heterotrophic production are yet to be successful. It is one of the best natural sources of β-carotene and a modern intensive plant of 50 000 m^2 can produce 3650kg/year. A proper growth medium for *Dunaliella* should contain around 1.5M NaCl, more than 0.4 M $MgSO_4$, and 0.1M $CaCl_2$ under pH control (Ben-Amotz, 2004).

Aphanizomenon sp, a fresh water cyanobacteria was first exploited in 1980s after first harvest of natural bloom in Klamath Lake (Orgon, USA) (Carmichael, 2000). 2000t was harvested in 1998 in Klamath Lake. Natural harvesting presents some obstacles such as chemical composition of the harvested biomass and contamination by other species or strains that produce neurotoxins or other undesirable substances. The development of

large-scale production photobioreactors for *Aphanimezonon* is therefore of utmost importance.

Nostoc sp can develop under various climatic conditions including Polar Regions, hot springs and deserts. Its optimum temperature for growth is from15°C to 25°C (Cui, 1983). It shows a great adaptability to a large temperature variation. Chinese have used Nostoc for consumption to survive during famine 2000 years ago (Han *et al.*, 2004). Its mass cultivation is still on experimental level.

In addition to its relatively high protein content *Scenedesmus s*p is mostly cultivated for biofuel production because of its high lipid content (31.7%) and high biomass productivity compared to other microalgae sources (Xia *et al.*, 2014). In a recent study carried out by Xia *et al.* (2014) in China, it was shown that *Scenedesmus obtutsus* (a strain of *Scenedesmus* species) gave the highest biomass productivity (20.2g/m^2.d) as compared to the other tested species. Microalgae species such *Scenedesmus* sp and *Chlorella* sp are being mostly produced for biofuel synthesis and the by-product obtained after lipid extraction can be readily valorised for its high protein content.

In another recent study on *Scenedesmus* mass production by Abomohra *et al.* (2014), *scenedesmus sp* was cultivated in a semi continuous culture for three (3) months using polyethylene transparent bags. From this experimental mass production, a maximum productivity of 0.14g/L.d was obtained. Harvesting methods were also investigated using different flocculants and a maximum flocculation of 82% using 250 mg/L of NaOH for 2 hours was achieved.

Investigations on the feasibility of growing *Porphyridium* biomass outdoors were done as earlier as 1985 by Vonshak *et al*. in a Laboratory study. It was found that, although the optimum temperature for growth is 25°C, there was no damage to the photosynthetic activity detected after exposure of the organism to higher temperatures, up to 35°C. Also, high O_2 evolution activity was observed at relatively high cell concentrations. And there was no inhibition of O_2 evolution detected at high light intensity. A production rate of up to 22g dry wt m^{-2} day^{-1} was obtained for several weeks in an outdoor cultivation.

In a recent study on the strain *Porphyridium purpureum by* Velea *et al.* (2011), the optimisation on culture growth using two variables experimental design (light and sodium bicarbonate feeding through amending ASW nutrient medium with additional amounts of $NaHCO_3$) was investigated. The study showed that more irradiance intensity and $NaHCO_3$ in ASW medium has led to substantial increases in the biomass production, as well as in the exopolysaccharide yields. Exo-polysaccharides and phycobiliproteins use to be the targeted substances in *Porphyridium sp* mass cultivation.

The biotechnology involved in *porphyridium* outdoors production was developed as early as the late 1970s by Gundin *et al.* (1991), cited by Arad *et al*. (2004).

Indoor bioreactors for mass production of *porphyridium* showed that the custom-built flat-sided photobioreactor with higher exposed surface area to volume ratio was the best production system (Iqbal *et al*., 1993).

Moreno *et al*. (2003) investigated on outdoor mass cultivation of the Nitrogen fixing marine cyanobacterium Anabaena *sp.* ATCC 33047 and found that, in open ponds

operated under semi-continuous regime biomass productivity values achieved ranged from 9 g (dry weight) m $^{-2}$ per day, in winter, to over 20 g m $^{-2}$ per day, in summer, provided that key operation parameters, including cell density, were optimized. Under these conditions, the harvested biomass was rich in high-value phycobiliproteins, namely allophycocyanin and phycocyanin, for which open cultures of marine Anabaena represent a most interesting production system.

An assessment of CO_2 fixation and biomass productivity of Anabaena sp ATCC 33047 was carried out by Clares *et al*. (2014). In this study, highest values achieved for CO_2 fixation rate and biomass productivity were 1 and 0.6gL^{-1}day^{-1}, respectively.

Table 1: Protein content (% dry basis), class, and kingdom of different microalgae

Alga	**Protein**	**Class**	**Domain and kingdom**	**Author**
Anabaena cylindrica	43-56	*Cyanophycea*	*Procaryota (Bacteria)*	Becker *et al*.(2007)
Aphanizomenon flos-aqua	62	*Cyanophycea*	*Procaryota (Bacteria)*	Becker *et al*.(2007)
Chorella pyrenoidosa	57	*Trebouxiophyceae*	*Eukaryota (Plantae)*	Becker *et al*.(2007)
Chlorella vulgaris	51-58			Becker *et al*.(2007)
	53,3			Sean *et al.* (2015)
Chlorella ellipsoidea	42.2			Servaites et al. (2012)
Chlorella ovalis	10,97			Scolombe et al.(2013)
Chlorella spaerckii	6,87			Scolombe et al.(2013)
Dunaliella salina	57	*Chlorophyceae*	*Eukaryota (Plantae)*	Becker *et al*.(2007)
Dunaliella primolecta	12.26			Scolombe *et al*.(2013)
Dunaliella tertiolecta	11.4			Barbarino and Lourenço (2005)
Porphyridium cruentum	35	*Porphyridiophycea*	*Eukaryota (Plantae)*	Cynthia *et al*.(2010)
	28-39			Becker (2007
porphyridium aerugeneum	31.6			Sean *et al.* (2015)
Scenedusmus obliquus	48	*Chlorophyceae*	*Eukaryota (Plantae)*	Cynthia *et al.*(2010)
	50-55			Becker (2007)
Scenedusmus almeriensis	41.8			Romero Gracia *et al*. (2012)
Tetraselmis	36	*Prasinophyceae*	*Eukaryota (Plantae)*	Schwenzfeier *et al*. (2011)
Tetraselmis chuii	31			Brown (1991)
	46.5			Sean *et al.* (2015)
Spirulina platensis	60-71	*Cyanophyceae*	*Procaryota (Bacteria)*	Paoletti *et al.* (1980)
	55.8			Sean *et al.* (2015)
Arthrospira maxima	56-77			Paoletti *et al.* (1980)

Anabaena has been subjected to an investigation for its ability of carbon dioxide removal. A maximum CO_2 fixation rate of 1.45 g CO_2 L^{-1} day^{-1} that can be increased up to 3.0 g CO_2 L^{-1} day^{-1} outdoors was experienced by the species investigated (Gonzalez Lopez *et al*., 2009).

Tetraselmis sp is also a species of halotolerant microalgae that can be either used as food, feed or for biofuel synthesis. A number of studies were carried out to assess and ameliorate its mass production conditions. The ability of the species to thrive high salt concentration is sustained by the variations of the starch productivity of the *Tetraselmis sp* biomass as a result of metabolic stress modifications under new growing media conditions. A study carried out by Yao *et al.* (2013) on *Tetraselmis subcordiformis* showed that decreased salinity combined with Nitrogen generated moderate stress to facilitate starch accumulation. They concluded that salinity manipulation can be effectively applied for enhanced starch production in marine microalgae.

In a recent pilot scale mass production study, Fon Sing *et al*. (2014) concluded that a peak productivity of 37.5 ± 3.1 g ash free dry weight (AFDW) $m^{-2}d^{-1}1^{-1}$ was reached in the recycled medium upon transition from 14% to 7% NaCl. The combination of high biomass-yielding mixotrophic growth under high salinity appeared to be a relevant strategy for sustainable cultivation.

Most microalgae exhibit high protein content (Table 1) with larger values reported for *Spirulina (Arthrospira)* species (55.8 to 77%). The variability within reported values for protein content by different authors is remarkable for some species such as *Dunaliella sp* when comparing for example the strains *Dunaliella tertiolecta* (11,4%) *(*Barbarino and Lourenço (2005) to *Dunaliella salina* (57%) (Becker *et al*., 2007) (Table1). Reported values also depend on the analytical methods used and the origin of the analysed biomass (growth medium, harvesting period, production method etc.).

Porphyridium sp is a eukariotic red microalgae species belonging to the family *porphyridiaceae* and the class of *porphyridiophyceae.* The microalga is known for its high carbohydrates content and especially for its sulphated polysaccharides species (Patel *et al.,* 2013) having beneficial health effects. The protein content of *porphyridium cruentum* varied from about 30% to 35% depending on the cell disruption pre-treatment applied (Cynthia *et al.,* 2010). A recent study on the chemical composition of some microalgae species carried out by Sean *et al.* (2015) revealed a crude protein content of the strain *porphyridium aerugeneum* to be 31.6% on dry weight basis.

Scenedesmus sp is also a eukaryotic microalga among the most common freshwater genera. *Scenedesmus almeriensis* is a novel strain of *Scenedesmus sp* that presents many advantages including its growth rate, its high tolerance to temperature and to high copper concentration. In addition to its high protein content, *Scenedesmus sp* is also an important source of carotenoids and especially lutein known for its protective effect to the eye macula region against photochemical damage (Landrum, 1997). Some studies revealed its strong antioxidant activities and its potential interest in reducing the incidence of cancer (Chew *et al*., 1996; Park *et al.,* 1999). The protein content of *Scenedesmus sp* is in the range of 30-50% depending also on the cell disruption pre-

treatment *(*Cynthia *et al.,* 2010). A protein content of 41.8% for the strain S*cenedesmus almeriensis* was reported by Romero Gracia *et al.* (2012).

In an algae composition data presented by Becker (2007), the protein content of *scenedesmus obliqus* was 50-55% while 48% protein content was reported by Cynthia *et al.* (2010) for the strain *scenedesmus obliquus.* As for most microalgae species, the protein content of *Scenedesmus* widely varies (30-55%) according to the cell disruption pre-treatment method, the strain of the species and probably the nature of the growth medium among others.

Dunaliella sp , the halotolerant biflagellated alga is known for its orange-red pigment β-carotene, a precursor of vitamin A. *Dunaliella* protein are reported to play a role in fighting against the transient intracellular salt fluctuations during hyperosmotic or hypoosmotic shocks (Chen *et al*., 2015). Results from a study by Travallaie *et al.* (2015) on the protein production of *Dunaliella salina* showed that protein production and pigmentation correlated well with the growth rate of the strain. *Dunaliella sp* is one of the few microalgae that are produced commercially on a relatively large-scale (Ben-Amotz *et al*., 2004). The protein content for the strain *Dunaliella salina* is 57% on dry basis Becker (2007) (table 1). Lower protein contents were reported for the strains *Dunaliella primolecta* (12.26%) (Slocombe *et al*., 2013) and *Dunaliella tertiolecta* (11.4%) (Barbarino and Lourenço, 2005).

Chlorella sp is a green microalga of the phylum *chlorophyta*. It has a spherical shape with about 2 to 10 µm in diameter (Scheffler, 2007). Protein content of *Chorella sp* has a wide range of variation among the strains of the species, starting from as low as 6.87% for the strain *Chlorella spaerckii* (Slocombe *et al.,* 2013) to as high as 58% for *Chlorella vulgaris* (Becker ,2007) (Table 1). *Chlorella spaerckii* and *Chlorella ovalis* (10.87%) (Slocombe *et al*., 2013) are revealed to be pour protein sources and the mass production of these strains should rather directed be towards other uses such as lipid extraction for bio fuel synthesis. Protein may be exploited as by-products after lipid extraction.

Anabaena sp is among the filamentous microalgae species also known as cyanobacteria. The species has a high ability to fix nitrogen and to remove CO_2 from polluted water (González López *et al.,* 2010). Despite of their relatively high protein content (43-56%) (Becker, 2007), they are one of the four genera of cyanobacteria biomass in which the presence of neurotoxins was revealed in their biomass (*Anabaena* sp., *Aphanizomenon sp*., *Planktothrix agardhii* and *Microcystis sp*.) (Pawlik-Skowrońska *et al*., 2013).

Aphanizomenon sp are filamentous free-floating, solitary, in few species joined into fascicle-like, microscopic or macroscopic (up to 2 cm long) colonies with trichomes oriented in parallel (Bornet and Flahault, 1886 '1888'). They are procaryotic cyanobacteria of the *Nostocacea* family. Compared to *Arthrospira*, *A. flos-aquae* has rather been recently used as food for human consumption. The exploitation of *A. flos-aquae* started on the early 1980s (Carmichael *et al*., 2000). The protein content of the strain *Aphanozomenon flos-aquae* on dry matter basis is 62% (Table 1) (Becker, 2007). Some studies revealed the presence of hepatotoxic microcystins in foods supplemented with *Aphanizomenon flos – aquae* at the level of 0.1–4.72µg/g (Saker *et al*., 2005). But

there was little evidence on the origin of the microcystins. These toxic substances may be present as a result of exogenous contaminations if the tested strain was cultivated outdoor or harvested from natural ponds or Lakes.

Spirulina sp or Arthrospira sp is a genus of free-floating filamentous cyanobacteria characterized by a bicylindrical, multicellular trichomes in an open left-hand helix. It is a ubiquitous microalga capable of adaptation to very different habitats and colonizes certain environments in which life for other microorganisms is, if not impossible, very difficult *(*Orio, 1983*).*

A French phycologist Dangeard (1940) reported that the material called *Dihé* in the local language (Kanembou), was eaten by the native population, and was obtained by sundrying mats of microscopic algae, floating on the surface of small lakes or ponds around Lake Chad *(Orio, 1983)*. The alga was identified as *Spirulina (Arthrospira) platensis*. At the same time another *Spirulina* strain *S. maxima* that was growing abundantly in the Lake Texcoco, near Mexico City was discovered *(Orio, 1983)*. Facts in the literature had clearly revealed the historical use of *Spirulina maxima* and *Spirulina platensis* strains as human food.

Spirulina protein contains a large quantity of phycobiliprotein (about 20% of the total protein content) among which are the phycocyanins known for their attractive blue colour and tremendous health effect on human organism demonstrated by a large volume of literature (Romay *et al*., 1998; González *et al*., 1999; Reddy *et al*., 2003; Hirata *et al*.,2000 ; Bhat and Madyastha , 2001). *Spirulina* appears to be among the best sources of protein with up to 77% for *Arthrospira maxima* (Paoletti *et al.,* 1980) (table1) on dry basis.

Tetraselmis sp is a genus of green unicellular flagellated eukariotic microalgae. It usually grows 10 µm long x 14 µm wide. Most strains possess 8 flagella and few possess 16 flagella. The specie is characterised by its high lipid content (22%) but its protein level is also relatively high (36%) when compared to some conventional high protein sources such as soya bean (37%) and milk (26%) (Becker, 1994; Schwenzfeier *et al*., 2011). Brown (1991) reported a protein content of 31% for the strains *Tetraselmis chui, Tetraselmis suecica.*

2-2 Nutritional and Functional properties of microalgae protein

Parameters measured for protein quality include the protein efficiency ratio (PER) expressed as the weight gain per unit protein consumed, tested on animal in short term trials. Other protein quality evaluations are the determination of its biological value (BV), the protein digestibility coefficient (DC) and the net protein utilization (NPU) equivalent to calculation of (BV) X (DC) (Becker, 2007). The drying method of the microalgae biomass seems to influence on their quality values. *Chlorella sp* DD and *Spirulina sp* SD and *Scenedusmus obliquus* DD showed highest values among the tested microalgae biomass, values that are not much far from those of the referenced sources casein and egg. To some extent, these microalgae can readily replace the conventional protein sources in a diet.

Table 2: Comparative data on biological value (BV), digestibility coefficient (DC) net protein utilization (NPU) and protein efficiency ratio (PER), of differently processed algae (Becker, 2007 based on Becker, 2004; Richmond, 2004)

Alga	Processing	BV	DC	NPU	PER
Casein		87.8	95.1	83.4	2.5
Egg		94.7	94.2	89.1	_
Scenedusmus obliquus	DD	75.0	88.0	67.3	1.99
Scenedusmus obliquus	DS	72.1	72.5	52.0	1.14
Scenedusmus obliquus	Cooked - SD	71.9	77.1	55.5	1.20
Chorella sp.	AD	52.9	59.4	31.4	0.84
Chorella sp.	DD	76.0	88.0	68.0	2.0
Spirulina sp.	SD	77.6	83.9	65.0	1.78
Spirulina sp	DD	68.0	75.5	52.7	2.10

One of the quality criteria of a protein is its amino acids content and specifically the essential amino acids. There are evidences in the literature showing that algae protein can readily compare with proteins from conventional protein sources .Values for amino acids content reported by Kent *et al.* (2015) compare well with those presented by Christaki *et al.*(2011) (Table 3). The slight differences may be due to the differences among the tested microalgae species or the origin of the analysed biomass. All essential amino acids levels reported for the various microalgae species also compared well to those of the conventional sources of protein soybean and egg. The essential amino acid cysteine appeared to be the same limiting amino acid for egg and for most of the analysed microalgae species. Microalgae as protein sources can readily substitute egg or other animal protein. Their amino acids profile is far better than that of soybean and probably that of other plant sources when considering the essential amino acids contents.

Table 3 : Amino acid profile of different microalgae as compared with conventional protein sources (g/100g protein)

Source	Ile*	Leu*	Val*	Lys*	Phe	Tyr*	Met*	Cys*	Try	Thr*	Ala	Arg	Asp	Glu	Gly	His*	Pro	Ser	Author
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0	–	6.2	11.0	12.6	4.2	2.4	4.2	6.9	Christaki *et al* (2011)
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8	
Dunaliella sp	4.5	9.3	6.0	6.2	6.0	4.0	2.5	4.0	-	5.0	7.8	6.6	10.5	13.6	5.7	2.5	4.9	4.4	Kent et al. (2015)
Dunaliella bardawil	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6	Christaki *et al.* (2011)
Chlorella	4.4	9.2	6.1	8.9		4.2	2.2	0.4	-	4.7	8.3	7.1	9.4	12.9	5.4	2.4	4.8	4.0	Kent *et al.* (2015)
Scenedesmus sp	4.7	9.4	6.0	6.8	5.5	4.0	2.4	0.1	-	4.9	6.8	6.0	9.2	13.8	5.2	2.6	8.3	4.2	
Scenedesmus obliquus	3.6	7.3	6.0	5.6	4.8	3.2	1.5	0.6	0.3	5.1	9.0	7.1	8.4	10.7	7.1	2.1	3.9	3.8	Christaki *et al.* (2011)
Spirulina	5.8	9.0	6.4	5.1	4.8	4.8	2.9	0.3	-	5.1	7.4	7.6	10.2	16.1	4.6	2.0	3.3	4.8	Kent *et al.* (2015)
Arthrospira maxima	6.0	8.0	6.5	4.6	4.9	3.9	1.4	0.4	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2	Christaki *et al.* (2011)
Spirulina platensis	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1	
Aphanizomenon sp.	2.9	5.2	3.2	3.5	2.5	–	0.7	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9	

* Means essential amino acid.

Some microalgae proteins exhibit functional properties and are readily integrated in food formulations for health management. Phycobiliproteins found in cyanobacteria such *Spirulina sp* and *Oscillatoria sp* are coloured proteins recognised for their large number of health applications and are intensively investigated for this purpose. These proteins include allophycocyanin, phycocyanin, and phycoerythrin.

A molecule of Phycocyanin is made up of two subunits linked together; a protein unit and a bilin chromophore unit (Figure 2). The chemical structure of the bilin chromophores in phycocyanin is close to bilirubin, a heme breakdown product. BiIirubin acts as an antioxidant and cytoprotector for tissues like myocardium and nervous tissue by scavenging oxygen free radicals (Temme *et al*., 2001).

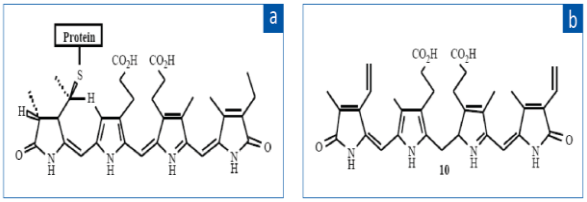


Figure 2: Chemical structure of phycocyanin bilin chromophore (open-chain tetrapyrrol) (a) and bilirubin (b) (Romay *et al*., 2003)

2-3 Technofunctional properties of algal proteins

Despite the recent interest gained by the microalgal product, the field still needs investigation to acquire its full knowledge. Although proteins are one of the most targeted substances obtained from algae, there remain serious gaps to fill in terms of algal protein characterisation. Only few scientific publications are available on technofunctional properties of algal protein.

Mahajan *et al*. (2010) investigated modified *Spirulina* proteins for their functional properties such as protein solubility, foaming properties; emulsification properties and viscosity. In this study, proteins were subjected to chemical treatment with succinic anhydride, acetic anhydride and formaldehyde. Results showed that protein solubility in unmodified water soluble *Spirulina* protein fraction was 23%. It decreased considerably when treated with any one of the three modifying reagents. Foam Stability was higher with methylation and acetylation. Maximum viscosity was obtained with the succinic anhydride modified protein fraction followed by acetylation.

Chronakis *et al.* (2000) investigated on the air/water interface of a protein isolated from *Spirulina platensis* using the Wilhelmy plate method. In this research the isolated material was characterised by determining the protein and lipid content, SDS-PAGE electrophoresis, isoelectric focusing, and visible spectroscopy. It was found that the protein is able to reduce the surface tension (air/water interface) at lower bulk concentration compared to common food proteins.

Another more recent investigation by Chronakis *et al.* (2001) on *Spirulina platensis* protein isolate was carried out on visible absorption spectra, dynamic oscillatory

rheological measurements, viscometry, and differential scanning calorimetry (DSC). The study revealed that the aggregation, denaturation, and gelation properties of *Spirulina platensis* algal protein isolate were rather likely to be controlled from protein-protein complexes than individual protein molecules.

Schwenzfeier *et al* (2011, 2013[a], 2013[b], 2014) assessed various technofunctional properties including polysaccharides interactions with protein isolated from *Tetraselmis sp*. The algal soluble protein isolated (ASPI) presented 59 ± 7% (w/w) protein and 20 ±6% (w/w) carbohydrates, the latter contributed for approximately one fourth of uronic acids (4.8 ± 0.4% [w/w]).

The superior foam stability of ASPI is suggested to be mainly due to the protein fraction (Schwenzfeier, 2013). Results of this study also showed that the charged carbohydrates present in ASPI contribute considerably to high emulsion stability in the pH range 5 – 7, while foam stability was mainly influenced by dissociated proteins and small glycoproteins.

2-4 Conclusion

Microalgae are quantitatively and qualitatively good sources of protein. Their use in human nutrition is not yet fully implemented and is most of the time limited to that of functional food ingredients. Some species such as *Chlorella sp*, *Aphanomezinon sp*, *Nostoc sp*, *Spirulina sp* had an historical use in human nutrition but are still more or less confined to their natural production areas and their industrial production is not up to the expected level. Mass production of microalgae should be encouraged by public authorities in other to overcome the food shortage for the growing global population. However, investigations must be carried out on complete toxicological, microbilogical and biochemical status of algal products before their full integration as basic ingredients in processed foods, be it for functional or solely nutritional purpose.

Literature on the technofunctional properties of algal proteins which is an important tool for industrial applications is also not enough. The need for more studies in this area is an emergency to speed up microalgae application in food process and their global mass production.

3. *Spirulina* protein extraction and purification

Spirulina biomass is worldwide recognised as a good source of protein for food industries (Vonshak, 1997). Protein extraction from *Spirulina* was so far focused mainly on the blue colour fraction known as C-phycocyanin (a phycobiliprotein) due to its various nutraceutical and biological applications (Deng and Chow, 2010; Ferreira *et al*., 2010).

Phycocyanins are light-harvesting pigments and nitrogen-storing proteins found in the prokaryotic cyanobacteria species, as well as in eukaryotic *chlorophyta*, *rhodophyta*, and *bacillariophyta* species (Eriksen, 2008). The phycocyanins include C-phycocyanin (C-PC), R-phycocyanin (RPC), and allophycocyanin. The hepatoprotective (Deng and

Chow, 2010), antioxidant (Ferreira *et al*., 2010) and anti-inflammatory effect of C-phycocyanin (Ferreira *et al*., 2010), as well as its behaviour as a free radical scavenger (Gantar *et al*., 2012) was shown. The chemical structure of this protein is presented (Figure 1).

Doke (2005) extracted phycocyanins using *Spirulina sp* dried biomass. This was done by incubating at 4°C for 24 h in phosphate buffer at pH 7. The use of dilute phosphate buffer for extraction can cause the breakage of cell wall due to osmotic shock. He found that biomass dried at low temperature allows most phycocyanins to be extracted. 80 mg C-phycocyanins per gram dried biomass from a biomass dried at 25°C was obtained compared to only 16.5 mg/g from biomass dried at 50°C. This finding was also confirmed by Oliveira *et al*. (2008) stating that high drying temperatures can decrease the level of extractable C-PC from *Spirulina platensis*. Many other researchers used a combination of EDTA-lysozyme to extract phycobiliproteins (Stewart and Farmer 1984). An alkaline solubilisation followed by acidic precipitation method was used by Chronakis *et al.* (2001) to extract the total *Spirulina* soluble proteins from dry biomass substrate. He also separated the green and blue fractions by ultracentrifugation of soluble proteins extract. The method was energy consuming and could not be easily applied at an industrial scale.

C-phycocyanins, the blue fraction is also being extracted from wet *Spirulina* biomass. This was carried out by subjecting the biomass to cycles of freezing at -15° to -25°C or in liquid nitrogen and thawing at 4°C to 30°C (Abalde *et al.,* 1998; Yi-Ming and Feng, 1999; Minkova *et al.,* 2003). Extraction was also carried out following mechanical cell disruption (Doke, 2005), high pressure exposure (Patil *et al*, 2006) or sonication (Abalde *et al*., 1998) of the wet biomass.

Sarada *et al.* (1999) tested various methods of phycocyanin extraction including extraction by homogenization of the wet biomass in a mortar and pestle in the presence of acid-washed neutral sand using 50 mM sodium phosphate buffer at pH 6.8; extraction with distilled water; extraction by homogenization in a Virtimixer in 50 mM phosphate buffer of pH 6.8, or extraction with various concentrations of hydrochloric acid (2 to 10 N) at room temperature. Of all the extraction methods tested in this study, water extraction was found to be a slow process.

Out of the above mentioned methods a mixture of *Spirulina* soluble protein fractions could be obtained and should be subjected to further purification steps. Purity of C-phycocyanin is evaluated via the ratio of the absorbance at 620 nm (A_{620}) to the absorbance at 280 nm (A_{280}) i.e A_{620}/ A_{280}. If the ratio $A_{620}/ A_{280} > 0.7$ the c-phycocianins obtained was considered food grade (Rito-Palomares *et al.*,2001), while A_{620}/ A_{280} of 3.9 was considered reactive grade and $A_{620}/ A_{280} > 4.0$ were analytical grade.

De Jesús *et al.* (2016) presented an exhaustive 50 years research results on extraction and purification methods of C-phycocyanins from *Spirulina* and other sources(Table 14).

Table 4 : Phycocyanins purification methods Phycocyanins purification methods (Source: Chamorro-Cevallos *et al.* 2016)

Extraction by cell disruption	Purification method			Observations	Reference
Thermal treatment	Density gradient by centrifugation			The phycobilisomes of N. muscorum were separated into two subunits containing C-phycocyanin and allophycocyanin. However, they had traces of phycoerythrocyanin, thus presenting low purity and low yields (data not shown).	Bekasova *et al.* (1984)
	Reverse phase chromatography using dicarboxylic acids and methanol-butanol washes			Traces of C-phycocyanin and phycoerythrocyanin were identified by mass spectrometry chromatography.	Fu *et al.* (1979)
Pressure homogenization	Aqueous two-phase system (ATPS)	Ionic exchange chromatography		C-phycocyanin was obtained at a 6.69 grade of purity from the aqueous extract of *Spirulina platensis*.	Patil *et al.* (2008)
		Polyethylene glycol 4000 and potassium phosphate saturation		C-phycocyaninwas obtained from *Spirulina platensis* in a single extraction step. With multiple extractions, the purity of the isolates increases from 3.23 to 4.02.	Patil *et al.* (2006)
		Ultra filtration	Salting out (precipitation crystallization)	C-phycocyaninwas obtained from *Spirulina* maxima with a purity of 3.8%.	Rito-Palomares, *et al.* (2001)
	Hexane extraction	SDS-PAGE electrophoresis		C-phycocyanin was obtained from *Spirulina* sp at a yield of 10.2% and a purity of 1.	Seo *et al.* (2013)
	Stirring-centrifugation	Expanded bed anion exchange with 80% ammonium sulfate		25.7 mg g^{-1}dm of C-phycocyanin was obtained from fresh *Spirulina platensis* at a purity of 4.8.	Moraes *et al.* (2013)
		Precipitation with ammonium sulfate Fast flow chromatography DEAE-Sepharose and hydroxyapatite columns		C-phycocyanin was isolated from *Spirulina* platensis with a yield of 30 mg g^{-1}dm and a purity of 3.94.	Ou *et al.* (2013)
Freeze-unfreeze agitation	Aqueous two-phase system (ATPS)	Gel filtration chromatography		C-phycocyanin was obtained from *Spirulina maxima* with a	Cruz de Jesús (2005)

			yield of 46.5 % and a purity of 3.4.	
		Ion exchange chromatography	C-phycocyanin was obtained from *Spirulina maxima* with a yield of 37.5% and a purity of 3.5 (determined by DEAE-Cellulose).	
		Ultra filtration	In this last stage, C-phycocyanin was isolated from fresh *Spirulina* maxima with a yield of 57% and a purity of 3.9.	
		Ultracentrifugation	C–phycocyaninwas isolated from cyanobacteria (*Spirulina maxima* and *Porphyridium cruentum*) with a yield of 98%, but with low purity (2.1).	Benavides and Rito-Palomares (2005)
	Organic solvents and buffer extraction	Centrifugation and filtration	C–phycocyanin obtained from *Spirulina* platensis had low yield and low purity (0.46).	Silveira *et al.* (2007)
		Polyethylene glycol systems 1500, 4000 and 6000/aqueous two-phase system (ATPS)	2.67 mg/g of C-phycocyanin was isolated from *Spirulina platensis* with a purity of 0.79.	Antelo *et al.* (2010)
Freeze-unfreeze cycles	Agitation-centrifugation		C-phycocyanin was extracted and isolated from some cyanobacteria (Synechocystisspp, Glueucopsaspp, *Anabaena sp* and *Lyngbya sp*) with a yield of 100 µg/g dm and a purity of 3.1.	Maurya *et al.* (2014)
	Ultra filtration	Ion exchange chromatography	A phycobiliprotein was obtained fromthe fresh biomass of *Spirulina sp* witha yield of 82.9 to 88.6% and a purity of 1.0.	Chaiklahan *et al.(*2011)
		Tri chloro acetic precipitation (TCA)/centrifugation Electrophoresis SDS-PAGE	C-phycocyanin was isolated from cyano- bacteria *Phorphyra columbina* with a yield of 19.9 mg/g dm and a purity of 0.08.	Cian *et al.*(2012)
	Cell disruption by pressure /agitation and centrifugation	Purification by hydroxyapatite column chromatography and anion exchange / ultra filtration / electrophoresis SDS-PAGE	C-phycocyanin was extracted from the cyanobacteria *Anabaena sp* with a yield of 10% and a purity of 2.7.	Ducret *et al.*(1996)
			C-phycocyaninwas isolated from *Spirulina sp* and purified, with a yield of 85 % and a purity of 3.66.	Yoshida *et al.* (1996)

	Precipitation with ammonium sulfate (25%)	Precipitation with ammonium sulfate (25%)	C-phycocyanin was extracted from lyophilized *Spirulina platensis* with a purity of 4.0.	Bermejo-Bescós *et al.* (2008)
			C-phycocyanin was isolated from *Synechococcus sp* and Aphanocapsa cyano bacteria with a yield of 50 % and a purity of 6.1.	Glazer and Cohen-Bazire (1971)
			C-phycocyaninwas extracted and purified from *Porphyrayezoensis* cyanobacterium with a yield of 20 % and a purity of 0.9.	He *et al.* (1997)
			C-phycocyanin was isolated and purified from the fresh biomass of *Spirulina platensis* with a yield of 13.1 % and a purity of 4.71.	Li *et al.* (2005)
			C-phycocyanin was extracted and isolated from *sp Chroomonas* cyano bacterium with a yield of 59 % and a purity of 0.92.	MacColl *et al.* (1973)
			C -phycocyanin was isolated from fresh *Spirulina platensis* with a yield of 95 µg/g dm and a purity of 3.9.	Piñero *et al.* (2001)
		Step chromatography with DEAE cellulose-11	The extract isolated from *Spirulina platensis* was identified asC–phycocyaninby SDS-PAGE, with a yield of 80% and a purity of 4.5.	Kumar *et al.* (2014)
			C-phycocyanin was extracted and isolated from *Anabaena* variabilis cyano bacteriumwith a yield of 36 % and a purity of 2.75.	Chakdar *et al.* (2014)
		Precipitation with ammonium sulfate (50 %)	C-phycocyanin was obtained from *Galdieria sulphuraria* cyanobacteria with a yield of 80 % and a purity of 4.	Moon *et a*l. (2014)
			C-phycocyanin was isolated and purified from *Spirulina maxima* with yield of 24% and a purity of 2.25.	Abd El-Baky and El-Baroty (2012)
			C-phycocyaninwas extracted and isolated from cyanobacterium of the *Nostoc sp* genus, with a yield of 59% and a purity of 2.8.	Gray *et al.* (1973)

			C-phycocyaninwas isolated and purified from *Spirulina fusiformis* with a yield of 60% and purity of 3.8.	Madhyastha *et al.* (2006)
		Ultracentrifugation	C-phycocyanin was isolated from *Cyanidium caldarium* cyanobacterium with a yield of 15 mg g^{-1}dm and a purity of 7.	Stec *et al.* (1999)
		Activated carbon and chitosan , flow filtration	High purity C- phycocyanin was obtained from *Limnotrix sp* with low ammonium sulfate concentrations.	Gantar *et al.* (2012)
	Ionic Exchange chromatography (DEAE-Sephadex)		A phycobili protein was obtained from *Spirulina platensis* under a three-step procedure, increasing its purity to 4.3 (identified by SDS–PAGE).	Liao *et al.* (2011)
	Anion exchange chromatography by hydrophobic interactions (butyl-Sepharose column)	Ion exchange chromatography (Q-Sepharose column) / filtration SDS-PAGE gel	C-phycocyanin was obtained from *Synechococcus sp* with high purity and good yield.	Abalde *et al.* (1998)
	Tricalcium phosphate gel chromatography		A set of phycobiliproteins was extracted from *Smithoranaiadum* microalga. Allophycocyanin, phycoerythrocyanin and C-phycocyanin were obtained after centrifugation, the latter at a low yield.	ÓhEocha and Haxo (1960)

4. Interfacial and functional properties of protein concentrates

Proteins in a food system contribute to its sensory characteristics and influence its acceptability to consumers. These characteristics include texture, flavour, taste, mouthfeel, colour and appearance of the food. Functional properties of proteins are broadly classified into two molecular aspects i.e. the protein surface related properties and the hydrodynamic properties (Damodaran, 1989). The hydrophobic, hydrophilic and steric properties of protein molecules lead to the change in behaviour of proteins at fluid interfaces while those related to the hydrodynamic properties are governed by shape, size, and flexibility of the protein (Table 5) (Damodaran, 1994).

Table 5: Functional properties of food proteins (Source: Damodaran, 1994)

Surface related properties	Hydrodynamic properties
Solubility	Viscosity
Wettability	Thickening
Dispersibility	Gelation
Foaming	Texturation
Emulsification	
Fat and flavour binding	

Our investigation in the present study is mostly oriented towards the surface related properties.

4-1 Surface properties: Concept and theory

4-1-1 Surface tension

The surface tension results from the manifestation of attractive intermolecular forces that keep cohesion of molecules within the liquid. These intermolecular interactions are also the framework that govern the different states of the matter. The resultant force to which a molecule is subjected as a result of interaction with neighbouring molecules within a liquid is zero except for the molecules located at the interfacial surface area (Figure 3). It is this asymmetric interactive force at fluid interfaces that makes molecules at interfaces to bear a higher potential energy than those in the subphase of the liquid.

$G = ng + Gs$

Where:

G: free energy of the system

n: number of liquid molecules

g: free energy of a molecule within the liquid

Gs: free energy at the interface

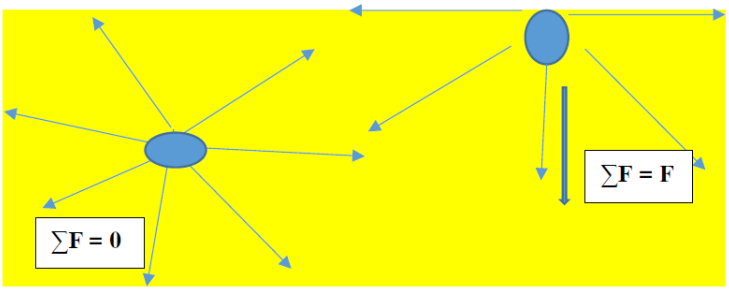


Figure 3: Net forces on molecules at surface and within bulk liquid

When an aqueous solution of surface active molecules is disturbed (bubble formation, drop formation etc.) its surface tension undergoes a decay within labs of time before reaching an equilibrium value. This is because after the disturbance of the liquid surface, the migration of the surfactant molecules towards the surface proceeds until the surface is saturated and the equilibrium free energy level is reached. Knowing that the disturbance is nothing but the change in surface or interfacial area of the solution, a mathematical relation between the interfacial area, the surface tension and the free energy, can be set.

When the interfacial area increases by dA, surfactant molecules will migrate from the subphase to the interface. To bring about this surface area change an energy equivalent to the energy difference between surface and subphase molecules is needed (Dagorn - Scaviner, 1986).

If **σ** is the free energy per unit surface area, we'll have:

$dG = dGs = \boldsymbol{\sigma} dA$ with $\boldsymbol{\sigma} = (dGs/dA)_{T,P}$

T: temperature

P: pressure

If we consider a lamina within a metallic wire frame with one side can glide parallel to the opposite side (Figure 4). The work done in increasing the surface area of the liquid lamina is: $dw = F.dl$

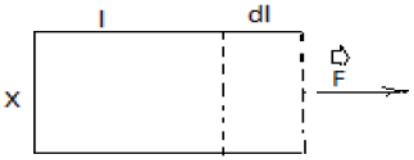


Figure 4: A liquid lamina within a metallic frame with one mobile side

But $xdl = dA$ and let's consider $\Upsilon = F/2x$

$dw = \Upsilon.dA$

A is the surface area of the liquid and Υ the surface tension.

Yet, at constant temperature and pressure: $dw = dG$

dG: change in GIBBS free enthalpy, thus $dw = dG = \sigma dA$ and $\Upsilon = \sigma$

$dG = \Upsilon.dA$

The surface free energy which is a thermodynamic variable can be expressed by using the surface tension which is measurable.

Υ in mN/m; erg/cm^2; or dyne/cm (Dargon Scavinier,1986).

4-1-2 interfacial tension

The interfacial tension is the tension between two immiscible liquids or between a liquid and a solid surface.

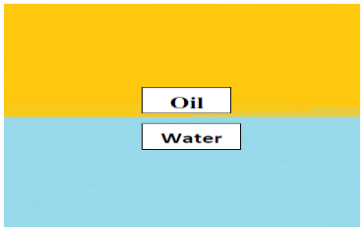


Figure 5: Two non-mixable liquids (water and oil)

Let's consider "a" a molecule of the liquid "A" (water here) and "b" a molecule of the liquid "B" (oil here) (Figure 5). At the interface, the potential energy of a molecule "a" at the interface is greater than that of a molecule "a" within the liquid "A" and it is equal to the potential energy of the molecule "a" within the liquid minus the interaction energy A_{ab} with "b" at the interface ($A_{aa} - A_{ab}$). Similarly the potential energy the potential energy of a molecule "b" of the liquid "B" at the interface is equal to that of a molecule "b" within the liquid ("A_{bb}") minus that of the interaction of "b" with "a" ($A_{bb} - A_{ab}$).

The free energy G at the interface is the potential energy difference between all the molecules at the interface and that of the molecules in the bulk liquid.

$G = (A_{aa} - A_{ab}) + (A_{bb} - A_{ab}) = A_{aa} + A_{bb} - 2A_{ab}$

Hence, the interfacial tension $\Upsilon_i = \Upsilon_a + \Upsilon_b - 2\,\Upsilon_{ab}$

Υ_a: surface tension of the liquid "A"

Υ_b: surface tension of the liquid B

Υ_{ab}: interaction energy a-b per unit area across the interface

(Dagorn-Scavinier, 1986)

4-1-3 proteins as food surfactants

As theoretically presented above, surfactants or surface active molecules are these molecules that can lower the surface tension of the liquid in which it is dissolved. They can be used as detergents, wetting agents, emulsifiers, foaming agents and dispersants. These properties in food systems are of utmost importance, especially in food formulations. Proteins are one of the food ingredients that readily contribute in reducing surface tension. They are able to adsorb to the surface of a liquid in which they are dissolved, and bring about a surface tension decay. This is made possible because protein molecules are amphiphilic molecules (possess hydrophilic and hydrophobic patches) and can properly orient their structures according their environment i.e the hydrophilic tail towards the water molecules and the hydrophobic patches away from the water molecules. Nevertheless as proteins are more complex macromolecules than other simple surfactants, their process of adsorption to interfaces is slower. Thus proteins in

food system facilitate the mixing of immiscible fluids such as water and oil thereby forming more stable emulsions and foams.

4-2 Protein solubility

Protein solubility is an important factor as far as its functionalities in food systems are concerned. It is the thermodynamic manifestation of an equilibrium between protein-protein and protein-solvent interactions (Blecker, 1998). Protein effectiveness in influencing surface related behaviour depends on its ease of solubilisation in an aqueous phase. Protein solubility in aqueous solution starts from a completely insoluble to highly soluble proteins (Kramer *et al*., 2012). For example, while serum albumin has a solubility greater than 500mg/mL (8), crambin is reported to be completely insoluble (Ahn *et al.*, 2006).

Protein solubility is influenced by many intrinsic (amino acids profile, chemical structure etc.) and extrinsic (pH, ionic strength, temperature etc.) factors. The extrinsic factors that affect protein solubility are: ionic strength, pH, temperature, and the solvent (Riès-kautt and Ducruix, 1997). Protein solubility is also directly affected by the electrokinetic potential of the protein molecules. The electrokinetic potential and thus the pH is among the factors that affect protein solubility (Riès-kautt and Ducruix, 1997). The intrinsic factors influencing protein solubility are mainly based on the amino acids on the protein surface (Kramer *et al*., 2012). Many studies on site-directed mutagenesis of surface residues have succeeded to improve protein solubility (Mosavi and Peng, 2003; Das and Georgiadis, 2001). There are some recent works tempting to elucidate these factors in an objective of developing mutation strategies to increase protein solubility (Trevino *et al*., 2007; Trevino *et al*. 2008).

The methods used to evaluate solubility of proteins in water include addition of the lyophilized protein to the aqueous solution and concentration of the protein solution (Kramer *et al*., 2012).

4-3 Foaming and emulsifying properties

Foams and emulsions can be defined as highly concentrated dispersions of gas (foams) or liquid (emulsions) in a liquid continuous phase. The term “concentrated emulsion” is often used as synonymous to “foam” in the literature because the phenomena responsible for their behaviour is essentially the same (Hartland, 2004). Foams and emulsions are colloidal systems characterised by large specific areas (surface area per unit mass) and are therefore deeply influenced by the interfacial properties (Schramm, 2014).

Protein foams are the basic components in many aerated structures in food products such ice cream, cakes and breads (Davis and Foegeding, 2004). The organoleptic quality of the processed foods is closely linked to the ability of the foam to withstand processing and other handling stresses. It is known that some low molecular weight surfactants and lipids can destabilize foams by competing with proteins because they are more surface active compared to proteins (Vaghela, 1996). Also, phospholipids and monoacylglycerides were reported to destabilize emulsions by reduction of proteins adsorption at the lipid interface (Yamauchi *et al*., 1980). It is worth noting that the

emulsifying abilities of proteins are strongly linked to their physiochemical properties (Moure *et al.*, 2006; Bueno *et al.*, 2009; Papalamprou *et al.*, 2010).

The Table 6 below shows some typical food emulsions with a brief presentation of their structures and production methods.

Table 6: Typical food colloids (O = oil; A= air; and W = water (Darling and Birketts, 1987)

Food	Type of emulsion	Method of preparation	Mechanism of stabilization
milk	O/W	Natural product	Protein membrane
cream	A+O/W	Centrifugation	Protein membrane + particle stabilization of air
ice-cream	A+ O/W	Homogenization	Protein membrane + particle stabilization of air + ice network
butter and margarine	W/O	Churning and in votator	Fat crystal network
sauces	O/W	High-speed mixing and homogenization	By protein and polysaccharides
fabricated meat products	O/W	Low-speed mixing and chopping	Gelled protein matrix
bakery products	A + o/w	Mixing	Starch and protein network

For long, egg white proteins were the only alternative for stabilizing food colloids (Asghari *et al*., 2016). However, investigations are carried out on protein from various non animal sources to partially or totally replace animal proteins in some applications. These include evaluation of the surface activities of some plant proteins in relation to their ability to stabilize foams and emulsions (Hu *et al.*, 2003; Uruakpa & Arntfield, 2005; Karaca *et al.*, 2011a). It should be beard in mind that animal proteins are expensive and less friendly to the environment as compared to protein from plant sources (Henchion *et al.*, 2017). Presently, proteins from byproducts are being investigated for their possible valorization as foams and emulsions stabilizers (Karamoko *et al*., 2013). Also, new protein sources such as insects, algae, microalgae and so on constitute the new research route to supplement the increasing protein demand. It is in this perspective that we intend to carry the present study as a contribution to the field.

Chapter 2

Materials and Methods

Introduction

This chapter presents the different materials and methods used to carry out the experiments of the present study. The different apparatus and their working principles are outlined in order to allow for a better understanding of the study.

An industrially feasible method used to obtain the soluble protein fractions from a spray-dried *Spirulina* biomass is described in this chapter. For the investigations on the fractions interfacial properties, protein fractions solution preparation is presented. Foam and emulsion preparation methods are also described and their evaluation methods presented.

1. Materials

Food grade spray dried *Spirulina* powder (2.5kg) is purchased in Belgium (Laboratoires BIORES Liège Belgium) and kept under freezing temperature (-20°C). Only part of it is sampled and kept in fridge (4°C) for the daily experiments. T

Solutions of sodium hydroxide and hydrochloric acid 0.1N and 1N are prepared from analytical grade sodium pellets and 37% fuming hydrochloric acid respectively. These solutions are used to adjust the pH value where necessary. All other chemicals used are of analytical grade made available by the laboratory of Food Science and formulation, Gembloux Agro Bio Tech, Université de Liège.

2. Methods

2.1 Extraction procedure

The extraction method is adapted from that used by Chronakis *et al* (2001). Protein solubilisation is carried out twice in order to extract the maximum quantity of protein. The different steps of the extraction are outlined in Figure 6A & B).

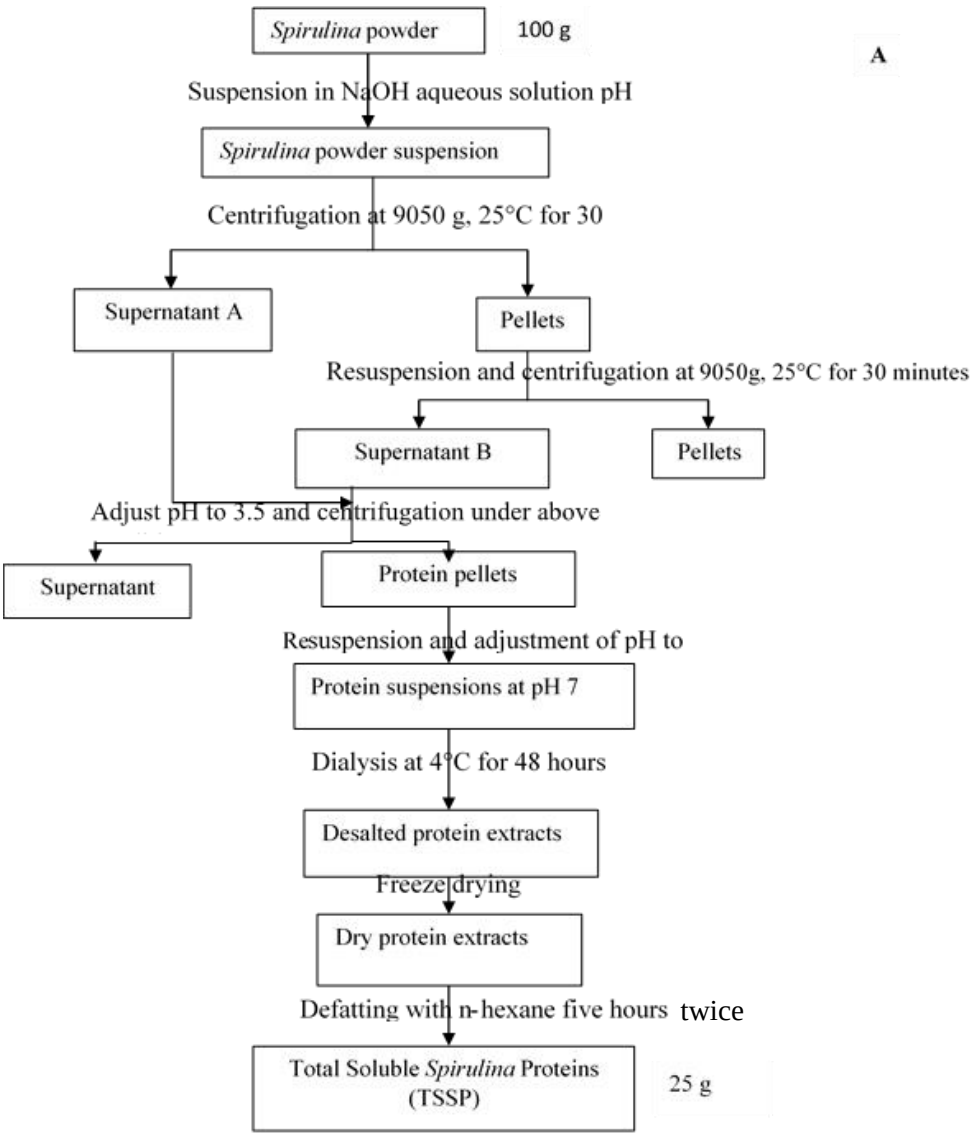
Spirulina powder is suspended in an alkaline solution at pH 10, where maximum solubility (Chronakis *et al.,* 2000) of the protein preparation occurs without substantial conformational changes (denaturation), and centrifugation for 30 min at 25°C and 9050*g*, followed by the supernatant collection (supernatant A). The pellet is collected and dissolved again in alkali followed by a new centrifugation at 25°C and 9050*g* and supernatant collection (supernatant B).

Both supernatants A and B are adjusted to pH 4.5 by titration with 0.1 M HCl and the proteins are precipitated and separated by centrifugation (9050*g* for 30 min, 25°C). The protein pellets are then suspended in milli Q water and their pH adjusted to 7. The suspension is dialyzed for 48 hours at 4°C before being freeze dried. The protein fraction obtained under these conditions is the green soluble fraction (GSSP) (Figure 6 B).

The pH of the dark blue supernatant from the previous step is readjusted to 3.5 and centrifuged under the same conditions as before. Pellets are subjected to the same treatment as pellets of the green fraction. This represents the blue soluble fraction of *Spirulina* proteins (Figure 6 B).

For the separation of the total fraction (Figure 6A), precipitation of the supernatants (A and B) is carried out once at pH 3.5 and the same steps are followed as for the other fractions (GSSP, BSSP) (Figure 6B).

The dry extracts obtained are then subjected to fat extraction using n-hexane (100 mL/20 g dry protein fraction) twice for five hours at 4°C. After a vacuum solvent evaporation, dry fractions awaiting analysis are kept at 4°C.



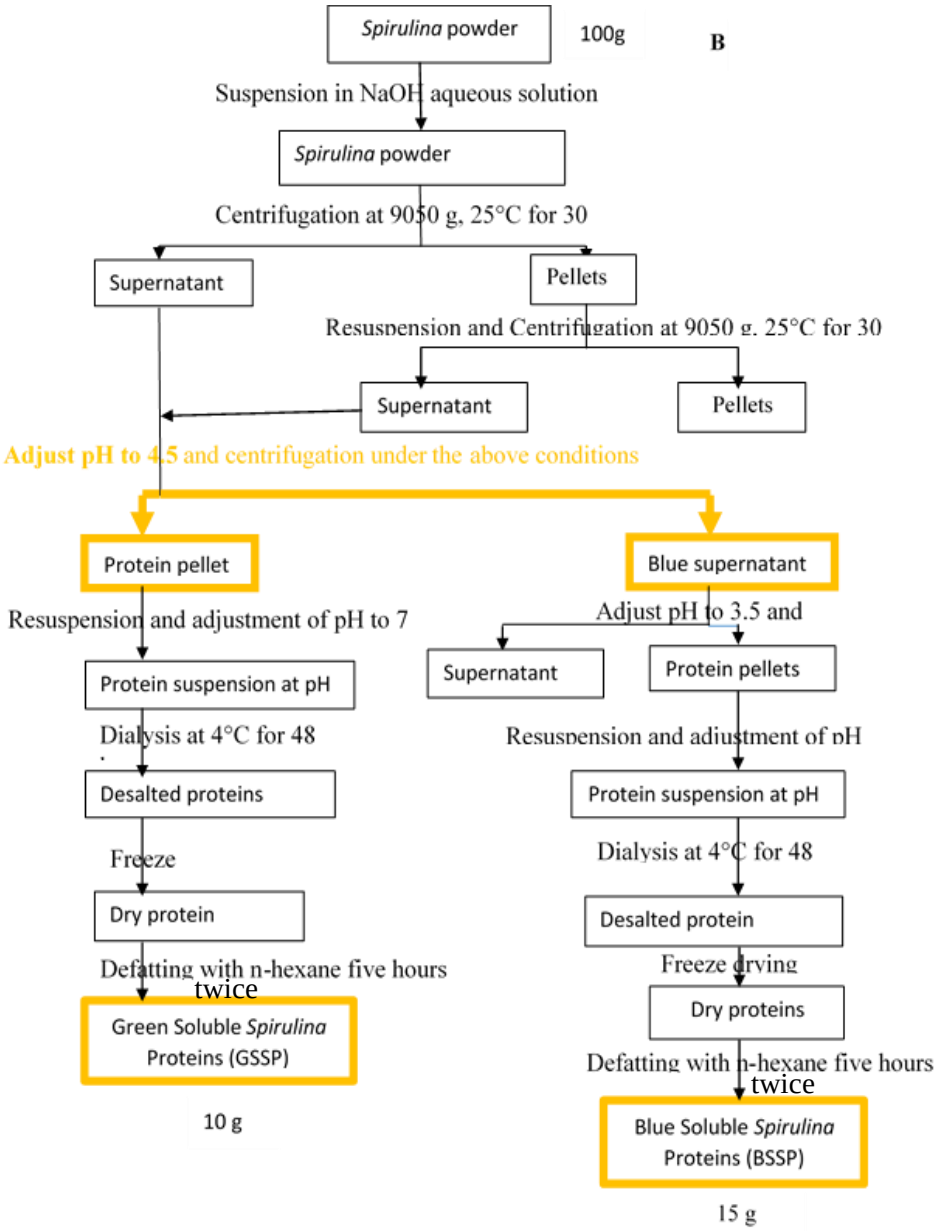


Figure 6: Extraction scheme of colored soluble *Spirulina* protein fractions from *Spirulina* powder, A: Blue soluble *Spirulina* proteins (BSSP), and Green soluble *Spirulina* proteins (GSSP); B: Total soluble *Spirulina* proteins (TSSP) (Adapted from Chronakis (2000))

2.2 Physicochemical properties of the protein fractions

2.2.1 Determination of total protein

The total protein content of the fractions and that of the *Spirulina* dry powder are evaluated using the DUMAS method with a nitrogen conversion factor of 6.25. The method is based on burning out 200 mg of sample and the nitrogen from the burned sample is quantified. The value obtained is converted to protein content using the conversion factor mentioned above.

2.2.2 Ash content determination

The ash content of the samples is evaluated using the NF V 04-384 (AOACI, 2000) by incinerating 3g of sample in a muffle furnace (Nabertherm, Bremen, Allemagne) at 500°C overnight. Experiments are repeated three times for every sample.

2.2.3 Fat content and dry matter

Fat content is determined by Soxhlet extraction method.

Dry matter content is determined using the AOAC (1990) method by drying the samples in an oven until a constant mass is obtained.

2.2.4 Zeta potential of the fraction solutions

The zeta potential (ζ potential) of the fractions solutions were measured using the Delsa Nano C Beckman Coulter (Miami, USA) coupled with an automatic titrator Delsa Nano AT (Miami, USA). Fraction solutions (0.05% (w/w) were prepared in milli Q water. Tested pH ranged from 2 to 10 and data were plotted in a graph ζ potential = f (pH). The isoelctric pH (pHi) of the fractions were identified as the point at which the ζ potential equals to zero.

2.2.5 Polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE protein analysis of colored *Spirulina* protein fractions was performed according to the method of Laemmli (1970) using 12 % acrylamide gel, under reducing conditions in a Mini-Protean II electrophoresis cell (BioRad, Hercules, CA). Gels were run at 160 V for 45 minutes, and stained with coomassie brilliant blue. Densitometry analysis of bands obtained in SDS-PAGE profiles was performed using ImageJ software.

2.2.6 Color of the extracts

The CIELAB coordinates (L*, a*, b*) are red with a spectrophotocolorimeter (Hunterlab, Hunter Associates laboratory, Reston, Virginia, USA). The colorimeter is calibrated before measurements using a white and a black standard plate ceramic tile. In this coordinate system, the L* value is a measure of lightness, ranging from 0 (black) to 100 (white), the a* value ranges from -100 (greenness) to + 100 (redness) and the b* value ranges from -100 (blueness) to + 100 (yellowness). The color of the powdered fractions and also those of the 0.5% solutions of the fractions at different pH values are measured. Color features are obtained as the average of three measurements performed on each sample.

2.2.7 UV absorption spectra

Spirulina colored protein fractions are dissolved in milli Q water a 0.05% (w/w) and adjusted to suitable pH using 0.1M HCl and/or 0.1M NaOH. The solutions are stirred for one hour with a magnetic stirrer until a limpid solution is obtained and the pH is adjusted. Absorption spectra (300 – 800 nm) measurements are carried at room temperature.

2.2.8 Differential scanning calorimetry

Calorimetric Measurements. DSC measurements are made using a Q1000 DSC (TA Instruments, New Castle, USA) equipped with a cooling system (TA Instruments, New Castle, USA) at a scan rate of 5 °C/min covering the temperature range from 30 to 150 °C. Aluminum coated sample pans from TA Instruments are used. DSC pans are filled with ý **4 mg** of the protein isolate powder, and **20** mg of milli Q water was added using a micropipette.

2.3 Interfacial properties

The three *Spirulina* protein fractions are subjected to various evaluations of their surface and interfacial properties. Their adsorption kinetics at interfaces (air-liquid and liquid-liquid) are studied in addition to the rheological properties at both interfaces. These include adsorption kinetics via the drop volume tensiometer (TVT1), the compression isotherms of monolayer films via the Langmuir film balance (Langumuir Film Balance), and the viscoelasticity at both interfaces using an automated drop volume tensiometer (Tracker). The non-polar liquid phase used for the evaluation at liquid-liquid interface is the n-dodecane 99+% (Alfa Aesar). The choice of pH of the

After presentation of the sample preparation, the different apparatus used are presented together with their operational principles.

2.3.1 Sample preparation

The proper quantity of protein powder is weighed and suspended in milli Q water. The suspension is stirred for one hour at ambient temperature using a magnetic stirrer afterwards a limpid solution is obtained. The pH of the solutions is adjusted to desired value using HCl 0.1M or NaOH 0.1M solutions. For the pH adjustment, a particular attention is paid to avoid dilution effect on the prepared protein solution. To do so, the graduated flask is not completely filled to gauge, the volume is then completed with some drops of an aqueous solution of the same pH prepared for the purpose. This is an alternative we have used to prepare solutions in milli Q without altering the concentration intended for the analysis. As such, we are able to exclude eventual effects of buffer (if solutions are prepared in a buffer) on the tested parameters while preparing protein solutions at desired pH without altering concentration. Different concentrations are tested including 0.05%, 0.1%, 0.3%, and 0.5% (w/w). All measurements are carried out at 25°C±0.1°C.

2.3.2 Adsorption kinetics (TVT1)

The adsorption kinetics at the air/water and *n*-dodecane/water interfaces is studied by measuring the changes in the surface tension as a function of time using the falling drop

method. The rate of surface tension decay is directly linked to the rate of surface active molecules adsorption to interface and thus, surface adsorption kinetics is evaluated via the measurement of the surface tension decay from the time the drop is formed to its detachment from the capillary tip. Graphs obtained from these measurements are then analysed using different mathematical models in order to determine the physical variable that characterises the adsorption kinetics. The method used is that of Jho and Burke (1983).

Principle of the method

A drop is created at a capillary tip of known geometry and its volume is progressively increased until it breaks free and falls. The surface of the drop is therefore expanding continuously. The maximum volume of the drop known as critical volume ν is determined, the diameter of the capillary is also known.

The surface tension (γ) ($mN.m^{-1}$) is calculated by:

$$\gamma = \Delta\rho g v / 2\pi r_{cap} f$$

Where:

v: critical drop volume

g: gravitational constant

Δρ: density difference between the two adjacent phases

r_{cap} :capillary radius

f: correction factor which depends on r_{cap} and *v*

$f = r_{cap}/a = \Phi.(r_{cap/v}^{1/3})$

Where a is the capillarity constant given by the relation: $\mathbf{a = (2}\ \gamma / \Delta\rho g)^{1/2}$

The relation $f = r_{cap}/a = \Phi.(r_{cap/v}^{1/3})$ is determined by using a polynomial function of the forth degree (Wilkinson, 1972) in addition to the tabled values of ($r_{cap/v}^{1/3}$) set by Harkins and Brown (1919).

The surface tension is determined by a drop formed in air. While for the interfacial tension the drop is formed within a non-polar liquid (*n*-dodecane in this study). The formation of a series of drops at various time intervals allows to evaluate the change in surface tension with the drop volume and the time taken to arrive at critical tension (i.e. the time at which the drop starts falling), the adsorption kinetic curve representing the change in interfacial or surface tension as a function of time (γ = f (t)) is drawn from the data obtained.

Apparatus used

TVT 1 tensiometer (Lauda Königshofen, Allemagne) is used and its picture is presented in the Figure 7. The measuring set is made up of two transmission shafts, a continuous current motor system, an air tight syringe, an apoelectrical detector, and also a receiving chamber (Miller *et al*., 1992; Razafindralambo, 1996). The syringe and the chamber can be thermostated. An electronic set ensures the settlement of the piston speed and the numbering of the encoding impulsions. The drops are formed with a syringe

whose flow rate is controlled by a microprocessor of the electronic set through a motor-encoder system. The apoelectric detector determines the time the drop is freed. The motion of the syringe piston is ensured by the press. The encoder serves to determine the number of rotations undergone by the transmission shaft (n_t). The volume of the drop can be determined with high accuracy from the number of rotations (*nt)*, the rate of transmission, the time needed for drop formation, and the capillary diameter. The measurement procedures and the speed of the motor system are chosen on a micro-computer connected to the electronic system via an interface RS232 (Lauda, Königshofen, Allemagne) which allows to observe the record of the measured values and the graphical results obtained. The dynamic equilibrium values during absorption can be determined by extrapolation.

Figure 7: TVT LAUDA Tensionmeter (Lauda Königshofen, Allemagne)

- **Analytical conditions**

Dynamic interfacial tension measurements are carried out at 25 ± 0.1 °C. The capillary radius is 1.055mm. A syringe (Lauda Königshofen, Allemagne) of 2.5 mL is used. Measurements of the surface and interfacial tensions are respectively carried out using an empty receiving cell or a cell filled with *n*-dodecane +99% (Alfa Aesar). The rate of drop formation varies from 0.07 to 0.8 s.μL^{-1} for the air/water interface and 0.07 to 0.40 s.μL^{-1} for the water/n-dodecane interface. Six drops of different volumes are formed and their interfacial tensions are being measured. Values of interfacial tension and drop formation time are averages of two values for every trial. Measurements are repeated three times.

- **Determination of adsorption kinetics parameters**

Two parameters are determined from the adsorption kinetic curves i.e. the initial adsorption rate and the equilibrium surface or interfacial tension. The initial rate of adsorption is defined as the tangent to the kinetic curve at time t = 0. The interfacial equilibrium tension is determined by extrapolation of the interfacial tension to infinity (t→∞) by using the linear part of the graph (Figure 8) (Blecker, 1998).

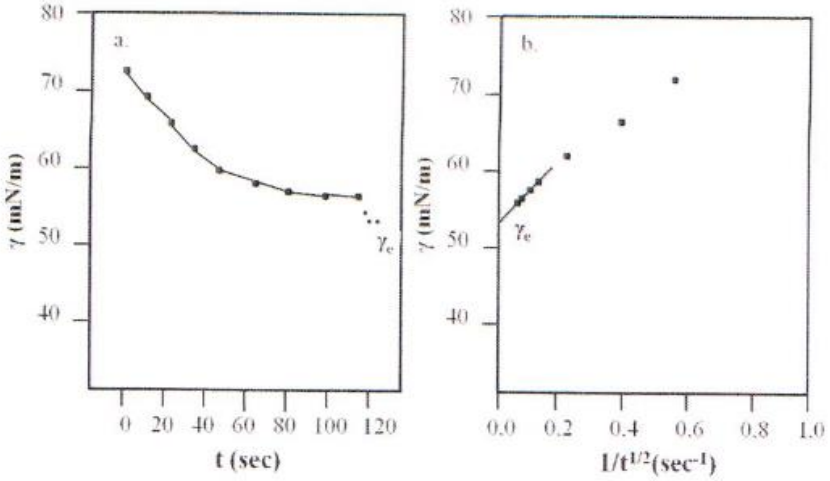


Figure 8: Determination of the equilibrium surface tension by plotting the curve of Ye= f (t-1/2) (a); surface tension is represented as Y= f(t) (b) (Blecker, 1998).

Figure 9: Bubble pressure tensiometer BP100

- **Adsorption kinetics using the Bubble pressure tensiometer (BP100)**

Apparatus

The bubble pressure tensiometer BP100 (Figure 9) is used to evaluate the dynamic surface tension of *Spirulina* colored soluble protein solutions. In this method, a gas bubble is being forced to expel from a capillary immersed in the protein solution to be tested. The expelling pressure is recorded just before the bubble emerges from the capillary into the liquid. Surface tension can be calculated from the pressure increase, the capillary radius and its immersion depth.

Measurements of the dynamic surface tension via the BP100 are done to complete those carried with TVT1. This is due to the ability of the apparatus to measure surface tension variations within shorter lapses of time (milliseconds) compared to TVT1.

Principle and Theory

The surface tension is derived from the quantity of energy required to increase the surface of a liquid under isothermal and isobaric conditions. It is in other words the change in free enthalpy linked to the change in surface area**.**

$\gamma = (\partial G / \partial A)_{p,T}$……..(1)

The pressure required to expel the air (i.e. to overcome the capillary pressure) from the capillary P_γ being the difference between the internal and external pressure of the bubble and the hydrostatic pressure Ph.

$P_h = h.\rho.g$……..…… (2)

Where Ph is the hydrostatic pressure

h immersion depth, g the gravitational constant, and ρ the density of the liquid

The work done to increase the bubble volume is:

$P_\gamma dV = P_\gamma 4\pi r^2 dr$……... (3)

Where r is the capillary radius

This work corresponds to the increase in the surface energy γdA, obtained from (1).

$\gamma dA = \gamma\ 8\pi dr$ ……..(4)

From (3) and (4) we can obtained:

$p_\gamma = 2\gamma / r$ ……(5)

The total pressure p_{max} at which a gas bubble emerges from the capillary tube into the liquid, and which is measued as the maximum pressure is the sum of the capillary pressure and the hydrostatic pressure.

$p_{max} = 2\gamma / r + h\ \rho\ g$ ……(6)

We can then obtained for the surface tension: $\gamma = r/2\ „(p_{max} - h,\rho,g)$

Analytical conditions

Measurement with bubble pressure tensiometer are carried out only on samples showing greater surface tension decay at the first drop age obtained by TVT1 measurements. The objective was to closely examine the adsorption kinetics (surface

tension vs time) within shorter lapses of time (milliseconds) at the begining of the decay. Samples were prepared in the same way as described above but only 0.3% and 0.5% (w/w) concentrations were tested for all the fractions. A glass flask was half filled with the sample solution and insuflated to allow for the formation of air bubbles at the surface. The surface tension decay kinetics of the freshly formed bubbles were measured in terms of milliseconds.

2.3.3 Monolayer films characterisation

The monolayer films of the TSSP, BSSP, and GSSP fractions are characterised by compression isotherms (π = f (A)) determined using a Langmuir film balance.

Principle

With the Langmuir method, surface pressure (π) was directly determined without using the relation $\pi = \gamma_0 - \gamma$ (where γ_0 is the initial surface tension and γ the actual surface tension). The principle is based on the measurement of the horizontal force applied to a floater that divides the surface into two parts. One of the parts is left clean and the other will serve for the formation of the film. By means of a mobile barrier, the total surface occupied by the film can be reduced or increased (Figure 10). The surface pressure is equal to the force per unit length needed to immobilise the floater ($\pi = F/L$).

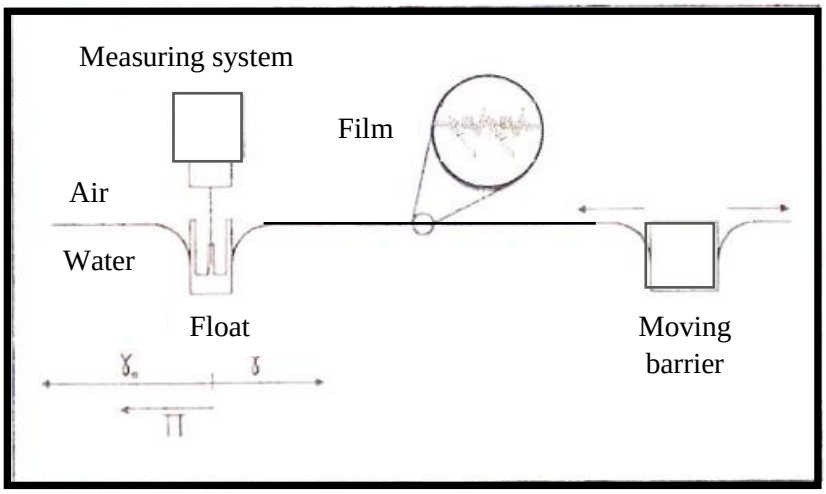


Figure 10: Principle of the Langmuir Film balance

When the mobile barrier is moved parallel to itself through a small distance dx, the work of the force applied is Fdx. In the presence of a film the work done will be

$(\gamma_0 - \gamma)$ldx. Thus at equilibrium we will have:

$Fdx = (\gamma_0 - \gamma).l.dx$

$\Longrightarrow \quad F/l = \gamma_0 - \gamma$

But we know: $F/l = \pi$ and $\gamma_0 - \gamma = \Delta\gamma$

Thus we will have the surface pressure defined as a function of the surface tension:

$\pi = \gamma_0 - \gamma = \Delta\gamma$

Where

π: surface pressure

γ_0: initial surface tension

γ: surface tension in presence of a surface active substance

F: resulting force

l: length of the perimeter

The graph representing the variation of the surface pressure (π) as a function of the area (A) occupied by the molecules at constant temperature is known as the compression isotherm.

Apparatus

The Langmuir film balance LFW2 (Lauda, Königshofen, Germany) is used. It is made up of a Teflon rectangular vat, a floater, a measuring system, a mobile barrier that serves in adjusting the useful surface, an aspiration system to clean the surface or empty the volume. The whole is placed in thermostatic system monitored through a micro-computer (Sanyo XT, software Lauda FW-29.5).

Samples preparation

Proper weights of TSSP, BSSP and GSSP protein fractions powder are dissolved in milli-Q water to obtain desired concentration (0.3% w/w). Details of the solution preparations are described in 2.3.1.

Analytical conditions

The vat is filled with mill-Q water and left until thermal equilibrium is set. The cleanness of the surface is then ensured by sweeping it many times. The surface is considered to be cleaned when the surface pressure is less than 0.5mN/m, otherwise the aspiration process continues until the surface is clean. When cleanness is achieved, 80 µL of the prepared protein solution is deposited drop by drop via a micro-syringe (Hamilton Bonaduz AG, Swizeland) on the surface, and allowed to spread (30 minutes). The film is compressed by the mobile barrier after 30 minutes. The film spreading surface is 927 cm^2 and the speed of compression is fixed at 185.4cm2/min. All measurements are done at 25 ± 0.1°C.

Analysis of the isotherm

The surface pressures and surfaces occupied by the protein extract solutions (mg) are represented in an isotherm $\pi = f(A)$ example (Figure 11).

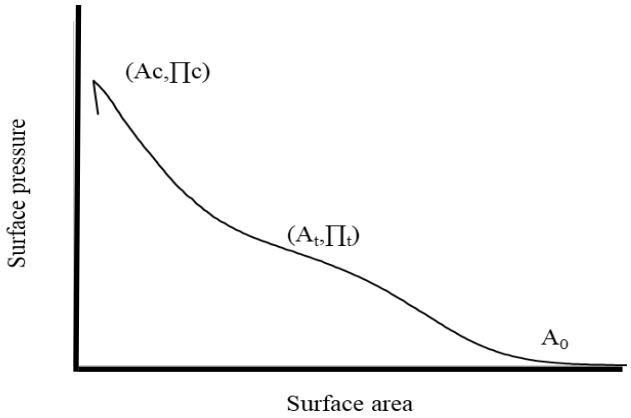


Figure 11: Typical compression isotherm; A0: initial area; (At, πt): transition point; maximum or collapse point (Ac, πc)

Elasticity

Elasticity (mN/m) was defined by Damodaran (1994):

ε = - A(dπ/dA)

Where A is the film surface

This is the inverse of the compressibility coefficient Cm defined by Bull (1945) and Gaines (1966).

The compressibility coefficient Cm (mN.m-1) was defined by Bull (1945) and Gaines (1966) with the formula: $C_m = dlnA/d\pi$. This coefficient represents the resistance offered by the monolayer during compression.

2.3.4 Interfacial rheology

Determination of viscoelastic properties

Viscoelastic properties are determined using an automatic falling and/or rising drop tensiometer known as Tracker (I.T. Concept Instrument, Longessaignes, France) by measuring oscillations of the surface tension at various interfacial surfaces during expansion-compression.

Experimental conditions

The oscillation frequency, the amplitude, and the volume of the drop are chosen after preliminary trials. From the results of these experiments, a drop volume of 5µL, an oscillation frequency of (period of 10 seconds) 100 milli Herz, and an amplitude of 10% are used. All experiments are carried out at 25°C in triplicates.

Principle

A drop of the fluid is formed at the capillary tip joined to a syringe whose piston is monitored by a motorised system. The drops are formed in a thermostated glass cuvette that contains air a non-miscible liquid depending on the interface to be studied. This can be a falling or rising drop via a straight or curved capillary respectively. Verticality of the capillary can be adjusted in other to form rigorously axisymmetric drops. The

capillary choice depends on the density of the two fluids studied. The shape of the drop depends on the combined effects of three forces that allow to maintain the system's equilibrium (Figure 12)

F_1, the sum of the surface tension forces is defined as:

$F_1 = 2\pi\gamma \sin\varphi r$

Where

r is the radius of the drop measured according to z axis;

γ the interfacial tension and, φ the angle between the tangent to the drop surface and the X axis.

F_2, the sum of the pressure forces acting on the plan P, is defined as:

$F_2 = \pi r^2 \Delta P$

Where, ΔP the difference between the hydrostatic pressures of the two interfaces caused by the bend of interface and is defined by the equation of Laplace-Young:

$\Delta P = \gamma (1/R + 1/R')$

But, $R = r/\sin\varphi$, and $R' = \infty$, the principal bending radii of the interface, thus

$F_2 = \pi r \gamma \sin\varphi$

P, the weight of the drop part situated under the inflexion plan (taking into account the Archimede pressure of the drop), is defined by:

$P = mg = V\Delta\rho g$

Where

m is the mass of the drop;

V its volume;

g the gravitational acceleration;

$\Delta\rho$ the density difference between the fluids studied.

The drop been at equilibrium, the sum of the forces acting on the plan P is equal to zero:

$0 = F_1 + F_2 + P$ and $\gamma = V\Delta\rho g / \Pi r \sin\varphi$

Thus, the calculation of interfacial tension between two fluids is made possible by measuring the shape characteristics of the drop as illustrated in the Figure 12 below (Benjamins *et al.*, 1996). When the interfacial tension decreases, the drop tends to increase in length and this allows monitoring the evolution of the interfacial tension by calculating its Cartesian coordinates. The larger the size of the drop, the accurate the measurement. In fact the more the drop is small the more it appears spherical and it gives lesser chances to apply the Laplace-Young equation.

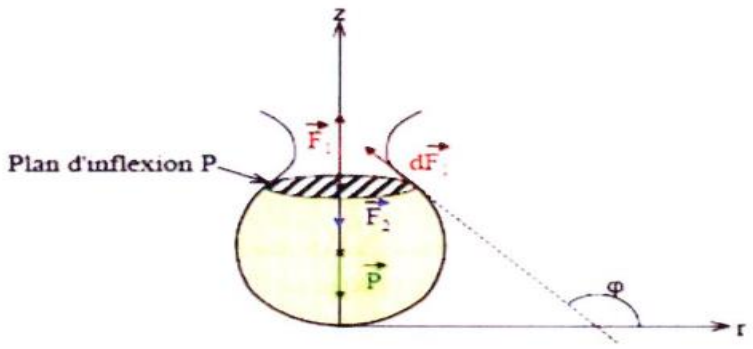


Figure 12: Illustration of the relationship between the different forces implicated at drop equilibrium. dF_1 surface tension force acting on the infinitesimal contour of the plan P (dl) (Benjamins *et al.,* 1996)

The rheological measurements are done by varying the drop surface, i.e. its volume at equilibrium. As a result, the surface or interfacial tension is recorded by the Tracker. The relation between the interface deformation and the recorded tension describes the rheological behaviour of the interface. During the expansion phase, the local concentration of the protein at the surface of the drop is reduced and this allows for a temporary increase in surface or interfacial tension. Molecules reorganise in a way to restore the initial concentration of the protein at the interface (Gibbs-Marangoni effect) (Fillery-Travis *et al.,* 2000). Changes in surface or interfacial tension as a function of drop surface allow for the calculation of the viscoelastic modulus that characterises the surface. The viscoelastic modulus of an interface is characterised by two parameters i.e. the elastic components (stored energy) and viscous (lost energy) of the surface that can be calculated as follows:

2 Interfacial elasticity (stored energy)
$E= A(d\gamma/dA)$
3 Viscosity (lost energy)
$E'= A(d\gamma/dA')$

Where, A is the interface area, dA' the rate of variation of the interfacial surface (dA/dt) and γ is the surface or interfacial tension.

When the drop surface variation is a sinusoidal one, the rheological characteristics at the interface are calculated with the Fourier analytical model. The deformation of the interface or surface ΔA/A as a function of time is therefore considered as an incoming signal of the system made up of the drop in its environment, while the response of the interfacial tension γ as a function of time is considered as an outgoing signal of the same system. The comprehensive analysis of the two signals allows the calculation of the complex viscoelastic modulus of which values are calculated.

Apparatus

The experimental set (Figure 13) is made up of a glass cuvette (8 mL) located in a thermostated mobile support, a syringe placed also in the thermostated bloc with the straight or bent capillary fixed to its end, a tele-centric system and, a camera CCD (Charge coupled Device) connected to a computer monitored by the software WinDrop. This software enables the treatment of the information based on the volume profile of the drop formed. A control system (function generator, amplifier, and piezoelectric actuator) allows the manual or automatic handling of the syringe.

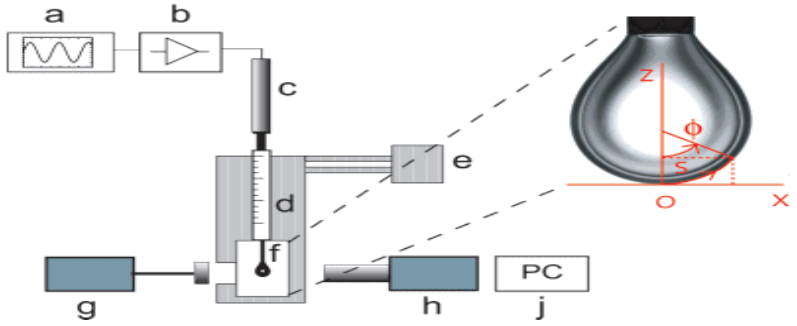


Figure 13**:** Oscillating pendant drop tensiometer (Tracker) (a, function generator; b, amplifier; c, piezoelectric actuator; d, glass syringe; e, heating/cooling system for cuvette and syringe; f, sample cell containing the continuous phase; g, light source with glass fiber and diffusor; h, CCD camera with macro zoom lens; j, personal computer for image acquisition and data analysis) (Erni *et al*., 2007)

2.4 Functional properties

2.4.1-Solubility

Nitrogen solubility profile is determined according to Nirmala *et al*. (1992). 1g samples of fractions powder are mixed with 10 mL of milli Q water and stirred for 10 min at room temperature. 0.1 M NaOH and 0.1 M HCl are used to adjust suspension pH to 2, 3, 5, 6, 7, 8, 9, and 10. The suspensions are once more magnetically stirred at 250 rpm for 30 min at room temperature and centrifuged at 8000g for 20 min. A 200uL aliquot of the supernatant are sampled for protein determination using DUMAS method. The solubilized protein is expressed as a percentage converted from nitrogen content using a conversion factor 6.25.

2.4.2 Oil absorption

The oil absorption capacity of the colored *Spirulina* soluble proteins is measured using the method adapted form Lin *et al*. (1974).Five milliliters of oil is added to 0.3g of fractions powder in 12 mL centrifuge tubes and agitated at room temperature for 30 minutes. The mixture is then centrifuged at 1600xg for 25 minutes. Part of the oil is retained within the protein at the bottom while the other part forms a free layer at the top. This layer is carefully removed by tilting the tube. The oil absorption capacity is expressed as g of oil held per 100g of sample.

2.4.3 Foaming capacity and stability

Foam capacity of the *Spirulina* protein fractions is evaluated using the method adapted from that used by Chew *et al*. (2003), Sciarini *et al*., (2009), and Timilsena *et al* (2016). 25 mL aqueous solutions of the *Spirulina* protein fractions are prepared at different pH values and stirred with an Ultra-Turrax (T25, IKA, Staufen, Germany) at 20000 rpm for 2 minutes in an 100 mL cylindrical flask (2.5 cm diameter). Foam volume is recorded and foam capacity is calculated as: FC (%) = (Ifv/Tsv)×100.

The decrease in the foam volume as a function of time is recorded within two hours and is considered to represent the foam stability (Sciarini *et al*., 2009).

2.4.5 Emulsion capacity

Two emulsion properties are evaluated, i.e. emulsion capacity and emulsion stability. For emulsion capacity, the method is based on an emulsification of a vegetable oil in a protein aqueous solution agitated with an Ultra turx until phase inversion. The inversion point is detected via electrodes joined to a multimeter and fixed at the bottom of the fask containing the mixture. As oil is a poor electrical conductor, when phase inversion occurs, the electrical resistance sharply increases, and the quantity of oil added is recorded at this very point. This method is adapted from that of Web *et al*. (1970)

A volume of 25 mL colored *Spirulina* protein fractions solutions are used, to which a sunflower oil is continuously added in the flask while agitation is going on until phase inversion is reached. Proteins solutions are tested at different concentrations (0.05%; 0.1%; 0.3%).

2.4.6 Emulsion stability

Emulsion stability is evaluated via a Turbiscan MA 2000 (Formulation, l'Union, France). 40 mL of *Spirulina* protein fractions (0.5% (W/W)) are added to 10 mL sunflower oil (Vandemoortelen, Izogem, Belgium) and mixed with an Ultraturax (T25, IKA, Staufen, Germany) at 13500tr/min for 2 minutes. Different pH levels of the protein suspensions w ere tested (3, 5, native pH). The emulsion was immediately transferred into Turbiscan tubes and their stability was followed for 24 hours.

2.4.7 Emulsion microscopy

The emulsion fat globules distribution is observed using a microscope, after emulsion preparation under conditions described in section 2.4.6. A droplet of freshly prepared emulsion is deposited on a glass slide and covered with a glass cover slip. Duplicates of slides are prepared for each sample. An image of the emulsion showing its particles distribution is obtained via temperature-controlled microscope stage (Nikon Eclipse E400, Kanagawa, Japan) (at 20°C), using a Linkam PE60 temperature controller and Peltier stage system (Linkam Scientific Instrument Ltd). Images record is done using a Nikon camera (Nikon DS-Fi2, Kanagawa, Japan) and digitized via NIS-D Nikon (Kanagawa, Japan) then a 20 times amplification is performed. Three visual fields are focused for each glass slider, of which only one is chosen to represent the observed particle size.

Chapter 3

Physicochemical characterization of *Spirulina* soluble protein fractions

Part of this chapter was published as cited below:
Barka A, Amal B A, Francis F, Blecker C, 2018. Physicochemical characterization of colored soluble protein fractions extracted from *Spirulina* (*Spirulina platensis*). *Food Sc. And Tech. Int.* doi/full/10.1177/1082013218786862.

1- Introduction

Spirulina platensis is an excellent source of proteins and other valuable nutrients (Kelly and Capelli, 2011). In order to evaluate the surface activity of its proteins, the soluble fractions are separated (extraction method described in previous chapter) from dry *Spirulina* biomass and characterized. Some of its proteins are soluble in water while others are insoluble even under the conditions used in this study.

Surface activity is a property of solutes (in an aqueous solution) that have the propensity to adsorb to interface and reduce interfacial tension. This behavior is linked to the amphiphilic nature of surface active molecules that allow them to direct their hydrophobic tails away from water molecules (surface or non-polar liquids), and their hydrophilic heads towards water molecules. For this to be possible, the molecule should be able to break free from its original cellular substrate and diffuse through the subphase to adsorb at interface. The influence of non-protein materials and the protein interactions with other molecules in its original substrate could be reduced by the protein separation. Soluble proteins separated from their original substrates are therefore further used to characterize the surface activity of *Spirulina* proteins. The change in surface tension is directly linked to concentration of adsorbed molecules at interface (Dagorn-Scaviner, 1986).

The aim of the present chapter is to determine some physicochemical properties of the separated soluble protein fractions. Three fraction colors are obtained using an industrially feasible method outlined in previous chapter (chap. 2, Figure 6A& B). The proximate compositions of the separated fractions are determined. The color and absorption spectra of the fractions are analyzed together with their solubility, zeta potential, SDS-page electrophoresis and DSC profiles evaluated.

2. Results and discussion

2.1 Proximate composition

Spirulina dry biomass reveals a relatively high protein content (62.55%) (Table 7) not usually found in known protein sources, be it animal or vegetable. The protein extraction method is presented in the second chapter of the thesis.This allows for its use as a protein supplement in its native form. Unlike other microalgae species, such as *Chlorella pyrenoidosa* (26%) (Nigam *et al.*, 2011), the lipid content of this *Spirulina* biomass is poor (3.75%), a fact that does not encourage its use as a raw material for fat production. Its relatively high ash content (6.12%) can be explained by its ability to thrive and grow under salty conditions that could increase its content in mineral elements such as sodium and potassium. The carbohydrate content of *Spirulina* biomass (17.55%) is rather close to that of vegetables, unlike its protein content, and this does not allow for an easy classification of the species (sometimes considered as a plant or a bacterium).

Table 7: Proximate composition of Spirulina powder and colored soluble protein fractions from Spirulina powder

Constituent	*Spirulina* powder	Blue fraction (%)	Green fraction (%)	Total Fraction (%)
Protein	62.55 ± 0.74^{a}	82.76 ± 0.20^{b}	82.29 ± 0.93^{b}	74.53 ± 0.58^{c}
Fat	3.75 ± 0.17^{a}	0.269 ± 0.06^{b}	1.02 ± 0.01^{c}	0.46 ± 0.12^{d}
water	10.01 ± 0.10^{a}	10.8 ± 0.09^{b}	10.29 ± 0.22^{c}	10.46 ± 0.05^{c}
Ash	6.12 ± 0.05^{a}	3.90 ± 0.00^{b}	3.34 ± 0.03^{c}	3.49 ± 0.07^{d}
Carbohydrates by difference	17.57	2.27	3.06	11.06

Values given are means of three repetitions ($\bar{x} \pm$ SD) (SD: Standard deviation) except that of carbohydrates obtained by difference. Values with same subscript letters within the same line are not significantly different ($P > 0.05$).

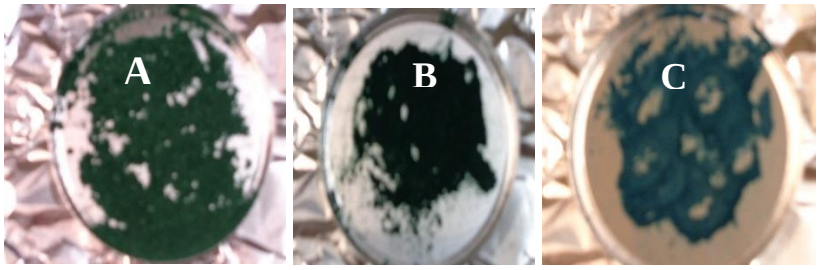


Figure 14: Picture of freeze dried *Spirulina* soluble protein fractions: A Green *Spirulina* soluble proteins; B Total soluble *Spirulina* proteins; C Blue soluble *Spirulina* proteins.

Out of every 100g of crude *Spirulina* powder (62.55% proteins) used, 25.5g of total soluble proteins fraction, or 14.75g blue fraction, and 10.10g of the green fraction are obtained by using the extraction method presented in the Figure 6. These represent the major soluble proteins fractions extractible under alkaline conditions (in aqueous solution pH 10) where solubility was reported to be maximum (Chronakis *et al*., 2000). This result is close to that of reported by Hedenskog and Hofsten (1970) with a 24% yield in an extraction carried out without cell disintegration. Most *Spirulina* proteins are embedded in complex cell structures such as photosynthetic thylakoids, and cyanophycin granules that hinder their solubilization in an extraction medium. The term soluble is used hear, in the denomination of the fractions to outline the difference with proteins not solubilizable under the extractions conditions used in the present study. It clearly appears that more than half of the proteins contained in the original biomass remained in the pellets obtained after extraction, together with other nutrients that could be used as food or feed ingredients.

Protein fractions (Figure 14) subjected to this study are mixtures of various proteins and other molecules such as carbohydrates and fat (Table 7) that may influence adsorption kinetic at interfaces. Our investigations are limited the whole contribution of

the fractions in surface tension reduction which is an important aspect as far the technological applications in foam and emulsion is concerned. Therefore, the fundamental mechanisms of molecular adsorption at interfaces cannot really be fully discussed under this study.

The total soluble fraction presented higher carbohydrates content (11.06 %) than the other fractions while the green fraction was rather higher in fat content. The protein contents of the green and the blue fractions are close (82.29 % and 82.76 % respectively) and higher than that of the total fraction (74.53%). The relatively low protein content of the total fraction can be attributed to its higher content in carbohydrates. In fact, for this fraction, precipitation is carried out once with probably a limited loss in soluble carbohydrates, leading to a higher carbohydrate proportion thereby reducing protein proportion in the fraction.

Table 8: CELAB color coordinates of Spirulina soluble proteins fractions aqueous 0.05% (w/w) at different pH levels

	L*	a*	b*
BSSP native pH	43.95±0.18^{a}	-26.07±0.16^{a}	-26.76±0.12^{a}
BSSP pH 5	32.59±0.01^{d}	-24.18±0.01^{b}	-25.13±0.00^{b}
BSSP pH 3	42.72±0.03^{e}	-27.51±0.01^{c}	-12.91±0.01^{c}
GSSP native pH	44.13±0.31^{ab}	-35.30±0.27^{d}	02.66±0.02^{d}
GSSP pH 5	45.54±0.18c	-36.10±0.22^{e}	04.66±0.02^{e}
GSSP pH 3	46.71±0.25^{f}	-29.63±0.11^{f}	08.53±0.09^{f}
TSSP native pH	43.87±0.12^{a}	-33.50±0.09^{g}	-07.92±0.00^{g}
TSSP pH 5	42.28±0.08c	-34.73±0.05^{h}	-05.89±0.00^{h}
TSSP pH 3	44.27±0.08^{b}	-29.14±0.01^{i}	02.18±0.02^{i}

For every color coordinate in a column, values affected with the same letter are not significantly different

Besides its vital role in maintaining life, food is also used for luxurious parties, and to satisfy comfort and attractiveness. Food producers today include the color attribute among the prime sensory characteristics needed to evaluate the quality of their products. Also, flavor perception is sometimes linked to the color and the taste of the food.

Sensory characteristics of food and food ingredients are therefore important parameters for consumer's appreciation. Although the visual aspect of the fractions (Figure 14) clearly shows the color differences ($P < 0.05$) between the fractions, measurable data may be useful in order to objectively decide on its color characteristics.

Aqueous solutions of the fractions are tested at different pH levels commonly found in foams and emulsions applications. The color tests results are presented in the Table 8.

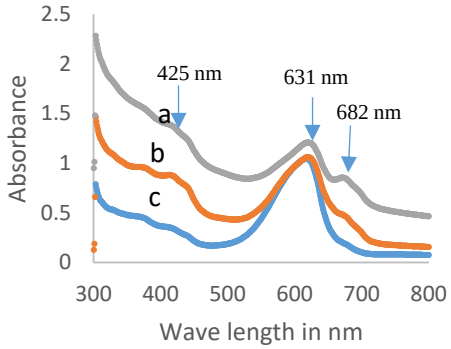
The L*, a* and b* are measurable color parameters based on the Opponent-Colors Theory. This suggest that color perception of the human eye can be ranked by the following opposites pairs colors i.e light-dark (L*), red-green (a*), and yellow-blue (b*) (Kosma *et al*., 2016b).

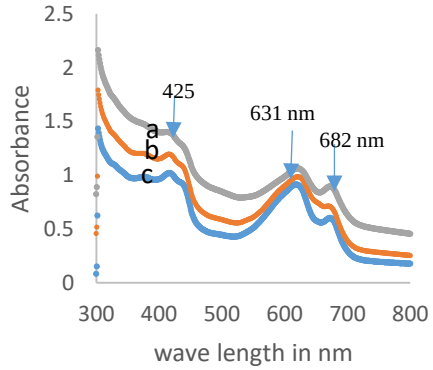
Important color changes are observed when changing the pH of the fractions 0.05% (w/w) solutions. As it shown in the Table 8, there are only few similarities in lightness at native pH and all other coordinates are significantly different for all the fractions solutions at tested pH values. The more important color change due to pH variation among the tested fractions solutions is that of the Blue fraction whose blueness changed from -26.76±0.12 (at its native pH) to -12.91±0.01 at pH 3 i.e. 13.85 units color change. Other fractions solutions also exhibit non-negligible color changes as a result of pH variation from their native pH to acidic pH (3 and 5). These results indicate that when either fraction is to be used as an ingredient, the pH of the resulting mixture pH should be controlled if a specific color of the mixture is targeted.

2.2- UV-visible absorption spectra

The UV-visible absorption spectra of the three colored *Spirulina* protein fractions presents peaks at the same wave lengths for every fraction (Figure 15) independently to the pH value. At the native pH, situated around the neutral pH for all the fractions, absorbance is greater at all tested wavelengths, followed by that at pH 5. At pH 3 fractions solutions showed lesser absorbance which approaches the absorbance at pH5 for wavelengths greater than 550 nm.

Most proteins are colorless in solution and the few colored proteins are conjugated proteins in which the protein carrier is colorless (Beaven and Holiday, 1952). *Spirulina* proteins are supra-molecules made up of a combination of protein with a pigment molecular fragment (Chronakis, 2001). Most *Spirulina* proteins are located in the photosynthetic thylakoids whose function is to convert light energy, a function usually played by chlorophyll pigments in plant cells. Unlike higher plants in which a clear distinction between leaves cells (photosynthetic sites) and stems cells with different functionalities, in *Spirulina* a single cell performs almost all its vital functions. Every *Spirulina* cell has its own light harvesting system that includes pigments. The soluble fractions of *Spirulina* proteins are probably chemically linked to pigments and methods to separate the colorless proteins fractions is not yet developed.The large peaks observed with the green (GSSP) and the total (TSSP) fractions spectra (~631 nm) is that of phycocyanobilin, and a shoulder (~670) that could be attributed to allophycocyanin (Chronakis, 2001). The blue fraction also presents a peak at ~630 nm but does not present the shoulder. Also, peak shoulders observed at ~425 nm corresponding to chlorophyll-pigment protein are not revealed on the BSSP spectrum. All the three fractions show high absorption at 300 nm much greater at native pH. The peak representing the chlorophyll-protein complex appears more clearly on the green fraction than on the total fraction. These results corroborate with those reported by Chronakis (2001).





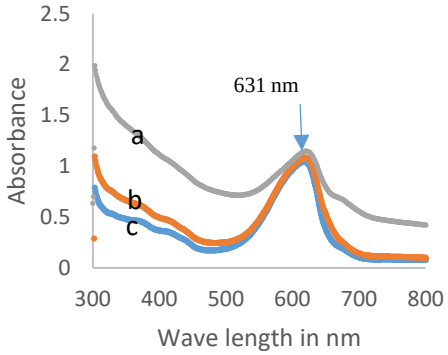


Figure 15: UV-visible absorption spectra of *Spirulina* colored soluble protein fractions aqueous solutions (0.05% w/w) at different pH levels: A: TSSP; B: GSSP; C: BSSP at a: native pH (6.5), b: pH 5: c: pH 3

Spirulina soluble proteins are therefore essentially made up of protein-pigment combinations that present color differences due to the variability of the chromophore linked to the protein. Protein-pigment complexes can exhibit different colors due to the color of the attached chromophore but similar other behaviors, if the protein segments are identical. Properties such as surface activity of these types of complexes are not expected to show significant differences because these are characteristic behaviors linked to the amphiphilic nature of the protein fragment in the complex. The effect of the chromophore could be limited and differently colored fractions would likely present the same behavior. Nevertheless, factors that can contribute to creation of steric or electrostatic barriers during adsorption processes may affect the overall protein adsorption to interfaces.

2.3- Electrophoresis of the different Spirulina colored protein fractions

The electrophoretic profile of the three *Spirulina* protein extracts (Figure 16) revealed bands of molecular weight ranging from 10 kDa to ~55 kDa. These results corroborate with those obtained by Benelhadj *et al.* (2016) who reported the presence of subunits of molecular weight within this range. Bands revealed at ~45kDa, ~38 kDa and ~55kDa are light and common to all the three fractions. Subunits weighing ~10kDa are revealed only in the total (Figure 16, Lane 2) and the blue (Figure 16, Lane 3) fractions, but absent in the green fraction (Figure 16, Lane 4). This may be due differences in fractions separation method based on pH adjustment and centrifugation.

The proteins subunits of~16kDa, ~17kDa, and ~19 kDa are the main bands of all the three fractions. The ~19 kDa band may correspond to α subunits of C-phycocyanins (Moreno *et al*., 1997).

Definitely, the composition of the two fractions (TSSP and BSSP) only differed in the concentrations of the protein subunits. In fact, based on densitometry analysis of protein bands in each fraction, it was found that the ~17kDa represented 25.91% of the TSSP fraction and 16.66% of the BSSP fraction; the subunits weighing ~19kDa made of the 30.60% TSSP (Figure 16 Lane 2) and 40.43% of the BSSP fraction (Figure 16, Lane 3) and 17.08% of the GSSP (Lane 4). The GSSP is devoid of the smaller subunits weighing (~10kDa). These light subunits probably remained soluble at the first precipitation pH (pH 4.5 in Figure1B) and were separated from the green pellets.

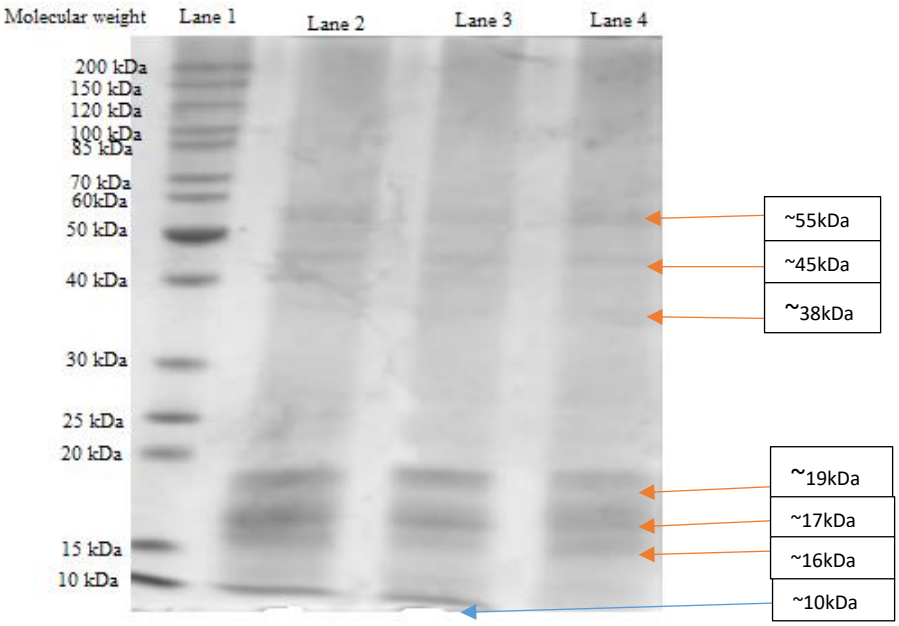


Figure 16: Electrophoretic profile of *Spirulina* protein fractions: 10- 200kDa molecular weight marker (Bio Basic Inc.) (Lane 1); Total soluble *Spirulina* protein fraction (Lane 2); Blue soluble *Spirulina* proteins fraction (Lane 3) and Green soluble *Spirulina* proteins fraction (Lane 4)

2.4- Zeta potential of Spirulina soluble fractions

The curves representing the variation of the electrokinetic potential with solution pH almost superimpose at the tested pH levels (Figure 17). All the fractions presented a maximum negative charge around pH 7 and a maximum positive potential around pH 2.5 (Figure 17). Their isoelectric pH (pHi) were revealed at 3.5 for the total and the blue fractions (TSSP, and BSSP), and 3.3 for the green (GSSP) fraction. These values were close to that reported by Chronakis *et al.* (2000). At these pH values, the net charge of the protein molecules is zero. Shaw *et al.* (2001) reported that proteins were expected to be least soluble near their isoelectric points, and the solubility should increase as the pH was raised or lowered with increasing net charge. Knowledge of zeta potential of the fractions was therefore important as far interfacial properties of proteins are concerned (Zhu *et al.*, 2006). Protein-protein and protein-solvent interactions were likely to be affected, as well as proteins ability to adsorb to interface. At electrokinetic potentials close to pHi protein-protein interaction was favored and protein molecules would tend to precipitate. Under these conditions, where solubility was low, protein could be separated from the solvent by centrifugation. In the present study, the protein powder was solubilized in an alkaline (NaOH) solution whose pH was initially at 10, from which a suspension measuring a pH around 8 was obtained. It could be observed that this pH value of 8 corresponded to a zeta potential of about – 45 mV which was close to the maximum negative potential of the total fraction i.e. the maximum net negative charge

of the *Spirulina* protein molecules. These results were the basis of the extraction method used in this study.

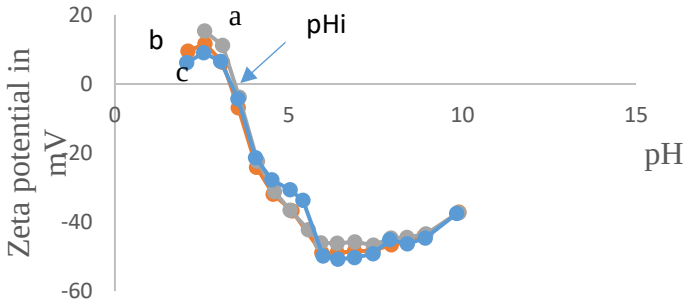


Figure 17: Zeta potential of *Spirulina* soluble protein fractions a: Green soluble *Spirulina* proteins; b Total soluble *Spirulina* proteins; c Blue soluble *Spirulina* proteins

2.5 DSC profiles of Spirulina protein fractions

DSC is an appropriate technique for studying the thermodynamic parameters controlling noncovalent bond formation (and therefore stability) in proteins and other macromolecules (CSC, 2006). It is an experimental method used to determine the difference in heat energy uptake in a sample relative to a reference during a regulated temperature change (Gill *et al*., 2010).

The relationship between enthalpy and entropy of the thermal denaturation of the protein can be determined in a short time (about one hour) and using a very low quantity of material (micrograms of protein). Correlating thermodynamic properties to stability is necessary for the rational design of engineered proteins and protein therapeutics (CSC, 2006). Also, DSC can be used to show protein sources or treatments differences (Arntfield and Murray, 1981).

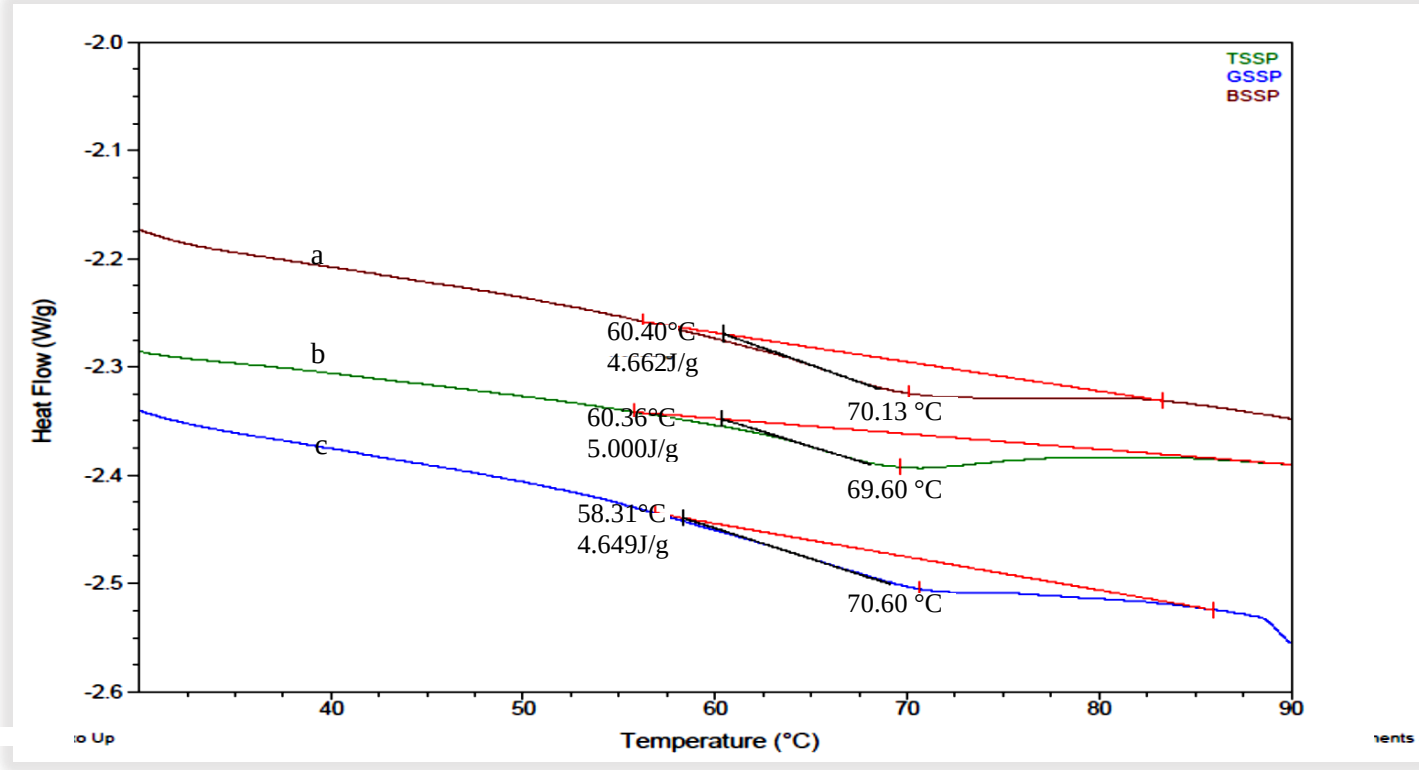


Figure 18: DSC of the three *Spirulina* protein fractions, a: BSSP, b: TSSP, and c: GSSP

Figure 18 presents the differential scanning calorimetry graphs of the three colored *Spirulina* protein fractions. The graph shows denaturation peaks midpoint temperatures around 70°C and precisely 69.60°C, 70.60°C, and 70.13°C for the TSSP, GSSP, and BSSP respectively. Peaks downwards indicate that denaturation is an endothermic process. Denaturation occurs under temperatures at which the folded protein starts unfolding. This denaturation is a combination of endothermic reactions, such as of hydrogen bonds breakup, and exothermic reactions, such as protein aggregation and the breakup of hydrophobic interactions (Privalov and Khechinashvili, 1974; Evans *et aI.,* 1979). The endothermic nature of the thermogram is an indication that hydrogen bonds disruption is the most important phenomenon (Arntfield and Murray, 1981). The closeness of the denaturation temperatures of the fractions once more confirm the fact that the protein fragment in the protein-pigment complexes making the *Spirulina* colored proteins are quite similar, their differences are likely due to the pigment fragments of the protein-pigment molecules in the various colored *Spirulina* protein fractions. It should be noted that the presence of the denaturation peaks on the three thermograms presented is an indication that the proteins are not denatured. In fact Arntfield and Murray (1981) tested heat treated plant proteins and concluded that at certain treatment temperatures the treated proteins did not show DSC thermograms (flat line without peak) , an indication that these proteins were denatured by an irreversible heat treatment.

2.6 Conclusion

The colored *Spirulina* protein fractions are extracted from *Spirulina* dry powder using an industrially feasible method, with fractions protein content close to that of commercial protein concentrates. Fractions obtained present different colors and their protein content are relatively high. Their electrokinetic potentials and DSC thermograms are close to each other. From these results, other related properties of the fractions are expected to be close. The extraction procedure allowed the separation of the soluble proteins from their original substrate, and also enabled the removal of all other insoluble substances that will impede the proper evaluation of the surface behavior of the proteins. It should be noted that the fractions obtained are not pure proteins, but a particular treatment of the fractions with n-hexane drastically reduced the fat content of the fractions thereby limiting eventual lipid interference during interfacial characterization of the fractions.

These basic physicochemical properties could allow for a better interpretation of the analysis to be carried in the coming chapters of the thesis. As interfacial and functional properties of *Spirulina* proteins are the main analysis of this work, knowledge of the protein content of the fractions together with their above tested properties is of great importance.

Chapter 4

Interfacial properties of *Spirulina* soluble protein fractions

Part of this chapter was: published as

Barka A, Amal B A, Francis F, Blecker C, 2018. Physicochemical characterization of colored soluble protein fractions extracted from *Spirulina* (*Spirulina platensis*). *Food Sc. And Tech. Int.* doi/full/10.1177/1082013218786862

And submitted as:

Barka A and Blecker C. *Spirulina* (*Spirulina platensis*) proteins at liquid-liquid Interface and their Emulsifying Properties.

1. Introduction

Food systems are colloidal mixtures usually made up of miscible and/or non-miscible liquids and solid particles. Proteins as ingredients have a significant effect on the textural outcome of processed foods. As surface active agents, they can contribute to foam and emulsions formation. These functionalities are linked to the ability of protein molecules to adsorb to interfaces and to form strong viscoelastic films that can contribute to the stabilization of foams and emulsions (Damodaran, 1994).

Presently, there are not enough investigations on surface and interfacial behaviors of colored *Spirulina* proteins. The few studies so far available on *Spirulina* protein's functional behaviors were focused on the total soluble *Spirulina* proteins. Chronakis (2001) evaluated visible absorption, differential scanning calorimetry, viscosimetry and dynamic oscillatory rheological properties of the *Spirulina* proteins aqueous solutions. Some functional properties of soluble *Spirulina* proteins including oil and water absorption capacities, foaming and emulsifying properties, surface hydrophobicity and nitrogen solubility were evaluated by Bashir *et al.* (2016). Benelhadj *et al.* (2016) investigated also on nitrogen solubility, oil and water absorption capacities, and emulsifying properties of the soluble *Spirulina* proteins. To the best of our knowledge, the only study on interfacial behaviors of colored fractions is that of Chronakis *et al*. (2000), who investigated the colored fractions obtained by ultracentrifugation. However, the separation method of the colored fractions used in this study was an analytical method that could not be industrially feasible for large quantity food processing, due to the high cost and energy requirements of ultracentrifugation. Also, other testing methods (dynamic methods of measuring adsorption kinetics) closer to processes of foam and emulsions preparation may be needed. This would allow for a better appreciation of the behavior of *Spirulina* colored proteins within colloidal systems during process.

In the previous chapter, a simple, industrially feasible method of separating *Spirulina* protein fractions of different colors is presented. For a proper industrial use of these fractions obtained by the developed method, their physicochemical evaluation is needed. Some of their chemical characterizations that can allow for proper interpretation of their interfacial behaviors are also determined in the second chapter of the thesis. Investigations on the interfacial properties of the fractions are the aim of this chapter. These include the dynamic method of surface tension measurement with the drop volume (TVT1), the bubble pressure (BP100) tensiometers under various concentrations and pH conditions, and the Langmuir film balance. Also, the fractions characterization at liquid-liquid interface is done using n-dodecane as the non-polar liquid. Evaluation of the adsorption kinetics at liquid-liquid interface is done with the drop volume tensiometer and viscoelasticity of the drop monolayer films using an automatic drop tensiometer (Tracker).

2. Results and discussions

2.1 Spirulina soluble protein fractions adsorption to air/water interface

2.1.1 Effect of concentration on adsorption kinetics

Adsorption kinetics of freshly formed drops of *Spirulina* colored protein fractions were measured at different concentrations (0.05%, 0.1%, 0.3%, and 0.5% w/w) using a dynamic method of drop volume tensiometer (Figure 19).

For all the three tested protein fractions (figures 19A, 19B, and 19C), the surface tension decay significantly increased with increasing protein concentration ($P < 0.05$). The rate of decay was low at lower protein concentration (0.05% (w/w)). At this protein concentration, the curves were almost linear for the three fractions. The initial rate of the decay can be considered to be the slope of the plotted curve i.e. the greater the slope of the curve, the higher the rate of surface tension decay.

A 20.7% surface tension decay from that of milli Q water (72 $mN.m^{-1}$) was observed within 6 seconds, when using 0.5% aqueous solution of total *Spirulina* protein fraction (figure 19A). The surface tension decay for the other *Spirulina* colored protein fractions underwent the same trend (about 20% within 6 seconds) at 0.5% (w/w) (Figure 19B and 19C). The same decay pattern was followed at 0.3% and 0.1% concentrations except for the 0.05% solution. For the later concentration, the decay was slower showing a lag phase at the beginning, before a uniformly fairly low decay rate was observed.

The diffusion speed of the subphase molecules towards the surface could be one of the factors influencing adsorption kinetics. Dargon-Scaviner (1986) presented protein adsorption to the interface as a two steps process. The first step characterized by the adsorption of the molecules to the surface and their concentration to form a monolayer film on the surface, thereby inducing rapid surface tension decay. The second step corresponded to penetration and conformational rearrangement of the adsorbed proteins. At the second phase, the slope of the surface tension decay curve dropped and the surface tension tends to stabilize to an equilibrium value. This was more or less verified by the behavior of all tested *Spirulina* colored protein fractions, for which the surface tension decay curves tend to be asymptotic towards the end of the measurement. Vogler (2013) reported that the decrease in protein adsorption (i.e. decrease in surface tension reduction) was due to the "energetic cost" of the surface adsorption-dehydration process. As interface water molecules are being replaced by proteins, the energy needed to remove the remaining water molecules became higher and the adsorption rate decreased.

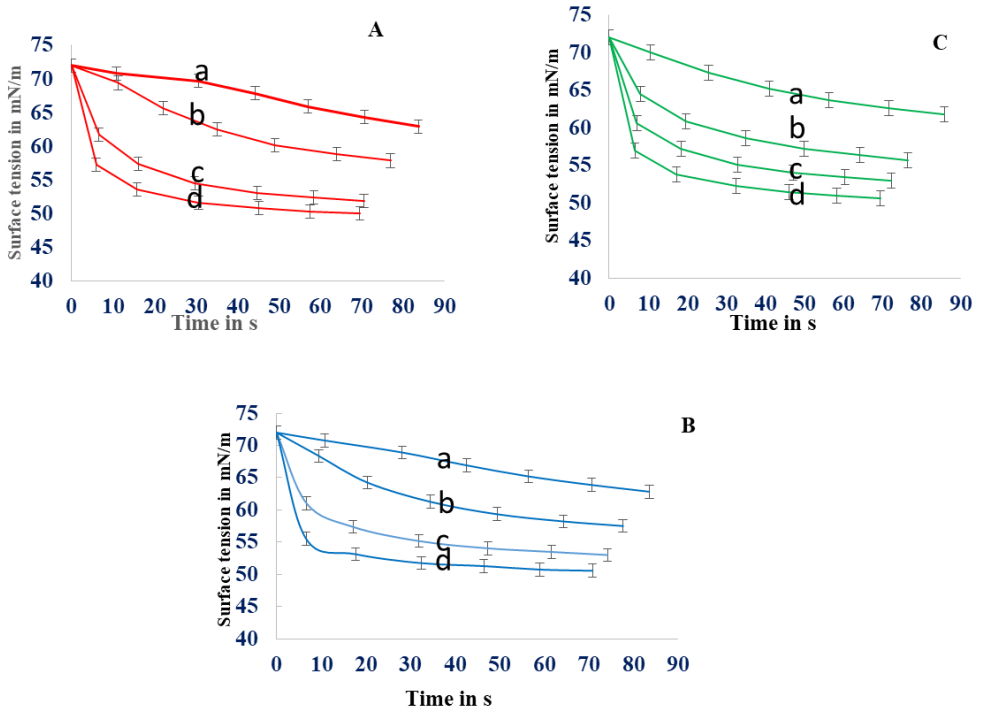


Figure 19: Surface tension decay kinetics of colored *Spirulina* soluble protein fractions at different concentration (a: 0.05%; b: 0.1%; c: 0.3%; d: 0.5% (w/w)) measured with TVT at 25°C; A: Total soluble *Spirulina* proteins; B: Blue soluble *Spirulina* proteins; C: Green soluble *Spirulin*a proteins

Another observation was that despite the color differences of the fractions, their behavior at the interface remains similar. Their color difference was just a matter of distribution of the various *Spirulina* protein molecules among the fractions with predomination of a given color agent in any of the fractions. This could be probably because fractions are not purified protein molecules, rather they are still mixtures with different color agents predomination.

The three fractions being all mixtures of *Spirulina* soluble proteins, they behave the same way on surface tension decay under these experimental conditions at the air/water interface.

All the fractions were made up of the same protein molecules and only the individual protein proportions varied from one fraction to another for example level of subunits weighing ~19kDa revealed in the SDS-PAGE and identified as phycocyanins subunits by Moreno *et al*. (1997), was higher in the blue (BSSP) fraction than in the other fractions. Another hypothesis could be that, the various *Spirulina* proteins behave the

same way as surface active agent. These results were quite different from those of Chronakis *et al*. (2000), who showed differences among the fractions. This was probably due to the fractions separation methods used. According to their study, one of the two blue fractions presented a higher surface tension reduction than the two green fractions. This means that the fractions performance as surface active agents is not an effect of a single type of molecule; but, rather a contribution of the various types of surface active molecules present in the mixture i.e. the more the fractions are purified the clearer will be their difference in reducing surface tension.

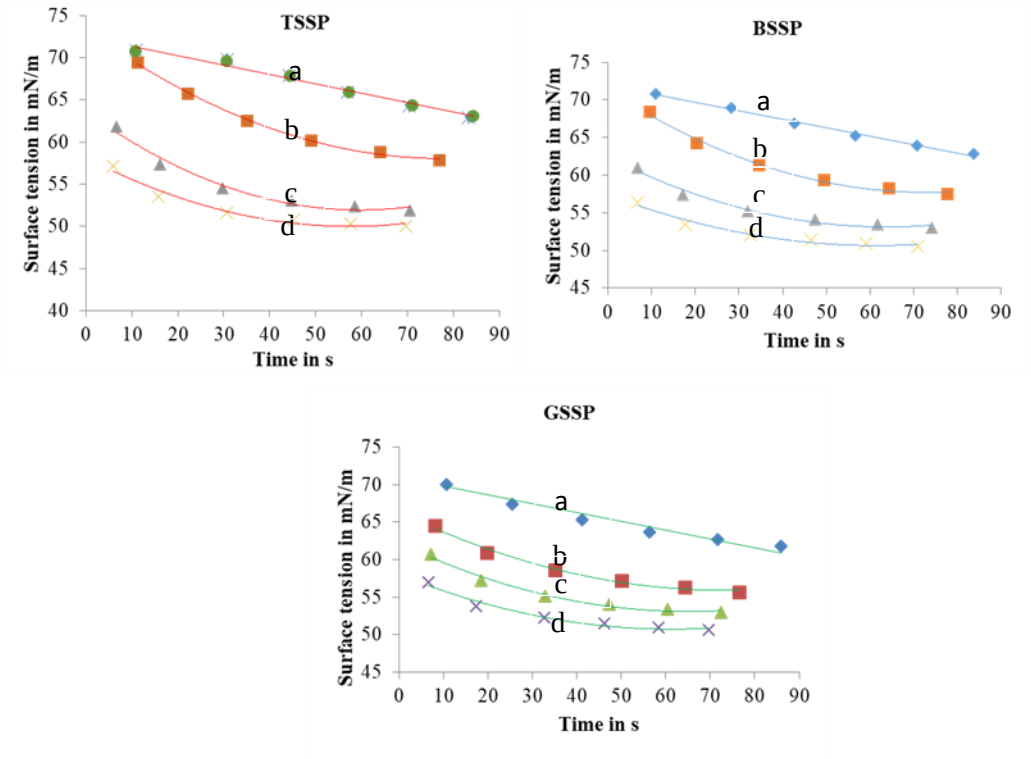


Figure 20: Linear and polynomial models fitting adsorption kinetics curves of colored *Spirulina* soluble protein fractions at different concentration at air/water interface (linear: 0.05% (a); polynomial: 0.1% (b); 0.3% (c); 0.5% (d) (w/w)) measured with TVT at 25°C

To further analyze the adsorption kinetics of the three *Spirulina* colored soluble protein fractions, we estimate the equilibrium surface tension and the initial rate of surface tension decay (Table 9).The equilibrium surface tension (γ_e) is obtained by extrapolating surface tension to infinity time. It is, in other words the least surface tension that can be brought about during the decay, if sufficient time is given to the molecules to diffuse through the subphase and adsorb to the surface at a given concentration. The initial rate

of surface tension decay is defined here as the decrease in surface tension per second. It is calculated as the ratio of the change in of surface tension (mN/m) from its initial value to the change in time(s). The easier the protein molecules diffuse, adsorb and rearrange at the surface, the faster the surface tension decay. While the equilibrium surface tension is the lowest possible surface tension obtainable under specific operating conditions (concentration, pH, temperature etc.), the rate reveals the quickness in attaining equilibrium under these conditions. Both are important parameters as far as foam and emulsion formation, and stability are concerned. It has been shown that the rate of surface tension decay is correlated to foaming capacity of either low molecular weight surfactants (Kitabatake et Doi, 1982) or proteins (German *et al*., 1985; Dagorn-Scaviner, 1986).

A theoretical analysis of the *Spirulina* protein adsorption kinetics to the surface, based on the fitting curves presented in Figure 20, and the parameters in Table 9 allows us to comfortably suggest that at low concentrations (0.05% w/w) *Spirulina* protein adsorption is a zero order kinetics that could be represented by a linear equation model

$\gamma_t = -kt + \gamma_0$ **or** $[\gamma_0 - \gamma_t] = kt$; where γ_0 is the initial surface tension (surface tension of pure water at 25°C) and γ_t is the surface tension at an adsorption time t, **k** is the initial adsorption rate. The regression coefficient of the fitting equations varies from 0.88 (GSSP) to 0.99 (BSSP).

At 0.1% (w/w) protein concentrations, the linear equation adsorption model does not longer fit the adsorption curves for all the fractions. It is rather a polynomial equation model that better fits the curves i.e. $\gamma_t = at^2 - kt + \gamma_0$; where **a** is constant that varies with the type of protein fraction. The BSSP fraction at 0.1% protein presents a value of **k** close the initial adsorption rate, with a regression coefficient of $R^2 = 0.99$.

Table 9: Linear and polynomial fitting equations from adsorption kinetics data obtained with a drop volume tensiometer (TVT1) at 25°C and native pH of Spirulina protein fractions

Spirulina protein Fractions	% proteins in aqueous phase	Model adsorption kinetic equation	Regression coefficient R^2	Theoretical adsorption rate k (from equation) in mN/m.s
Total Soluble *Spirulina* Protein	0.05	$\gamma = -0.10t + 72$	0.98	0.10
	0.1	$\gamma = 0.0018t^2 - 0.33t + 72$	0.99	0.33
	0.3	$\gamma = 0.0033t^2 - 0.39t + 63.61$	0.98	0.39
	0.5	$\gamma = 0.0025t^2 - 0.28t + 58.15$	0.96	0.28
Blue Soluble *Spirulina* Protein	0.05	$\gamma = -0.11t + 72$	0.99	0.11
	0.1	$\gamma = 0.0029t^2 - 0.40t + 72$	0.99	0.40
	0.3	$\gamma = 0.0023t^2 - 0.29t + 62.38$	0.97	0.29
	0.5	$\gamma = 0.0018t^2 - 0.22t + 57.38$	0.97	0.22
Green Soluble *Spirulina* Protein	0.05	$\gamma = -0.13t + 72$	0.88	0.13
	0.1	$\gamma = 0.0049t^2 - 0.57t + 72$	0.67	0.57
	0.3	$\gamma = 0.0022t^2 - 0.28t + 62.19$	0.98	0.28
	0.5	$\gamma = 0.002t^2 - 0.24t + 58.03$	0.97	0.24

Where γ is the surface tension in mN/m, and t the adsorption time in seconds

The polynomial fitting curve equations still present quite acceptable regression coefficients R^2 (0.96 to 0.99) at 0.3% and 0.5% (w/w) concentrations, but the constants are very different from the initial rate of adsorption *k* (Table 10), and surface tension of pure water γ_0 (72 mN/m). At these concentration the theoretical polynomial model could not be applied to the whole adsorption process, probably due to the difference in the adsorption rates during the different adsorption phases. In fact, an adsorption model kinetics suggested by Panizzolo *et al* (2014) presented a first order kinetics model of protein adsorption with two sections giving two different kinetic constants corresponding to different pheonmena occurring during the adsorption processes. The first phase is linked to the increase of the surface protein concentration by adsorption, and the second phase is related to the constant surface protein concentration.

The *Spirulina* protein adsorption kinetics could therefore be considered to be a zero order kinetics at lower concentration (0.05% w/w) but, at higher concentrations (0.1%, 0.3%, 0.5% w/w) it appears to be closer to first order kinetics as reported in the literature (Boutaric and Berthier,1939; Kitabatake and Doi, 1982).

Table 10: Equilibrium surface tension and initial adsorption rates of Spirulina protein fractions solutions calculated from TVT1 measurements at different concentrations and native pH at 25°C

Sample	% protein	Equilibrium surface tension γ_e (mN/m)	Initial adsorption rate k' in mN/m.s from data
Blue Soluble *Spirulina* Protein	0.05	53.54 ± 0.01a	0.11
	0.1	50.78 ± 0.07b	0.38
	0.3	49.39 ± 0.01c	1.63
	0.5	48.17 ± 0.19d	2.49
Green Soluble *Spirulina* Protein	0.05	55.41 ± 0.12e	0.11
	0.1	51.98 ± 0.27f	0.31
	0.3	48.46 ± 0.23d	1.63
	0.5	47.53 ± 0.03g	2.35
Total Soluble *Spirulina* protein	0.05	54.89± 0,29h	0.19
	0.1	50.02± 0,14i	0.23
	0.3	47.16 ± 0.41gj	1.59
	0.5	46.84 ± 0.13j	2.28

Values affected with the same letter are not significantly different

2.1.2 Effect of pH

Although comparison of the overall surface tension decay did not show significant difference ($p > 0.05$) among the samples at 0.3% (w/w), the total fraction showed lowest equilibrium tension at 0.3% (w/w) (Table 10). However, when considering performances at all tested concentrations, the blue fraction shows better performances in terms of equilibrium surface tension and initial rate of surface tension reduction.

A plot of surface tension (mN/m) against drop age (in seconds) is presented (Figure 21) for the three proteins fractions at different pH levels (7, 5, and 3). The choice of the three tested pH is based on the pH levels currently found in food products such as salad sauces, mayonnaise, meat products etc. whose pH is within this range. The kinetic curves show similar patterns for the three fractions at the tested pH values. During the first ten seconds no significant difference is observed ($p < 0.05$) and the three curves almost superimpose. All fractions appear to be less effective in reducing surface tension at pH 3. At pH 7, the surface tension reduction is lower compared to pH 5 during the first 30 seconds, and tended towards that of pH 5 at longer drop ages. Whereas at pH 3, and at these drop ages, surface tension reduction is clearly revealed to be less effective than the other two tested pH levels.

To further investigate the effect of pH on the surface tension decay, we chose to test two pH values (pH 3 and pH 5) that showed important differences in affecting surface tension decay kinetics. This evaluation is done via another dynamic measurement with the bubble pressure tensiometer within the first seconds of which differences are not clearly revealed with the first method (TVT1). Results for this analysis are presented in Figure 22.

The kinetics of the surface tension decay for the three fractions follow the same pattern, and also for all the fractions solutions at pH 3 the proteins are less effective as surface active agents. This confirms previous results obtained with the drop volume tensiometer for longer drop ages. The closeness of this pH value to isoelectric pH of the protein fractions found to be between 3.5 for the GSSP, and 3.3 for TSSP and BSSP, could justify this observation.

These results corroborate with observations reported on pure milk proteins such as proteose – peptones, for which the pH level significantly affects surface tension decay (Karamoko, 2014). For the *Spirulina* proteins, the significant difference of the effect on surface tension decay is revealed only at the isoelectric pH. The decline in electrostatic barrier due to the isoelectric pH would rather contribute to the protein precipitation and does not favor adsorption to the surface. At this pH level, the proteins are less soluble and the diffusion of individual molecules to the surface is expected to be limited, compared to the almost completely solubilized molecules at pH 5 (far away from isoelectric pH). Also, at isoelectric pH surface protein molecules may aggregate and desorb back to the subphase. Despite the presence of a relatively important electrostatic barrier at pH 5, molecules are more susceptible to remain adsorbed to the surface and bring about more pronounced surface tension decay. This is clearly observed within the few milliseconds of measurement by the bubble pressure tensiometer (Figure 22 A, B, C). Other adsorption factors such as

the steric barrier during longer adsorption periods of time may contribute to minimize the effect of pH change on surface tension decay. This is observed on the drop volume tensiometer (TVT1) measurements for which the differences due to pH (5 and 7) variations are not revealed at longer drop ages.

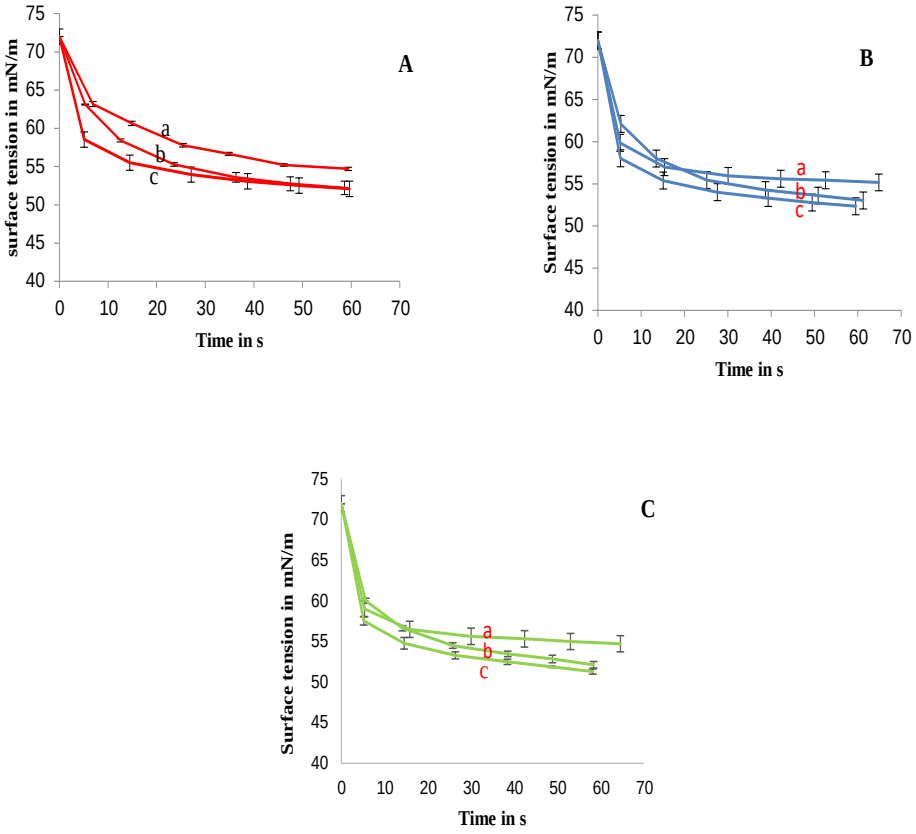


Figure 21: Effect of pH(a: pH 3; b: pH 7; c : pH 5 on surface tension decay of *Spirulina* colored soluble protein fractions aqueous 0.3% (w/w) solutions measured with TVT1 at 25°C A: total soluble; B: Blue soluble *Spirulina* proteins; C: green soluble *Spirulina* proteins

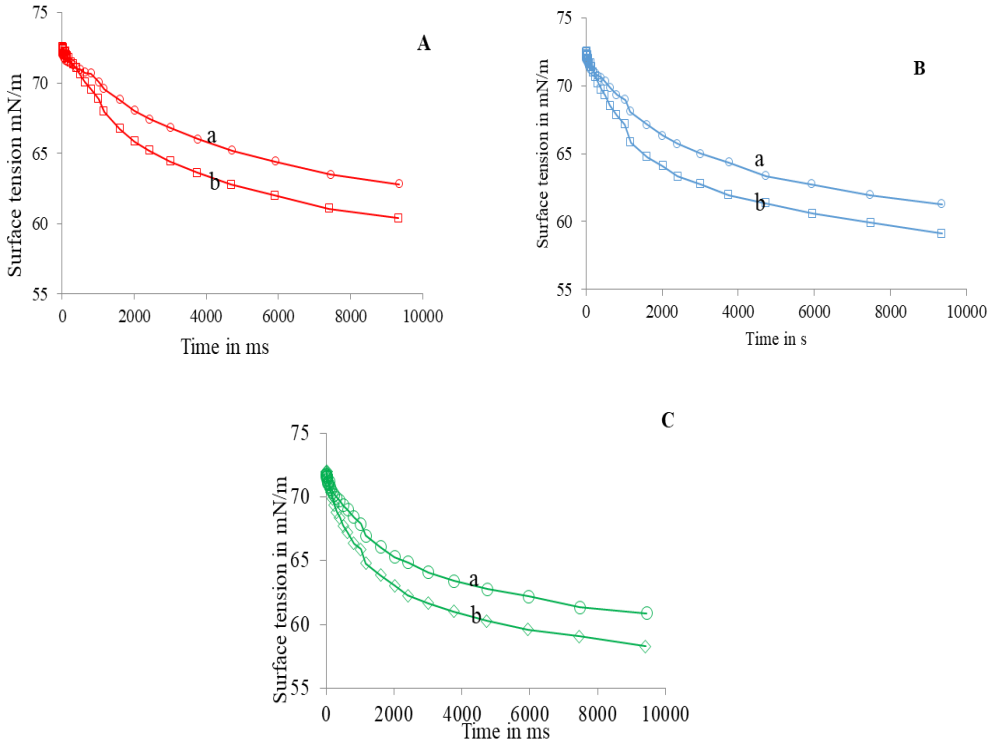


Figure 22: Effect of pH on surface tension decay of aqueous solutions of *Spirulina* colored soluble proteins (0.3% w/w) measured with a Bubble pressure tensiometer (BP100) at 25°C A*: TSSP; B: BSSP; C: GSSP. a: pH 3; c : pH 5*

2.2 Mechanical behavior Spirulina soluble proteins

Compression isotherms of monolayer films formed by spreading of surface active molecules to fluid interfaces are important factors, as far as foams and emulsions stability is concerned (Kinsella and Phillips, 1989). Mechanical behavior of monolayer films at air-water interface can be evaluated via a spreading and compression method using Langmuir film weight (Boyd *et al*., 1973). The objective here was to evaluate the ability of the colored protein fraction to form cohesive films when spread on the water surface. Compression isotherms for the three samples at 0.3% (3mg/mL) were illustrated in the Figure 23. A monolayer film was formed by spreading aqueous solutions of the fractions on a milli Q water surface. When molecules of the film were compressed, the surface area per mg of protein decreased, and the surface pressure (π) increased from an initial value A_0.

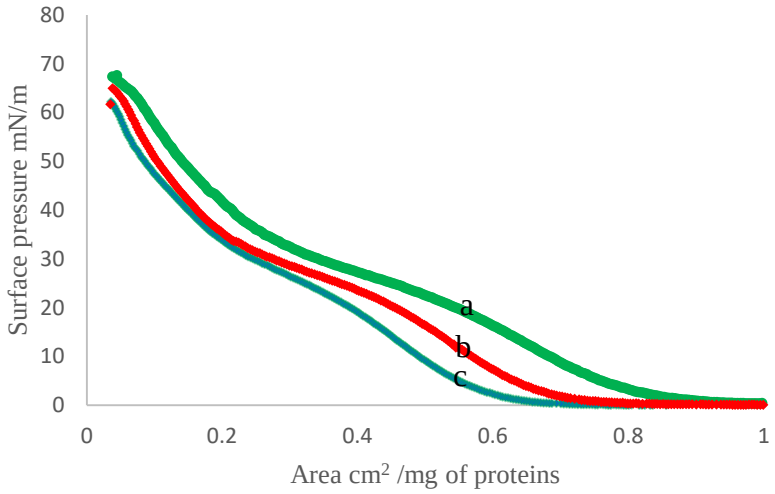


Figure 23: Compression isotherms of different *Spirulina* colored soluble protein fractions dissolved in milli Q water (3mg/mL) at 25 ±1°C on a milli Q water subphase via Lamgmuir film balance: a blue soluble *Spirulina* proteins (BSSP); b Total soluble *Spirulina* proteins; and c Green soluble *Spirulina* proteins (TSSP)

The value of A_0 (Table 11) obtained from the compression isotherm, was an indication of the degree of expansion of the monolayer film at the surface during compression (MacRitchie and Ter-Minassian-Saraga, 1983). A greater expansion of the monolayer film was observed for the Blue fraction (A_0 = 0.861 cm^2/mg protein). All values of A_0 obtained for the various *Spirulina* soluble proteins were within the range reported for whey protein concentrates by Blecker *et al* (1997). The film elasticity is the expression of its individual molecules flexibility under compression or expansion constraints. The films elasticity (ε) was estimated at the linear part of the curve following the transition point and ending at the collapse point (Blecker *et al*., 1997). The Green fraction presented the least elasticity (12.96

mN/m); while elasticity of 17.08 mN/m and 17.59 mN/m were observed for the Total soluble and the blue soluble fractions, respectively (Table 10). Highly flexible molecules exhibit poor elasticity (Damodaran, 1994). Less flexible film molecules are less susceptible to conformational change from loops to trains and vice versa during compression and expansion of the film, thus preventing the film from local stresses (Phillips, 1981). This will delay liquid drainage and reduce film thinning. Consequently, high elasticity (low flexibility) could positively contribute to foam stability.

All the three isotherms obtained followed the same pattern with the BSSP fraction showing higher surface pressure for equivalent surface areas. The general shape of the isotherms was comparable to those reported for other proteins, such as those from milk e.g. proteose-peptones (Karamoko *et al*., 2013), and whey proteins (Blecker *et al*., 1997). The surface pressure almost linearly increased and then changed at the transition point (A_t, π_t) to a greater slope until it reached its collapse value (A_c, π_c). This later point is lower for the green and the total fractions, compared to that of the blue fraction.

The transition point (A_t, π_t) is a point usually observed on protein compression isotherms (MacRitchie and Ter-Minassian-Saraga, 1983). As compression proceeds, available surface area for individual molecules at the surface becomes narrower. Molecules at the surface underwent spacial rearrangement and conformational change. Further compression would bring about an increase in the steric barrier and desorption of the monolayer film molecules towards the subphase. At the end of the compression, the monolayer film was not able to support compression constraints anymore, and it collapses thereby making the pressure to brutally fall. The transition point (A_t, π_t) could therefore be the point from which the increase in pressure during compression was determined by the steric barrier. No further spacial rearrangement and conformational changes were possible and compressibility of the film was limited. Many authors reported this point as that of minimum compressibility of the monolayer film (MacRitchie and Ter-Minassian-Saraga, 1983; Nitsch and Maksymiw, 1990; Blecker *et al*., 1995a).

The blue soluble fraction showed the highest collapse pressure ($\boldsymbol{\pi_c}$ = 67.03 mN/m) followed by the total soluble ($\boldsymbol{\pi_c}$ = 64.28 mN/m) and the green fraction ($\boldsymbol{\pi_c}$ = 61.60 mN/m) (Table 11). This showed that the blue fraction forms a stronger film that better resist the mechanical constraints due to compression. The film formed by the blue are expected to be more cohesive under these conditions. Indeed, phycocyanins, expected to be present in the blue fraction at a higher proportion was reported to improve emulsion stability; a property linked to viscoelastic film formation at interfaces (Batista *et al.,* 2006). This property greatly contributes to foam and emulsion stability (Blecker, 1997). A more cohesive film could readily hold gaz bubbles in foams and oil droplets in emulsions matrices, usually encountered in foams and emulsions.

Table 11: Characteristic parameters of three Spirulina soluble protein fractions determined from their compression isotherms

Spirulina protein fractions	A_0 (cm^2/mg protein)	A_t (cm^2/mg protein)	π_t (mN/m)	π_c (mN/m)	Ɛ (mN/m)
Green Soluble	0.62 ± 0.01^a	0.36 ± 0.03^a	22.68 ± 0.30^a	61.70 ± 0.07^a	12.96 ± 0.94^a
Blue soluble	0.86 ± 0.00^b	0.45 ± 0.02^b	25.34 ± 0.55^b	67.03 ± 0.59^b	17.08 ± 0.23^b
Total Soluble	0.67 ± 0.05^a	0.39 ± 0.01^a	23.87 ± 0.71^c	64.28 ± 0.71^c	17.59 ± 0.30^c

All values given are means of three repetitions ($\bar{x} \pm$ SD) (SD: Standard deviation). Values with same subscript letters within the same column are not significantly different ($P > 0.05$).

A0: Expansion area; At: Transition area; πt: Transition pressure; πc: collapse pressure; Ɛ: film elasticity

2.3 Adsorption kinetics at water/n-dodecane interface

2.3.1 Effect of concentration

In order to evaluate the fractions behavior at liquid-liquid interface, n-dodecan is chosen as the non-polar liquid. It is preferred to vegetable oils because it is devoid of other surface active molecules such as mono or diglycerides that may alter the results of the protein performances. Interfacial tension measurements are carried out by expelling a drop of the protein suspension via a capillary immersed in the n-dodecan filled in the cuvette of the TVT1. The drop detaches from the capillary and falls to the bottom of the cuvette when the interfacial tension is not strong enough to support the weight of the drop.

Adsorption kinetic of the proteins is evaluated through interfacial tension decay measurement because both processes are tightly linked. Indeed, the interfacial tension is a measurable expression of the progressive diffusion and adsorption of protein to the interface.

Figure 24 shows the adsorption kinetics (interfacial tension γ as a function of time t; γ = f (t)) of the three *Spirulina* protein fractions (TSSP, BSSP, GSSP) at different concentrations (0.5%; 0.3%; 0.1% (w/w)). The interfacial tension decreases with the age of the drop. Greater reduction of interfacial tension is obtained with higher protein concentration. At 0.1% (w/w) the BSSP shows the highest initial adsorption rate (0.88mN/m.s) (Table 1) by bringing about a 30.46% reduction of the interfacial tension from its initial value (44.53 mN/m). The interfacial tension revealed for the BSSP fraction at this concentration is also the lowest (24.01mN/m) compared to those of the TSSP and GSSP fractions (25.28mN/m and 25.15 mN/m respectively). Also, at 0.5% (w/w) protein concentration, the BSSP shows the highest initial adsorption rate (1.96 mN/m.s) and the lowest equilibrium interfacial tension (16.21 mN/m).

Despite their high molecular weight compared to LMWS (low molecular weight surfactants), protein molecules in bulk aqueous solution have the propensity to adsorb to interfaces. The amphiphilic protein molecules migrate towards the interface once the drop is formed thereby causing the decay of the interfacial tension (Damodaran, 1994). Newly adsorbing molecules should overcome an energy barrier (Tornberg, 1978a) by creating the necessary space ΔA for adsorption at the interface. Ward and Torday (1952), Graham and Phillips (1979a), and Dagorn-Scaviner *et al*. (1986) suggested that ΔA for every phase is the slope of the segment representing this phase on the graph – $\ln (d\pi/dt) = f(\pi)$ (Figure 24). These authors sectioned protein adsorption to interface into three steps. This stepwise fractionation of the protein adsorption process depends on the size and the molecular structure of the protein. The general adsorption pathway suggested by these authors divides the protein molecular adsorption into different phases - diffusion of the molecules from the aqueous phase to the interface - penetration into interfacial layer by new molecules and development of an energy barrier opposing this process - and then rearrangement of adsorbed molecules by conformational modifications (Tornberg, 1978a). The decrease of the curve slopes towards the end of the measurement can be explained by the fact that adsorption becomes more difficult when the interface is saturated. In fact, the barriers created by the already adsorbed molecules, and the need for the molecule to rearrange and change their conformations at interfaces, hinder adsorption process. Adsorption proceeds at a speed higher at the beginning (freshly formed drops) and progressively slows down as the barriers are becoming stronger.

Under the present experimental conditions (dynamic method of surface tension measurement) the adsorption kinetics is evaluated within periods of time that do not allow for the implementation of all the three phases suggested by Ward and Torday (1952), and Dagorn-Scaviner *et al* (1986). According to Tornberg (1978a) the occurrence of the rearrangement phase is revealed when ΔA calculated from the plot of $- \ln (d\pi/dt) = f(\pi)$ curve is null. By considering this statement, only the first two phases of diffusion and penetration are revealed (Figure 25).

Both the initial adsorption rate and the equilibrium interfacial tension are affected by the protein concentration in the aqueous phase. While the equilibrium interfacial tension is lower at higher concentrations, the initial adsorption rate increases with concentration (Table 10). The BSSP fraction presents the higher adsorption rates at the concentrations (0.1% and 0.5% (w/w)) and native pH. This may be attributed to its high solubility in water.

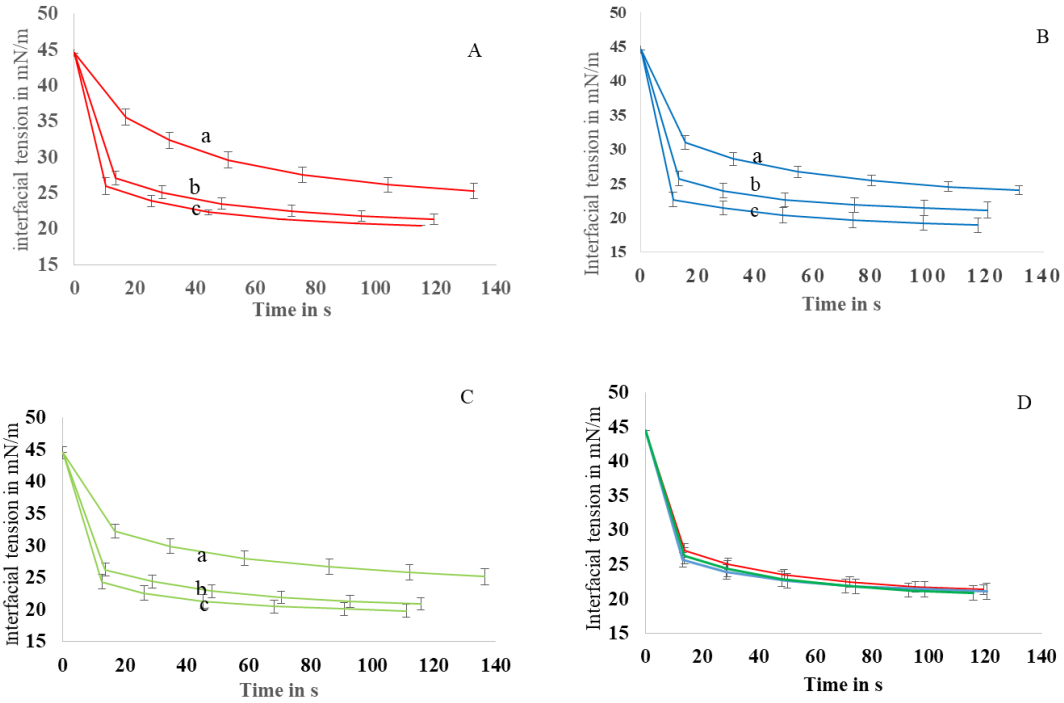


Figure 24: Effect of concentration on adsorption kinetics of different *Spirulina* colored soluble protein fractions at water/n-dodecane interface measured with drop volume tensiometer (TVT1) at 25°C and native pH: A TSSP; B BSSP; C GSSP: (a: 0.1% ; b: 0.3%; c:0.5% (w/w)); D: 0.3% (TSSP, BSSP, GSSP)

The adsorption kinetics comparison of the three fraction at 0.3% (w/w) concentration is presented in the Figure 23D. No significant difference is observed among the three tested *Spirulina* protein fractions. Despite their color difference, the extraction method used does not give pure fractions containing exclusively one type of protein. Also the differences in color of *Spirulina* protein fractions are due to the nature of the pigment phycocyanobilin linked to the protein fragment. The pigment may not necessarily significantly influence the adsorption process of the pigment-protein complex (phycobiliprotein).

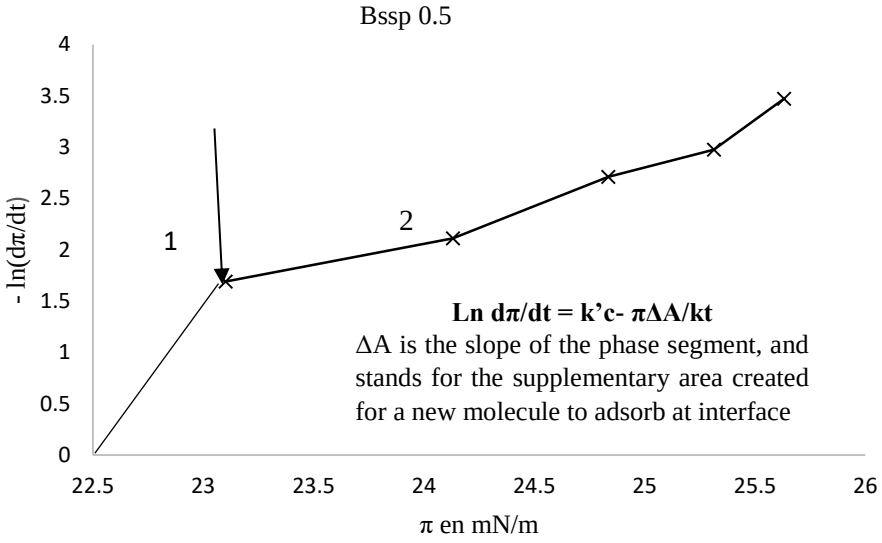


Figure 25: A plot of –ln (dπ/dt) = f (π) calculated from the adsorption kinetic Y= f (t) of the BSSP fraction aqueous solution (0.5% w/w) at water/n-dodecane interface. 1: diffusion phase 2: penetration phase

2.3.2 Effect of pH

Suspensions (0.3% (w/w)) are prepared from the three *Spirulina* protein fractions and tested at three pH levels (native pH, pH 5 and pH 3). Figure 26 presents their adsorption kinetics (interfacial tension as a function of time, f (t) = γ). These results show that the interfacial tension decreases with the drop age fairly quickly at the beginning, and slowly towards the end of the experiment. At the same concentration (0.3% w/w), the interfacial tension decay kinetics for all the three fractions are close to each other at pH 3 and the native pH (~ 6.5), while at pH 5 the decay is significantly important ($p < 0.05$) and differs from the first two pH levels.

The interfacial tension measured at the milli-Q water/n-dodecane interface (44.53 ± 0.08 mN/m) is considered as the initial point of the interfacial tension decay kinetics during the protein suspensions drop ageing. Knowing that surface tension decay with the drop age is the manifestation of the adsorption kinetics at the interface, the fast decay within the first ~12 seconds observed with the suspensions means that proteins adsorption is faster within this period of time. Ward and Tordai (1946) stated that within the first instants of adsorption processes, the surface (or interfacial) tension decay reflects adsorption of solutes that diffuse from the aqueous phase to the interface. The interfacial tension is reduced from its initial value (44.53±0.08 mN/m) by 47.48% (23.38±0.06 mN//m) within 12.4 seconds when using the BSSP (0.3% (w/w)) at pH 5.

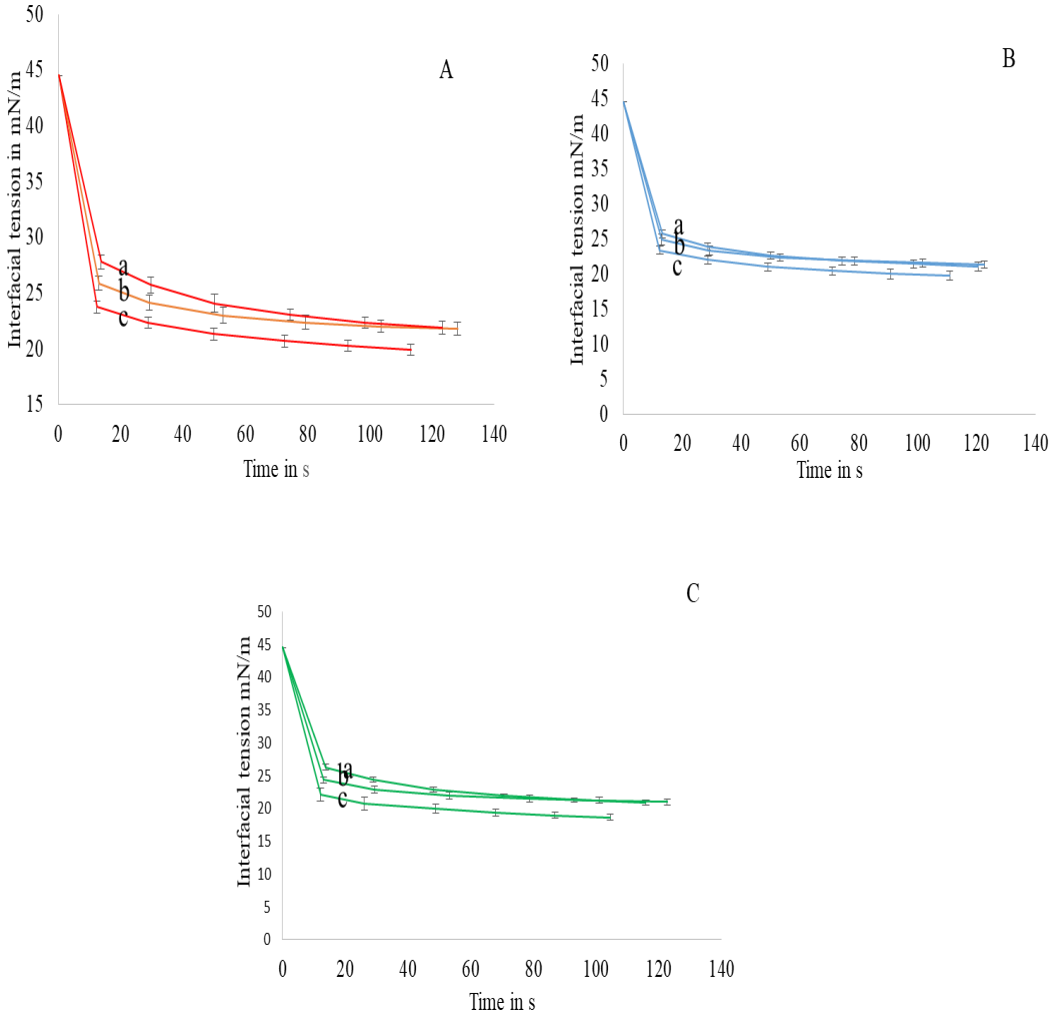


Figure 26: Effect of pH on adsorption kinetics of different *Spirulina* colored soluble protein fractions at water/n-dodecane interface measured by the drop volume tensiometer (TVT1) at 25°C: A TSSP; B BSSP; C GSSP; a: pH 3, b: native pH, and c :pH 5

Once a drop is formed, the free energy of the system reaches its highest value and the protein diffusion towards the interface (the tendency to lower the free energy) is at the same time accelerated by the gravitational force (for pendant drop measurements), the pressure exerted by the expelling system, and the acceleration brought about by the change in free energy of the system due to the disturbance associated with the new drop formation. This fast adsorption to interface during the dynamic surface tension measurement is one of the peculiarities of the dynamic surface tension measurement

method as compared to the static method. Measurements are carried out both on freshly formed interfaces, and during their ageing. The method is closer to real colloid systems during emulsions formation, far from being equilibrium systems. The needed property in colloids as far as surface active agents are concerned, is not only their ability to adsorb to interfaces but also the rate at which the adsorption proceeds i.e. the rate of interfacial tension reduction in addition to the strength of the film formed at interfaces.

The initial adsorption rates to the water/n-dodecane interface of protein suspensions at different (0.1%, 0.3%, and 0.5% w/w) concentrations and pH (pH 3, pH 5, and native pH) are presented in the Table 12 A and B respectively. The adsorption rate is expressed as the change of interfacial tension from its initial value (44.53± 0.08 mN/m) to the first measured value (about 12 seconds after drop formation depending on pH and concentration) during drop formation. It is therefore an estimation of the interfacial tension reduction per unit time and expressed as the differential of the interfacial tension over that of the first measurement time ($d\gamma/dt$). This gives an idea on how fast the interfacial tension decreases during drop formation and ageing. The initial adsorption rate increases with increasing concentrations for all the fractions and is higher at pH 5 for all the fractions. At 0.5% the blue fraction (BSSP) shows the highest initial speed (1.96 $mN.m^{-1}.s^{-1}$) (Table 11A), and this performance is followed by the rates obtained at pH 5 for the blue green and total fractions respectively at 0.3% (w/w) concentration. This shows that interfacial adsorption of *Spirulina* proteins rate could be significantly improved by reducing the suspensions pH from its native value (around 6.5) to pH 5. While expecting higher adsorption rates at the native pH that showed higher protein solubility, adsorption rates at this pH remain lower compared to those obtained at pH 5 for all the fractions. This suggests that protein molecules diffusion and adsorption to interface is more affected by electrostatic interactions than the concentration gradient. In a previous study we obtained a greater electrokinetic potential at pH range 6.5 to 7 (-50 mV) compared to -35 mv at pH 5 (Barka *et al*, 2018). At pH 5 solubility is fairly high and the molecular repulsion is not high enough to prevent other protein molecules to adsorb to interface, thus adsorption rate is favored. On the other hand at native pH, solubility is higher but protein adsorption to interface is hindered by the high electrostatic barrier between protein molecules. Otherwise, pH 5 seems to be a good compromise that brings about better adsorption performances for *Spirulina* proteins. Thus, lower equilibrium interfacial tensions and higher initial adsorption rates are observed with all the three fractions at pH 5 as compared to those values obtained at pH 3 and native pH at the same concentration (0.3% w/w). At these later pH values, the curves representing the adsorption kinetics are close while expecting to see different behaviors when capitalizing an argument on the protein solubility presented in Figure 24. In fact, the higher solubility of *Spirulina* proteins at their native pH (~ 6.5) is expected to favor protein adsorption thereby effectively lowering interfacial tension. This limited interfacial tension reduction at pH 3 and native pH can be attributed to two different phenomena. At pH 3; a pH value close to the isoelectric pH of the *Spirulina* proteins (pH 3.4-3.5), the low protein solubility could be the major factor hindering adsorption. Ultimately, at the native pH (around pH 6.5), solubility does not appear as a limiting factor, but the highly charged protein molecules

may build an electrostatic barrier (molecular repulsion) that could hinder adsorption of protein molecules.

Table 12: Initial rate of adsorption (in mN.m-1.s-1) of different Spirulina colored proteins suspended in milli Q water calculated from data obtained by a drop volume tensiometer at water/n-dodecane interface TVT1: A: different concentration levels at native pH

A			
Sample	**Concentration**	**Equilibrium interfacial tension ϒe in mN/m**	**Calculated initial rate of adsorption dϒ/dt in mN/m.s**
TSSP	0.1	20.00±0.05a	0.52
	0.3	19.00±0.04b	1.22
	0.5	18.05±0.39c	1.77
BSSP	0.1	19.80±0.16a	0.88
	0.3	19.00±0.60b	1.43
	0.5	16.21±0.81d	1.96
GSSP	0.1	20.75±0.47e	0.72
	0.3	18.80±0.30b	1.20
	0.5	17.30±0.35f	1.59

B			
Sample	**pH**	**Equilibrium interfacial tension ϒe in mN/m**	**Calculated initial rate of adsorption dϒ/dt in mN/m.s**
TSSP	Native pH	19.00±0.04a	1.22
	pH 5	18.00±0.15b	1.68
	pH 3	20.30±0.53c	1.44
BSSP	Native pH	19.00±0.60a	1.43
	pH 5	17.80±0.29b	1.71
	pH 3	20.00±0.21c	1.51
GSSP	Native pH	18.80±0.30a	1.20
	pH 5	17.90±0.20b	1.66
	pH 3	20.01±0.05c	1.55

Values with same subscript letters within the same column of the same table are not significantly different ($P > 0.05$).

2.4 Viscoelastic properties of Spirulina colored protein fractions

2.4.1 Effect of concentration

The oscillating pendant or rising drop tensiometer (Tracker) is used to characterize dilatational interfacial rheometry by periodically varying the volume (surface area) of drop. It is based on the formation of a pendant or rising drop at the tip of a capillary immersed in a continuous liquid phase (n-dodocane) filled in a thermostatically protected cuvette. The theoretical Laplace equation is applied on the profile image of the drop contour through a numerical procedure. Interfacial dilatational modulus can therefore be determined from $\partial\pi/(\partial A/A_0)$ (where $\partial\pi$, $\partial\pi$ are variations of drop interfacial pressure and area respectively, and A_0 the initial drop area) . The dilatational modulus is a complex quantity, $E = E' + iE''$, with a real part E' (storage or elastic modulus) and an imaginary part E'' (loss or viscous modulus). In small sinusoidal area perturbations, E is measured in analogy to bulk rheology from the stress response π to a given strain function $(\Delta A/A_0)$ at a given time t (Erni *et al.*, 2007).

To the best of our knowledge, the determination of the interfacial viscoelastic properties (elasticity and viscosity) of *Spirulina* proteins at liquid-liquid interface have not yet been investigated in the literature. Elasticity (E'), and viscosity (E'') moduli of the proteins aqueous solution at different concentrations are presented in Table 13. For all the fractions the elasticity decreases with concentration. However, the viscosity increases when increasing concentration within the range of concentration tested. At 0.1% and 0.3% concentrations, the GSSP fraction shows lower elasticity compared to the TSSP and the BSSP fractions. At this tested concentration range and at 25°C *Spirulina* protein suspension are revealed to be more elastic than viscous (E' is almost ten times E''),.

The blue fraction (BSSP) appears to be more sensitive to concentration changes followed by the total fraction (TSSP) and the green fraction (GSSP) as far their elastic moduli are concerned. These results are somehow contradictory to what we expected during our investigations. The elastic modulus is rather expected to increase with increased solution concentration but the reverse is the case within the range of the tested concentrations, unlike the viscosity. In fact, the interfacial film elasticity is nothing but the ability of the adsorbed proteins to desorb and readsorb to interface during drop compression/expansion i.e. the system returns back to its initial state. The effectiveness of these processes (interfacial adsorption/desorption) may depend on the protein molecules flexibility and their concentration at the interface. For instance, a solid-like film will be less elastic but most viscous. Thus, the increase in protein concentration expected to bring about formation of more solid-like interfacial film may explain the drop in the elastic moduli and the increase in the viscous moduli observed. The dilatational (viscoelastic) moduli E resulting from the elastic and viscous contributions rather decrease with concentration within the tested concentration range.

While strong, viscous, and elastic interfacial films are needed to stabilize colloidal systems such as foams and emulsions, a proper combination of the film elasticity and viscosity may be required for a good stability. To achieve this, proteins are sometimes

structurally modified (Yann, 2011) or associated to other emulsifiers such as gums (Erni *et al.*, 2007).

Table 13: Effect of concentration on the elasticity (E′) and viscosity (E″) moduli of Spirulina colored soluble protein fractions at water/ndodecane interface measured with an automated drop tensiometer (Tracker) at native pH and 25°C

Sample	Protein concentration (% w/w)	Elasticity (E′), mN/m	Viscosity (E″) mN/m
TSSP	0.1	24.21 ± 0.30a	2.27 ± 0.01a
	0.3	23.32 ± 0.69b	2.75 ± 0.10b
	0.5	19.67 ± 0.03c	2.93 ± 0.12cd
BSSP	0.1	24.31 ± 0.03a	2.74 ± 0.16b
	0.3	23.26 ± 0.06b	2.83 ± 0.04d
	0.5	19.46 ± 0.09c	3.37 ± 0.04e
GSSP	0.1	21.29 ± 0.06d	2.61 ± 0.00e
	0.3	20.03 ± 0.04e	2.79 ± 0.04bd
	0.5	19.95 ± 0.02ec	3.03 ± 0.03c

All values given are means of three repetitions ($\bar{x}$ ± SD) (SD: Standard deviation). Values with same subscript letters within the same column are not significantly different (P > 0.05).

2.4.2- Effect of pH

The viscoelastic moduli are also evaluated for their response to the suspension pH variations at 0.3% (w/w) concentration. Results are presented in the Table 14. The viscoelastic moduli increase when reducing pH. Three pH values are tested i.e. pH 3, pH 5 and the native pH (~ pH 6.5) of the suspensions prepared by dissolving the fractions in milli Q water. Unlike concentration variation, the response of the interfacial viscoelastic moduli (interfacial elasticity and viscosity) to pH variation is revealed to be more important. Another striking observation on these results is that at the same protein concentration, the pH reduction from its native value to pH 3 leads to an increase of the elastic modulus (E') by about 4mN/m, and an increase in viscosity modulus (E") by about 1.5 mN/m. From pH 3 to pH 5 the decrease in the elastic and viscosity moduli (E' and E" respectively) is greater than when comparing pH 5 to the native pH. In fact, as we reported previously, the isoelectric pH of the *Spirulina* soluble protein fractions is within the range of 3 to 3.5. At this pH where electrostatic repulsion between protein molecules is supposed to be negligible, it is expected to show a behavior different from that of the two other pH values. Proteins under this condition can easily come together in the subphase and precipitate or at the interfacial layer and form stronger monolayer interfacial films with higher elasticity and viscosity. On the other hand, at pH values far away from the isoelectric pH, proteins molecules are charged and they need a supplementary work to overcome electrostatic barrier and adsorb to the interface (Macritchie and Alexander 1963c). When considering this later phenomenon, adsorption

is favored when the pH value of the aqueous phase is close to the isoelectric pHi (around pH 3), but this is not the case for experiments carried in this study and in our previous studies at air/water interface. We are therefore tempted to believe that, absence of electrostatic barrier due to closeness to isoelectric pH does not compensate the shortcomings brought about by the limited solubility of proteins at isoelectric pHi.

Table 14: Effect of pH on the elasticity (E′) and viscosity (E″) moduli of Spirulina colored soluble protein fractions (0.3% w/w) at water/ndodecane interface measured with an automated drop tensiometer (Tracker) at 25°C

Sample	Protein suspension pH	Elasticity (E′), mN/m	Viscosity (E″) mN/m
TSSP	Native	23.32 ± 0.69a	2.75 ± 0.10a
	pH 5	24.56 ± 0.07b	3.56 ± 0.07b
	pH 3	27.38 ± 0.07c	4.25 ± 0.14c
BSSP	Native	23.26 ± 0.06a	2.83 ± 0.04a
	pH 5	23.96 ± 0.60a	3.72 ± 0.07d
	pH 3	27.92 ± 0.05c	4.23 ± 0.30c
GSSP	Native	20.03 ± 0.04d	2.79 ± 0.04a
	pH 5	22.45 ± 0.05e	4.10 ± 0.02c
	pH 3	27.82 ± 0.03c	4.67 ± 0.01e

All values given are means of three repetitions ($\bar{x}$ ± SD) (SD: Standard deviation). Values with same subscript letters within the same column are not significantly different (P > 0.05).

3- Conclusion

The three colored soluble protein fractions extracted from *Spirulina* biomass, exhibited surface and interfacial tension reduction.

All fractions appear to be more surface active at pH 5 but at the water/n-dodecane the difference between pH 3 and the native pH is less pronounced compared to that observed at air/water interface. Within the tested concentrations, the viscoelastic moduli of the fractions decrease with increasing concentration. However, the viscous and elastic moduli are rather higher at lower pH (pH 3 and pH 5) than at the native pH. Thus, to obtain more viscous and elastic interfacial film at this concentration, one just has to reduce the pH of the protein suspension. Nevertheless, the performance of the *Spirulina* suspension at its isoelectric pH (or close to its isoleclectric) may not guaranty the stability of the colloidal (foams and emulsions) system they form.

The blue soluble fraction showed a greater initial adsorption rate and better cohesive films, with higher elasticity compared to the other fractions. Their use in foam and emulsion may be boosted by their ability to form strong organic films which could be needed in coating sensitive molecules. The different fractions separation improved

surface activities and gave out different colored protein products that could be attractive for some specific industrial applications.

For a better appreciation of the fractions as surface active agents, and for their proper implementation in foams and emulsions, knowledge of their functional properties sustained by their interfacial behavior should be evaluated. These include, solubility, foaming and emulsion properties, and also their comparison to conventional protein under the same experimental conditions may also be instructive.

Chapter 5

Functional properties of *Spirulina* soluble protein fractions

Part of this chapter is submitted as:
Barka A and Blecker C. *Spirulina* (*Spirulina platensis*) proteins at liquid-liquid Interface and their Emulsifying Properties.

1. Introduction

The growing need to satisfy consumer's preferences instigates researchers in looking for more appropriate raw materials that can improve product quality and boost consumers' acceptability. With the advent of industrialization, processed foods appear to be more convenient to consumers, due to their "ready to eat" aspect, the ease of identification of what one is eating as food, and what ingredients are used in its formulation. Natural ingredients are preferred in processed food, as compared to synthetic ingredients (Rozin *et al.*, 2004).

Proteins can be used as ingredients in a processed food for a dual purpose; to improve the nutritional value of the food, and to give desired organoleptic properties to the processed food. Conventional proteins are for long being used for this purpose, but the limited protein availability and their high production cost are the key factors encouraging the search for alternative protein sources.

In food colloids, especially foams and emulsions, proteins are used as emulsifiers or stabilizers, a role naturally played by milk proteins to maintain the stable colloidal aspect of milk. In some products such as ice cream, proteins are added to improve quality and stability (Lee and white, 1991). Tremendous investigations are carried out on milk proteins and other conventional proteins, concerning their ability to act as surface active molecules that can stabilize foams and emulsions (Wilde, 2000). This is not the case for proteins from new sources such as *Spirulina* proteins.

An industrially feasible *Spirulina* protein extraction method is developed and presented in previous chapter, and protein fractions of different colors are obtained from a spray dried powdered *Spirulina* biomass. Evaluation of their surface activity is also carried in previous chapters. In this chapter, we intend to evaluate the applicability of these protein fractions in foams and emulsions stability, a function expected to be sustained by their surface properties.

Specifically the aim of this chapter is to evaluate the foam and emulsion capacity and stability of the colored *Spirulina* protein fractions, their solubility, oil absorption and the microscopy of their emulsion particles.

2. Results and discussions

2.1 Solubility

Protein solubility is a determinant physical property, sometimes considered as a prerequisite factor for other functional behaviors to manifest. It is defined here as the ratio of protein that dissolves in water under the described experimental conditions. Protein solubility is reported to be the most important factor and an excellent index for protein functionalities (Kinsella, 1976). Its knowledge can give useful information on the potential utilization of proteins and their functionalities, especially in foams, emulsions and gels (Zayas, 1997). For a protein to be used as a surface active molecule at a water/fluid interface, its solubility in water is in fact required.

Solubility curves of the three *Spirulina* protein fractions are presented in Figure 27. All the fractions appear to be less soluble at pH 3, probably due to the proximity to the isoelectric pH of the fractions. A fairly stable solubility is obtained for the BSSP and the TSSP fractions right from pH 7. The GSSP is revealed to be less soluble at pH 5 while the BSSP almost reaches its maximum solubility at this pH level. Solubility of GSSP keeps increasing up to pH 10 unlike the two other fractions that arrive at a steady solubility between pH 7 and pH 10.

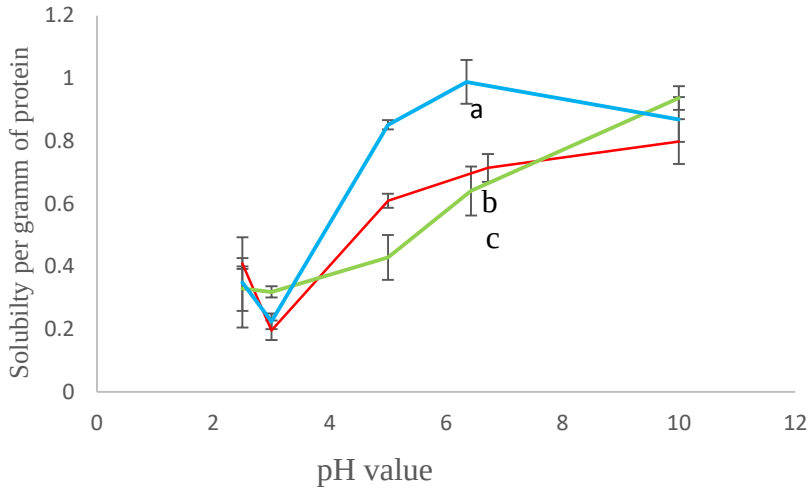


Figure 27: Nitrogen solubility of three *Spirulina* protein fractions at different pH values:

a: BSSP; b:TSSP; c: GSSP

As it can be observed on the curves, the pH of the aqueous medium is a determining factor as far as protein solubility is concerned. As pH affects the electrokinetic potentials of the protein molecules in the medium, the electrostatic interactions between the molecules within the solution is affected. When the electrostatic repulsion between protein molecules is favored by the pH, solubility increases. However, when hydrophobic interactions are favored protein-protein attraction increases and solubility decreases. It seems that the balance between hydrophobic and hydrophilic protein interactions with its environment governs the protein solubility.

For a protein to be soluble, it should be able to interact as much as possible with the solvent (Zayas, 1997). The least solubility observed on the figure is located around the

isoelectric pH of the fractions. At this pH, the electrokinetic charge of the molecules is zero and there is no electrostatic repulsion between protein molecules. Protein molecules tend to aggregate and solubility is hindered. The reverse is the case when the pH is far away from the isoelectric point.

2.2 Oil absorption

Oil retention within a processed food is an important factor as far the texture and mouthfeel of the food are concerned. According to Kinsella (1979), oil retention is important in many applications including meat replacement in some foods to improve flavor retention and mouthfeel. Table 15 shows the % oil retention of the three colored *Spirulina* protein fractions.

Table 15: Oil absorption capacity of the three Spirulina protein fractions

Spirulina protein fraction	Oil absorption capacity g of oil per 100g of powdered protein fraction
BSSP	342,70 ± 1,60a
GSSP	333,38 ± 1,67b
TSSP	266,76 ± 4,48c

All values given are means of three repetitions ($\bar{x}$ ± SD) (SD: Standard deviation). Values with same subscript letters within the same column are not significantly different (P > 0.05).

A significant difference ($p < 0.05$) in oil absorption capacities of the three *Spirulina* protein fractions is observed with the BSSP showing a higher oil absorption capacity of 342.7g of oil/100g protein followed by the GSSP (333.38g of oil/ 100g of protein). The TSSP on the other hand shows the lower oil absorption capacity (266.76g of oil/100g of protein). This later value of TSSP obtained is close to that reported by Benelhadj et al. (2016) on the *Spirulina* proteins obtained without any fractionation.

This result shows that the fractionation of the soluble *Spirulina* proteins into its individual green and blue fractions improves its ability to absorb oil to values greater than that reported on soy protein isolates (119-154%) (Kinsella, 1979).

2.3 Foaming capacity and stability

Proteins ability to form foams is a property contributing to texture of foods processed from colloidal mixtures. This property is essentially due to their ability to reduce surface tension. Air bubble distribution within foam is clearly revealed on the texture of the processed solid food. The more the air bubble size distribution is regular, the uniform is the texture of the final food. Body and smoothness of food foams is brought about by volatilization of flavors through air bubbles, and palatability of the processed food is improved (Zayas, 1997). Small bubbles will impart a smooth texture to the food.

Measurements of stability and capacity of foams produced from 0.5% (w/w) aqueous solutions of the three *Spirulina* soluble proteins are presented in Figure 28 and Table 16. For this concentration (0.5% (w/w)), the higher foam capacity is obtained at the native pH (~ pH 6.5) that decreases with decreasing p H. The BSSP presents the best foaming

capacity at its native pH (pH 6.42) follow by the TSSP and then the GSSP with the least foaming capacity.

The foam stability on the other hand is revealed to be the best at pH 3 and for GSSP fraction. In other words, while the GSSP fraction presents the worst foaming capacity among the fractions, its ability to form a relatively stable foams is revealed at the tested pH levels. At pH 3, at least 10% of the foam persists for more than ten hours later. Greater volumes of foam with less stability are formed at native pH for all the fractions.

We didn't consider intrinsic factors such surface hydrophobicity, surface flexibility of protein molecules in our comparison of the colored *Spirulina* soluble proteins as foaming agents. This is because of the similarities of the proteins contained in the different fractions. They have a common source, and as we stated early, their difference in color is a matter of proportion of color agents or protein-pigment complexes. Their differences in foaming behavior may be principally linked to the environment of the proteins such as lipid content and differences in responses to pH changes.

A relatively more stable foam is also obtained with the GSSP solution at pH 5 as compared to the other fractions at this pH value. This can be attributed to the fact that precipitation of this fraction (Figure 6B) is obtained at a pH close to this value (pH 4.5) while the BSSP fraction is separated as supernatant.

The high foam stability observed at pH 3 for all the fractions can be attributed to the closeness of this pH to the isoelectric point of the fractions. At this pH, electrostatic repulsion is minimal, protein molecules are tightly packed at interfaces, and more viscous films are formed around the imprisoned air. Zayas (1997) reported that the surface elasticity of protein films which is critical for foam stability was at a maximum near the isoelectric point.

Table 16: Foaming capacity of 0.5% (w/w) colored Spirulina protein fraction solution

Protein fraction	Foaming capacity mL foam/mL liquid
GSSP pH 3	1.57±0.06a
GSSP pH 5	1.65±0.03b
GSSP native	1.7±0.00c
TSSP pH 3	1.39±0.01d
TSSP pH 5	1.62±0.03ab
TSSP native	1.77±0.03e
BSSP pH 3	1.40±0.03d
BSSP pH 5	1.69±0.08c
BSSP native	1.79±0.05e

All values given are means of three repetitions ($\bar{x} \pm$ SD) (SD: Standard deviation). Values with same subscript letters within the same column are not significantly different ($P > 0.05$).

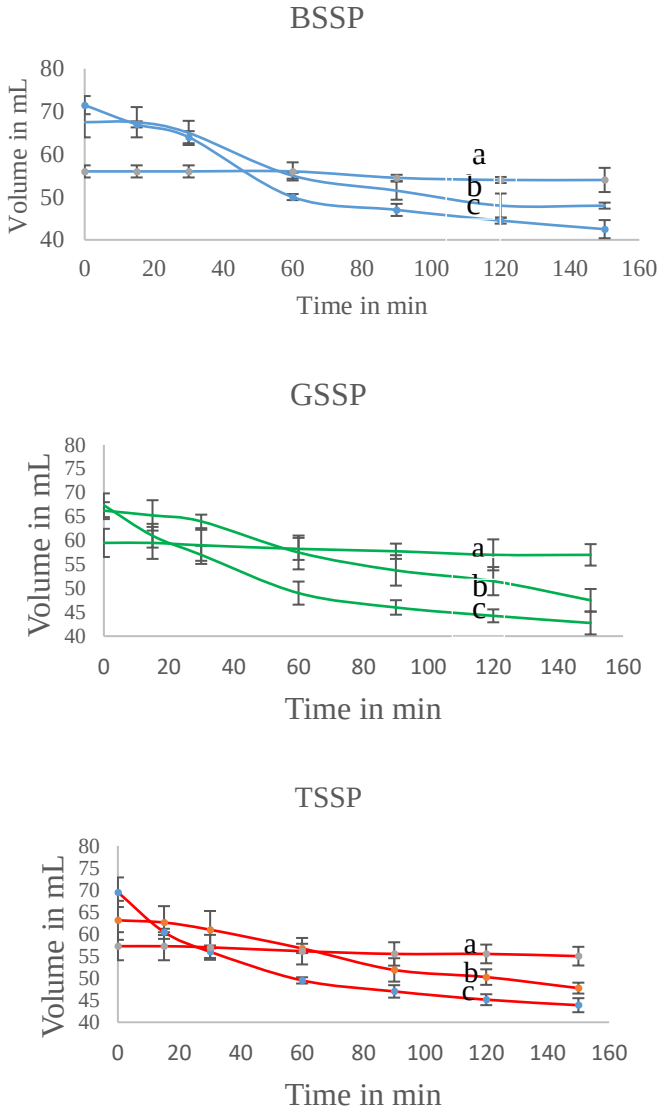


Figure 28: Foam stability of 0.5% (w/w) solutions of colored *Spirulina* protein fractions

a: pH 3; b: pH 5; c: pH 5

2.4 Emulsifying capacity

As foaming properties, protein molecules are able to enhance an emulsion stability due to: (1) their propensity to adsorb to interfaces, thereby increasing its pressure, (2) their ability to form a rigid viscoelastic film at interface (3), and to bring about an electrostatic

stabilization (Dalgleish, 1997). Interfacial tension reduction (or pressure increase) by colored *Spirulina* protein fractions is proven in previous chapters. All fractions are able to reduce interfacial tension therefore expected to act as emulsifying agents in foods emulsions. They can be used together with other food emulsifying agents to play a dual role i.e. improving the nutritional quality of the food and the stability of the food emulsion.

The emulsion capacities of the *Spirulina* protein fractions in terms of grams of emulsified oil per milligrams of protein in the aqueous solution as defined by Vuillemard *et al*. (1990) are presented in Figure 29. At 0.05% (w/w) a higher emulsion capacity is obtained (1.02 to1.31g oil/mg protein), and this value decreases with increasing concentration.

However, the quantity of emulsified oil increases with increase in the protein concentration. Larger oil quantities are emulsified by the BSSP fraction compared to the other fractions at 0.1% and 0.05% (w/w) concentration. At 0.3% there is no significant difference in emulsified oil quantity ($p < 0.05$) between the BSSP and the total fraction. The GSSP is able to emulsify lesser quantities of sun flower oil at all tested concentrations.

Better emulsion capacities (Figure 29) are achieved by the BSSP fraction, an observation that could be attributed its high solubility and interfacial properties revealed in the previous experiments. Thermodynamically, emulsion formation regardless of the emulsification method used, is favored when there are more solubilized protein molecules that would form viscoelastic films around the fat globules dispersed in the continuous phase (O/W emulsion).

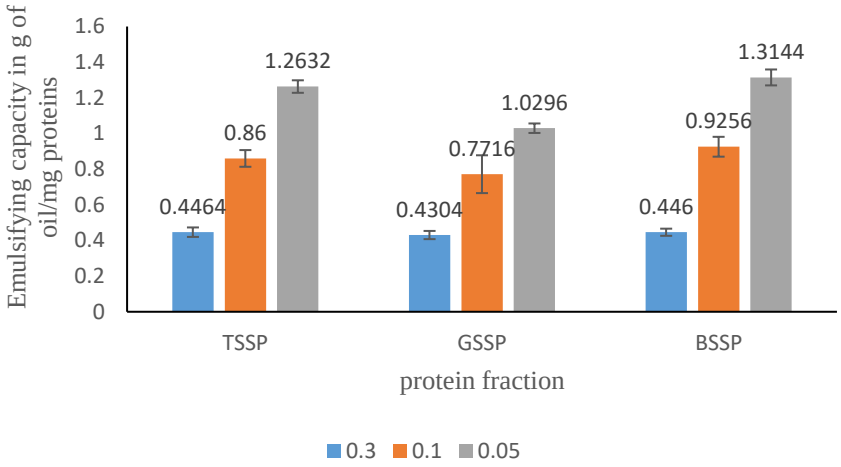


Figure 29: Emulsifying capacity (EC) of Spirulina protein fractions at different concentrations (0.05%; 0.1%; 0.3%) determined by phase inversion detection on progressive addition of sunflower oil to 2 5 mL Spirulina protein suspension

2.5 Emulsion stability

Like foams, emulsions are unstable colloidal systems due to their high entropy. In an oil /water emulsion, fat globules suspended within the aqueous phase have the tendency to coalesce due to the thermodynamically unstable conditions (Damodaran, 2005). In the absence of a stabilizer, an emulsion will quickly lead to two separate phases i.e. the aqueous phase at the bottom of the container and the oil phase at the top. It is in an objective to reduce phase separation rate and to ease the emulsification process that proteins are added to foods alone or supplementing other emulsifiers (Tadros, 2013). *Spirulina* colored soluble proteins can play many roles when added to such food systems. They can improve emulsion stability and facilitate emulsification as a result of interfacial tension reduction, they can increase the nutritional value of the food, and then can change the color of the processed food. The later property is not always desired for some foods known with specific desired colors.

The stability of the emulsions containing *Spirulina* protein fractions at different concentrations and at different pH levels is evaluated using light backscattering of freshly prepared emulsions under natural conditions. Migration of the oil droplets is therefore monitored via a light backscattering record (Figure 30). Data records are made punctually at 0, 15, 30, 60, 90, 120 minutes, up to 25 hours to monitor the creaming process.

Graphs in the Figure 30 show model backscattering measurements of the emulsions prepared from sunflower oil and the aqueous solutions of the total *Spirulina* protein fractions at pH 3 and pH 5 levels. The backscattering profile of the emulsions at native pH (~6.5) and pH 5 showed two phenomena occurring at two height sections of the Turbiscan tube. From the bottom to about 40 mm a clarification occurs, and from 40 mm to 55 mm the emulsion is creaming at all the two pH levels. The isobestic point separates the two sections. Meunier and Mengual (1996) reported that at this point, destabilization of emulsions occurs by both coalescence and creaming processes.

At pH 3, the destabilization process starts right from the bottom and covers almost all the height of the emulsion in the test tube (Figure 30). The behavior of the other two fractions (BSSP and GSSP) emulsions follows the same trend with minor individual differences. This behavior is later confirmed by the emulsion microscopy (Figure 32) that shows larger fat globules resulting from coalescing smaller globules at pH 3.

The least emulsifying capacity of the proteins at pH 3 is revealed here by the phase separation process occurring all along the height of the emulsion in the test tube. This is due to the closeness of this pH to the isoelectric point of the proteins at which the net charge of the protein molecules is zero and no electrostatic repulsion between protein molecules can occur, protein-protein interactions are favored and the propensity of protein molecules to aggregate between them reduce their chances to adsorb to interface, thereby maintaining the emulsion network unstable leading to a rapid phase separation. To the other known phenomenon commonly identified as those destabilizing emulsions, the electrostatic neutrality of proteins at their isoelectric pH should also be considered. In fact, proteins aggregation or precipitation at their isoelectric pH does not only hinder the adsorption process at interfaces, but can also create a steric effect that favors

coalescence and creaming. As such, the effect of pH change on the emulsion behavior is more apparent than that on the interfacial tension at this pH.

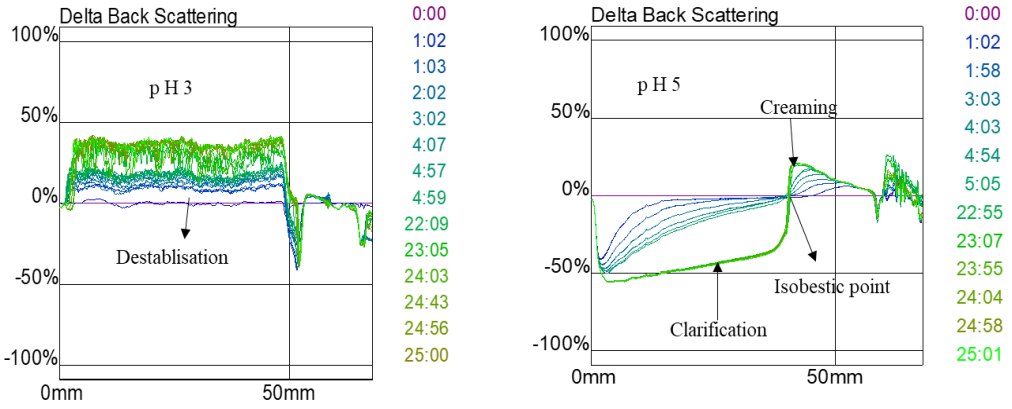


Figure 30: Backscattering profile of emulsion stabilized with 0.3% (w/w) TSSP at different pH levels obtained with TURBISCAN

Figure 31 presents a comparative creaming kinetics of the emulsions prepared with the three *Spirulina* colored protein fractions solutions (0.3% (w/w) at native pH) and sunflower oil. We evaluate the emulsion stability based on the creaming kinetics which is in turn directly related to the backscattering profile. It is in fact the comparison of the backscattering brought about by emulsions prepared from the *Spirulina* protein fractions at the maximum creaming point of the emulsion filled in the Turbiscan tubes (~45 mm). The higher the backscattering, the rapid is the creaming process. Creaming is therefore an indication of an emulsion instability. The BSSP shows the lower rate of creaming, closely followed by the TSSP, and then the GSSP. However, the difference between the creaming behaviors of emulsions from the BSSP and the TSSP is not significant ($p>0.05$).

Despite the similarities in terms of interfacial tensions reported previously, the functional behaviors in general, and particularly the ability of the protein fractions to stabilize emulsions, may not give hundred per cent expected performances. The physical properties of protein molecules expected to show a particular function in a colloidal system could be influenced by other parameters within their environment, thereby hindering or favoring their implementation.

As reported early concerning the emulsion capacity of the fractions at different pH levels, the closeness of this pH value to the isoelectric point of the *Spirulina* protein limits the stability of the emulsions due to the propensity of the protein molecules to precipitate instead of forming a protective film around the fat particles. The system is thermodynamically unstable, and the phase separation is visible once the mixing process stops. Dark spots representing protein precipitates are apparent on the image (Figure 32). The films surrounding the fat globule are rather attracted by neighboring proteins, a phenomenon that leads to the film rupture. Coalescence of fat globules is therefore favored, and a complete phase separation does not last long.

Spirulina proteins at pH 3 are poor stabilizers, despite their behavior at interfaces (oil/water).They adsorb to interface to form monolayer films whose role in the emulsion system is to keep the fat particles apart as long as possible. The stability of an emulsion depends on the density difference between the continuous phase and the dispersed phase (Tadros, 2013). Also, the affinity of the surface active agent (protein in the present case) to the dispersed phase changes. The difference in the steric effect of the dispersed phase should also be taken into account when considering the pressure exerted on the monolayer films separating the different fat globules) of the emulsion.

McClements (1999) reported that Protein-stabilized emulsions are particularly sensitive to pH and ionic strength effects and they are likely to flocculate at pH values close to the isoelectric point of the adsorbed proteins and when the ionic strength exceeds a particular level, because the electrostatic repulsion between the droplets is then no longer sufficiently strong to overcome the various attractive interactions.

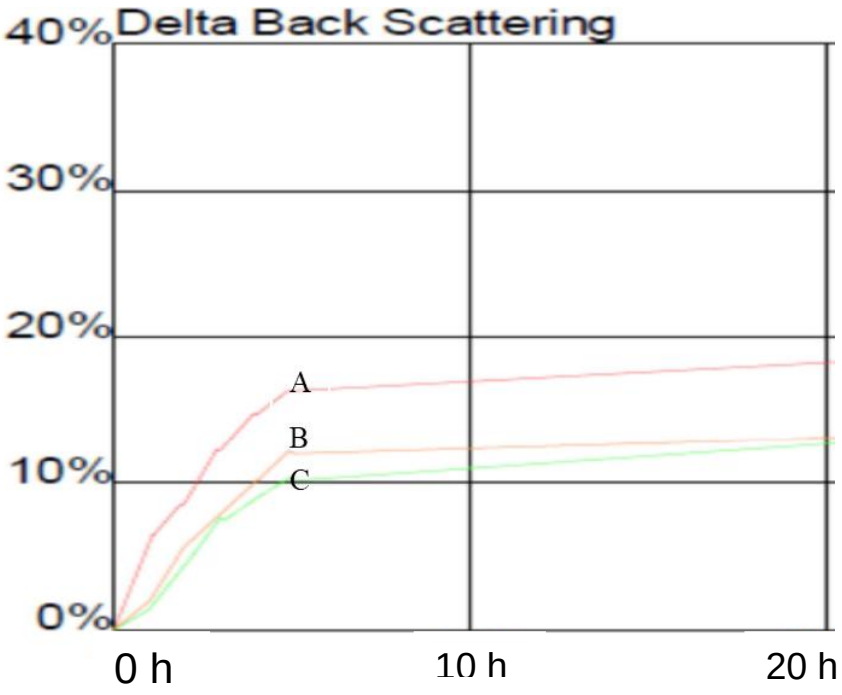


Figure 31: Creaming kinetics of emulsions made from Spirulina protein fractions solutions (0.3% (w/w)) and sun flower oil and colored (10 oil/40 protein solution), A: GSSP; B: TSSP and C: BSSP

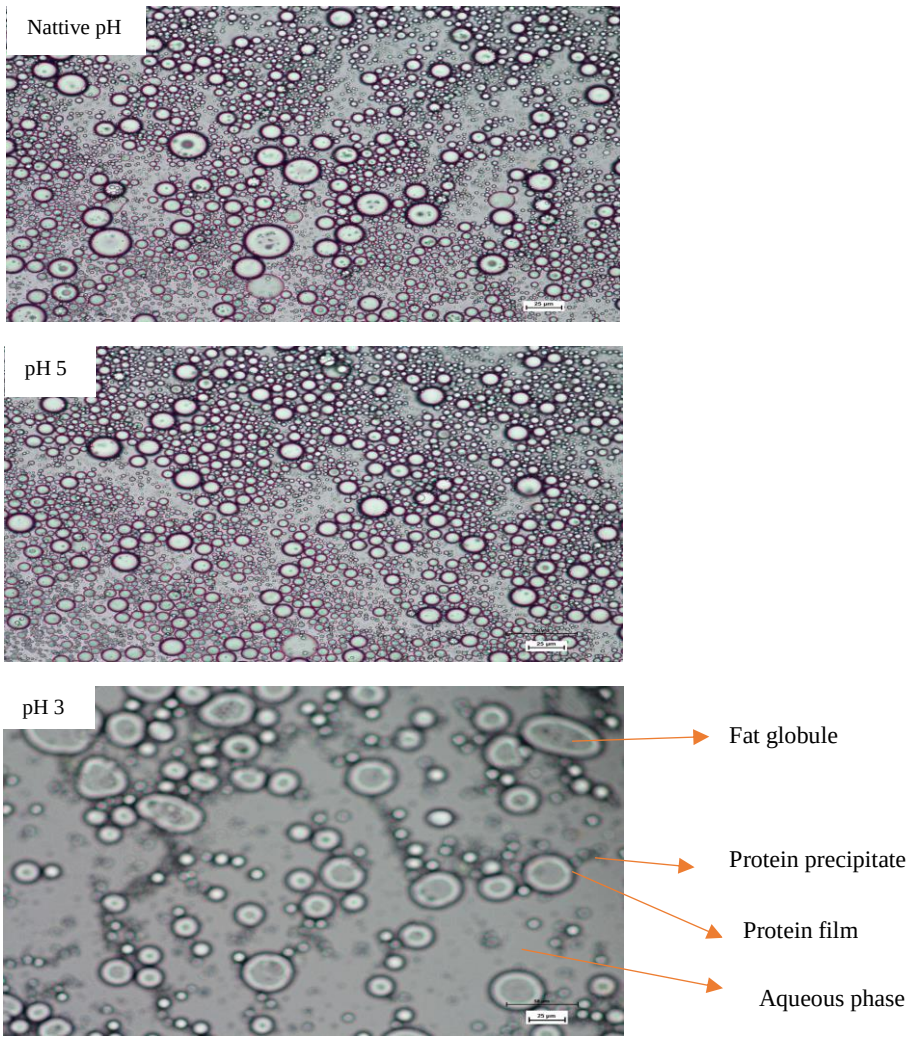


Figure 32: Microscopy of emulsions based on a mixture of aqueous solutions of the TSSP fraction (0.3% (w/w)) at different pH levels (native pH, pH 5, and pH 3) and sun flower oil 40:10

2.6 Microscopic observation of emulsion

Figure 32 shows microscopic pictures of the emulsions prepared from the total soluble *Spirulina* fraction solutions and sun flower oil (40:10) at different pH levels. It can be observed on the various pictures that smaller oil globules are obtained with the corresponding emulsion at pH 5. Pictures of emulsions at the native pH appear to be

closer to those obtained at pH 5. At pH 3 larger fat globules are observed for all the fractions.

As reported early concerning the emulsion capacity of the fractions at different pH levels, the closeness of this pH value to the isoelectric point of the *Spirulina* protein limits the stability of the emulsions due to the tendency of the protein molecules to precipitate instead of forming a protective film around the fat particles. The system is thermodynamically unstable, and the phase separation is visible once the mixing process stops. Dark spots representing protein precipitates are apparent on the image (Figure 32). The films surrounding the fat globules are rather attracted by neighboring film proteins, a phenomenon that leads to the film rupture. Coalescence of fat globules is therefore favored, and a complete phase separation does not last long.

Unlike the foam stability, the *Spirulina* proteins at pH 3 are poor emulsion stabilizers, despite the closeness of their behavior at interfaces (oil/water and air/water). In both cases they adsorb to interface/surface to form monolayer films whose role in the colloidal system (emulsion or foam) is to keep the particles separate as long as possible. This allow to suggest that the stability of a colloidal system depends on both the nature of the continuous phase and that of the dispersed phase. In fact, the affinity of the surface active agent (protein in the present case) to changes with the dispersed phase. The difference in the steric effect of the dispersed phase should also be taken into account when considering the pressure exerted on the monolayer films separating the different particles (air bubbles or fat globules) of the colloidal mixtures (foams and emulsions).

McClements (1999) reported that Protein-stabilized emulsions are particularly sensitive to pH and ionic strength effects and they are likely to flocculate at pH values close to the isoelectric point of the adsorbed proteins and when the ionic strength exceeds a particular level, because the electrostatic repulsion between the droplets is then no longer sufficiently strong to overcome the various attractive interactions.

3. Conclusion

A prediction of the functional properties of the three colored *Spirulina* protein fractions based on their physicochemical properties investigated in previous parts of the work can be reliable for most cases such as the foam stability at pH 3. Foams are revealed to stay longer at this pH. The fractions solubility increases with pH right from the isoelectric point for all the tested pH values. Emulsions at pH 3 are very susceptible destabilizing phenomena such as coalescence, flocculation, and creaming. The functional behaviors of the three fractions follow the same trends. However, the BSSP fraction shows a better emulsifying effect.

For a better appreciation of the functional abilities of the colored *Spirulina* protein fractions, a comparative evaluation with the conventional proteins may be needed.

Chapter 6

Overall discussions

1. Introduction

The amphiphilic nature of proteins allows them to act, not only as nutrients in a formulated foods, but also as functional ingredients that could affect the processed food's organoleptic properties. These properties are the fingerprints of the functional behaviors linked to proteins surface activities. For long, conventional proteins are being used as emulsifiers in foods prepared from foams and emulsions (Kralova and Sjöblom, 2009). Today, the "protein gap" due to the increasing global population and the cost of the conventional protein concentrates, the new research route is oriented towards exploration of new conventional protein substitutes or supplements. Microalga are potential contenders that can fulfill this goal.

The relationship between basic physicochemical properties of proteins and their functional behaviors are reported by many researchers (Dargon-Scavinier, 1986; Wouters *et al*., 2016). Properties such as surface/interfacial activities, solubility and viscoelasticity of monolayer films formed by surface adsorption of these proteins are the physicochemical characteristics usually evaluated together with their related functional behaviors. Also, intrinsic structural properties of the protein molecules are investigated, being that their effect on the protein functionalities in food systems is not negligible (Utsumi *et al*., 2002). These include the protein isoelectric pH, availability of hydrophilic patches on the protein molecular structure etc.

This study is a non-exhaustive approach intended to relate some functional properties of the colored *Spirulina* protein fractions to some of their physicochemical properties including: isoelectric potential, interfacial tension, viscoelasticity of protein monolayer films.

Through this chapter we are therefore aiming at relating the experimental observations on the fractions' physicochemical properties reported in previous chapters to their tested functional behaviors. This will enable the food technologist to operate a rational choice of a protein fraction for its proper application in food colloids.

2. Foaming properties and related physicochemical properties

Foams are all colloidal systems in which air is trapped within the aqueous phase that forms thin film layers around the gas phase. The films forming the gas cavities are made up of adsorbed protein molecules and water. This statement shows that the interfacial protein adsorption process is the fundamental step in creating the foam. We also stated in the previous work that surface tension decay can be evaluated through adsorption kinetics. In other words, there is an evidence that shows the relationship between surface tension and the properties of interfacial films surrounding the gas bubbles within a foam system. Indeed, there are a lot literature demonstrating the relationship between surface tension and foaming (Wouters *et al*., 2016, Patino *et al*., 2007a, Patino *et al*., 2007b, Patino *et al*., 2008).

Foams destabilization is therefore brought about by all processes that can break the thin film. This can be due to the ease of departure of water and/or proteins that constitute this film. Even without any external effect, various phenomenon can intervene in the foam destabilization that could be separated into two groups: Those directly induced by the water departure from the film enrobing the gas phase (drainage due to gravity, electrostatic forces etc), and those linked to the adsorbed protein molecules (desorption, aggregation, repulsion, competition).

Colored soluble *Spirulina* protein fractions are investigated on their ability to reduce surface tension at different concentrations and pH levels. For related functional properties the foaming behavior is investigated to compare the fractions and also the effect of pH is tested. Like the surface tension, fairly better foaming capacity results are obtained with the BSSP fraction.

Considering the pH, best foaming capacity is revealed at the native pH of the fractions solutions found to be around 6.5 for all the three fractions. At pH 3, foams obtained have the least volume (poor foaming capacity), but last for longer time (even after 24 hours 5% foam remained on the top of the liquid). It is the same trend observed with ability of the protein fractions to reduce surface tension. The higher surface tension reduction rate (surface tension decay) is obtained at the native pH and pH 5. While at pH 3, the fractions appear to be less efficient in reducing surface tension. Functional properties are sometimes qualified as the macroscopic manifestation of the basic physicochemical characteristics of the proteins (Blecker, 1998). Many researchers reported a positive correlation between surface tension reduction ability of a protein and its foaming capacity (German *et al*., 1985; Dagorn-Scaviner, 1986; Karamoko *et al.*, 2013).

As far as the relationship between foaming behaviors and rheological properties is concerned, an expected positive correlation is not revealed. Within the tested concentration range, viscoelasticity of surface films for all the fractions solutions is rather reduced at these pH levels (native, pH5) and higher protein concentrations (within the tested concentration range) expected to bring about greater reduction in surface tension.

Unlike foaming capacity, foam stability is revealed to be higher around isoelectric pH (pH 3) at which fractions are less surface active. This could be explained by the formation of thicker “solid films” around the gas bubbles that are able to maintain the foam structure even when the gas has escaped. We previously stated that at the isoelectric pH, protein-protein interactions is favored compared to protein-water, due to the absence of electrostatic repulsion between protein molecules. A process leading to expulsion of most water molecules from the protein network and aggregation of protein molecules to form a precipitate. Thus, the films encapsulating gas within the foam network are poor in water (solid-like). These films may be poor in terms of airtightness ability (less flexible) but strong enough to withstand stresses (gravitational, steric etc.) even when the gas escapes from the initial bubbles.

3. Emulsion behavior and related physicochemical properties

Emulsions are made up of fat globules suspended in an aqueous continuous phase or water droplets within an oil continuous phase (O/W or W/O respectively). More complex emulsions are made up of more than two phases e.g. O/W/O. These systems are subject to destabilization due their thermodynamic properties. The thermodynamic stability of a system will be acquired only when its free energy is at its minimum level. The use of emulsifiers is one of the methods often employed in food and chemical industries to keep emulsion properties stable for a reasonable period of time. In the case of an O/W emulsion, destabilization will lead to a complete separation between the aqueous and the oil phases (the aqueous phase at the bottom). Food systems are expected to be ideally stable to guaranty its desired organoleptic and sensory characteristics during distribution, storage, and consumption.

For an emulsion to be stable, the fat globules suspended in the aqueous phase (oil in water emulsion) should be maintained separate. In fact, due to their hydrophobicity, fat globules have the propensity to come close to each other, combine and form larger droplet; a phenomenon known as coalescence. These fat globules can either form aggregates without combining their contents, and this is known as flocculation. Also, under the impulsion of the density difference between the oil and the aqueous phase, the oil droplets move towards the surface resulting in the creaming process. As such, the system inevitably results to a complete separation of the two phases.

Proteins in food formulations are known to give more stable emulsions compared to surfactant-based emulsions, due to their interfacial organization (Mackie *et al*., 2007). This superior function is attributed to their slower conformational changes at the interface, which lead to a lower density network, in comparison to surfactant-based emulsions (Mackie *et al*., 2007).

Physiochemical properties of proteins are the key factors affecting their emulsifying abilities (Bueno, *et al*., 2009; Moure, *et al*., 2006). At the interface, proteins direct their surface hydrophobic amino acids towards the oil phase and hydrophilic amino acids within the aqueous phase thereby forming the protecting film around the fat globule (or water droplet in the case of W/O emulsion) (Walstra, 2003). The role of proteins in the O/W emulsion is therefore to enrobe the fat globules, owing to their amphiphilic nature, thereby reducing coalescence phenomenon. The adsorbed protein film (made up of protein and water molecules) limits the phase separation of the system by creating a physical barrier between the fat globules (limitation of coalescence), and also by reducing the density difference (reducing rate of flocculation). The monolayer film should therefore be strong enough to perform this task.

In the present work, the physicochemical properties including the electrokinetic potential and adsorption kinetics of the *Spirulina* protein fractions are evaluated together with the monolayer film properties.

Similarly to the foam capacity, emulsion properties are revealed to be better at the pH values (native and pH 5) far away to the isoelectric pH. Emulsions at these pH values present fat globules of smaller sizes compared to emulsions prepared with protein

solutions at pH 3 (close to the isoelectric pH). Unlike foam stability, emulsion at pH 3 is highly unstable leading to a complete phase separation within shorter laps of time. The absence of electrostatic repulsion between protein molecules due to isoelectric pH rather tends to create spots of protein precipitate resulting from aggregation of neighboring film protein molecules. Fat globules coalescence is therefore accelerated by this protein precipitation process. McClements, (2005) reported that pH conditions close to the protein's isoelectric point (or high ionic strength) fat globules flocculation/aggregation dominates and can lead to coalescence and instability.

Protein adsorption to interface is what makes it to behave as emulsifier. Results obtained from the study of the adsorption kinetics at model liquid/liquid interface (interfacial tension reduction) are in accordance with the emulsion properties. Adsorption is revealed to be better at pH 5 of the fractions solutions compared that pH 3, although the difference is not as pronounced as that observed between the emulsions at these pH levels ((native pH, pH 5)/ pH 3). This can be explained by the fact that interfacial tension reduction by adsorbed protein molecules is not the only factor affecting emulsion properties. Protein molecules may adsorb to interface, cause interfacial tension reduction, but if the film formed is not strong enough to support the destabilization stresses of the environment, the system could not be stable enough. Also, the interfacial tension measurement system is quite different from an emulsion in which there are many neighboring encapsulated fat globules that may influence each other, unlike the measuring system with a single drop expelled.

With the Langmuir film balance, the compression isotherms of the aqueous solutions of the colored *Spirulina* protein fractions are investigated. This allows for a comparison between the three fractions. Out of this investigation, the BSSP fraction appears to present the best behaviors compared to the others. The same trend is followed by the evaluated emulsion properties (emulsion capacity and stability) although isotherms are evaluated at air/water interface.

On the other hand the measurement of the viscoelastic moduli via the automated drop tensiometer rather showed less viscoelastic moduli at pH and concentrations expected to give greater values of viscoelastic properties. However, an elastic and viscous monolayer film is required to maintain emulsion stability by protecting fat globules from coalescence and flocculation. Nevertheless, Lucassen-Reynders (1993) reported a drop in the viscoelasticity of a surfactant monolayer above a certain concentration. The same interfacial behavior was reported by Graham (1976) on BSA (Bovine serum albumin)**.** He reported that the increase in interfacial protein concentration and formation of multilayers could bring about a decrease in viscosity and elasticity to limiting lower values.

It is clear that, for an emulsion to be stable, a stronger film is needed to enrobe the fat droplets in other to protect them from coalescence and flocculation; processes that will end up giving two distinct phases from the emulsion. It should be noted that the electrostatic effect on emulsion stability is remarquably revealed by the highly destabilized emulsions at pH 3 (close to isoelectric pH). Results from the Langumuir film balance are in accordance with the expected behaviors of the three *Spirulina* fractions in emulsions when considering the investigated properties of the monolayer

film of adsorbed proteins. However, it should be kept in mind that in most applications, emulsion properties are not solely linked to the proteins used but rather to the whole emulsion system, the equipment, and method used to produce this emulsion (Tornberg and Hermansson, 1977).

4. Solubility and physicochemical properties of the fractions

Solubility is the starting point of almost all the functional properties linked to the surface activities of proteins. For a protein molecule to easily adsorb to interface/surface, the first step is to break free from its original substrate, so that it can conveniently orient its hydrophobic patches and hydrophilic tails thereby reducing interfacial tension. In other words, the amphiphilic property of the protein molecule should be made available for intermolecular interactions with solvent.

Solubility here is defined as the ability of the protein to dissolve in an aqueous phase. This occurs when the protein-protein interactions are overcome by the water-protein interactions. The protein molecule is rather surrounded by water molecules when solubilized. The presence of hydrophobic patches on the structure of the protein molecule allow it to move to the interface where it can orient these hydrophobic patches away from the aqueous subphase. It is therefore this electrostatic interaction between protein and water molecules that, at the same time allow for protein solubilization and protein adsorption to interface (or surface).

Considering the above statement, the relationship between protein solubility and surface tension is obvious. Surface tension reduction is nothing but, the replacement of water molecules at the liquid water surface by other molecules. The more the protein is soluble in water, the easy its ability to diffuse through the subphase and adsorb to the interface (or surface).

Under certain conditions (pH, concentration etc.) protein solubility is affected and all other properties of the protein linked to interfacial adsorption are affected. At pH levels close to the isoelectric pH, protein solubility decreases surface activity of the protein is also affected. Results of the effect of pH surface and interfacial tension reveal that at pH 3 all the fractions poorly reduce surface or interfacial tension. This means that the effect of pH on the other functional behaviors of the colored *Spirulina* proteins can be considered as an indirect effect of solubility. Thus solubility can be considered both as a physicochemical property and a functional property.

Protein solubility is directly affected by the electrokinetic potential of the protein molecules. The electrokinetic potential and thus the pH is among the intrinsic factors that affect protein solubility (Riès-kautt and Ducruix, 1997). Being that solubility is just the direct functional expression of the protein-solvent (water molecule) interaction which is essentially an electrostatic affinity, the more this interaction is favored, the more soluble is the protein. At the isoelectric pH and other closer pH values, the electrokinetic potential of the protein is close to zero (equals to zero at isoelectric pH) and the solubility is at its minimum value. When the net protein charge within the solution is zero, there is no repulsion between protein molecules, protein precipitation occurs thereby reducing

solubility. On the other hand, at pH values far away from the isoelectric point, the high electrokinetic potential on protein molecules brings about an electrostatic repulsion between protein molecules thereby favoring proteins interaction with water molecules. Under these conditions, solvation of protein molecules is rather favored. Definitely, *Spirulina* proteins are less soluble when their net charge is close to zero (pH 2.5-3.5) and their solubility increases with increase pH level, right from the isoelectic pH. This solubility trend is also reported with other proteins such as soya proteins (Malhotra and Coupland, 2004). This is the property usually exploited to separate water soluble proteins from their original substrates.

5. Overall conclusion and perspectives

This study was aiming at evaluating the potentials of microalgae in general and particularly *Spirulina platensis* as a source of protein and other valuable nutrients. Its productivity is revealed to be high and its ability to develop under harsh climatic conditions is reported. The use of these microalgae in human consumption experience an historical background but was limited to its production areas. Its valorization as a food or food ingredient needs a lot of sacrifices both from researchers and public authorities.

Spirulina protein fractions are extracted and separated into two different color fractions in addition to the total soluble fraction. With the extraction conditions used, most of the initial biomass is left behind together with the protein fraction not water-soluble under the extraction conditions. This means that the search for an alternative to valorize the byproduct is unavoidable. Also, economic considerations for the industrial implementation of these experimental results should be taken into account. It is however known that *Spirulina* biomass is used in feeding fish and other animals. As such, valorization of the byproduct in feed or feed formulations should be subjected to investigations. High protein (74-82%) content fractions are obtained with different colors (green and blue).

As researchers, our task is to investigate on the microalgae on various aspects including: - nutrients content – digestibility- toxicity - functionalities…to allow the public authorities to consider microalgae, and particularly *Spirulina* as an alternative food for the growing global population.

Nutrient functionalities are some of the qualities that attracts food technologist who always need to formulate new products with improved nutritional and sensory qualities for additional reasons to convince consumers. Food industries are in fact one of the important partners of the public authority in disseminating information about a new food to the population. But the first step forward is the evaluation of the nutrient content and the functionalities of the “new” food source.

Spirulina platensis is a good source of protein, which has higher protein content than that reported in some foods considered as good sources of proteins such as meat, egg and meat. Almost 25% of its proteins are soluble in an alkaline solution and can be separated base on this property. The proteins are linked to pigments and two fractions of different colors (blue and green) can be obtained out of the dark green color soluble

proteins. The two fractions present completely different colors but most of their physicochemical properties appear very close despite their color difference. All the fractions are able to bring about interfacial tension reduction to some level, depending on their initial concentration and pH. The higher the protein concentration, the lower the equilibrium interfacial/surface tension that could be reached.

A synthetic presentation of the relationships between the studied surface and functional properties of the three *Spirulina* protein fractions are presented in Table 17. This could allow for an easy choice of the ideal fraction and pH condition for the best functional and interfacial properties. The fractions show the best interfacial and functional properties at their native pH, and pH 5 except the "*very high (++++)"* foam stability observed at pH 3 for all the three fractions. Nevertheless, the BSSP fraction shows some demarkations in terms of emulsion stability, foam capacity and solubility.

Table 17: Synthesis of the relationship between the measured interfacial and functional properties

Fractions	pH	Surface tension reduction	Solubility	Foaming capacity	Foam stability	Emulsion capacity	Emulsion stability
TSSP	pH 3	++	+	++	++++	nd	+
	pH 5	++++	++++	+++	++	nd	+++
	Native pH	+++	+++	++++	++	+++	+++
BSSP	pH 3	++	+	++	++++	nd	+
	pH 5	++++	++++	+++	++	nd	+++
	Native pH	+++	+++	++++	++	++++	+++
GSSP	pH 3	++	+	++	++++	nd	+
	pH 5	++++	+++	++	++	nd	+++
	Native pH	+++	++	+++	++	+++	+++

+ + + + very high; + + + high; ++ average; + low; nd not determined

The BSSP fraction presents a striking high solubility to which the observed fairly improved emulsifying properties could be imputed. The poor emulsifying properties of

all the fractions at pH 3 could be partly due their poor performances as interfacial tension reduction agents, and mostly on the low solubility of the fractions at this pH (close to their isoelectric point). The creaming behavior of BSSP emulsions is close to that of the intermediate TSSP fraction. In conclusion, separation of the *Spirulina* proteins into different color fractions leads to fractions with improved properties. Indeed, the BSSP fraction presents improved emulsifying properties in addition to its high solubility. The green fraction is less efficient, and logically, the total fraction has an intermediate emulsifying behavior between its constitutive fractions.

In addition to the fundamental knowledge brought about by the study, this stands for the starting point to studies that could lead to the industrial implementation of the separation method presented. Protein fractions of different colors comparable to the protein concentrates currently used in food formulations are obtained. Despite its apparent feasibility stated elsewhere in our discussions, its implementation needs to take into account the various economic aspects thereon. Alternatives to the enrgy-consuming freeze-drying process should be investigated. The spray-drying method is usually preferred to freeze-drying in an industrial food processing to reduce production costs linked to energy consumption. Other aspect such as the *Spirulina* harvesting costs, and marketability of the coproducts should be evaluated for successful industrial implementation of these results. There is also a need for improving extraction efficiency by associating a simple alkaline solubilization of *Spirulina* proteins to other extraction methods such as sonication whose success was proven by Hedenskog & Hofsten (1970).

For a better appreciation of the fractions as surface active agents, and for their proper implementation in foams and emulsions, their comparison to conventional protein under the same experimental conditions will be highly instructive.

References

Abalde J, Berancour L, Torres E, *et al*., 1998.Purification and characterization of phycocyanin from the marine cyanobacterium *Synechococcus sp*. 109201. *Plant Science* 136(1): 109-120.

Abd El-Baky H H, El-Baroty G S, 2012. Characterization and bioactivity of phycocyanin isolated from *Spirulina* maxima grown under salt stress. *Food & Function* 3(4): 381-388.

Abomohra Abd El-Fatah, El-Sheekh M & Hanelt D., 2014. Pilot cultivation of the chlorophyte microalga *Scenedesmus obliquus* as a promising feedstock for biofuel. *Biomass Bioenergy*, 64, 237-244

Ahn H C, Juranic N, Markley J L, 2006. Three-dimensional structure of the water-insoluble protein crambin in dodecylphosphocholine micelles and its minimal solvent-exposed surface. *J. Am. Chem. Soc*. 128, 4398–4404.

Antelo F S, Anschau A, Costa J A, *et al*. 2010. Extraction and purification of C-phycocyanin from *Spirulina* platensis in conventional and integrated aqueous two-phase systems. *J Braz Chem Soc* 21(5): 921-926.

AOAC, 1990. Official methods of analyses. Washington DC: Association of Official Analytical Chemist.

AOACI (Association of Official Analytical Chemists International), 2000. Official Methods for Analysis. Fat in milk (modified Mojonnier method)-method 989.05; 33.2.26, ash of dried milk method 9330.30; 33.5.05.AOACI 17th edn, Gaithersburg.

Arad S & Richmond A, 2004. Industrial production of microalgal cell-mass and secondary products – species of high potential: *Porphyridium* sp. *In*: Richmond A. *Handbook of microalgal culture. Biotechnology and applied phycology*. Oxford, UK: Blackwell, 289-297.

Arntfield S D, and Murray E D, 1981. The Influence of Processing Parameters on Food Protein Functionality 1. Differential Scanning Calorimetry as an Indicator of Protein Denaturation. *Can. Inst. Food Sci. Technol J*., 4 (4), 289·294.

Asghari A K, Norton I, Mills T, Sadd P and Spyropoulos F, 2016. Interfacial and foaming characterization of mixed protein-starch particle systems for food-foam applications. *Food Hydrocolloids*, 53, 311-319. DOI: 10.1016/j.foodhyd.2015.09.007

Barbarino E & Lourenço S O, 2005. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. *J. Appl. Phycol.*, 17, 447-460.

Barka A, Amal B A, Frederic F and Blecker C, 2018. Physicochemical characterization of colored soluble protein fractions extracted from *Spirulina (Spirulina platensis*). *Food Science and Technology International* (accepted 12/06/2018).

Bashir S, Mian K S, Masood S B and Shahid M, 2016. Functional Properties and Amino acid Profile of *Spirulina* platensis Protein Isolates. *Pak. j. sci. ind. res. Ser. B: biol. sci*. 59(1) 12-19

Batista A P, Raymundo A, Sousa I, Empis J, Franco J M, 2006. Colored food emulsions – Implications of pigment addition on the rheological behavior and microstructure. *Food Biophysics* 1: 216–227.

Beaven G H, and Holiday, E R, 1952. Ultraviolet Absorption Spectra of Proteins and Amino Acids Adv. *Protein Chem*., 7, 319-387.

Becker E W, 1994. *Microalgae: biotechnology and microbiology*. Cambridge, UK: Cambridge University Press.

Becker E W, 2004. Microalgae in human and animal nutrition. *In*: Richmond A. *Handbook of microalgal culture. Biotechnology and applied phycology.*Oxford, UK: Blackwell, 312-351.

Becker E W, 2007. Micro-algae as a source of protein. *Biotechnol. Adv*., 25, 207-210.

Bekasova O D, Muslimov I A, Krasnovskii A A, 1984. Fractionation of phycobilisomes from the blue-green alga Nostoc muscorum. *Molekuliarnaia Biologiia* 18(1): 262-271.

Ben-Amotz A, 2004. Industrial production of microalgal cell-mass and secondary products. Major industrial species: *Dunaliella*. *In*: Richmond A. *Handbook of microalgal culture. Biotechnology and applied phycology*. Oxford, UK: Blackwell, 273-280.

Benavides J, Rito-Palomares M, 2005. Potential aqueous two-phase processes for the primary recovery of colored protein from microbial origin. *Engineering in Life Sciences* 5(3), 259-266.

Benelhadj S, Gharsallaou A, Degraeve P, Attia H, Ghorbel D, 2016. Effect of pH on the functional properties of *Arthrospira* (*Spirulina*) *platensis* protein isolate. *Food Chemistry*, 194, 1056-1063.

Benjamins J, Cagna A, & Lucassen-Reynders E H, 1996. Viscoelastic properties of triacylglycerol/water interfaces covered by proteins. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *114*, 245-254.

Berenice F-R, Hernández-Juárez J, Pedraza-Chaverri J, 2014. Nutraceutical properties of phycocyanin. *Journal of functional foods*, 11,375–392.

Bermejo-Bescós P, Piñero-Estrada E, Villar del Fresno Á M, 2008. Neuroprotection by *Spirulina* platensis protean extract and phycocyanin against iron-induced toxicity in SH-SY5Y neuroblastoma cells. *Toxicol In Vitro* 22(6), 1496-1502.

Bhat V B & Madyastha K M, 2001. Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from *Spirulina platensis*: protection against oxidative damage to DNA. *Biochem. Biophys. Res. Commun*., 285, 262-266.

Blecker C, 1998. Etude de la modification des propriétés fonctionnelles du lactoserum par hydrolyse enzymatique de la matière grasse résiduelle. *Thèse de Doctorat soutenue à la faculté Universitaire de Gembloux, P 239.*

Blecker C, Cerne V, Paquot M, Lognay G, Sensidoni, A, 1997. Modification of the interfacial properties of whey by enzymatic hydrolysis of the residual fat fraction. In: Lorient, D, Dickinson, E (eds) Food Macromolecules and Colloids, Cambridge: Royal Society of Chemistry, pp. 85–89.

Blecker C, Razafindralambo H, Paquot M, Cerne V, Lognay G, Severin M, 1995a. Two forms of lipase from Mucor michei exhibit a different behavior at the air-water interface. *Colloids Surfaces*. 3, 271-279.

Bornet É & Flahault C (1886 '1888'). Revision des Nostocacées hétérocystées contenues dans les principaux herbiers de France (quatrième et dernier fragment). *Annales des Sciences Naturelles, Botanique, Septième Série* 7: 177-262.

Boutaric, M. A., and Berthier, P. 1939. "Act Decay of the Surface Tension of Solutions in Function of Time."*Journal of Physical Chemistry* 36 (1): 1-4. (in French)

Boyd J V, Mitchell J R, Irons I, Musselwhite P R., Sherman P, 1973. The mechanical properties of milk protein films spread at air-water interface. *J. Colloid interface Sci*., 45, 478-486.

Brown M R, 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculutre, *J.Exp.Mar. Biol. Ecol.*, 145, 79–99.

Bueno A S, Pereira C M, Menegassi B, Arêas J A G, & Castro I A, 2009. Effect of extrusion on the emulsifying properties of soybean proteins and pectin mixtures modelled by response surface methodology. *Journal of Food Engineering*, 90, 504–510.

Bull H, 1945. Monolayers of β-lacto globulin on concentrated salt solution. *Journal of the American Chemical Society*, 67 (1), 8-10.

Carmichael W W, Drapeau C & Anserson D M, 2000. Harvesting of Aphanizomenon flos-aquae Ralfs ex Born. & Flah. var. flos-aquae (cyanobacteria) from Klamath lake for human dietary use. *J. Appl. Phycol*., 12, 585–95.

Chaiklahan R, *et al*., 2011. Separation and purification of phycocyanin from *Spirulina sp*. using a membrane process. *Bioresour Technol.,* 102(14): 7159-7164.

Chakdar H, Saha S, Pabbi S, 2014. Chromatographic and spectroscopic characterization of phycocyanin and its subunits purified from Anabaena variabilis CCC421. *Applied Biochemistry and Microbiology*, 50(1), 62-68.

Chamorro-Cevallos G, *et al*., 2016. Methods for Extraction, Isolation and Purification of C-phycocyanin: 50 years of Research in Review. *Int J Food Nutr Sci* 3(1): 275-284.

Chen X-J, Wu M-J, Jiang Y, Yi Y, Yong-Bin Y, 2015. Dunaliella salina Hsp90 is halotolerant. *International Journal of Biological Macromolecule*, 75, 418-425.

Chen F & Zhang Y, 1997. High cell density mixotrophic culture of *Spirulina platensis* on glucose for phycocyanin production using a fed-batch system. *Enzyme Microb. Technol*., 20, 221-224.

Chew B P, Wong M W, Wong T S, 1996. Effects of lutein from marigold extract on immunity and growth of mammary tumors in mice. *Anticancer Res.*, 16, 3689-3694.

Chew P G, Andrew J C, Stuart K J, 2003. Protein quality and physico-functionality of Australian sweet lupin (*Lupinus angustifolius* cv. Gungurru) protein concentrates prepared by isoelectric precipitation or ultrafiltration. *Food Chemistry*, 83 (4): 575-583

Christaki E *et al*., 2012. Effect of dietary *Spirulina platensis* on milk fatty acid profile of dairy cows. *Asian J. Anim. Vet. Adv*., 7, 597-604.

Christaki E, Florou-Paneri P & Bonos E, 2011. Microalgae: a novel ingredient in nutrition. *Int. J. Food Sci. Nutr.* 62 (8), 794-799.

Chronakis I S, 2001. Gelation of Edible Blue-Green Algae Protein Isolate (*Spirulina platensis* Strain 387 Pacifica): Thermal Transitions, RheologicalProperties, and Molecular Forces Involved. *J. Agric. Food Chem.,* 49 (2), 888-898.

Chronakis I S, Galatanu A N, Nylander T & Lindman B, 2000. The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface. *Colloids Surf. A*, 173 (1-3), 181-192.

Cian R E *et al*., 2012. Immunomodulatory Properties of the Protein Fraction from Phorphyra columbina. *J Agri Food Chem* 60(33): 8146-8154.

Clares M E, Moreno J, Guerrero M G, & García-González M, 2014. Assessment of the CO_2 fixation capacity of *Anabaena* sp. ATCC 33047 outdoor cultures in vertical flat-panel reactors. *J. Biotechnol*., 187, 51-55

Cohen Z, 1999. Production of polyunsaturated fatty acids by the microalga *Porphyridium cruentum*. In: Cohen Z (Ed). Production of chemicals by microalgae, *Taylor and Francis*, London, 1-24.

Cruz de Jesús, V., 2005. Optimizing a process for purifying C - phycocyanin from *Spirulina* maxima. Thesis, School of Higher Studies Cuautitlán Izcalli. *Autonomous University of Mexico.*

CSC (Calorimetry Sciences Corporation), 2006. Life Sciences Application Note, *Document No. 20211021306 - February 2006, p 2.*

Cui Z, 1983. Culture trial of Facai in soil-soaked solution. *Sci. Technol. Lett. Inner Mongolia*, 4, 10-38.

Cynthia V G L, *et al*., 2010. Protein measurements of microalgal and cyanobacterial biomass. *J Bioresource Technology*, 101, PP 7587–7591.

Dagorn-scaviner C, 1986. Etudes des propriétés tensioactives des gliadines du pois. Application aux isolats protéiques utilisés comme agents d'émulsification ou de moussage. *Thèse de Doctorat, Université de Nantes.*

Dagorn-Scaviner C, Guecuen J and Lefebvre J. (1986). Comparison of interfacial behaviours of pea (Pisum sativum L.) legumin and vicilin at air/water interface. *Die Nahrung* 30(3–4): 337–347.

Dalgleish D G, 1997. Adsorption of protein and the stability of emulsions. *Trends in Food Science & Technology*, *8*(1), 1-6.

Damodaran S, 1989. Interrelationship of molecular and functional properties of food proteins. In *Food Proteins: Structure and Functional Relationships*, J.E. Kinsella and W. Soucie (Ed.), p. 21-51. Am. Oil Chem. Soc. Am. Oil Chem. Soc., Champaign, IL.

Damodaran S, 1994. Structure-function relationship of food proteins. In: Protein functionality in food systems. Marcel Decker Inc. New York, eds, N.S. Hettiarachchy, G.R. Ziegler, P1-38.

Damodaran S, 2005. Protein Stabilization of Emulsions and Foams. *Journal of Food Science* 70, 3, R54-R66.

Dangeard P, 1940. Sur une algue bleue alimentaire pour l'homme : *Arthrospira platensis* (Nordst.) Gomont. *Actes Soc. Linn. Bordeaux Extr. P.V.*, 91, 39-41.

Darling DF, Birkett RJ. In: Dickinson E, editor. Food emulsions and foams, 1987. London: Royal Society of Chemistry; p. 1

Das D, & Georgiadis M M, 2001. A directed approach to improving the solubility of Moloney murine leukemia virus reverse transcriptase. *Protein Sci*. 10, 1936–1941.

Davis J P, and Foegeding E A, 2004. Foaming and Interfacial Properties of Polymerized Whey Protein Isolate. *Journal of Food Science*, 69, 5, C404-C410.

De Jesús *et al.*, 2016. Methods for Extraction, Isolation and Purification of C-phycocyanin: 50 years of Research in Review. *Int J Food Nutr Sci*, *3*(3), 1.

De Pauw N, Morales J & Guido P, 1984. Mass culture of microalgae in aquaculture systems: Progress and constraints. *Hydrobiologia*, 1 16/117, 121-134.

Deng R & Chow T J, 2010. Hypolipidemic, antioxidant, and antiinflammatory activities of microalgae *Spirulina. Cardiovascular therapeutics*, *28*(4), e33-e45.

Devendra K, Dolly W D, Sunil P, Neeraj K, Suresh W, 2014. Extraction and purification of C-phycocyanin from *Spirulina* platensis (CCC540*), Ind J Plant Physiol. 19(2):184–188 DOI 10.1007/s40502-014-0094-7*

Doke J M Jr., 2005. An Improved and Efficient Method for the Extraction of Phycocyanin from *Spirulina* sp. *Inter J of Food Eng.* 1 (5). DOI: 10.2202/1556-3758.1037

Ducret A, *et al.*, 1996. Isolation, characterization and electron microscopy analysis of a hemidiscoidal phycobilisome type from the cyanobacterium Anabaena *sp*. PCC 7120. *Eur J Biochem* 236(3): 1010-1024.

Eriksen N T, 2008. Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. *Applied microbiology and biotechnology*, *80*(1), 1-14.

Erni P, Windhab E J, Gunde R, *et al.*, 2007. Interfacial Rheology of Surface-Active Biopolymers: *Acacia senegal* Gum versus Hydrophobically Modifed Starch. *Biomacromolecules*, 8, 3458-3466.

Evans M T A, Phillips, M C and Jones, M N, 1979. The conformation and aggregation of bovine, β-casein A. II. Thermodynamics of thermal association and the effects of changes in polar and apolar interactions on micellization. *Biopolymers*, 18 (5), 1123-1140.

Ferreira HA, Torres DPV and Juarez-Oropeza MA, 2010. Hepatoprotective effects of *Spirulina* maxima in patients with nonalcoholic fatty liver disease: a case series. *J Med Case Reports*, 4,103.

Fillery-Travis A, Mills E N C, and Wilde P, 2000. Protein-lipid interactions at interfaces. *Grasas y Aceites*, 51 (1-2), 50-55.

Fon Sing S, Isdepsky A, Borowitzka M A & Lewis D M, 2014. Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production. *Bioresour. Technol.*, 161, 47-54.

Fu E, Friedman L, Siegelman H W, 1979. Mass-spectral identification and purification of phycoerythrobilin and phycocyanobilin. *Biochem J*, 179(1): 1-6

Gaines G L, 1966. Insoluble monolayers at liquid-gas interface. Prigogine, Interscience, New York, p 1-386.

Gantar M *et al.*, 2012. Isolation, characterization and antioxidative activity of C-phycocyanin from Limnothrix sp. strain 37-2-1. *J Biotechnol* 159(1–2): 21-26.

German J B, O'Neil T E, Kinsella J E, 1985. Film forming and foaming behavior 365 of food proteins. Journal of the American Oil Chemists'Society, 62(9), 1358-1366.

Gill P, Moghadam T T, Ranjbar B, 2010. Differential scanning calorimetry techniques: applications in biology and nanoscience. *J Biomol Tech.,* 21(4), 167-93.

Glazer A N, Cohen-Bazire G, 1971. Subunit structure of the phycobiliproteins of blue-green algae. *Proc Natl Acad Sci* U S A, 68(7): 1398-1401.

González López C V, *et al.*, 2009. Utilization of the cyanobacteria Anabaena sp. ATCC 33047 in CO2 removal processes. *Bioresource Technology*, 100, 5904–5910

González López C V *et al*., 2010. Protein measurements of microalgal and cyanobacterial biomass. *Bioresour. Technol*., 101, 7587-7591.

González R *et al*., 1999. Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. *Pharmacol. Res*., 39 (1), 55-59.

Gouveia L, *et al*., 2008. Microalgae in novel food products. In: Papadopoulos KN, editor. Food chemistry research developments. Nova Science Publishers, Inc., Hauppauge NY, USA.

Graham D E, 1976. Structure of adsorbed protein films and stability of foams and emulsions. phD thesis, Unilever Research Laboratory/Welwyn, The Frythe, Welwyn, Hertfordshire.

Graham D E, Phillips M C, 1979a. Proteins at liquid interfaces. 1 Kinetics of adsorption and surface denaturation. *J. Colloid Interface Sci*., 70, (3), 403-414.

Gray B H, Lipschultz C A, Gantt E, 1973. Phycobilisomes from a blue-green alga Nostoc species. *J Bacteriology*, 116(1), 471-478.

Gudin C & Chaumont D, 1991. Cell fragility – the key problem of microalgae mass production in closed photobioreactors.*Bioresour. Technol*., 38, 145-151.

Han Danxiang, Bi Yonghong and Hu Zhengyu, 2004. Industrial Production of Microalgal Cell-mass and Secondary Products – Major Industrial Species: Nostoc. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology* Edited by Amos Richmond 2004 by Blackwell Publishing Ltd, 304-331.

Harkins W D, and Brown F E, 1919. The determination of surface tension (free surface energy), and the weight of falling drops: the surface tension of water and benzene by the capillary head method. *Journal of the American Chemical Society*, 41(4), 499-524.

Hartland Stanley, 2004. Surface and interfacial tension: Measurement, Theory, and Applications. *Surfactant science series vol 119. Marcel Dekker, Inc., 270 Madison Avenue, New York, NY 10016, U.S.A.*

He J A, Hu Y Z, Jiang L J, 1997. Photodynamic action of phycobiliproteins: in situ generation of reactive oxygen species. *Biochimica et Biophysica Acta (BBA) Bioenergetic*s 1320(2): 165-174.

Hedenskog G & Hofsten A V, 1970. The Ultrastructure of *Spirulina* platensis—A New Source of Microbial Protein. *Physiologia Plantarum*, *23*(1), 209-216.

Henchion M, Hayes M, Mullen A M, Fenelon M, and Brijesh T, 2017. Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods*, 6, 53, doi: 10.3390/foods6070053

Hirata T *et al.,* 2000. Antioxidant activities of phycocyanobilin prepared from *Spirulina platensis. .J. Appl. Phycol 12*, 435–439.

Hu M, McClements J, & Decker E A, 2003. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. Journal of Agricultural and Food Chemistry, 51, 1696–1700

Iqbal M, Zafar S I, Stepan-Sarkissian G & Fowler M W, 1993. Indoor mass cultivation of red alga *Porphyridium cruentum* in different types of bioreactors: effect of scale-up and vessel shape. *J. Ferment.Bioeng.*, **75**(3), 76-78.

Iwamoto H, 2004. Industrial production of microalgal cell-mass and secondary products – major industrial species: *Chlorella*. *In*: Richmond A. *Handbook of microalgal culture. Biotechnology and applied phycology*. Oxford, UK: Blackwell, 255-263.

Jho C, and Bruke R, 1983. Drop weight technique for the measurement of dynamic surface tension. Journal of colloid and interface Science, 95 (1), 61-71.

Karaca A C, Low M N 2011a**.** Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction *Food Research International*, 44, 2742-2750.

Karamoko G, 2014. Etude des propriétés de surface et techofonctionnelles des fractions proteose-peptones. Thèse de Doctorat soutenue à la Faculté agronomique de Gembloux, Université de Liège.

Karamoko G, Danthine S, G Olive, Blecker C, 2013. Interfacial and foaming properties of two types of total proteose-peptone fractions. Food and Bioprocess Technology, Springer, 6 (8), pp.1944-1952.

Kelly M and Bob C, 2011. Nature's Superfood. *3rd edition* by Cyanotech Corporation, PP 73.

Kent M, Welladsen M H, Mangott A & Li Y, 2015. Nutritional evaluation of Australian microalgae as potential human health supplements. *PLoS One*, 10 (2), e0118985.

Kinsella J E, 1979. Functional properties of soy proteins. Journal of American Association 484 of Oil Chemists' Society, 56, 242-258.

Kinsella J E, Phillips L G , 1989. Structure function relationships in food proteins: Film and foaming behaviour. In: Kinsella, JE, Soucie, WG (eds) Food Proteins: Structure and Functional Relationships, Champaign, IL: The American Oil Chemists Society, pp. 52–77.

Kinsella, J E, 1976. Functional properties of proteins in food: a survey, *Crit. Rev. Food Sci. Nutr.*, 7 (3), 219-280.

Kitabatake N, Doi E, 1982. Surface tension and foaming of protein solutions. J. Food Sci., 47(4), 1218-1221.

Kosma I, *et al*., 2016b. Characterization and Classification of Extra Virgin Olive Oil from five less well-Known Greek Olive Cultivars. *J Am Oil Chem Soc*, 93 (6), 837–848.

Kralova I and Sjöblom J, 2009. Surfactants Used in Food Industry: A Review. *Journal of Dispersion Science and Technology*, 30:1363–1383 DOI: 10.1080/01932690902735561.

Kramer R M *et al*., 2012. Toward a Molecular Understanding of Protein Solubility: Increased Negative Surface Charge Correlates with Increased Solubility, *Biophysical Journal* (102) 1907–1915.

Kumar, D., Dhar, D., Pabbi, S., *et al.,* 2014. Extraction and purification of C-phycocyanin from *Spirulina platensis* (CCC540). *Indian J Plant Physiol* 19, 184-188

Laemmli U K, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.

Landrum J T, Bone R A, Kilburn M D, 1997. The macular pigment: a possible role in protection from age-related macular degeneration. *Adv Pharmacol*. 38, 537–556.

Lee F Y and White C H, 1991. Effect of ultrafiltration and whey protein concentrates on ice cream quality during storage. *J. Dairy Sci.* 74, 1170-1180.

Lee Y K, 1997. Commercial production of microalgae in the Asia-Pacific rim. *J. Appl. Phycol.*, 9, 403–11.

Li B, *et al.,* 2005.Effects of CD59 on antitumoral activities of phycocyanin from *Spirulina* platensis. *Biomed Pharmacother* 59(10), 551-560.

Liao X *et al*., 2011. Purification of c-phycocyanin from *Spirulina* platensis by single-step ion-exchange chromatography. *Chromatographia*, 73(3): 291-296.

Lin M J, Humbert E S, & Sosulski F W, 1974. Certain functional properties of sunflower meal products. *Journal of Food Science*, 39, 368–370.

Lucassen-Reynders E H, 1993. Interfacial Viscoelasticity in Emulsions and Foams.*Food Structure,* 12, l-12.

MacColl R, Habig W, Berns D S, 1973. Characterization of Phycocyanin from Chroomonas Species. *J Biol Chemistry* 248(20): 7080-7086.

Mackie A R, Ridout M J, Moates G, Husband F A, & Wilde P J, 2007. Effect of the interfacial layer composition on the properties of emulsion creams. *Journal of Agricultural and Food Chemistry*, 55, 5611–5619.

MacRitchie and Ter-Minassian-Saraga, 1983. Stability of compressed spread monolayers l-labelled and cold BSA.*Progress colloid polymer science*, 68 14-19.

MacRitchie F, Alexender A E, 1963c. Kinetics of adsorption of proteins in interfaces. Part III. The role of elctrical barriers in adsorption. *J. Colloid Sci*, 18,464-469.

Madhyastha H K *et al.,* 2006. Purification of c-phycocyanin from *Spirulina* fusiformis and its effect on the induction of urokinase-type plasminogen activator from calf pulmonary endothelial cells. *Phytomedicine*, 13(8): 564-569.

Mahajan A & Ahluwalia A S, 2010. Effect of processing on functional properties of *Spirulina* protein preparations. *Afr. J. Microbiol. Res*., 4 (1), 55-60.

Malcata F X, 2011. Microalgae and biofuels: A promising partnership? *Trends Biotechnol.* 29, 542- 549.

Malhotra A, Coupland J N, 2004. The effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates. *Food Hydrocolloids,* 18 (1), 101-108

Margulis L, 1981. *Symbiosis in cell evolution*. New York, NY, USA: W.H. Freeman.

Marshall H, 2007. Micro-algae as a superfood source: phytoplankton for future nutrition. Vegetarian Issues (Jun):1–2.

Maurya S S, Maurya J N, Pandey V D, 2014. Factors regulating phycobiliprotein production in cyanobacteria. *Int J Curr Microbiol App Sci* 3(5): 764-771.

McClements D J, 1999. Food Emulsions: Principles, Practice and Techniques

McClements D J, 2005. Food emulsions: Principles, practice and techniques (2nd ed). Boca Raton, FL, USA: CRC Press Taylor & Francis Group.

Meunier G, Mengual O, 1996. A new concept in stability analysis of concentrated colloidal dispersions (emulsions, suspensions, foams, gels). Proceedings 4th World Surfactant Congress, Barcelona Spain, 300-314

Miller R., Hoffmann R, Schano K H, and Halbig A, 1992. Measuring dynamic surface and interfacial tensions. *Advanced Materials*, 4 (5), 370-370.

Minkova K M *et al.,* 2003. Purification of C-phycocyanin from *Spirulina* (Anthrospira) fusiformis. *J of Biotechnology* 102, 55-59.

Moon M *et al*., 2014. Isolation and characterization of thermostable phycocyanin from Galdieria sulphuraria. *Korean Journal of Chemical Engineering*, 31(3): 490-495.

Moraes C C *et al*., 2013. Modeling of ion exchange expanded-bed chromatography for the purification of C-phycocyanin. *J Chromatogr* A 1281: 73-78.

Moreno A *et al.*, 1997. Purification, crystallization and preliminary X-ray diffraction studies of C-phycocyanin and allophycocyanin from *Spirulina* platensis. *Acta Crystallographica* Section D, 53, 321–326

Moreno J et al., 2003. Outdoor cultivation of a nitrogen-fixing marine cyanobacterium, *Anabaena* sp. ATCC 33047. *Biomol. Eng*., 20 (4-6), 191-197.

Mosavi L K, & Peng Z Y, 2003. Structure-based substitutions for increased solubility of a designed protein. Protein Eng. 16, 739–745.

Moure A, Sineiro J, Domínguez H, & Parajó J C, 2006. Functionality of oilseed protein products: A review. Food Research International, 39, 945–963.

NAS-NRC (National Academy of Sciences—National Research Council), 1963. The Growth of World Population. Analysis of the problems and Recommendations for Reseach and Training. Committee on science and public policy – National Academy of Sciences, Washington, D.C.

Nigam S, Prakash M R and Sharma R, 2011. Effect of Nitrogen on Growth and Lipid Content y of Chlorella pyrenoidosa American *Journal of Biochemistry and Biotechnology* 7 (3), 124-129.

Nirmala C, Prakash V, Venkataraman L V, 1992. Physico-chemical and functional properties of proteins from spray dried algae (*Spirulina platensis*). *Molecular Nutrition Food Research*, Volume 36, (6), 569-577.

Nitsch W, Maksymiw R, 1990. Catalase monolayers at the air/water interface I. Reversibilities and irreversibilities. *Colloid Polym. Sci.* 268, 452-459.

ÓhEocha C, Haxo F T, 1960. Some atypical algal chromoproteins. *Biochimica et Biophysica Acta*, 41(3), 516-520.

Oliveira E G, Rosa G S, Moraes M A. & Pinto L A A, 2008. Phycocyanin content of *Spirulina* platensis dried in spouted bed and thin layer. *J of Food Process Eng*., 31(1), 34-50.

Orio Cifferi, 1983. *Spirulina*, the Edible Microorganism. *Microbiological Reviews*, 551-578.

Ou Y, *et al*., 2013. Antidiabetic potential of phycocyanin: Effects on KKAy mice. *Pharm Biol.*, 51(5), 539-544.

Panizzolo L A, Mussio L E, & Añón M C, 2014. A kinetic model for describing the effect of proteins on the Air-Water interface tension. *Journal of Food Science and Engineering*, 4, 282-290.

Paoletti C, Vincenzini M, Bocci F & Materassi R, 1980. Composizione biochimica generale delle biomasse di *Spirulina platensis* e S. Maxima. *In*: Materassi R., ed. *Prospettive della coltura di Spirulina in Italia*. Roma: Consiglio Nazionale delle Ricerche, 111-125.

Papalamprou E M, Doxastakis G I, and Kiosseoglou V, 2010. Chickpea protein isolates obtained by wet extraction as emulsifying agents. *Journal of the Science of Food and Agriculture*, 90, 304-313.

Park J S, Chew B P, Wong T S, Zhang J X, Magnuson N S, 1999. Dietary lutein but not astaxanthin or â-carotene increases pim-1 gene expression in murine lymphocytes. Nutr. Cancer 1999, 33, 206-212.

Patel A, Mishra S, Pawar R, Ghosh P K, 2005. Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expression and Purification*, 40(2), 248-255.

Patel AK *et al*., 2013. Separation and fractionation of exopolysaccharides from *Porphyridium cruentum*. *Bioresour. Technol*., 145, 345-350.

Patil G *et al.*, 2006. Method to obtain C-phycocyanin of high purity. *J Chromatogr* A 1127(1–2), 76-81.

Patil G *et al*., 2008. Fractionation and purification of the phycobiliproteins from *Spirulina* platensis. *Bioresour Technol*, 99(15), 7393-7396.

Patino R J M *et al.*, 2007a. Interfacial and foaming properties of enzyme-induced hydrolysis of sunflower protein isolate. *Food Hydrocolloids*, 21, 782-793.

Patino R J M, Carrera S C, & Nino R M R, 2008. Implications of interfacial characteristics of food foaming agents in foam formulations. *Advances in Colloid and Interface Science*, 140, 95-113.

Patino R J M, Nino R M R, & Carrera S C, 2007b. Physico- chemical properties of surfactant and protein films. *Current Opinion in Colloid & Interface Science*, 12, 187-195.

Pawlik-Skowrońska B, Kalinowska R, & Skowronski T, 2013. Cyanotoxin diversity and food web bioaccumulation in a reservoir with decreasing phosphorus concentrations and perennial cyanobacterial blooms. *Harmful Algae*, 28, 118-125.

Phillips M C, 1981. Protein conformation at liquid interfaces and its role in stabilizing foams and emulsions, *Food Technol.*, 35(1): 50.

Piñero Estrada *et al.*, 2001. Antioxidant activity of different fractions of *Spirulina platensis* protean extract. Il Farmaco, 56(5–7): 497-500.

Praveenkumar R *et al.*, 2014. Improved biomass and lipid production in a mixotrophic culture of *Chlorella* sp. KR-1 with addition of coal-fired flue-gas. *Bioresour. Technol*., 171, 500-505.

Privalov P L and Khechinashvili N N, 1974. A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J. Mol. BioI*. 86 (3), 665-684.

Qian Hu, 2004. Industrial production of microalgal cell-mass and secondary products major industrial species: *Arthrospira* (*Spirulina*) *platensis*. *In*: Richmond A. *Handbook of microalgal culture. Biotechnology and applied phycology*. Oxford, UK: Blackwell, 265-272.

Razafindralambo H, 1996. Contribution à l'étude des propriètés tensioactives des lipopeptides de bacillus sutilis. Thèse de doctorat, Faculté des Sciences Agronomiques de de Gembloux, Belgique.

Reddy M C *et al.*, 2003. C-Phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem Biophys Res Commun*. 304 (2), 385-92.

Richmond A, 2004. Handbook of microalgal culture: biotechnology and applied phycology. Blackwell Science Ltd.

Riès-kautt M, Ducruix A, 1997. Inferences drawn from physicochemical studies of crystallogenesis and precrystalline state. In: Carter C.W. Jr., editor. Methods in Enzymology. Academic Press; New York: pp. 23–59.

Rito-Palomares M, Nuñez L, Amador D, 2001. Practical application of aqueous two-phase systems for the development of a prototype process for c-phycocyanin recovery from *Spirulina* maxima. *Chem Tech Biotech*, 76(12), 1273-1280.

Romay Ch. *et al*., 1998. Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. *Inflammation Res*, 47, 36-41.

Romay Ch. *et al.*, 2003. Phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Curr. Protein Pept. Sci*., 4, 207-216.

Romero García J M, Acién Fernández F G & Fernández Sevilla J M, 2012. Development of a process for the production of L-amino acids concentrates from microalgae by enzymatic hydrolysis. *Bioresour. Technol*., 112, 164-170.

Rozin P *et al*., 2004. Preference for natural instrumental and ideational/moral motivations, and the contrast between foods and medicines. *Appetite*, 43, 147- 154.

Saker M L *et al.*, 2005. Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*. *Toxicon*, 46 (5), 555-562.

Sarada R M G P, Pillai M G, & Ravishankar G A, 1999. Phycocyanin from *Spirulina sp:* influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. *Process Biochemistry*, *34*(8), 795-801.

Scheffler J, 2007. Underwater habitats. *Illumin*, 9 (4).

Schramm L L, 2014. Emulsions, Foams, Suspensions, and Aerosols: Microscience and Applications. Wiley-VCH Verlag GmbH & Co. KGaA p 492.

Schwenzfeier A, Lech F, Wierenga P A, Eppink M H M and Gruppen H, 2013[b]. Foam properties of algae soluble protein isolate: Effect of pH and ionic strength, *Food Hydrocolloids* 33 111-117.

Schwenzfeier A, Wierenga P A, Eppink M H M and Gruppen H, 2014. Effect of charged polysaccharideson the technofunctional properties of fractions obtained from algae soluble protein isolate. *Food hydrocolloids* 359-18.

Schwenzfeier A, Wierenga P A, & Gruppen H, 2013a. Emulsion properties of algae soluble protein isolate from *Tetraselmis* sp. *Food Hydrocolloids*, 30 (1), 258-263.

Schwenzfeier A, Helbig A, Wierenga P A, & Gruppen H, 2011. Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. *Bioresour. Technol*., 102 (19), 9121-9127.

Sciarini L S, Maldonado F, Ribotta P D, Perez G T, & Leon A E (2009). Chemical composition and functional properties of Gleditsia triacanthos gum. Food Hydrocolloids, 23, 306–313.

Sean M T & Milley J E, & Lall S P, 2015. Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors. Appl Phycol 27, 1109-1119.

Seo Y, *et al*., 2013. Stable Isolation of Phycocyanin from *Spirulina* platensis Associated with High-Pressure Extraction Process. *Int J Mol Sci* 14(1), 1778-1787.

Servaites J C, Faet J L, & Sidhu S S, 2012. A dye binding method for measurement of total protein in microalgae. *Anal Chem*, 421, 75-80.

Shaw K L, Grimsley G R, Yakovlev G I. Makarov A A, & Pace C N, 2001. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein Science*, 10(6), 1206–1215.

Silveira S, *et al*., 2007. Optimization of phycocyanin extraction from *Spirulina* platensis using factorial design. *Bioresour Technol*, 98(8), 1629-1634.

Simpore J, *et al*., 2005. Nutrition Rehabilitation of HIV-Infected and HIV-Negative Undernourished Children Utilizing *Spirulina*. *Annals of Nutrition and Metabolism*, 49, 373-380.

Simpore J, *et al*., 2006. Nutrition rehabilitation of undernourished children utilizing *Spiruline* and *Misola*. *Nutr. J*., 5**(**3), doi: 10.1186/1475-2891-5-3.

Slocombe S P, *et al*., 2013. A rapid and general method for measurement of protein in microalgal biomass. *Bioresour. Technol*., 129, 51-57.

Stec B, Troxler R F, Teeter M M, 1999. Crystal structure of C-phycocyanin from Cyanidium caldarium provides a new perspective on phycobilisome assembly. *Biophys J*, 76(6), 2912-2921.

Stewart D E, Farmer F H, 1984. Extraction identification and quantitation of phycobiliprotein pigment from phototrophic plankton. *Limnol Oceonogr*, 29, 392- 397.

Tadros T F, 2013. Emulsion Formation and Stability. Published by Wiley-VCH Verlag GmbH & Co. KGaA, P 262.

Temme E H M, Zhang J, Schouten E G, & Kesteloot H, 2001. Serum bilirubin and 10-year mortality risk in a Belgian population. *Cancer Causes Control*, 12, 887-894.

Timilsena YP, Adhikari R, Barrow C J, & Adhikari B, 2016. Physicochemical and functional properties of protein isolate produced from Australian chia seeds. *Food Chem.* 212**:** 648-656.

Tornberg E and Hermansson A M, 1977. Functional characterisatio of protein stabilized emulsions: effect of food processing. *Journal of Food Science*, 42 (2), 468-472.

Tornberg E, 1978a. The application of the drop volume technique to measurements of the adsorption of proteins at interfaces. *J. Colloid Interface Sci.*, 64, (3), 391-401.

Travallaie S *et al*., 2015. Comparative studies of β-carotene and protein production from *Dunaliella salina* isolated from Lake Hoze-soltan, Iran. *J. Aquat. Food Prod. Technol*., 24, 79-90.

Trevino S R, Scholtz J M, and Pace C N, 2007. Amino acid contribution to protein solubility: Asp, Glu, and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa. *J. Mol. Biol.*, 366, 449–460. 17.

Trevino S R, Scholtz J M, and Pace C N, 2008. Measuring and increasing protein solubility. *J. Pharm. Sci.* 97:4155–4166.

Uruakpa F O, & Arntfield S D, 2005. Emulsifying characteristics of commercial canola protein-hydrocolloid systems. *Food Research International*, 38, 659-672.

Utsumi S, Maruyama N, Satoh R, & Adachi M, 2002. Structure-function relationships of soybean proteins revealed by using recombinant systems. *Enzyme and Microbial Technology*, 30, 284e288.

Vaghela M N, & Kilara A, 1996. Foaming and emulsifying properties of whey protein concentrates as affected by lipid composition. *Journal of Food Science, 61*(2), 275-280.

Velea S, Ilie L, & Filipescu L, 2011. Optimization of *Porphyridium purpureum* culture growth using two variables experimental design: light and sodium bicarbonate. *U.P.B. Sci. Bull. Ser. B*, 73 (4), 81-94.

Vogler Erwin A, 2013. Protein Adsorption in Three Dimensions. *Biomaterials* 33(5): 1201–1237.

Vonshak A, Cohen Z & Richmond A, 1985. The feasibility of mass cultivation of *Porphyridium*. *Biomass*, 8, 13-25.

Vonshak, A. (Ed.). 1997. *Spirulina platensis arthrospira: physiology, cell-biology and biotechnology*. CRC Press.

Vuillemard J C, Gauthier S F, Richard J P, and Paquin P, 1990. Development of a method for the measurement of the maximum value of emulsifying capacity of milk protei. *Milchwissenschaft*, 45 (9), 572-575.

Walstra, P, 2003. Physical chemistry of foods. New York: Marcel Dekker, Inc.

Ward A F H, Tordai L, 1946. Time-dependence of boundary tensions of solutions. The role of diffusion in time effects. *J. Chem. Phys*. 14, (7), 453-461.

Ward A F H., & Tordai L, 1952. Time-dependence of boundary tensions of solutions: IV. Kinetics of adsorption at liquid-liquid interfaces. *Recueil des Travaux Chimiques des Pays-Bas, 71*(6), 572-584.

Web N B, Ivey F J, Craig H B, Jones V A, and Monroe R J, 1970. The measurement of emulsifying capacity by electrical resistance. Journal of Science, 35 (4), 501-504.

Wilde P J, 2000. Interfaces: their role in foam and emulsion behavior. Current Opinion in *Colloid & Interface Science*, 5, 176-181.

Wilkinson M, 1972. Extended use of, and comments on, the drop-weight (drop-volume) technique for the determination of surface and interfacial tensions. Journal of Colloid and Interface Science, 40 (1), 14-19.

Wouters A G B, Rombouts I, Legein M, Fierens E, Brijs K, Blecker C, Delcour J A, 2016. Aire/water interfacial properties of enzymatic wheat gluten hydrolyzates determine their foaming behavior Food Hydrocolloids 55 (2016) 155e162.

Xia L *et al*., 2014. Selection of microalgae for biodiesel production in a scalable outdoor photobioreactor in north China. *Bioresour. Technol*., 174, 274-280.

Yamani E *et al*., 2009. Intérêt de la spiruline chez les personnes vivant avec le VIH à Bangui (RCA). *Med. Trop.*, 69 (1), 66-70.

Yamauchi K, Shimizu M, and Kamiya T, 1980. Emulsifying properties of whey protein. *J. Food Sci,* 45, 1237–1242.

Yan-Jiao Li *et al.*, 2016. C-phycocyanin protects against low fertility by inhibiting reactive oxygen species in aging mice. *Oncotarget, 7* (14), 17393–17409.

Yann D, Saint-Jalmes A, Anniina S, *et al.*, 2011. Strong Improvement of Interfacial Properties Can Result from Slight Structural Modifications of Proteins: The Case of Native and Dry-Heated Lysozyme. *Langmuir*, 14947-14957.

Yao C-H, Ai J-N, Cao X-P, & Xue S, 2013. Salinity manipulation as an effective method for enhanced starch production in the marine microalga *Tetraselmis subcordiformis*. *Bioresour. Technol.*, 146, 663-671.

Yi-Ming Z, and Feng C, 1999. A simple method for efficient separation and purification of c-phycocyanin and allophycocyanin from *Spirulina platensis*. *Biotechnology Techniques* 13, 601-603.

Yoshida A, Takagaki Y, Nishimune T, 1996. Enzyme Immunoassay for Phycocyanin as the Main Component of *Spirulina* Color in Foods. *Biosci Biotechnol Biochem* 60(1), 57-60.

Zayas J F, 1997. Functionality of Proteins in Food. Springer-Verlag Berlin Heidelberg P382.

Zhu X, Hongsong F, Li D, Xiao Y, Zhang X, 2006. Protein Adsorption and Zeta Potentials of a Biphasic Calcium Phosphate Ceramic under Various Conditions *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* 82(1), 65-7.