Investigation of natural submicron capsules and soluble proteins extracted from oleaginous seeds for environmental application

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PhD 2014

Investigation of natural submicron capsules and soluble proteins extracted from oleaginous seeds for environmental application

by

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This thesis is submitted to Dublin City University for the degree of Doctor Philosophy (PhD) in the School of Biotechnology

January 2014

Under the supervision of Professor Ian W. Marison

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of the others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate) ID No.:_____ Date:_____

Ai miei genitori
il cui amore e supporto
non è mai mancato anche
attraverso la distanza

To my parents
whose love and encouragement
never faltered despite the distance

ñConsiderate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza"

"Consider your breed:
You were not made to live like beasts,
but to pursue virtue and knowledgeñ

Inferno, vv 118-120,
Canto XXVI
Dante Alighieri

Acknowledgments

It is very hard to summarize (the past) 3 years of ñPhD lifeò in a few words, so many people to thank!

First of all, I would like to thank my supervisor Prof. Ian Marison who gave me the opportunity to join and do my PhD in the LiB group. It was a very intense period that taught me a lot, not only on a professional, but even on a personal level.

I also would like to thank my colleagues of LiB for their encouragement throughout the course of this project, in particular Dr. Karen Byrne (my ñtwinò in this PhD adventure), Dr. Roisin Foley and Tanja Buch with whom I shared all of the ups and downs, from which a strong ñLiBò bond was created. Thank you girls! I also need to extend a large thanks to Sharon Conaghan and Micheal Whelehan whose support during the first year was very precious.

I would like to thank Mary Rafter for the help in all bureaucratic matters, especially at the beginning when I was still getting used to the new Irish life!

This research was funded by Science Foundation Ireland (Research Program). I would like to express my gratitude to SFI for providing me with such an opportunity.

I also would like to thank Linda Hughes for proofreading my thesis.

I probably would have not reached such a big achievement without the support of great people in my personal life. Italians are well known to have big, strong families, and mine is for sure not an exception! Thanks to all of my wonderful family who understood my reasons for choosing to do a PhD abroad, supported me at any stage and uplifted me when I felt down.

Thanks to Stefano, my rock, whose support was fundamental during these years of PhD (and even more). You always made me smile and laugh even in the hard times, but most of all, you believed in me every single day. Ah, I almost forgot to mention the delicious

meals which you cooked for me, that played a key role in keeping my synapsis working!!!!!

DCU means not only PhD, but even friendship for me. One of the best gifts, the ñAlma Materò brought me is Claudia! Thank you ñciccinaò for the help, the chat, the laughs and the tears which you shared with me! We have known each other for 3 years, but it seems like forever! ñI love youò!

A big thank to my Italian friends ñWinxò Chiara, Giulia and Valeria: we have always managed to get together and support each other during these years.

Thanks to Max too for your support, I know you are very busy but you always find a few minutes to talk with me and give me your suggestions.

I also need to extend a big thanks to my ñsistersò from Bologna University: Francesca and Daniela! We kept in contact and supported each other despite the distance and the different paths, and it is wonderful to reminisce with you about our time in Bologna!

It was an incredibly great period of my life and I cannot be more proud of myself and grateful for all of the people I knew, the lessons I have learned and the achievement I have reached!

Thank you Ireland, youôll always be in my heart.

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Abstract

The treatment of water is needed for the supply of adequate quality of drinking water and before the discharge of wastewater to the environment.

Since the flocculants used in the water and wastewater plants have been shown to be potentially hazardous for the human health, it has become necessary to find flocculants less dangerous and more eco-friendly. This thesis consists of a systematic study to investigate the applications of natural submicron capsules (oil-bodies) and proteins extracted from oleaginous seeds for water treatment.

Submicron capsules were extracted from different oleaginous sources and their flocculation activity has been investigated and characterized. We showed that they possess flocculation activity for clay suspensions comparable or even higher than the standard flocculants as alum and ferric salts. Moreover, the capsules have been tested for their ability to remove a range of pharmaceuticals from water.

The investigation of oleaginous seed materials was further extended to the extraction of soluble proteins from press-cakes, the by-product of seeds after oil extraction. Thus, the flocculation activity of extracts from several press-cakes has been tested and compared to conventional flocculants, for example, alum and ferric salts.

Finally, since lab-scale flocculation tests are usually off-line, in order to improve this methodology an on-line quantification method was developed. To do that, a typical jar test apparatus was equipped with six turbidity probes connected to a computer to get on-line measurements.

Abbreviations

AD	Alzheimerôs disease
ANOVA	Analysis of variance
BSA	Bovine serum albumin
DI	Deionised Water
ΔT	Total turbidity removal
EPA	Environmental Protection Agency
FA	Flocculation activity
HOP	Hydrophobic organic pollutants
IL-α	Interleukin 1α
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
NFTs	Neurofibrillary tangles
NF-κB	Nuclear factor-κB
NSC	Natural submicron-capsules
NTU	Nephelometric turbidity units
OB	Oil-body
PAH	Poly-aromatic hydrocarbons
PC	Press-cakes
PhAC	Pharmaceutical active compound
PL	Phospholipid
ppm	Parts per million
RSM	Response surface methodology
RT	Room Temperature

SD	Standard Deviation
SDSPAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
STP	Sewage treatment plants
TAG	Triacylglycerols
TNF-α	Tumour necrosis factor-α
UN	United Nations
UP	Ultrapure water
WHO	World Health Organisation

CHAPTER 1

Introduction

1.1 Water treatment

In industrialized countries it can be often taken for granted that tap water is safe, clean and potable. However, in developing countries the universal access to safe drinking water at an acceptable level is still a concern. Eighty eight million people do not have access to safe drinking water and more than 2.6 billion do not have basic sanitation, while approximately 1.5 million children under 5 years of age die and 443 million school days are lost each year as a result of water and sanitation related disease (Emblidge and DeLorenzo, 2006, U.N., 2010). Thus, in 2002 the Committee on Economic, Social and Cultural Rights adopted General Comment No. 15 on the right to water. Article I stated that "The human right to water is indispensable for leading a life in human dignity. It is a prerequisite for the realization of other human rights". Comment No. 15 also defined the right to water as the right of everyone to sufficient, safe, acceptable and physically accessible and affordable water for personal and domestic uses (U.N., 2002). This concept was confirmed in 2010 through the Resolution 62/292 when the United Nations General Assembly explicitly recognized the human right to water and sanitation and acknowledged that clean drinking water and sanitation are essential to the realisation of all human rights (U.N., 2010). The Resolution calls upon States and international organisations to provide financial resources, help capacity-building and technology transfer to developing countries in order to guarantee safe, clean, accessible and affordable drinking water and sanitation for all (U.N., 2010).

1.1.1 Colloidal particles suspended in water

Clean water is rarely found in nature and for health reasons it is not advisable to drink untreated water. The unwanted impurities and particles dissolved in water result from

land erosion, the picking up of hazardous materials such as decay from plants, airborne contamination, sewage, industrial discharges and animal waste. Thus, surface water sources, polluted by man and nature, are likely to contain suspended and dissolved organic (plant or animal origin) and inorganic (mineral) material, and biological forms such as bacteria, spores, cysts and plankton (EPA, 2002, Castiglioni et al., 2006).

The particulate impurities cover a broad size range and are usually in the macroscopic to submicroscopic size range and at the pH of water are usually negatively charged (Bratby, 2006).

Larger sized particles such as sand and heavy silts can be removed from water by slowing down the velocity of flow to allow for simple gravity settling. These particles are often called settleable solids. Settling of intermediate sized particles occurs naturally when surface water is stored for a sufficient period in a reservoir or a lake (**Table 1.1**).

Table 1.1: Classification of particles size (Bratby, 2006)

Particle size mm	Classification	Examples	Total surface area m^2/cm^3	Time required to settle 100 mm if specific gravity = 2.65
10	Coarse dispersion (visible to naked eye)	Gravel, coarse sand, mineral substances, precipitated and flocculated particles, silt, macroplankton	6×10^{-4}	0.1 s
1			6×10^{-3}	1 s
10^{-1}			6×10^{-2}	13 s
10^{-2}	Fine particulate dispersion (visible under microscope)	Mineral substances, precipitated and flocculated particles, silt, bacteria, plankton, and other organisms	0.6	11 min
10^{-3}			6	20 hours
10^{-4}			60	80 days
10^{-5}	Colloidal dispersion (submicroscopic)	Mineral substances, hydrolysis and precipitated products, macromolecules, biopolymers, viruses	600	2 years
10^{-6}			6000	20 years
$<10^{-6}$	Solution	Inorganic simple and complex ions, molecules and polymeric species, polyelectrolytes, organic molecules, undissociated solutes		

Particles smaller than approximately 10^{-5} mm are referred to as colloids (Brown, 2007), while materials smaller than approximately 10^{-6} mm are referred to as solutes. These types of particles are able to retain a dispersed state and remain as independent entities thanks to the surface charge and degree of hydration of the particlesô surface layer. Therefore, the smaller the particles the more predominant become such interfacial phenomena and the lesser become the influences of gravity effects associated with mass (Bratby, 2006). As shown by **Table 1.1**, for colloidal size range particles it may take 2 to 20 years to settle down, so it is necessary to find a treatment which produces larger particles that are more amenable to removal (EPA, 2002, Bratby, 2006).

Different techniques exist for the removal of particles, including adsorption and filtration (**Figure 1.1**).

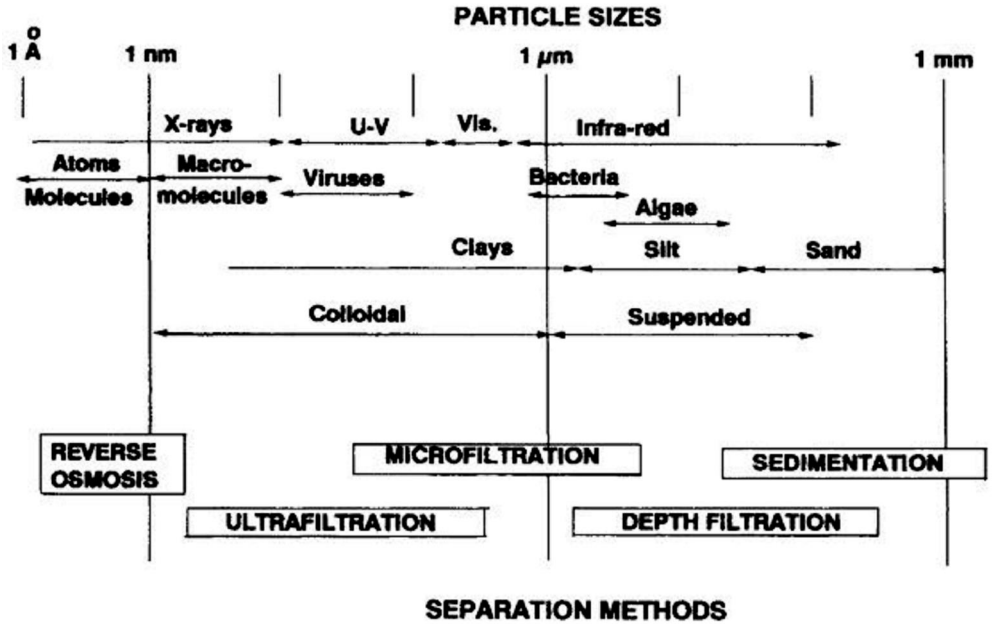


Figure 1.1: Typical size ranges of particles and appropriate solid-liquid separation processes for different particle sizes (Jia et al., 1998).

However, these methods can be expensive, thus alternative feasible methods that alter the surface properties of colloid material in order to form larger particles to separate by sedimentation and/or filtration have to be considered.

The processes which are able to accomplish destabilisation and subsequently aggregation of colloidal particulate are coagulation and flocculation.

1.1.2 Coagulation and flocculation

Four thousand years ago, in the area of potable water treatment, the Egyptians used to clarify water by using smeared almonds and alum was used as a coagulant by the Romans in 77 A.D. (Bratby, 2006).

As is evident, coagulation and flocculation are extremely important and are interdependent stages of the solid separation phase of a typical water treatment (**Figure 1.2**).

The two processes can be often confused and the terms sometimes are used interchangeably, but it is important to specify the differences between them (**Figure 1.3**):

- Coagulation is the process whereby destabilization of a given suspension or solution is effected. Basically, the function of coagulation is to overcome the factors that promote the stability of the colloidal particles.
- Flocculation is the process whereby destabilized particles are induced to come together, make contact and thereby form larger agglomerates, called flocs (Bratby, 2006), that are able to settle out and to be removes as sludge (EPA, 2002).

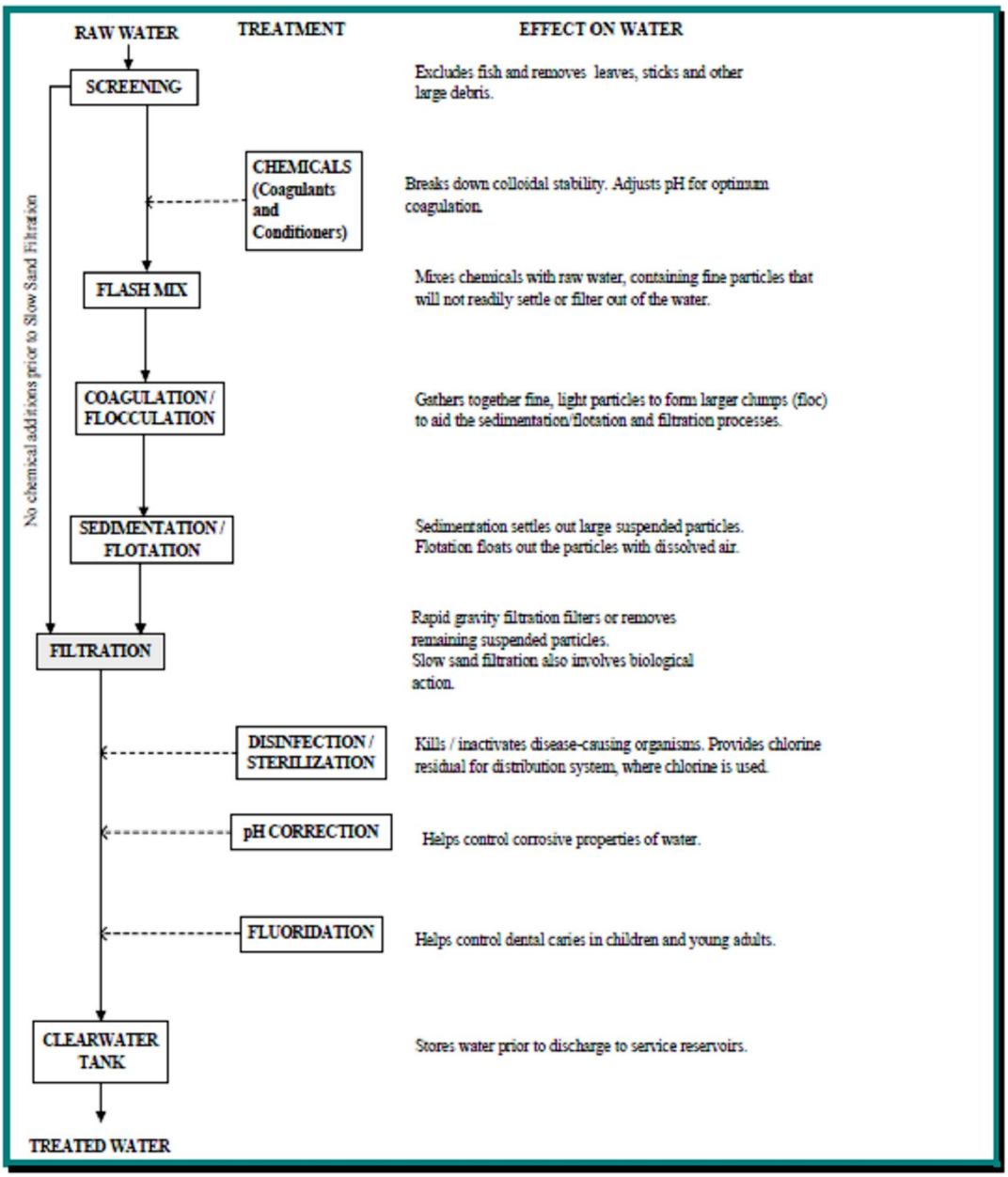


Figure 1.2: Typical water treatment process (EPA, 2002).

During these processes two different types of agents are usually used:

- Primary coagulant is a chemical or substance added to a suspension or solution to effect destabilization
- Flocculant aids are the chemicals or substances added to a destabilized suspension to accelerate the rate of flocculation, or to strengthen flocs formed during flocculation.

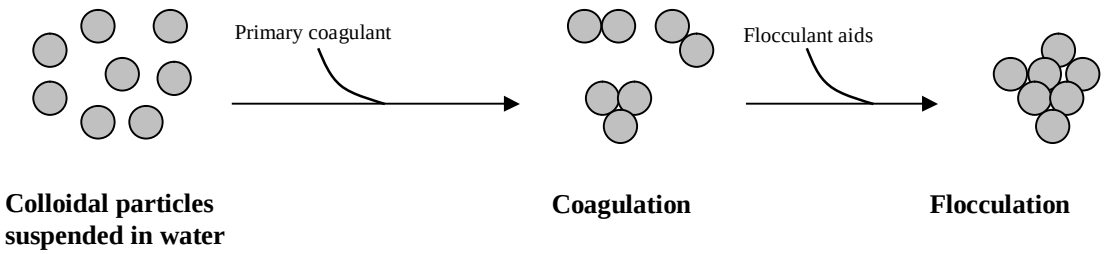


Figure 1.3: Diagram of coagulation-flocculation.

However, since there might be lack of agreement on the distinction between coagulation and flocculation, some authors prefer to use the term flocculation as a generic term covering all aggregation processes (Gregory and O'Melia, 1989).

1.1.2.1 Commonly used primary coagulants

Metals are commonly used as coagulants and they fall into two categories: alum based and iron based. Their popularity arises not only from their effectiveness as coagulants, but also from their ready availability and relatively low cost.

When a metallic salts such as aluminium sulphate[1] [$Al_2(SO_4)_3 \cdot 18H_2O$] or ferric sulphate [$Fe_2(SO_4)_3 \cdot 9H_2O$] are added to water, a series of reactions occur with water and the other ions present. Sufficient quantities of the chemicals must be added to water to exceed the solubility limit of the metal hydroxide and result in the formation of flocs. The resulting flocs will then adsorb on particles in the water.

The reaction of aluminium in water is complex, but a simple representation is given below:

Al^{3+} Ÿ $Al(OH)^{2+}$ Ÿ $Al(OH)_2^{+}$ Ÿ $Al(OH)_3$ Ÿ $Al(OH)_4^{ï}$

Briefly, all the water ions are hydrated to some extent. In the case of Al^{3+} and Fe^{3+}, there is a primary hydration shell with six octahedrally coordinated water molecules

[1] Aluminium sulphate is often referred as alum and ferric sulphate as ferric

$[Al(H_2O)_6]^{3+}$. Hydrolysis of such ions is often represented as a sequential replacement of the water molecules by hydroxyl ions, and can also be thought of as progressive deprotonation of water molecules in the primary hydration shell (Gregory and Duan, 2001). Since each step involves the loss of a proton, increasing the pH causes the equilibria to be shifted to the right. Aluminium hydroxide has low solubility in water and precipitation occurs at intermediate pH values. Further increase of pH causes the soluble aluminate ion to be formed (Gregory and Duan, 2001).

In natural alkaline water, the formation of an aluminum hydroxide floc is the result of the reaction between the acidic coagulant and calcium bicarbonate, as expressed by the equation below (EPA, 2002):

$Al_2(SO_4)_3 + 3Ca(HCO_3)_2 \leftrightarrow 2Al(OH)_3 + 3CaSO_4 + 6CO_2$

A similar sequence can be written for Fe^{3+}. In this case, the hydroxide is much less soluble than aluminium hydroxide (Duan and Gregory, 2003).

1.1.2.2 Mechanism of coagulation

Charge Neutralisation

As previously mentioned, colloidal particles are subject to interfacial phenomena which keep them as a stable solution. The principal mechanism controlling this stability is electrostatic repulsion (Montgomery and Engineers, 1985, Gregory and Duan, 2001, Tripathy and De, 2006). Oppositely charged ions in an electrical solution are attracted to the surface of a charged particle and can either be closely associated with the surface or distributed some way in the solution. This produces a diffuse cloud of ions surrounding the particle known as an electrical double layer (Tripathy and De, 2006).

This electrical double layer is divided into two parts: an inner layer (named Stern layer) and an outer layer (diffuse layer), as shown in **Figure 1.4**. The interactions between charged particles is governed predominantly by the overlap of diffuse layers (Gregory

and O'Melia, 1989, Tripathy and De, 2006), so the most important potential is that at the boundary between stern and diffuse layer, called the Stern potential.

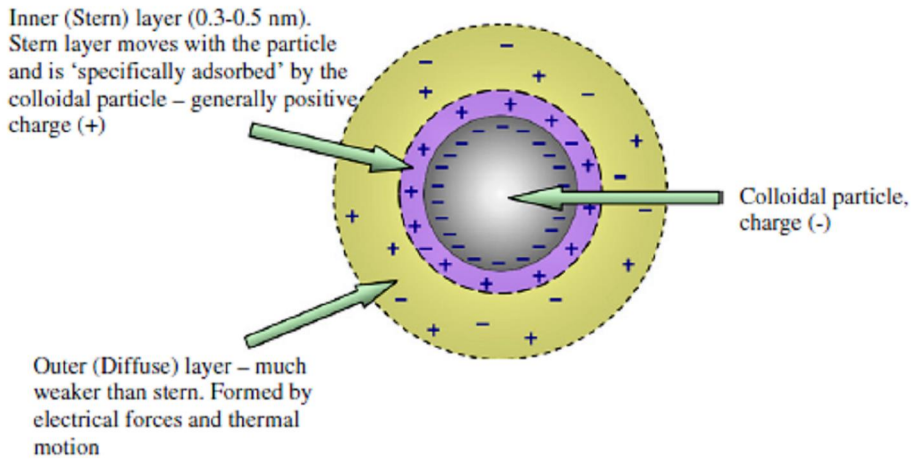


Figure 1.4: Electrical charge surrounding a colloidal particle (Pritchard et al., 2010a).

The colloidal particle is assumed to be negatively charged. Counter-ions are located in the inner layer. This is considered to be 0.3/0.5 nm which is the diameter of an hydrated counter-ion, while in the diffuse layer ions are distributed according to the influence of electrical forces and random thermal motion.

According to the DLVO theory (Derjaguin and Landau, 1993, Verwey and Overbeek, 1999), the stability of colloids is due to a balance between attractive and repulsive forces (van der Waals and electrical repulsion, respectively) between particles. When the kinetic energy of the particle is large enough to surmount the potential hump created between them created by the double layer, the particles would coalesce otherwise they would remain as a stable suspension (**Figure 1.5**)(Tripathy and De, 2006).

Practically to overcome the potential created by the double layer, it is necessary to increase the ionic strength. Indeed, the expression for the Debye-Huckel length (the distance over which significant charge separation can occur) is given as

$$K = \sqrt{\frac{4e^2 N \cdot I}{\varepsilon kT \times 10^3}} \qquad \textbf{Eq.1.1}$$

where, e is electronic charge, N is Avogadroôs number, I is the ionic strength, ε the electrical permittivity of the solvent, k the Boltzmann constant and T the absolute temperature.

From the **Equation 1.1**, the double layer thickness is approximately 1/K, therefore increasing the ionic strength decreases the double layer thickness (Bratby, 2006). This compression allows the approach of the colloidal particles to where the attractive forces predominate over electrostatic repulsive forces. In the presence of hydrolyzable metal ions, such as Al^{3+} and Fe^{3+}, the charge is neutralised by adsorption of these species onto the particle surface (Tripathy and De, 2006).

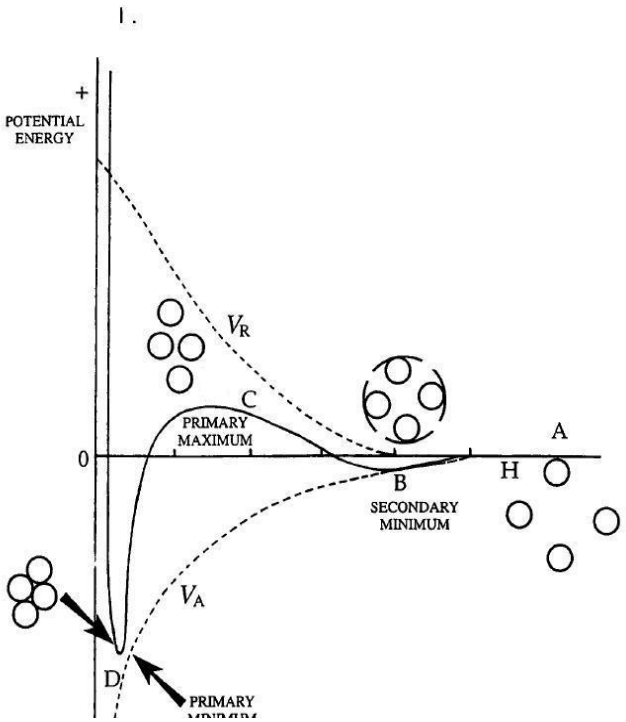


Figure 1.5: Potential energy diagram for the interaction of colloid particles adapted from Islam et al. (1995).

V_A is the energy resulting from the van der Waals attractive forces; V_R is the energy resulting from the electrostatic repulsive forces; at long particles distance the energy of the van der Waals and the electrical double layer have no effect on the particles (A). When the particles approach one another, there is an increase in V_A and V_R. As the V_R term increases more slowly than V_A at large inter-particle separations, there is then a slight domination of the attractive (B). As the inter-particulate distance decreases even further, V_R has a much stronger effect on the particles, resulting in a large energy barrier which inhibits the close approach of particles (C). This occurs until a critical distance is reached, where the van der Waals attractive forces dominate the repulsive electrical double layer forces, resulting in strong, irreversible coagulates (D).

Sweep flocculation (Colloid entrapment)

Although particles may be effectively destabilized by charge neutralisation, there should be a stoichiometric relationship between particle concentration and optimum coagulant dosage (Werner and O'Melia, 1968). At low particle concentrations, low coagulant dosages should be required, but under these conditions, the coagulation rate can be very low and it has been found that higher coagulant dosages are much more effective where extensive hydroxide precipitation occurs (Yukselen and Gregory, 2004). Thus, impurity particles can be enmeshed in a growing hydroxide precipitate and hence can be effectively removed from water by sedimentation. This process has become known as sweep flocculation, since particles are ñswept outò of water by amorphous hydroxide precipitate (Gregory and Duan, 2001, Yukselen and Gregory, 2004).

Bridging mechanism

Bridging occurs when a coagulant (typically a long chain polymer (Gregory and Barany, 2011)) forms threads or fibres which attach to several colloids, capturing and binding them together. In practice, a polymer adsorbs to a particle. The unattached polymer segment forms ñloopsò, ñtrainsò and ñtailsò into the liquid phase.

Subsequently, another particle attaches to another segment of the polymer to form a bridge (**Figure 1.6**). Generally several polymer chains will form bridges between adjacent particles and more than one particle will be attached in the resultant floc (Hocking et al., 1999).

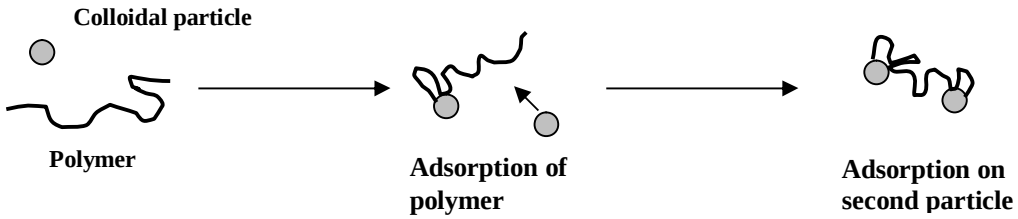


Figure 1.6: Bridging flocculation (Hocking et al., 1999).

1.1.2.3 The Jar Test

The jar test is one of the most common pieces of bench test apparatus for the study of coagulation-flocculation (Farrow and Swift, 1996, Szpak et al., 1996). This test is used to investigate the type of coagulant used and to identify the optimum concentration, as well as pH. A typical jar test consists of six identical jars with a stirrer submerged in each (**Figure 1.7**). The stirrers are connected to a motor to ensure control over stirring speed. Each jar contains the same volume of water to be treated, but a different dose of coagulant is added. The suspensions are mixed rapidly, slowly and then are allowed to sediment. These three stages correspond to the fundamental process of dispersing the coagulant onto the particles surface, gently sweeping the colloidal particles together to form large flocs and allowing the flocs to settle down (EPA, 2002, Tripathy and De, 2006). At the end of the settling period, test samples are drawn from the jars and turbidity of supernatant is measured. A plot of turbidity against coagulant dose gives an indication of the optimum dosage.

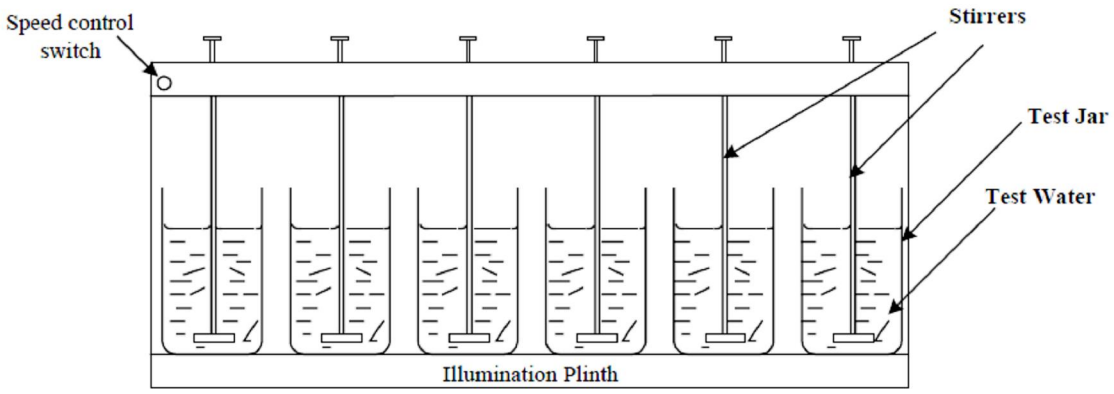


Figure 1.7: A typical jar test apparatus.

1.1.2.4 Factors affecting coagulation

The characteristics of water, such as pH and ionic strength, along with dosing and mixing of coagulants, are important achieving a good coagulation. The main factors affecting the particle-coagulant interactions are reported.

pH effect

pH has an influence on the net charge of the flocculant, but also on the particles within water. For example, the effective species of inorganic coagulants can be solvated metal ions, which affect flocculation through double-layer compression. Thus, increasing the pH of the solution would affect the charge of these species and the mechanism of action (Tripathy and Singh, 2001). In addition, a common turbidity model in coagulation tests is a suspension containing kaolin. Kaolin usually exists as hexagonal platelets with different chemistry occurring at the basal face than at the edge face. The basal face has a net negative charge and is usually unaffected by pH. However, the edge face is pH-dependent due to the presence of the metal oxide bonds which are able to neutralize their charge by dissociating water molecules to produce metal hydroxide (Chukwudi, 2008). Having a pI around 5.5, the kaolin edge displays positive charges and negative charge in acidic and basic pH values, respectively (Hocking et al., 1999, Chukwudi, 2008), as shown in **Figure 1.8**.

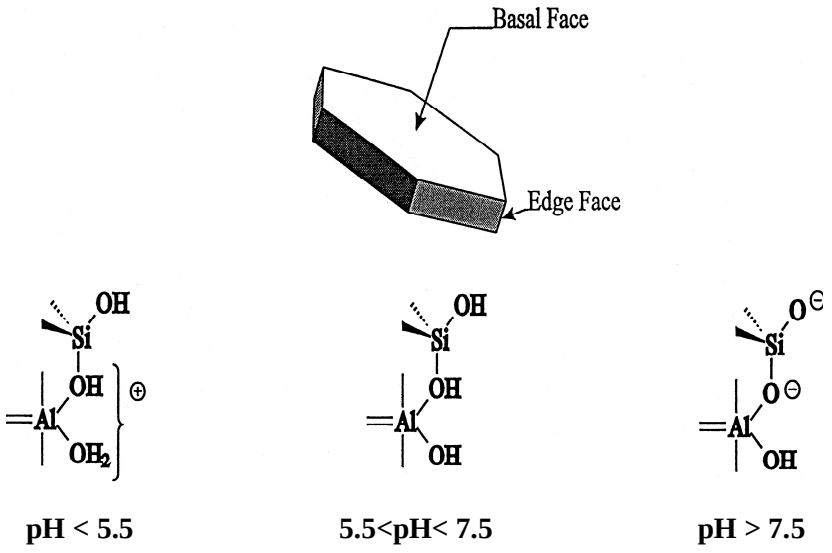


Figure 1.8: Kaolin clay platelet and pH related surface chemistry surface at the edge (Hocking et al., 1999).

Alkalinity

Alkalinity refers to the acid-neutralizing capacity of water, and is a general indication of a waterôs buffering capacity. Alkalinity and pH are related and is needed to provide anions, such as -OH for forming insoluble compounds to precipitate them out. Metal coagulants are acidic, and coagulant addition consumes alkalinity. For low alkalinity waters, coagulant addition may consume all of the available alkalinity, depressing the pH to values too low for effective treatment. Thus, alkalinity is added as hydroxides or bicarbonates. High alkalinity waters may require high coagulant additions to depress the pH to values favourable for coagulation.

Ionic Strength

As explained **Equation 1.1**, ionic strength helps to compress the double layer of particles and promotes the charge neutralisation where flocculant and surface are oppositely charged (Gregory and O'Melia, 1989, Tripathy and Singh, 2001). However, ionic strength can also affect the conformation of polymer by compressing the charge sites. This brings to a folding of the polymer tail which is not able to expand in solution and, consequently, to remove particles (Tripathy and Singh, 2001).

Temperature

Temperature significantly affects coagulation operations. Low temperature affects coagulation and flocculation processes by altering coagulant solubility, increasing water viscosity and retarding the kinetics of hydrolysis reactions and particle flocculation. On the other side, higher temperature makes faster and the more effective the coagulation.

Mixing and dosing

The degree of flocculation achieved can be markedly affected by dosing and mixing conditions. It is well know that excessively high dosage of coagulant might promote restabilization of charge and prevent coagulation (Hocking et al., 1999, Bratby, 2006). On the other hand, mixing is a key step that might determine whether or not a good flocculation occurs because it will ensure collisions between particles and floc formation. Mixing of coagulant involves quite intense agitation (órapid mixö) for a short time, followed usually by a longer period of gentler mixing. The purpose of the second phase is to promote collisions of particles and hence floc growth. However, this can not be too rapid as fast moving water can limit flocculation and break apart the larger flocs that form (Rossini et al., 1999, Duan and Gregory, 2003).

1.2 Water treatment and potential risks for human health and environment

1.2.1 Effects of metal coagulants on human health

Metallic coagulants such as alum and its derivates are frequently employed due to their proven performance and cost effectiveness (Raghuwanshi et al., 2002). However, in recent years, the use of alum as a coagulant raised some concerns about human health and even the World Health Organisation (WHO) analysed the potential risks of using alum as a coagulant in drinking water treatment and stated that the concentrations of this metal should be Ò0.2 mg/L in treated drinking water (WHO, 2003).

In the first instance, the use of alum as coagulant in water treatment increases its concentration in finished water (Barnett et al., 1969, Miller et al., 1984), so that it may result in a decrease in the effectiveness of disinfection due to enmeshment of microorganisms by alum floc (Hoff, 1977).

Moreover, alum exposure at high concentrations has been proven to have a profound effect on the neurological systems of organisms (Flaten, 2001) and can exert toxicity on neurological, skeletal and haematological systems in humans with advanced renal failure (Nayak, 2002, Zatta et al., 2003) .

It is well accepted that alum is a causal agent in dialysis encephalopathy, a fatal brain disorder occurring in some patients with chronic renal failure (Alfrey, 1993). In these patients, tissue accumulation of aluminium to levels high enough to cause toxicity is mainly due to a combination of (1) high exposure, since alum goes directly into the bloodstream (thus bypassing absorption in the gastrointestinal tract, which is generally below 1%) and (2) these patientsô lack of kidney function, which is the main excretion route for aluminium (Ganrot, 1986).

Several studies also linked large amounts of aluminium intake to various neurodegenerative diseases including Alzheimerôs disease (AD) and senile dementia

(Rondeau et al., 2000, Rondeau et al., 2009), as well as amyotrophic lateral sclerosis, Parkinsonism dementia in the Kii Peninsula and Guam and Gulf War syndrome (Kawahara and Kato-Negishi, 2011). In particular, a possible association between aluminium and the pathogenesis of AD has been discussed for several decades (Crapper et al., 1973, Martyn et al., 1989, Flaten, 1990, Doll, 1993, Mclachlan, 1995, Rondeau et al., 2000). AD is a neurodegenerative cerebral disorder defined as a progressive deterioration of cognitive function and loss of autonomy.

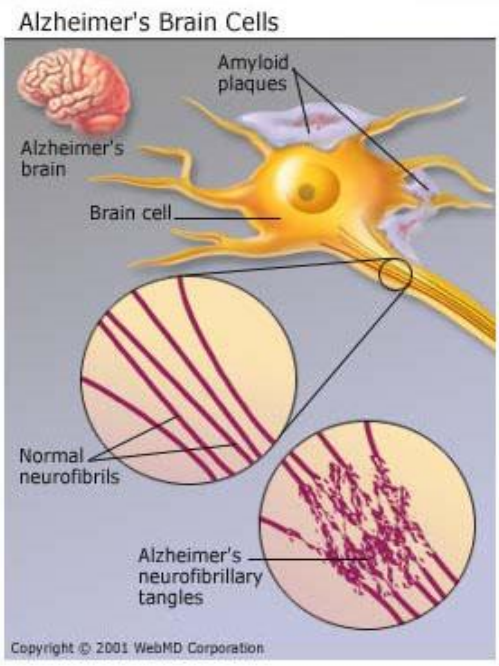


Figure 1.9: Diagram of modifications occurring in an Alzheimerôs brain (WebMD, www.webmd.com).

The gradual loss of brain function that characterizes Alzheimer's disease seems to be due to nerve cells develop tangles (neurofibrillary tangles, NFT) and protein deposits known as beta-amyloid plaques build up in the brain

The pathological hallmarks of AD are the deposition of extracellular senile plaques, intracellular neurofibrillary tangles (NFTs) and the selective loss of synapses and neurons in the hippocampal and cerebral cortical regions (Kawahara et al., 2001), **Figure 1.9**.

The hypothesis that aluminium might be an environmental contributor to the pathogenesis of AD (called ñaluminium hypothesisò) was proposed in 1965 when Klatzo et al. (1965) found that the intracerebral administration of alum to experimental animals induced neurofibrillary degeneration and the appearance of tangle-like structures that were similar to the NFTs found in brains of AD patients. Some years later, Crapper et al. (1973) reported increased levels of aluminium in brains of AD patients, thus supporting this hypothesis.

The possibility of such an association was also suggested by the presence of aluminium in senile plaques and neurofibrillary degeneration, two histologic lesions that are characteristic of the disease (Candy et al., 1992, Edwardson et al., 1992, Yumoto et al., 2009). Moreover, it was pointed out that alum promotes amyloid β-peptide (Aβ) aggregation in vitro (Bondy and Truong, 1999, Kawahara et al., 2001), another characteristic of the disease (**Figure 1.9**).

Aluminium is present in all food-stuffs, drinking water and other beverages as well as dust in the air (WHO, 2003). At an average adult intake of alum from food of 5 mg/day and a drinking-water alum concentration of 0.1 mg/litre, the contribution of drinking-water to the total oral exposure to alum will be about 4%. This data led to a plethora of epidemiological studies about the association between aluminium and AD, with emphasis on drinking water (Doll, 1993, Mclachlan, 1995, McLachlan et al., 1996, Flaten, 2001, Rondeau et al., 2009). Even though the debate is still open, these studies demonstrated that high concentrations of aluminium in drinking water (equal to 100 μg/L or greater) are associated with a higher risk of developing AD.

An interesting study by Campbell et al. (2004) correlates high concentrations of aluminium to an increased level of Nuclear factor-κB (NF-κB) as well as tumour necrosis factor-α (TNF-α) and interleukin 1α (IL-α), all proteins involved in inflammation. This finding is noteworthy since inflammatory processes are basally

elevated within aging brain and this may provide the substrate upon which aluminium can act and thus accelerate the progression of age-related neurodegenerative disease.

Further evidence of the correlation between alum and AD is represented by the studies of Walton (2012). This showed translational aging rats to which high alum doses were administered had a significantly cognitive deterioration in old age and the metal was accumulated in the entorhinal cortical and hippocampal cells resulting in microtubule depletion and dendritic dieback. Analogous pathological change in humans leads to the destruction of the perforant pathway and AD.

1.2.2 Effects of metal coagulants on environment

Along with human health concerns, it has to be considered that alum and alum based coagulants create potential hazardous sludge containing residual aluminium which may cause side effects when discharged into the open water course, landfill or spread on agricultural land (Ahmad et al., 2006, Renault et al., 2009, Zheng, 2010).

This use may have adverse effects on plant growth. It has been studied that aluminium in acidic soil is solubilised into ionic forms which may be very toxic to plants causing inhibition of root elongation by destroying the cell structure of the root apex and thus affecting the water and nutrients uptake (Upjohn et al., 2005, Zheng, 2010), **Figure 1.10**. Moreover, aluminium reduces the availability of calcium and magnesium and phosphorous by immobilising them (Upjohn et al., 2005).

This can affect the crop yields and have devastating effect on farming industry.

Figure 1.10: The effect of aluminium toxicity on the roots of wheat plants.
From left to right the plants were grown in solutions containing 0, 5 and 10 ppm aluminium (Upjohn et al., 2005)

Aluminium and iron contaminated water can seep into surface and ground waters, contaminating rivers and lakes and affecting fish and other aquatic organisms. For example, Sotero-Santos et al. (2005) demonstrated that both alum and ferric sludge caused chronic toxicity characterized by low fecundity on *Daphnia similis*, a planktonic crustacean, and fish (Sotero-Santos et al., 2007, Mortula et al., 2008). It has been determined that fish tend to be more sensitive to aluminium toxicity than aquatic invertebrates (RobertYokel and Golub, 1997). An interesting study performed by Dussault et al. (2001) on rainbow trout reported that alum accumulates in the gills in

direct proportion to the concentration in water. In addition, a decreased sodium, calcium and chlorine blood levels along with an increased potassium hematocrit, hemoglobin, glucose, and lactate levels were detected, indicating increasing ionoregulatory disturbance with increasing aluminium concentration. In severe cases, the blood pressure of the trout can rise and it can go into heart failure. This problem can lead to mass fish deaths in heavily contaminated water.

Thus, it has become a necessity to develop a more efficient, **environmental friendly** coagulant which has similar potential as aluminium coagulants with an enhanced economic profile.

1.3 Water treatment by plants and plant-based material

Plants and plant-based materials are good candidates for developing novel technologies for water treatment as they are a renewable resource, cheap and environmentally friendly.

Indeed, the use of plant materials for water treatment is not a novel concept: in particular, plant seeds have been used by rural societies to treat river water prior to consumption: as mentioned previously, crushed almonds and nirmali (*Strychnos potatorum*) seeds were used by the Egyptians and Indians respectively in the past to clarify water. Nowadays rural populations of Sudan used crushed seeds of *Moringa Oleifera* lam as coagulants (Jahn and Dirar, 1979), while nirmali seeds are still in use in some rural villages in India (Babu and Chaudhuri, 2005). A study performed in Tanzania showed that in local villages the population use seed powder from *Vigna unguiculata*, *Voandzeia subterranea*, *Arachis hypogaea*, *Vicia faba*, and *Parkinsonia Aculeate* (all of them belonging to the family of Fabaceae) to purify water.

Coagulation activity was also reported from other plant materials such as *Prosopis juliflora* (mesquite bean), *Cactus latifaria* (Diaz et al., 1999) and *Opuntia* spp (cactus) (Miller et al., 2008). Coagulant agents were also found in chestnut (*Aesculus hyppocastanum*) and acorn (*Quercus robur*) by Sciban et al. (2009).

However, a great interest has risen from the study of seed materials as coagulants. Sanghi et al. (2002) showed that seeds of *Cassia angustifolia* (belonging to the family Fabaceae) are good coagulants for the decolourisation of acidic and direct dye solutions, while grape seeds (*Vitis vinifera*) were found to induce fast decolourisation followed by coagulation and resulted in the removal of cationic dyes (Jeon et al., 2009).

Of special interest, though, are the aforementioned Moringa seeds, whose coagulant characteristics are well known (Gassenschmidt et al., 1995, Muyibi S, 1999, Pritchard et al., 2010b).

1.3.1 Oleaginous plant seeds and press-cakes in water treatment

Oleaginous plants are all those species with high oil content in their seeds. In seeds, the plant stores oil as main source of carbon and energy for the germination and growth of seedlings into small and discrete organelles called oil-bodies (OBs) (Tzen and Huang, 1992a). Each OB contains a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids (PL) embedded with proteins termed oleosin (Tzen et al., 1992b).

In Europe, the principal oleaginous plants are oilseed rape, sunflower, olive, soya-bean and flax. This group of plants are cultivated essentially for oil production, for either nutritional or industrial consumption since they are also rich in proteins. After the oil extraction, the protein-rich residues are transformed into oil-seed cakes, called press-cakes (PC) and used as animal feed.

1.3.1.1 *Moringa oleifera* as coagulation agent

Moringa oleifera is a tropical plant belonging to the family of *Moringaceae* (**Figure 1.11**). Many parts of the tree are used as traditional medicines, the seeds contain up to 40% by weight of quality edible oil (greater than 80% unsaturated fatty acid content (Mohammed et al., 2003)) and the seeds (and oil free presscakes) yield proteins capable of acting as effective coagulants in water and wastewater treatment.

Figure 1.11: *Moringa oleifera* adapted from Pritchard et al. (2010a).

The *M. oleifera* tree can produce about 2000 seeds per year. This number of seeds would be able to treat about 6000 L of water using a 50 mg/L dose. Trees can, however, be cultivated to produce about five to ten times this (i.e. 10000-20000 seeds). This would produce up to 60000 L of treated water per year. Simplistically it has been estimated that a tree could be cultivated in a rural village to provide enough seeds for four families to use for a whole year (Pritchard et al., 2010a).

In terms of water treatment applications, *M. oleifera* seeds in diverse extracted and purified forms has proved to be effective at removing suspended material (Folkard et

al., 1995, Muyibi and Evison, 1995a, Ndabigengesere and Narasiah, 1998b), generating reduced sludge volumes in comparison to alum (Folkard et al., 1995, Ndabigengesere et al., 1995), softening hard waters (Muyibi and Evison, 1995b) and acting as effective adsorber of cadmium (Sharma et al., 2006).

It has been shown that the active components of the *M. oleifera* seeds is a cationic peptide of relatively low molecular weight (6ï 16 kDa) with an isoelectric pH value of 10, named MO 2 (Gassenschmidt et al., 1995, Ndabigengesere et al., 1995). It has been proposed that MO 2 is in its native form a dimeric protein linked by disulphide bonds and the coagulation mechanism occurs through destabilisation of the negatively charged colloids combined with a neutralisation or bridging mechanism (Ndabigengesere et al., 1995). Interestingly, this peptide possesses also an antimicrobial effect as shown in the study performed by Ghebremichael et al. (2005).

In 2002, a recombinant peptide called FLO, corresponding to the sequence of one of the MO 2 polypeptides, was produced and was demonstrated to have flocculation and antibiotic activity (Suarez et al., 2003). Recently, other peptides from moringa seed extracts have been described as having flocculation activity (Doerries, 2005), indicating that the family of flocculating proteins might be large.

1.3.1.2 Oleaginous seeds by product (presscakes) in water treatment

Moringa is the best example of oleaginous seed applied to a water treatment process, but oleaginous seeds may have a greater impact on improving this field.

These seeds are particularly interesting as they are grown for their oil content, making the oil pressing by-product very rich in residual proteins. PC, often considered as a waste, may thus constitute a possible candidate for developing novel sustainable water treatment technologies as a source of coagulants and/or novel biosorbents.

The extraction of coagulants from moringa PC was successfully achieved by Doerries (2005), while their use as biosorbents is a recent and expanding field.

The presence of hydrophobic organic pollutants (HOP), highly toxic and hard to remove from the environment represents a serious concern for the remediation of contaminated water. HOP includes many categories of chemical compounds such as poly-aromatic hydrocarbons (PAH) or other aromatic compounds, dyes, drugs and many pesticides.

The application of PC to HOP removal from aqueous solutions seems appropriate because of the residual oil left behind. Indeed, the adsorptions of atrazine (a moderately hydrophobic pesticide) into PC from rapeseed, moringa and soybean show an efficiency of 77, 95 and 84%, respectively (Boucher et al., 2008a).

Moreover, thanks to its composition (cellulose, ligninin and hemi-cellulose) PC harbours many potential metals binding functional groups, which makes them a promising biosorbent for removing metals from contaminated water: for example, cadmium and copper, two potentially hazardous metals (Spurgeon et al., 2003, Akar and Tunali, 2006), were removed by PC originating from three oleaginous species (rapeseed, moringa and soybean) (Boucher, 2006, Tofan et al., 2011), chromium was also removed using Jatropha oil cake (Garg et al., 2008).

Efforts to remove dyes, potentially hazardous for the environment and human health, by replacing the coal-based activated carbon with activated carbon from renewable sources as oil palm fibres or sunflower PC has been achieved (Tan et al., 2007, Karagoz et al., 2008).

The successful application of oleaginous seeds and PC suggests an inexpensive, environmentally friendly method applicable to water treatment process.

1.4 Aim and objective of the thesis

The aim of the present work is to investigate the possible application of extracts from seeds and PC in water treatment (**Figure 1.12**). Special attention is given to those seeds (and PC) that are commonly found in Europe (such as rapeseed, sunflower and hemp) as an alternative source to moringa.

1. Do OBs extracted from a range of plants seeds have flocculation activity under different conditions of pH and ionic strength?
2. Development and optimisation of a quantitative on-line method using a jar test apparatus connected to turbidity-meters in order to measure the kinetics of flocculation.
3. Do proteins extracted from a range of PC have flocculation activity as well?
4. Is it possible to remove a range of drugs from water using OBs?

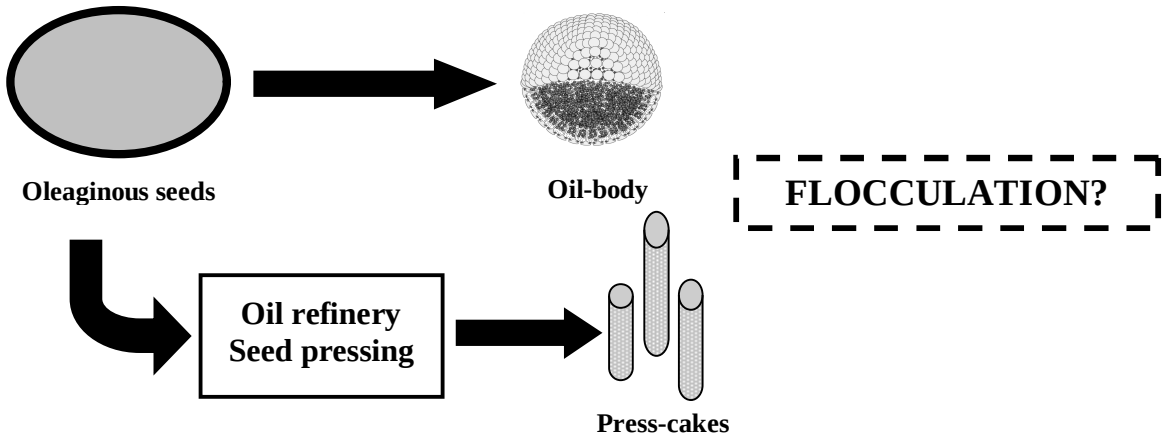


Figure 1.12: Schematic representation of the aims of the thesis.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Seeds and Press-cakes

Earth Oil: Sunflower (*Helianthus annuus*) press-cakes, moringa (*Moringa oleifera*) press-cakes.

Harnettôs Oil: Rapeseed (*Brassica napus*) seeds and press-cakes, hemp (*Cannabis sativa*) seeds and press-cakes.

Quinns of Baltinglass: Linseed (*Linum usitatissimum*) seeds, sunflower (*Helianthus annuus*) seeds.

Syngenta: Soybean (*Glycine max*) seeds.

2.1.2 Reagents

Acorn Water: Aluminium sulphate 8% and ferric sulphate 11%.

BioRad: Kaleidoscope Polypeptide Standards (Cat. no. 161-0325), 16.5%, Mini-PROTEAN Tris-Tricine Precast Gel, 10 well, 30 µl (Cat. no. 456-3063), Leammli Buffer (Cat. no. 161-0737).

Hach Lange: 1000 NTU StablCal® (Cat. no. 26606-49), 100 NTU StablCal® (Cat. no. 26602-49), 20 NTU StablCal® (Cat. no. 26601-42).

Novex®: Novex ® NuPAGE® 4-12% Bis-Tris Precast Gel (Cat. no. NP0322PK2), SimplyBlueÊ SafeStain (Cat. no. LC6065).

Sigma-Aldrich: Ammonium acetate (Cat. no. 17836), Bovine Serum Albumin (Cat. no.A8022), Carbamazepine (Cat. no. C4024), Casein (Cat. no. C3400), Chloroform

(Cat. no. C2432), Clofibric Acid (Cat. no. 90323), Brilliant Blue G (Cat. no. B2025), Diclofenac sodium salt (Cat. no. D6899), Diethyl ether (Cat. no. 179272), Gelatin from cold fish skin (Cat. no. G7041), ɔ-Globulins (Cat. no. G5009), Formic Acid (Cat. no. F0507), Furosemide (Cat. no. F4381), Hexane anhydrous (Cat. no. 296090), Hydrochloric Acid, 37% (Cat. no. 320331), Lactalbumin (Cat. no. L7252), Linseed oil (Cat. no. 430021), Magnesium Chloride (Cat. no. M8787), Monobasic Phosphate (Cat. no. S9638), Methanol CHROMASOLV® (Cat. no. 34860), Metoprolol tartrate salt (Cat. no. M5391), Phenylmethanesulfonyl fluoride solution ~0.1 M in ethanol (Cat. no. 93482), Potassium Chloride (Cat. no.60130), Proteinase K from *Tritirachium* album lyophilized powder, Ó30 units/mg protein (Cat. no. P6556), Rapeseed oil (Cat. no. 83450), Sodium Chloride (Cat. no. S7653), Sodium Phosphate Dibasic Anhydrous (Cat. no. 71640), Sodium azide (Cat. no. S2002), Soybean oil (Cat. no. S7381), Sulfamethoxazole (Cat. no. 76177), Tris (Cat. no. T1503), UREA (Cat. no. U5378), Total Protein Kit, Micro Lowry, Petersonôs Modification (Cat. no. TP0300), Warfarin (Cat. no. UC213).

Thermo Scientific: Protein assay kit, BCA Thermo Scientific Pierce (Cat. no. 10741395)**,** Calcium Chloride Dehydrate (Cat. no. 10050070), Sodium acetate anhydrous (Cat. no. 10010500), Kaolin (Cat. no. 10772441), Acetic acid glacial (Cat. no. 10384970)**.**

2.2 Methods

2.2.1 Extraction of oil-bodies from rapeseed and hemp seeds

Oil-bodies, defined natural submicron capsules (NSC) in this thesis, were extracted from intact dried seeds of the rapeseed and hemp plant using an aqueous extraction-method which has being previously described (Iwanaga et al., 2007).

Briefly, 30 g of mature seeds were homogenized in 100 ml of buffer (homogenization) solution containing 3 mM $MgCl_2$ and 100 mM Tris-HCl, pH 8.6, using a Polytron homogenizer (PT 2100, Kinematica, Luzern- CH). Seeds were homogenized for 20 s at low speed and then for a further 40 s at high speed. The resultant slurry was filtered through three layers of cheesecloth (Mediswab N/S swab, Flemings, Ireland), and the remaining filter cake pressed and the filtrate collected and centrifuged (Avanti®J-26 XP centrifuge, Beckman Coulter, Fullerton, CA, USA) at 20,000 rpm for 30 min in 50 mL centrifuge tubes (Beckman Coulter, Fullerton, CA, USA). The resulting cream layer, containing the NSC, was collected with a spatula and homogenised in a chilled buffer solution (10 mM Tris-HCl buffer, pH 8.6) using a Polytron homogeniser for 20 s at low speed. The homogenized sample was recovered in a clean centrifuge tube and the centrifugation step repeated. The collection of the cream layer, suspension, and centrifugation represents one complete wash cycle, for all samples a total of three wash cycles were performed to purify NSC to a very high level. A hexane wash was then carried out to remove defective NSC in which the resultant cream layer formed after three wash cycles was re-suspended in chilled buffer and diluted at a ratio of 1:2 in hexane (Tzen et al., 1997). The sample was then centrifuged; the upper hexane layer was removed and the interface layer containing NSC collected, re-suspended in chilled buffer and centrifuged. The final cream layer of NSC, sometimes referred to as the ñpadò, was collected and re-suspended in chilled buffer at a concentration of 0.5 g/mL.

NSC, within the pad, were stored until required in 0.02% (w/v) sodium azide at 4 ºC to prevent bacterial contamination.

2.2.2 Extraction of oil-bodies from linseed and soybean seeds

Linseed and soybean NSC were extracted following the method described by Iwanaga et al. (2007) with the following modifications.

Seeds were soaked overnight (45 g) in buffer solution (10mM Tris HCl, pH 8.6) at 4°C. The soaked seeds were homogenised in 200 mL of homogenisation buffer (100mM Tris-HCl, 3mM $MgCl_2$, pH 8.6) with a blender for 2 mins and then with a Polytron homogenizer (PT 2100, Kinematica, Luzern- CH) for 20 s at low speed and 40 s at high speed. The resultant slurry was filtered through three layers of cheesecloth (Mediswab N/S swab, Flemings, Ireland) and the remaining filter cake pressed and the filtrate collected and centrifuged (Avanti®J-26 XP centrifuge, Beckman Coulter, Fullerton, CA, USA) at 20,000 rpm for 30 min in 50 mL centrifuge tubes (Beckman Coulter, Fullerton, CA, USA).

The resulting cream layer was collected and dispersed (using the Polytron) in 30 mL of chilled buffer (10mM Tris-HCl, pH8.6) and centrifuged at 20,000 rpm for 30 min. The collection of the cream layer, suspension, and centrifugation represents one complete wash cycle, for all samples a total of three wash cycles were performed to purify NSC to a very high level. The cream layer was then resuspended in chilled buffer and diluted (1:2) with hexane to remove defective NSC (Tzen et al., 1997. The upper hexane layer was decanted and the cream layer recovered, resuspended in chilled buffer and centrifuged at 20,000 rpm for 20 min. The final cream layer was collected in chilled buffer containing 0.02% NaN_3 to give final concentration of 0.5 g/mL.

2.2.3 Extraction of oil-bodies from sunflower seeds

Sunflower seeds (30g) were homogenised with the blender and the Polytron in ultra-pure (UP) water (1:5 w/v, 0.02% w/v sodium azide) (Millichip et al., 1996). The slurry was filtered through three layers of cheesecloth (Mediswab N/S swab, Flemings, Ireland), the remaining filter cake pressed and the filtrate collected and centrifuged (Avanti®J-26 XP centrifuge, Beckman Coulter, Fullerton, CA, USA). The resulting cream layer was collected and dispersed (using the Polytron) in 30 mL of UP water and centrifuged at 20,000 rpm for 30 min. The pad was washed with UP water and centrifuged twice more. The cream layer was resuspended in chilled UP water and diluted (1:2) with hexane to remove defective submicron-capsules (Tzen et al., 1997) and then centrifuged at 20,000 rpm for 20 min. The upper hexane layer was decanted and the cream layer was recovered, resuspended in UP water and centrifuged at 20,000 rpm for 20 min. The final cream layer was collected in UP water containing 0.02% NaN_3 to give a final concentration of 0.5 g/ml.

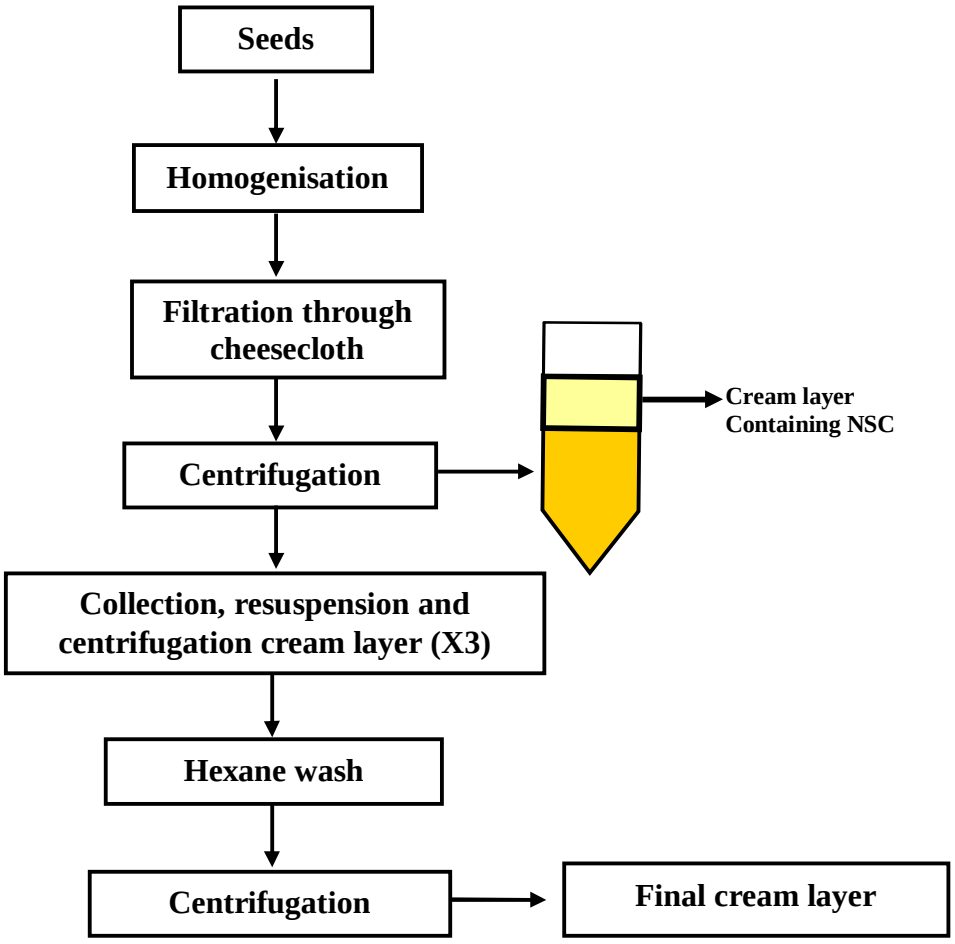


Figure 2.1: Schematic representation of the NSC extraction procedure.

2.2.4 Urea wash to remove contaminant proteins

To determine if NSC extracted from seeds are responsible for the flocculation activity, a urea wash was performed on the cream layer extract (after the first homogenization and centrifugation steps) to dissociate and remove peripheral proteins which might be present in the NSC preparation but not embedded within the membrane (Tzen et al., 1997). The cream layer was suspended in 8 M urea and agitated (IKA platform shaker, Ireland) at 60 rpm for 10 min at room temperature. The suspension was centrifuged at 7500 rpm for 45 min using Amicon®-Ultra Centrifugal Filter Devices, 3 kDa (Millipore, Ireland) to remove all urea and any residues. The cream layer was collected,

re-suspended in chilled buffer and centrifuged twice using Amicon®-Ultra Devices, 3 kDa. A hexane wash as above was then performed to remove any defective NSC.

2.2.5 Oleosin extraction

Defatted NSC were prepared by a diethyl ether extraction (Tzen et al., 1997). NSC within the fat pad were diluted to a ratio of 1:3 in diethyl ether and centrifuged at 10,000 rpm for 15 min (Centrifuge 5415 R, Eppendorf, Germany). The upper diethyl ether layer was removed with an Eppendorf pipette while the lower layer consisting of the oleosins and phospholipids, was collected and stored at -80ºC for further investigations.

2.2.6 Protein determination in NSC

Diluted samples of the final cream layer were analysed for total protein using a Total Protein assay kit, Micro Lowry, Peterson's Modification following the manufacturer's instructions, using bovine serum albumin (BSA) as standard (Sigma-Aldrich, Dublin, Ireland), **Appendix A**.

2.2.7 Oil determination of NSC

The oil content into NSC suspensions was evaluated by diethylether extraction that yield defatted NSC (Tzen et al., 1997). A quantity of 0.2 mL NSC was mixed with 1.2 ml of diethylether (1:6) in Eppendorf tubes. After vortexing, the suspension was centrifuged for 10 min at 13000 rpm (Eppendorf Centrifuge 5415 R, Eppendorf, Germany), the upper diethylether layer was carefully sampled and evaporated before finally weighing the residual oil. Extractions were repeated three times, all weights were added together to obtain the oil content in the submicron-capsules suspension. A

standard curve between the volume of oil and the weight of oil was previously done. For all the LLE experiments the amount of 0.1 mL of oil into NSC was used.

2.2.8 Water determination of NSC

The water amount was determined gravimetrically. A sample of 0.2 mL of NSC was placed in an eppendorf tube and evaporated in a desiccator under vacuum for 24h. The difference between the initial weight and final weight was used to determine the amount of water present in a suspension containing submicron-capsules.

2.2.9 Proteinase K treatment

A defined amount of NSC was incubated with 8 U of Proteinase K in 25 mM Tris HCl pH 8, 150 mM NaCl, 2mM $CaCl_2$ for 30 min at 37ºC. The reaction was stopped with 5mM PMSF and then this solution was used for experiments. The dilution introduced by this solution was taken into account.

2.2.10 Extraction of soluble proteins from PC

PC were ground to a fine powder using a blender and stored in the dark at room temperature. Press-cake powder (30 g) was added to 150 mL of mixed salt extraction buffer (411 mM NaCl, 10 mM KCl, 7 mM $CaCl_2$ and 20 mM $MgCl_2$) and gently stirred (50 rpm) for 1 h. The suspension was then centrifuged at 5000 rpm (Thermo Electron FL 40R centrifuge, CD1 rotor, Thermo Scientific, France) for 15 min and decanted through two layers of cheesecloth (Mediswab N/S swab, Flemings, Ireland). The supernatant was transferred to a Duran bottle, sealed and incubated at 85 °C for 5 min in a water bath. The suspension was allowed to cool at room temperature, centrifuged for 5 min at 5000 rpm and filtered through a 0.2 μm filter using a syringe. The filtered extract was finally stored at room temperature in the dark.

2.2.11 Protein determination in PC extracts

To determine the total protein content of the PC extracts the bicinchoninic acid (BCA) method (Thermo Scientific, USA) was used according to manufacturerôs instructions, using bovine serum albumin (BSA) as standard.

2.2.12 SDS-PAGE

The samples to analysed were precipitated with methanol/chloroform/water precipitation (Wessel and Flugge, 1984) with the following modifications: 100 µg of protein was dissolved in 400 µL of methanol, 200 µL of chloroform and 400 µL of water and centrifuged for 5 min at 8000 rpm (Centrifuge 5415 R, Eppendorf, Germany). The upper layer was carefully removed and discarded, 600 µL of methanol was added and centrifugation repeated at 13000 rpm for 30 min. The supernatant was removed and the pellet air dried for 10 min. Finally, the pellet was resuspended in Leammli buffer (BioRad, UK) and boiled for 10 min at 90ºC.

A total of 15 µg of protein was loaded onto a 16.5% Mini-PROTEAN® Tris-Tricine precast gel (BioRad, UK) and run for 180 min at 120 Volts. After electrophoresis, the gel was stained with Brilliant Blue colloidal following manufacturerôs instructions (Sigma-Aldrich, Dublin, Ireland)

2.2.13 Atomic force microscope (AFM)

The NSC suspension was poured onto microscope glass slides, incubated for adsorption for approx. 20 min, washed with ultraclean water and dried in a nitrogen flow leading to a linear surface coverage of oil-bodies on the slide. Hence, the time searching for groups or single NSC could be minimized by combining AFM with optical microscopy. Suitable capsule samples could then be chosen in transmitted light DIC contrast prior to scanning. Optical microscope images were obtained at room temperature in

combination with an Asylum Research AFM-3D instrument. The AFM head was placed onto a Zeiss Axiovert 200 inverted optical microscope. AFM analysis was relatively slow since the NSC were delicate and easily damaged mechanically if the force exerted by the AFM tip was too high. Microscope images were obtained in intermittent contact mode with standard silicon non-contact cantilevers (NCH) provided by Asylum Research, UK. Resonance frequency was approx. 300 kHz and spring constant of 40 N/m. Digital resolution was 512 x 512 pixels. The tip radius of cantilever was less than 10 nm and the opening angle between 40° and 50° according to the manufacturer. The applied force between the cantilever tip and oil-bodies was minimised in order to alleviate the influence of the AFM tip on the capsule surface topography during scanning. Measurements were started by scanning a random area of 1μm. These images were used to evaluate the morphology of NSC. Scan size was decreased gradually until NSC could be viewed clearly.

2.2.14 Zeta potential and particle size analysis of NSC

NSC were diluted 1:1000 using DI water (pH 6).Diluted samples were injected directly into a folded capillary cell (DTS 1061, Malvern Instrument, Worcester, UK). The Zeta potential was then determined by measuring the direction and velocity of the NSC movement in an applied electrical field using a Zetasizer Nano ZSP (Malvern Instrument, Worcester, UK). The Zeta potential measured was reported as the average calculated from three readings.

The particle size distribution was measured using a Mastersizer 3000 with Hydro MV (Malvern Instrument, Worcester, UK). Particle size measurements are reported as the average of three readings per sample.

2.2.15 Synthetic colloidal (turbid) water

Synthetic water samples were used in this study and prepared using kaolin at a concentration of 2 g/L to obtain the colloidal solutions. Suspensions were prepared in three different buffers (50 mM Acetate, 46 mM Phosphate and 50 mM Tris Buffer) at a pH of 5, 7 and 9 respectively in order to test the flocculation and establish the optimum conditions of samples. Prior to analysis, fresh kaolin suspensions were prepared and were gently agitated for 24 h on a magnetic stirrer to ensure complete hydration (Muyibi S, 1999).

2.2.15.1 Zeta potential and particle size analysis of synthetic colloidal water

Kaolin suspensions (pH 5, 7 and 9) were let sediment 10 min and a sample was taken from the top and diluted 1:4. Diluted samples were injected directly into a folded capillary cell (DTS 1061, Malvern Instrument, Worcester, UK). The Zeta potential was then determined by measuring the direction and velocity of the kaolin particles movement in an applied electrical field using a Zetasizer Nano ZSP (Malvern Instrument, Worcester, UK). The Zeta potential measured was reported as the average calculated from three readings of each pH. The particle size distribution was measured using laser diffraction instrument: Mastersizer 3000 with Hydro MV (Malvern Instrument, Worcester, UK). This instrument measures the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample. The angular scattering intensity data is then analysed to calculate the size of particles responsible for creating a scattering pattern, using the Mie theory of light scattering. Particle size measurements are reported as a volume equivalent sphere diameter. The average of three readings per sample is indicated.

The Zeta potential of kaolin is -45.5 mV, -62.8 mV and -49.4 mV, for pH 5, 7 and 9 respectively.

The particle size distribution of kaolin is between 1 and 100 μm.

2.2.16 Flocculation assay using the spectrophotometric method

To determine the flocculation activity of samples under different conditions a simple jar test-like procedure was developed using a 1 L beaker, a mechanical overhead stirrer with adjustable speeds and a spectrophotometer (Spectronic, Helios Alpha UV-Vis, Thermo Fisher Scientific, USA), which was used to quantify the colloidal particle within the kaolin solution. The beaker was filled with 1 L of turbid suspension, and the required volumes of flocculants were added to stirred suspensions using Eppendorf pipettes. The amount of NSC was calculated as total protein present in water after the addition of the flocculation agent. The suspension was mixed at a high rate (500 rpm) for 2 min, followed by a low mixing rate of 50 rpm for 5 min and finally by a period of 5 min where no mixing occurred to allow sedimentation. These three stages correspond respectively to the fundamental processes of dispersing the flocculant onto the particle surface, gently sweeping the colloidal particles together to form flocs and allowing the flocs to separate. After stirring (high rate) began (t=0), 3 ml samples were removed at 30 s intervals, over a total time of 12 min and the optical density of each sample was measured at 600 nm spectrophotometrically. To ensure reproducibility, all samples were removed from the same position (2.5 cm below the surface of the suspension) in the centre of the beaker. Controls containing no flocculant were run to ensure that any activity detected was due to the presence of added coagulant and not to any other factors. All experiments were performed at room temperature.

Flocculation activity (FA), which corresponds to the percentage removal of the initial concentration of colloidal particles, measured spectrophotometrically, in the turbid water after addition of the coagulant agent was calculated using the following equation:

$$FA(\%) = \frac{OD_c - OD_s}{OD_c} \cdot 100 \qquad \textbf{Eq. 2.1}$$

where OD_c is the optical density of the control (after 12 min) and OD_s is the optical density of the sample after 12 min.

A graph of optical density versus time was plotted to display the activity of the material under examination.

2.2.17 Flocculation assay using the on-line quantification method (modified jar test apparatus with turbidity probes)

In order to test the turbidity probes for measurement of flocculation activity a series of flocculation assays were performed to quantify sedimentation rate and turbidity removal in NTU. The jar test was set up using six 1L kaolin suspensions. The amount of flocculant to be tested was added while the suspensions were mixed at high rate (300 rpm) for 2 min followed by a low mixing rate (30 rpm) for 5 minutes and finally no mixing for 5 min. Continuous on-line measurement readings were taken using the data acquisition system (LabView).

The sedimentation rate was calculated by determining the initial slope of the turbidity readings (NTU). The linear range of the curve after minute 7 was identified and slope calculated as fallowed:

$$Slope = \frac{y_2 - y_1}{x_2 - x_1} \qquad \textbf{Eq. 2.2}$$

where y_2 and y_1 are the values in NTU of the linear slope and x2 and x_1 are the time period associated with these values. The data were taken with r^2 Ó0.80.

The total turbidity removal (ΔT) was determined from the turbidity value of the kaolin solution after the addition of flocculant (time 0) minus the turbidity value at 12 min after the addition of flocculant:

$$\Delta T = \frac{(NTU_0 - NTU_{12})}{NTU_0} \times 100 \qquad \textbf{Eq.2.3}$$

where NTU_0 is the time at the time 0 and NTU_{12} is the time at the end of the sedimentation (after 12 minutes).

CHAPTER 3

Submicron capsules extracted from rapeseed as novel flocculant agents for the treatment of turbid water

Related publication

Tassinari, B., Doherty, S., and Marison, I. W. (2013). "Submicron capsules extracted from rapeseed as novel flocculant agents for the treatment of turbid water." *Water Res.*, 47(14), 4957-4965.

Poster

Poster at 15th European Congress on Biotechnology together with ESBES and ISPP (Istanbul, Turkey), September 2012. ñSelf-assembling nanocapsules as natural flocculantsò. Tassinari B., Whelehan M., Doherty S. and Marison I.

Abstract

Flocculation[2] is an important step in water treatment as it is responsible for the separation of suspended solids and colloids. The currently used flocculants have certain limitations with respect to environmental impact and disposal as well as potentially being harmful to human health, which has encouraged the study of natural flocculants originating from oleaginous plants. Oil-bodies are individual small organelles in which oleaginous seeds store triacylglycerols reserves. In this paper, the flocculant properties of oil-bodies have been investigated. Oil-bodies flocculate at pH 5, 7 and 9 and high ionic strength (100 mM NaCl) and it was demonstrated that their intact structure is necessary for the flocculation activity as treatment with protease K and diethyl ether, that remove the protein coat and the oil core, respectively, dramatically decreased the flocculation activity. This study shows that oil-bodies have the potential to be novel, natural, sustainable, environmentally friendly and biodegradable flocculant candidates for water treatment.

[2] Note: in this thesis the term flocculation refers to both the process of coagulation and flocculation

3.1 Introduction

The combination of coagulation and flocculation is one of the most commonly used methodologies to remove suspended and colloidal particles from water in drinking water treatment plants (Ndabigengesere and Narasiah, 1998a, Bouyer et al., 2005, Ma et al., 2008). In the coagulation process, the suspended and colloidal particles are neutralized (destabilized) after the addition of the coagulant agent (Pritchard et al., 2010a), while in the flocculation process the resulting destabilized particles are induced to aggregate and form large stable agglomerates (flocs), which come out of suspension and can be separated by subsequent sedimentation, flotation or filtration (Braga et al., 2005, Pritchard et al., 2010a). However, in most practical applications, the term flocculation is used as generic term to cover all aggregation processes (Gregory and O'Melia, 1989) and this is the sense in which the term is used in the present thesis. In addition the activity of both mechanisms are usually combined and measured together (to give a single value representing both processes under certain conditions) by carrying out sedimentation tests and determining the residual turbidity which is then related to the activity (Szpak et al., 1996). This method is referred to as the jar-test and is the standard procedure for determining coagulation-flocculation activity (Szpak et al., 1996, Tripathy and De, 2006).

Various kinds of coagulant agents have been employed to generate safe drinking water for human consumption (Tripathy and De, 2006, Ma et al., 2008) and aluminium (in the form of aluminium salt) is by far the most widely used (Ndabigengesere and Narasiah, 1998a, Raghuwanshi et al., 2002, Ma et al., 2008, Sciban et al., 2009). This metallic coagulant is frequently employed due to it being relatively cheap (Raghuwanshi et al., 2002) and easy to handle as well as being suitable for the treatment of many different types of water (Boucher, 2006).

Nevertheless the employment of aluminium as a flocculation material has a number of disadvantages. These include potential health ramifications for consumers, as its employment in the water treatment process increases the metal concentration in final drinking water (Miller et al., 1984, Raghuwanshi et al., 2002). Exposure to the metal has being associated to numerous human neurological disorders such as dialysis encephalopathy syndrome (Alfrey, 1976, Parkinson et al., 1979). It has also being linked to the development of neuropathological diseases including presenile dementia and Alzheimerôs disease (Crapper et al., 1973, Martyn et al., 1989, Doll, 1993, Mclachlan, 1995, Flaten, 2001, Frisardi et al., 2010), and has also being shown to be harmful to dialysis patients even at low accumulated concentrations within the body (Altmann et al., 1988). The low selectivity of the metallic coagulant yields flocs with fragile structures and creates large sludge volumes (James and Omelia, 1982, Ndabigengesere et al., 1995, Ndabigengesere and Narasiah, 1998a). Aluminium also affects the natural alkalinity of the water by depressing the pH (Ndabigengesere and Narasiah, 1998a).

These problems have led to research studies examining the capability of using novel flocculant and coagulant materials. These include other inorganic metallic ions and polyelectrolyte structures either on their own (Renault et al., 2009) or in conjugation with another polymer (Tripathy and Singh, 2001) or a metallic coagulant. Unfortunately these alternative methods have limited success (Ndabigengesere et al., 1995) attributable to their toxic nature towards the environment and human health relative to aluminium based coagulants (Letterman and Pero, 1990).

In light of these issues, there is a clear need for alternative water treatment technologies, which are efficient, cost-effective, sustainable, and environmentally safe for human and animal health. In this regard, natural flocculants (referred to as bioflocculants) from plant and plant-based materials are highly viable alternatives to presently used

techniques (Kawamura, 1991, Ndabigengesere and Narasiah, 1998a, Pritchard et al., 2010a), and they have already being used to treat water for many centuries in small towns and villages in tropical countries (Sciban et al., 2009, Pritchard et al., 2010a)

Among them, the pan-tropical *Moringa oleifera* has received much attention (Sciban et al., 2009) since these extracts have a flocculation activity comparable to that of aluminium and other commonly used coagulant agents, without presenting any reported toxic effects (Madsen et al., 1987, Suarez et al., 2003). Furthermore, extracts produce readily biodegradable and less voluminous sludge, which represents 20-30% volume equivalent relative to aluminium agents (Sciban et al., 2009), as well as having a negligible effect on the pH (Ndabigengesere et al., 1995). It has been shown that the flocculation activity of the plant is due to the presence of small molecular weight cationic proteins within the seed extract (Ndabigengesere et al., 1995, Ndabigengesere and Narasiah, 1998a, Doerries, 2005). Bioinformatics analysis on MO 2 proteins, the best characterised flocculant proteins of Moringa, suggested a sequence homology with 2S albumin proteins, which are one of the storage protein group present in seeds (Suarez et al., 2005)

However, in spite of the numerous advantages, the employment of Moringa extracts to purify water in the northern hemisphere has some disadvantages: (1) the process is often sometimes perceived as a crude water treatment methodology due to a lack of scientific understanding (Ndabigengesere and Narasiah, 1998a), and (2) the methodology is not seen to be financially viable due to high transport costs, as the plant is only found in sub-tropical regions (Pritchard et al., 2010a), usually in widely dispersed, small scale plantations. The latter problem has led to researchers screening numerous seeds (protein extracts) from plants which are abundantly available and widespread in the Northern Hemisphere countries, to establish if these indigenous plants also produce extracts which can be used as flocculation agents (Sciban et al., 2009).

In oleaginous plants, oleosins are a family of proteins which are found abundantly, and can account for several percent of the total protein within seeds (Tzen et al., 1993). Oleosins are insoluble alkaline proteins of molecular mass between 15-26 kDa depending on the plant species (Lee et al., 1991b). Oleosins function as structural proteins within oil bodies (Lee et al., 1991b). Oil-bodies (which also referred to as oleosomes) (Tzen et al., 1997) act as a storage structure for triacylglycerols (TAGs) (Lee et al., 1991b), which are used as food reserves for germination and post-germinative growth of the seedlings (Tzen et al., 1993, Tzen et al., 1997). They are small discrete and stable intracellular structures ranging between 0.5 ï 2.5 µm in diameter and consist of a TAG matrix surrounded by a monolayer of phospholipids and oleosins (Tzen et al., 1993, Tzen et al., 1997).

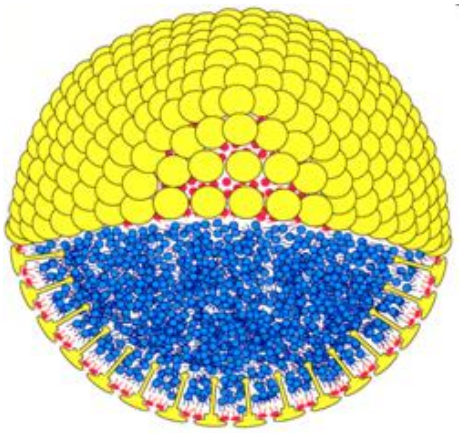


Figure 3.1: Diagram of an oil-body (Huang, 1996)
Blue: TAG core; pink: phospholipid monolayer; yellow: oleosins proteins.

The oleosin proteins help maintain the structural integrity of oil-bodies by shielding the phospholipid layer and the TAG matrix, and also by embedding the hydrophobic tail into the oil-core (Tzen and Huang, 1992a, Huang, 1996). In addition oleosin proteins harbour negative surface charge that prevent oil-bodies from coalescing by electrostatic repulsion (Tzen and Huang, 1992a) resulting in freely suspended, individually dispersed submicron-structures. The presence of charges at the surface of the oil-bodies, along

with their very high molecular mass, makes the oil-bodies a possible candidate for use as novel flocculation agents for the treatment of drinking water.

In this study, we investigated the flocculant activity of oil-bodies extracted from seeds of the rapeseed plant (*Brassica napus*), using a synthetic turbid water. Initial work focused on the extraction of oil-bodies and subsequent determination of flocculation activity in a standard, defined assay procedure. Optimum conditions for flocculation were then determined over a range of pH and ionic strengths. Subsequently the oil-bodies were treated with different reagents in order to gain a better understanding of the mechanism behind the flocculation activity.

3.2 Results and discussion

3.2.1 Extraction of oil-bodies from rapeseed

The first goal of this study was to extract oil-bodies from the seeds of the rapeseed plant in a form which could be used in future experiments to treat turbid water. In this work such preparations were prepared using the following steps: (1) mechanical dispersion and grinding of seeds to release oil-bodies embedded within the seeds; (2) filtration and centrifugation to remove larger particles and yield semi-purified preparations; (3) further purification of the samples by washing three times with a chilled buffer to remove any soluble and contaminating material and (4) a washing step with hexane to remove any defective or damaged oil-body structures which may be present. Defective and/or damaged particles would be expected to be found in these preparations due to the high shear forces generated by the homogenization and grinding steps of the extraction procedure; (5) a final wash with chilled buffer to remove the hexane

Figure 3.2 shows sub-micron images of the oil-body preparations obtained after step 5 of the extraction technique. This image shows oil-bodies as discrete individual entities

composed of a defined shell (membrane) of oleosin proteins and phospholipids fully enclosing a liquid-core of oil (TAG), which is characteristic for each kind of seed.

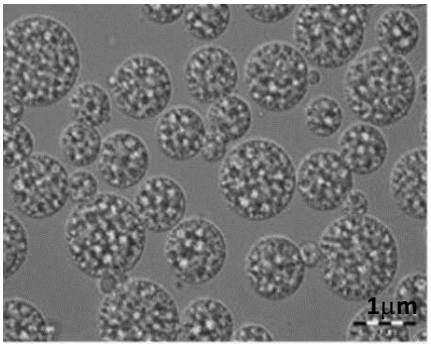


Figure 3.2: Sub-micron image generated using AFM and showing natural nanocapsules (NSC) preparations obtained from the seed of the rapeseed plant.
The size of the particles can be estimated from the scale bar (1 µm).

Moreover, particle size distribution measurements indicate that the majority of particles in oil-bodies suspensions are relative small (around 500 nm) (**Figure 3.3**). On the other hand, there are few larger particles around 10 µm. The ratio may be estimated to be 5000:1 between the 2 populations by number.

Due to the size (around 500 nm) and the structure of oil-bodies, we suggest that natural oil-bodies extracted from oleaginous plants should be termed as ñnatural submicron capsulesò (NSC). In fact, we believe that this term represents the true structure of the particles more accurately compared to the presently used term (oil-bodies), which can give the impression that the structure is compressed solely of oil, freely suspended in an aqueous solution and not incorporated within a membrane.

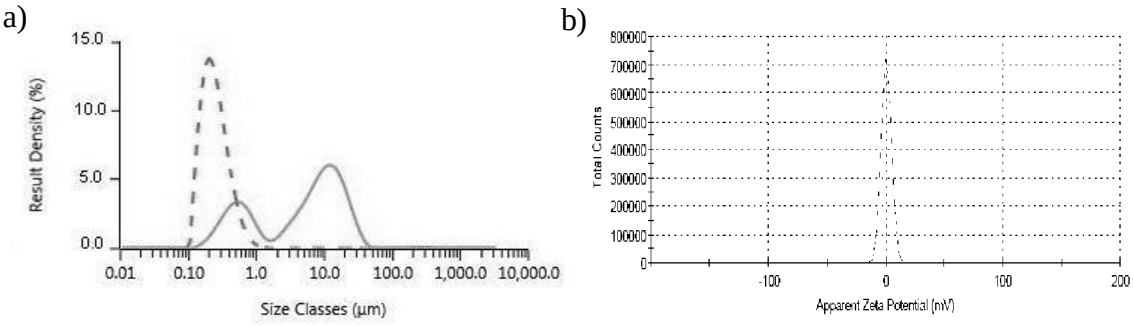


Figure 3.3: Zeta potential and particle size of natural submicron capsules.

a) Particle size distribution of NSC at pH 6 and b) Zeta potential at pH 6 of a diluted solution of NSC. Dash line: number distribution of particles; straight line: volume distribution of particles

In addition, these results highlight how compression of the NSC within the cells of the seeds did not lead to aggregation and/or coalescence of the particles after extraction using the outlined procedure. This is due to the repulsion of individual NSC due to the negative charges of the oleosin proteins on the outer layer (Tzen et al., 1997). To determine the effect of the purification process, samples from each of the five steps were run on SDS-PAGE gels. Results show an extremely enriched band at a molecular weight of 20 kDa in all the 5 samples (**Figure 3.4**). According to previous studies this band corresponds to an oleosin protein (Tzen et al., 1993). Nevertheless, the SDS-PAGE indicates that NSC extracts still contain significant non-oleosin, non-specifically bound proteins which could potentially affect with the flocculation activity of NSCs and/or the ability to measure this activity accurately.

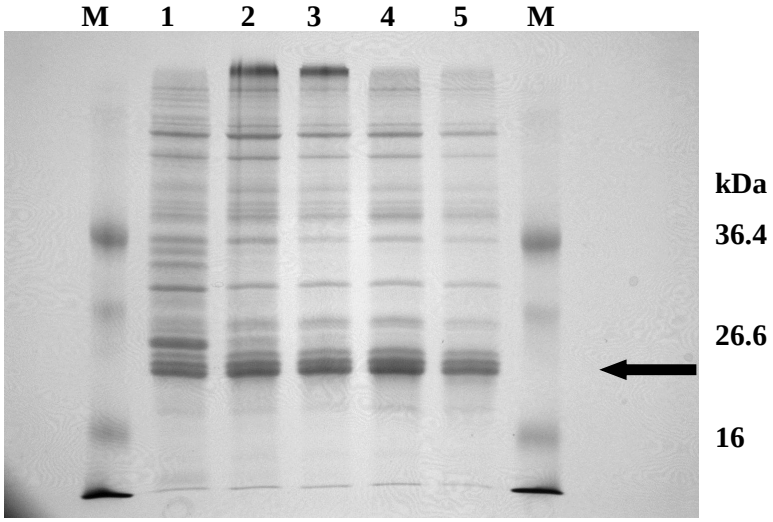


Figure 3.4: SDS-PAGE of extracts obtained from the five different stages of the NSC extraction and purification.

Lane M represents the low molecular weight standard (BioRad, UK), and lane 1-5 represents the corresponding samples from the extraction procedure.

We assumed that these proteins were not removed by the extraction process because they are firmly associated with the NSC surface. Previous studies showed that peripheral and associated proteins can be denatured by washing with chaotropic agents such as urea (Millichip et al., 1996, Tzen et al., 1997). This aspect will be further investigated in **Section 3.2.4**.

3.2.2 Flocculation activity of NSCs

In the next stage the potential of NSC to act as flocculation agents to treat turbid water and remove the suspended and colloidal particles was tested and the optimal dosage determined. Dosage is a very important factor to consider within a water treatment plant, as over/under dosing can lead to insufficient removal of colloidal particles (Sciban et al., 2009). In addition over-dosing can have a negative impact on process cost

and depending on the agent used, can substantially increase the organic load within the water leading to an increase in microbial growth (Sciban et al., 2009). Kaolin was selected as the colloidal material model to simulate turbid conditions.

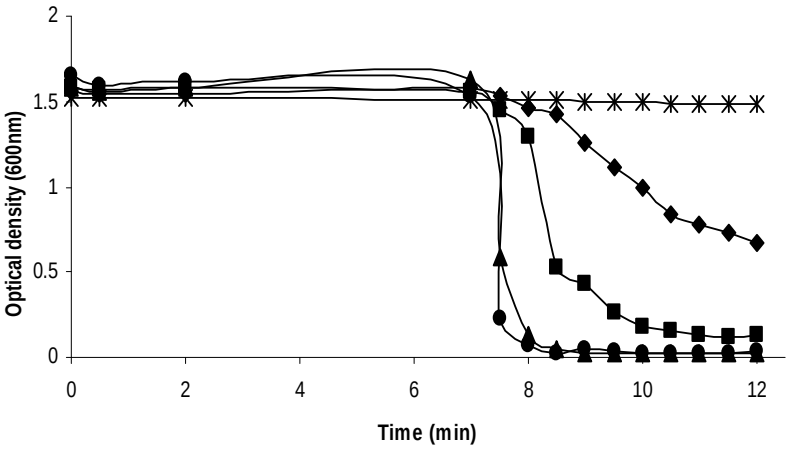
Figure 3.5 shows the effect of dosing turbid water, containing 2 g/L kaolin, with different concentrations of NSC extracted from rapeseed. The results clearly show that NSC preparations from rapeseed exhibit both high flocculation rate and sedimentation rate in the standard assay. The flocculation activity (percentage reduction in OD) increased with increasing NSC concentration in the range 5 ï 30 ppm with 5, 10 and 30 ppm yielding a 60%, 90% and 100% fall in the optical density. The reduction in OD reached a maximum at 30ppm.

Furthermore, the rate of sedimentation also increased with increasing NSC dosage with 30 and 50 ppm dosages resulting in full clarification of the suspensions within 1.5 min after the agitation had been stopped, whereas 3 ï 4 min at 10 ppm NSC and greater than 5 min for 5 ppm. These results suggest that higher concentrations of flocculation agent in water would result in shorter average distances between colloidal particles and flocculation particles, hence enabling the flocculation agent to exert its destabilizing mechanism on the particles at a quicker rate because of a higher frequency of collisions between NSC and colloids. These results are in contrast to those previously generated with polymers (Razali et al., 2011) and water soluble proteinaceous extracts from Moringa sp. and other plants (Sciban et al., 2009). In these cases higher levels of dosing actually resulted in a decrease in the removal of the colloidal particles (Sciban et al., 2009, Razali et al., 2011). The authors suggest that this may be the result of charge reversal, at higher coagulation doses, on the colloidal particles which become re-stabilized and repel each other; hence causing the colloidal particles to remain in solution (Muyibi and Evison, 1995a, Razali et al., 2011). Alternatively the authors suggest a saturation of the polymer bridge sites, which results in the restabilization of

the destabilized particles due to an insufficient number of particles being available to form inter-particle bridges (Muyibi and Evison, 1995a).

In the work presented here, the increase in FA with higher concentrations of NSC could be explained by more charges being available to neutralise and destabilise the colloidal particles, compared to the solutions containing lower concentrations of the flocculation agent. However, NSC have been shown to have a point of zero zeta potential at pH 6, as reported by previous studies which indicates 6 as isoelectric point of NSC (Tzen et al., 1992). This means that NSC have a net negative charge above pH 6, yet still present a FA with the negatively charged kaolin.

a)



b)

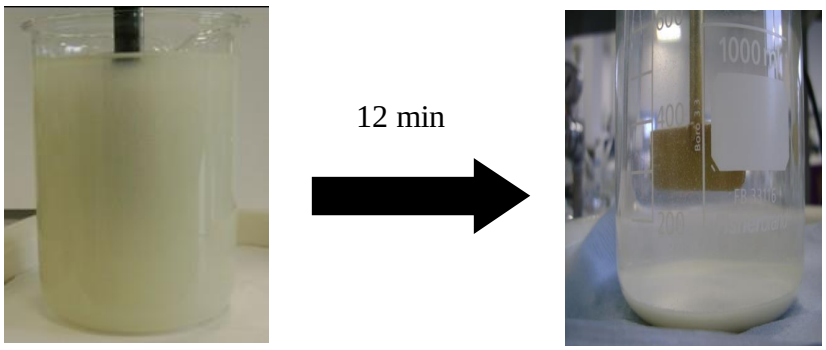


Figure 3.5: Flocculation activity of NSC.

a) Graph displaying the rate and amount of flocculation in 2 g/L kaolin solutions (in 5 mM phosphate buffer, pH 8) as a function of the dosage.

The mixing was stopped at min 7. Symbols (NSCs dosage): Diamonds: 5 ppm, Squares: 10 ppm, Triangles: 30 ppm, Circles: 50 ppm and Crosses: Control.
b) The images represent the clarity of water obtained with 10 ppm of NSC at the beginning of the process (on the left) and after 12 min (on the right).

From these results it can be hypothesized that the FA is probably not based on an electrostatic interaction charge, like other commonly used flocculants, but through other interactions, which are addressed in **Section 3.2.3**.

It has been reported that the concentration of colloid particles affects the flocculation of metal flocculants, such as alum and ferric salts. For low colloid concentrations, there is less contact opportunity for colloid-colloid interactions and when a quantity of metal flocculant is introduced, uneven adsorption occurs and, therefore, a certain number of particles remain stable. At higher colloid concentrations, particles are in closer proximity and can be destabilized and start forming flocs (Bratby, 2006).

The FA under high and low kaolin concentrations at pH 5, 7 and 9 was tested (**Figure 3.6** shows the results at pH 7, while **Appendix B** at pH 5 and 9). In general, the flocculation activity and the rates of sedimentation increased with increasing the kaolin concentration for all three pH values. This is in agreement with previously studies performed on natural and synthetic polymers (Muyibi S, 1999, Divakaran and Sivasankara Pillai, 2001, Bolto and Gregory, 2007). However, it was possible to get the 50% of turbidity removed even at medium-low kaolin concentration after 12 min. This is in contrast with moringa extracts which need more than 1 h to achieved the same removal at low kaolin concentrations (Muyibi S, 1999). Following these results, in order to standardize the flocculation assays, the kaolin concentration was kept at 2 g/L and NSC concentration at 10 ppm.

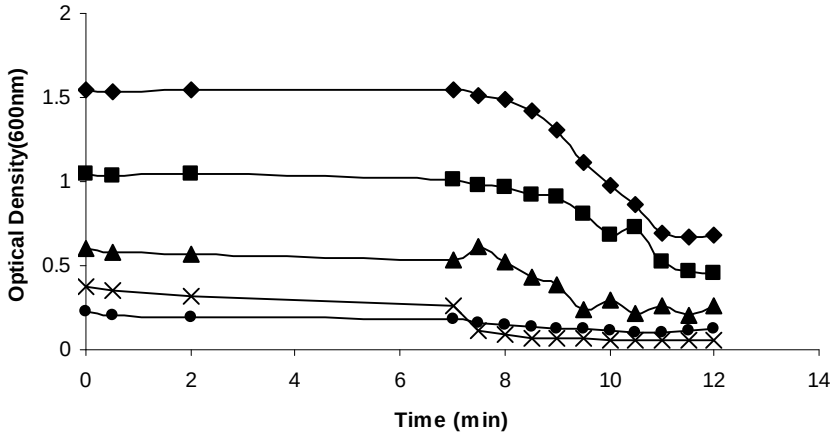


Figure 3.6: Flocculation activity of 5 ppm NSC at different kaolin concentrations at pH 7.

The dosage of 5 ppm of NSC was added to the kaolin suspensions, mixed for 2 min at high rpm, 5 min at slow rpm and let sediment for 5 min. Diamonds: 2 g/L; Squares: 1 g/L, Triangles: 0.5 g/L; Crosses: 0.250 g/L and Circles: 0.125 g/L.

3.2.3 Effect of pH and ionic strength on FA

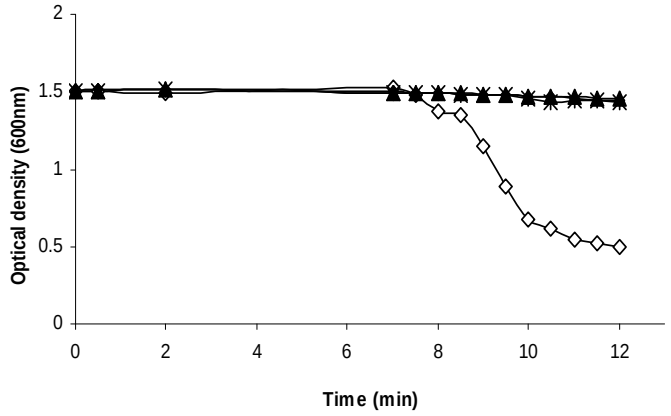
The effect of pH and ionic strength on flocculation activity of NSC, aluminium sulphate and ferric sulphate, the most commonly used flocculants, was measured and compared in order to gain a better understanding of the flocculation mechanism as well as to optimise the conditions. These two parameters would be expected to play an important role in flocculation since they affect the process through destabilisation of the colloidal particles and the net charge of both particles and flocculant**.**

Figure 3.7 shows that under high ionic strength conditions at pH 7, NSC had a 65% FA while aluminium sulphate and ferric sulphate showed no activity at all. Similar results were obtained at pH 5 and 9, where activity was better than or comparable to the inorganic standards (**Appendix C**). However, at low ionic strength the FA was significantly reduced. Thus, according to the Debye-Huckel theory, NaCl interacts and

neutralises the electrical double layer of kaolin particles, enabling the kaolin to aggregate in the presence of NSC. Such interactions clearly inhibited the reaction of the iron and aluminium salts, but did not affect the affinity of the NSC for the kaolin particles. From these results we hypothesize that the NSC FA could be compared to polymers which flocculate through a mechanism of bridging and charge neutralisation combined. This is particularly true for anionic polymers, where the ionic strength reduces the repulsion between particles and subsequently facilitates adsorption (Bratby, 2006), and would explain the decrease in activity at low NaCl concentrations.

In the mechanism underlying such flocculation, the nature and effect of pH and ionic strength on NSC has to be considered. In acidic and basic environments NSC are charged positively or negatively respectively, thereby inhibiting the coalescence and aggregation (Tzen et al., 1992b). However, the addition of cations such as Na^{+} and Ca^{2+} stimulates aggregation by presenting electrostatic repulsions (Tzen et al., 1992b, Iwanaga et al., 2007) which can also improve the formation and density of flocs.

a)



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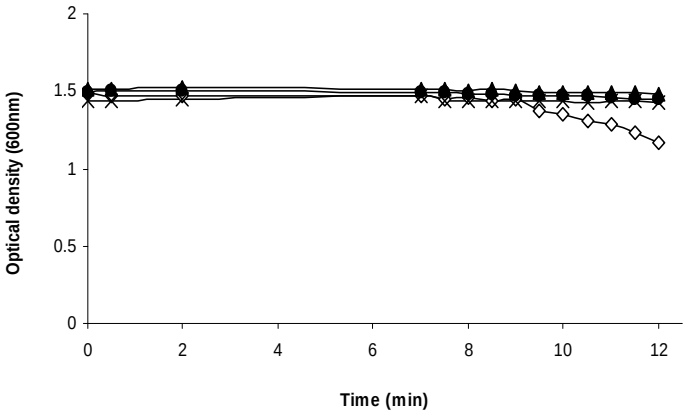


Figure 3.7: Effect of ionic strength on NSC flocculation activity.

a) 10 ppm of NSC is compared to 10 ppm of aluminium sulphate and 10 ppm of ferric sulphate at pH 7 at high ionic strength. b) 10 ppm of NSCs is compared to 10 ppm of aluminium sulphate and 10 ppm of ferric sulphate at pH 7 at low ionic strength. The mixing was stopped at min 7. Open diamonds: NSC; Circles: aluminium sulphate; Triangles: ferric sulphate.

3.2.4 Mechanism behind flocculation activity of NSCs

Since aqueous extracts of certain oleaginous seeds are reported to contain proteins that may be responsible for the FA (Gassenschmidt et al., 1995, Sciban et al., 2009, Asrafuzzaman et al., 2011) it is possible that it is not the NSC themselves which are responsible for the flocculation, but proteins bound to the NSC surface by electrostatic

and non-covalent interactions (Tzen et al., 1997). In order to test this and to further understand the flocculation mechanism, NSC preparations were washed with the chaotropic agent urea at high concentration to denature and dissociate any protein-NSC interactions without affecting the oleosins which are held and protected within NSC (Murphy and Cummins, 1989, Tzen and Huang, 1992a, Tzen et al., 1997).

In **Figure 3.8** it can be seen that urea treatment of NSC preparations actually increased the FA with over 90% of turbidity removed within 5 min of the cessation of agitation compared with less than 60% removal in untreated preparations. These results show that peripheral proteins are not responsible for the FA, and that their presence is actually detrimental to the colloidal removal process, due to partial shielding of the surface of the NSCs.

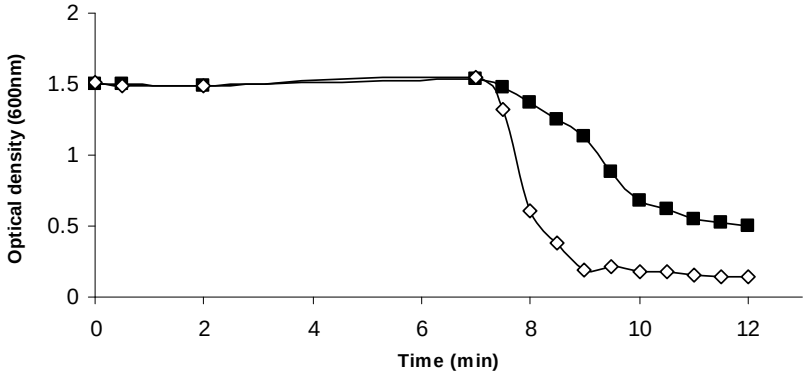


Figure 3.8: FA of NSC extracted in presence of 8M urea in the extraction buffer. Kaolin solution at pH 7, 100 mM NaCl. The mixing was stopped at min 7. Triangles: presence of urea; Open diamonds: absence of urea.

However, it is noteworthy that the aqueous layer obtained from the urea wash does show flocculation activity as well (**Figure 3.9**), suggesting that in oleaginous seeds

more than one flocculant might be present. This subject will be further discussed in **Chapter 5**.

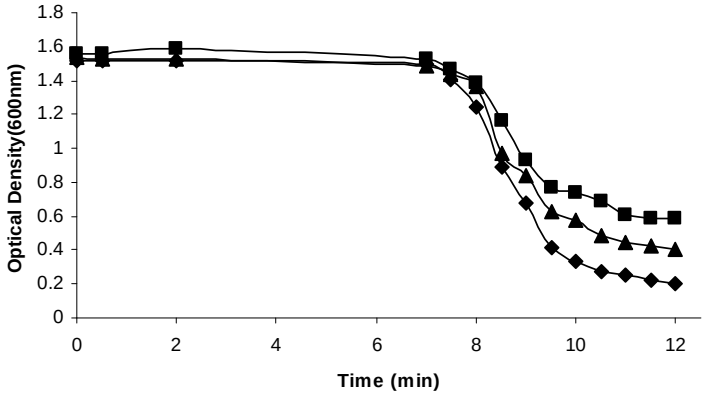


Figure 3.9: FA of the aqueous layer after urea wash under different pH conditions at 100 mM NaCl.

The concentration added to the kaolin suspension (2 g/L) was 10 ppm of total proteins. The mixing was stopped at min 7. Diamonds: pH 5, Square: pH 7 and Triangles: pH 9.

The surface of the NSC is believed to be coated with the hydrophilic, charged domains of the amphipathic oleosin proteins (Tzen and Huang, 1992a), which are largely responsible for maintaining the structural integrity of the NSC structures (Tzen et al., 1992b, Huang, 1994, Frandsen et al., 2001).

The role of these surface domains in the flocculation mechanism was determined by treatment of NSC preparations with Proteinase K, an endopeptidase of the serine protease family, which hydrolyzes peptide bonds following hydrophobic residues, and is commonly used to completely hydrolyze proteins (Sweeney and Walker, 1993).

After treatment of NSC suspension with Proteinase K, all flocculation activity was lost, thereby confirming that the oleosin surface (hydrophilic domains) was essential for FA (**Figure 3.10**). As described by Tzen (1997), the TAG core is protected by the hydrophilic coating from hexane extraction so that only more polar solvents (such as

diethylether) are able to penetrate and extract the TAG which represents the 97% (w/w) of the oil-body (Tzen and Huang, 1992a).

Interestingly, defatting of NSC with diethyl ether also resulted in a complete loss of activity. This suggests that the extraction of the oil results in an important change in the conformation of the oleosins, resulting in an inability to successfully link with and bridge the kaolin particles.

This also confirms that it is likely that the intact NSC both attach to the kaolin surface and form bridges between the kaolin particles, thereby creating a dense floc which sediments rapidly. This is in contrast to the standard flocculating/coagulating agents such as aluminium and iron salts, as well as soluble Moringa sp. extracts and proteins, which function optimally in highly turbid water (Asrafuzzaman et al., 2011). At lower turbidities it is often necessary to add particulate matter, such as lime, and/or to add polymers, such as acrylamide derivates (Aguilar et al., 2005).

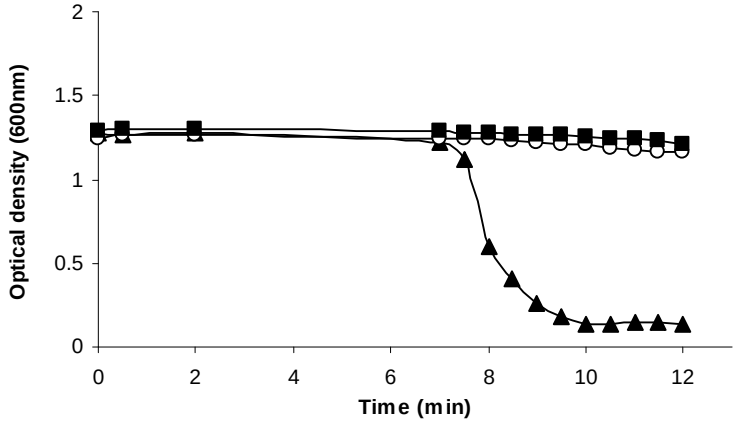


Figure 3.10: FA of NSC treated with 8 U of Proteinase K and diethyl ether in kaolin solutions (sodium phosphate buffer pH 7, 100 mM NaCl).

The mixing was stopped at min 7. Open circles: de-fatted NSCs; Squares: Proteinase K treated; Triangles: Control.

3.3 Conclusion

In the present study it is demonstrated that in a turbid water model such as 2 g/L of kaolin suspension, NSC (oil-bodies) extracted from the seeds of oleaginous rapeseed plant are highly effective biological flocculants capable of being used alone, in the absence of inorganic compounds or artificial polymers. The flocculation activity increases with the dosage of NSC, but is already effective at low concentrations and results in complete removal of turbidity in the standard flocculation assay.

This work showed that the flocculation activity of NSC is influenced by the ionic strength: the presence of high NaCl concentration results in higher turbidity removal compared to the standards used, while low concentrations resulted in reduced activity.

The activity was shown to require both an intact NSC structure with exposed oleosin and the oil core, since no activity was observed when this structure was modified.

In subsequent studies it remains to be shown whether the flocculation activity is widespread throughout oleaginous plant seeds, whether the activity varies as a function of plant origin and conditions, and whether FA in this standard reference assay occurs under actual water-treatment conditions.

Although this is a pilot study, considerable advantages of using NSC as bioflocculants are the ease of widespread production and sustainability, as well as the biodegradability of the NSC-flocs, which would avoid the costly, highly energy-requiring incineration or landfilling of current sludges produced from drinking water treatment processes with possible dangerous side effects for humans and environment.

CHAPTER 4

Application of turbidity-meters for the quantitative analysis of flocculation and sedimentation in Jar test apparatus

Related publication

Tassinari, B., Conaghan, S., Freeland, B., and Marison, I. W. "Application of turbidity-meters for the quantitative analysis of flocculation in Jar test apparatus". Submitted to Journal of Environmental Engineering (*under review*).

Abstract:

Turbidity is an important water quality indicator as it does not only indicates the presence of suspended solids but also shows the possible presence of algae, microorganisms, organic matter and micro- particulates. Generally turbidity is removed through two very important steps in a typical water treatment process: coagulation and flocculation, followed by floc separation.

At lab-scale the measurement of flocculation activity may often be difficult and imprecise, since reproducible water samples need to be taken offline, inserted into a turbidity meter or spectrophotometer, while the standard jar test system is relatively imprecise and measurements discontinuous and/or subjective. In order to allow routine quantification, an online quantitative method for the analysis of flocculation activity was developed which can measure sedimentation as a function of time. This method involved modification of a standard, commercial jar test apparatus by the addition of six turbidity meters coupled with a data acquisition system operating with LabView software. This system has been tested using a standard, reference turbid water suspension containing kaolin, with aluminium sulphate and ferric sulphate as coagulants.

4.1 Introduction

The American Public Health Association (APHA) defines turbidity as ñexpression of the optical property that causes light to be scattered and absorbed rather than transmitted in straight lines through the sampleò(APHA, 1985). The intensity of the scattered light increases with the suspended solids (Grayson et al., 1996). When a beam of light is passed through ultra-pure water the water path remains relatively undisturbed. However, in samples containing suspended solids, the manner in which the sample interferes with light transmittance is related to the size, shape and composition of the particles in the solution and to the wavelength of the incident light. A minute particle interacts with incident light by absorbing the light energy and then, as if a point light source itself, re-radiating the light energy in all directions. This omnidirectional re-radiation constitutes the ñscatteringò of the incident light (Sadar, 1998). The unit used to describe turbidity is nephelometric turbidity unit (NTU) and it measures the scattered light at 90º from the incident light beam detected by a nephelometer, the instrument for measuring concentration of suspended particulates in a suspension.

Turbidity is an important water quality indicator; it not only indicates the presence of suspended solids, such as clays, but also shows the possible presence of algae, microorganisms, organic matter and other micro particulate (Packman, 1999, Huey, 2010). Excessive turbidity in drinking water is aesthetically unappealing, and may also represent a human health concern (EPA, 1999). Turbidity can provide nutrients and shelter for pathogens. If not removed, it can even promote re-growth of pathogens in distribution systems, leading to waterborne disease outbreaks, such as gastroenteritis (D'Antonio et al., 1985, LeChevallier et al., 1991, Schwartz et al., 1997, Schwartz et al., 2000, Aboytes et al., 2004). Although turbidity is not a direct indicator of health risk, numerous studies have shown a strong correlation between the removal of turbidity and removal of protozoa (LeChevallier et al., 1991, Goldstein et al., 1996, Betancourt and

Rose, 2004). Indeed, turbidity particles provide ñshelterò for microbes by reducing their exposure to disinfectants (LeChevallier et al., 1984, LeChevallier et al., 1991).

For these reasons, the World Health Organisation (WHO, 2011) states that water turbidity levels to be disinfected must be <1.0 NTU.

Coagulation and flocculation followed by floc separation are key steps in water treatment as these are the most effective in removing turbidity from water. This involves the removal of suspended micro-particulate matter by the addition of highly charged cations such as aluminium and iron salts, usually in the form of aluminium sulphate ($Al_2(SO_4)_3$) and ferric sulphate ($Fe_2(SO_4)_3$), commonly termed alum and ferric respectively, to water (Matilainen et al., 2005, Pritchard et al., 2010a).

These ions neutralise the electrical charges on the suspended solids and colloids which in turn form flocs. The flocs grow in size, merge and due to the increased mass and density result in sedimentation which can ease their removal by filtration or clarification (Bratby, 2006, Barbot et al., 2010).

Effective coagulant dosage is mainly determined by the results of the standard jar test method. A typical jar test consists of six identical jars with a stirrer submerged in each. Each vessel contains the same volume of water to be treated but different doses of flocculant. After the mixing and sedimentation, the supernatant of the treated sample is collected to measure the residual turbidity for evaluating the coagulation efficiency (Rossini et al., 1999, Franceschi et al., 2002, Xiao et al., 2008). Accurate measurement of flocculation activity can often be difficult as samples need to be taken off line and inserted to into a turbidimeter or spectrophotometer (Rossini et al., 1999, Franceschi et al., 2002), making these methods extremely time consuming and difficult to reproduce due to sample heterogeneity.

Moreover, the selection of an effective coagulant and dosage is usually dependent on the operator experience based on the results of jar tests (Tse et al., 2011).

This often leads to wasteful over-dosing of chemical flocculants in water treatment plants causing greater operation costs and increased chemical sludge (Franceschi et al., 2002, Cheng et al., 2008). For these reasons, online turbidity meters attached to the standard jar test apparatus would render analysis more reliable and efficient.

To date there are reports of on-line methods that focus on measuring floc size by image analysis and microscopy (Gregory, 1985, Cheng et al., 2008, Tse et al., 2011), however these methods tell us very little about the sedimentation rate and more about floc formation. Sedimentation rates and residual turbidity values are vital to any water treatment plant, as they tell the operator how efficient the flocculant is for the required water on any given time (Tse et al., 2011).

Although the use of on-line turbidity-meters it is not new (Cheng et al., 2008, Cheng et al., 2011), this chapter focuses on the development of an on-line quantitative method using commercial turbidity sensors to a standard six jar test apparatus, its validation and applicability. Subsequently, the instrument was tested for the study of flocculation activity of alum and ferric, and their efficiency characterised by sedimentation rate and total turbidity removal.

4.2 Experimental equipment and methods

4.2.1 Preparation of synthetic water

Synthetic water samples were used in this study and prepared using kaolin at concentrations of 2 g/L, 1 g/L, 0.5 g/L, 0.250 g/L and 0.125 g/L to obtain the colloidal solutions. Prior to analysis, fresh kaolin suspensions were prepared and were gently agitated for 24 h on a magnetic stirrer to ensure complete hydration (Muyibi S, 1999)

4.2.2 Jar test

Figure 4.1 shows a schematic representation of the equipment used. To a standard jar test system (Model PB-700, Phipps and Bird, USA), six Mettler-Toledo InPro 8000 series turbidity sensors (Mettler Toledo, Switzerland) were attached in order to give an online measurement of flocculation and sedimentation. These sensors were equipped with a light emitting diode (LED) in the near infra-red range (880nm) via a fibre optic cable. These sensors function on the basis of back-scattered light: the incident light is scattered at an angle of 180º, captured and fed back to a photodiode in the turbidity transmitters (Model Trb 8300, Mettler Toledo, Switzerland), processed as photocurrent and the signal transformed to give a NTU value. The height and distance of the probe position was optimised to 80 mm and 25 mm from the base of the vessel in order to give consistent and reproducible turbidity values. All six turbidity transmitters were connected to a data acquisition system, LabView, by the means of RS232 cards. The data were transferred at 3 sec intervals and processed in 15 s MpA in order to reduce background noise. The data was then transferred to Microsoft EXCEL to enable graphical representation of turbidity as a function of time as well as determination of sedimentation and total turbidity removal. Each test was performed in triplicate and the standard deviation calculated for each sensor.

Prior to use, the six turbidity sensors were calibrated via multipoint calibration, following the manufacturerôs instructions (Mettler Toledo, Switzerland) using a set of standard formazin suspensions: 2000, 1000, 500, 100 and 20 NTU.

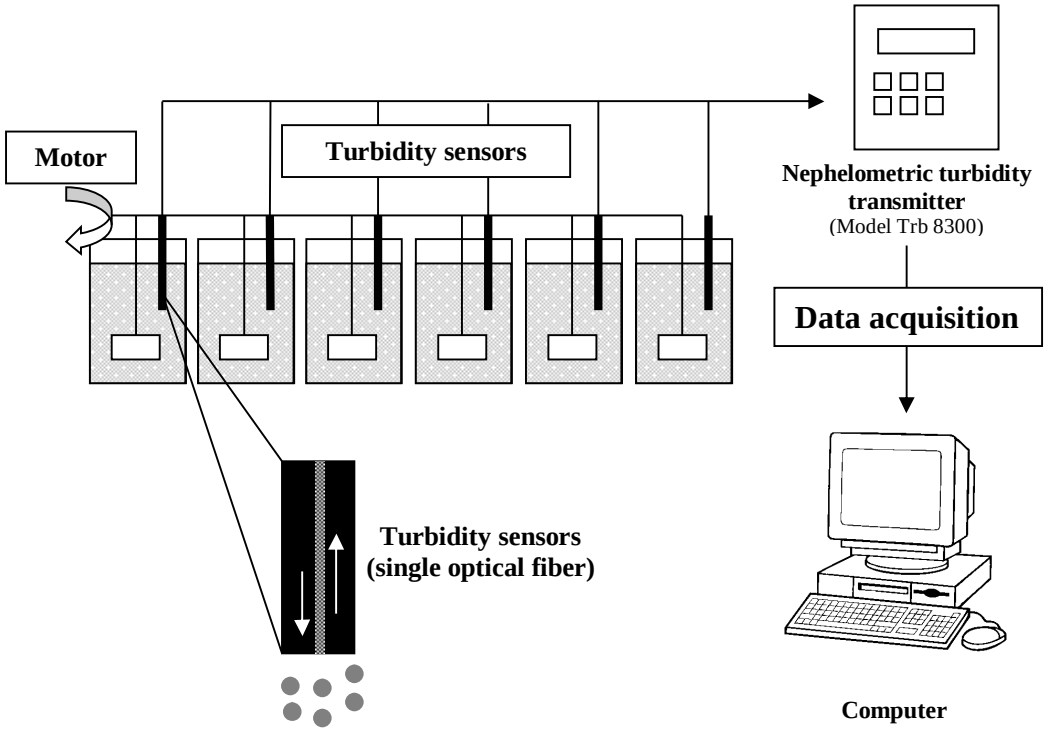


Figure 4.1: Schematic representation of the jar test connected to turbidity meters

4.2.3 Instrument validation

4.2.3.1 Light Interference

The experiments were run in completely darkened conditions using a black screen around the jar test system, in order to eliminate any outside light and in natural ambient light, i.e. by removal of the screen. The two formazin suspensions (100 NTU and 1000 NTU) were left to mix at 200 rpm for 2 min and at 30 rpm for 3 min and the data collected by LabView. In order to show that the data from the six turbidity probes were normally distributed over a low turbidity of 100 NTU and a high turbidity of 1000 NTU, a Normality Q-Q plot was completed (Bremer and Doerge, 2010).

4.2.3.2 Reproducibility test

To establish repeatability between the six probes and each probe themselves, a reproducibility test was carried out using 100 NTU and 1000 NTU formazin suspensions, in triplicate and in darkened conditions by means of a black screen placed around the beakers. Solutions were constantly mixed at high speed (200 rpm) and the data were collected for 2 min.

The data were analysed to determine the percentage error of each turbidity meter calculated as standard deviations and a 95% confidence interval of the offset within the turbidity sensor was determined.

4.2.4 Sensitivity and Limit of detection (LOD)

Kaolin (0.05 g) was added to a beaker with 1 L of DI water and allowed to mix at high rpm (200 rpm) for 2 min while the NTU values were recorded. A further 0.05 g/L of kaolin was added at 2 min intervals until a final concentration of 2 g/L was achieved and the NTU was determined. This procedure was repeated three times. From these results the sensitivity of the turbidity sensors was determined and an NTU value for kaolin concentration was calculated. The limit of detection was calculated based on calibration and regression statistics where the *y*-intercept and standard deviation of regression were used:

$$LOD = 3.3 \times (SD / S) \qquad \textbf{Eq. 4.1}$$

where the *SD* is the standard deviation of *y*-intercepts of regression lines and *S* is the slope of the calibration curve.

4.2.5 Kaolin calibration and reproducibility test

The probes were calibrated in darkened conditions using kaolin suspensions of 0, 0.25, 0.5, 1 and 2 g/L. In order to validate this process, a reproducibility study was performed using 0.125 g/L to 2 g/L of kaolin. The suspensions were stirred 5 min before reading to

ensure complete mixing of the kaolin before recording results. The reproducibility between the six probes and for each probe was investigated. The procedure was repeated three times. The data were then analysed to determine the percentage error of each sensor as a standard deviation and a 95% confidence interval of the offset within the turbidity probes was determined.

4.2.6 Effect of flocculants colour on the reading

Alum and ferric sulphate were added to 1 L of deionised water from 0 to 100 ppm in 5 ppm intervals at high rpm (300). This was repeated in kaolin suspensions from 0.125 g/L to 2 g/L. The experiments were performed in darkened conditions and repeated three times. For the effect of colouring on the kaolin suspensions a Two Way ï Analysis of variance (ANOVA) showed the interaction between the coagulants and the DI water and kaolin solutions (McDonald, 2011). For kaolin suspensions a surface response graph was plotted in order to determine the effect of colour of coagulants on the reading.

4.2.7 Flocculation activity

In order to test the turbidity probes for measurement of flocculation activity a series of flocculation assays were performed to quantify sedimentation rate and change in NTU. The jar test was set up using six 1L kaolin suspensions at 2 g/L. A defined amount of alum or ferric (in ppm) was added while the suspensions were mixed at high rate (300 rpm) for 2 min followed by a low mixing rate (30 rpm) for 5 minutes and finally no mixing for 5 min. Continuous on-line measurement readings were taken using the data acquisition system (LabView).

The sedimentation rate and total level of sedimentation was determined from the turbidity value of the kaolin solution as described in **Chapter 2, Section 2.2.15**.

The jar test samples were analysed every 30 sec at the spectrophotometer (λ= 600 nm) to test whether there might have been significant differences in the detection of flocculation between the two methods.

4.3 Results and Discussion

4.3.1 Effect of light on turbidity meters

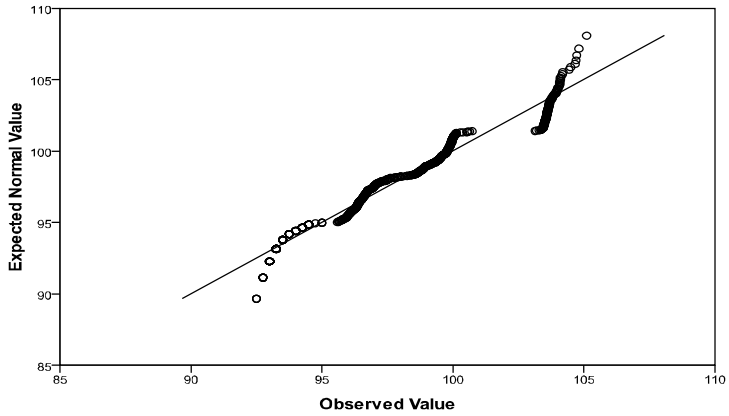
The modified jar test apparatus consists of six turbidity sensors immersed in turbid solutions and connected to a computer (**Figure 4.1**). These commercial sensors possess a single optical fibre where emitted and backscattered light travel along the same fibre.

The technique of scattering laser light from quiescent solutions of macromolecules is widely used as a method of determining the translational and rotational diffusivity of isolated molecules and thereby extracting information about their size and shape. As the particles of the solution or suspension move under the influence of Brownian impacts from the solvent molecules, so the instantaneous scattered light changes (Rallison and Hinch, 1986). Light scattering intensifies as particle concentration increases. But as scattered light strikes more and more particles, multiple scattering occurs and absorption of light increases as well (Sadar, 1998).

The sources of errors and variations in turbidity measurements might be many; among them the most significant might be ambient and stray light. Stray light is any light from a source other than scattering by particulates in the samples and has always a positive interference. Sources of stray light include reflections and refractions that, reaching the detector, could affect the reading (APHA, 1985). To verify whether the turbidity meters are influenced by light two repeatability tests were performed. Two formazin solutions,

100 and 1000 NTU were run three times, with all probes in ambient light conditions and darkened conditions. The data were then analysed for normality distribution (**Figure 4.2** and **Table 4.1**).

a)



b)

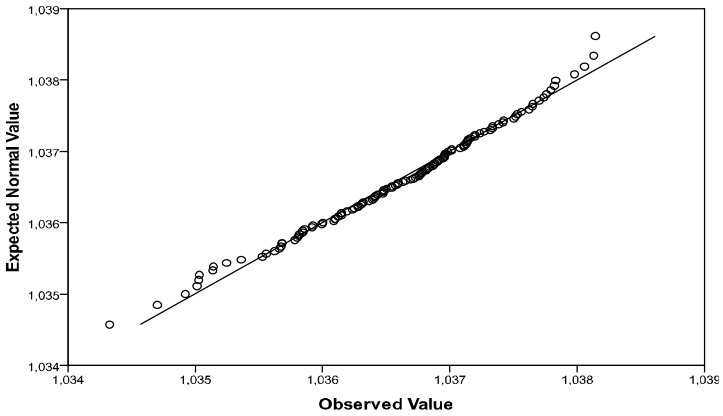


Figure 4.2: Normality Q-Q plot for 100 NTU (a) and 1000 NTU (b) formazin suspensions in stray light conditions.

The formazin suspension were mixed at 300 rpm and recorded for 2 min.

Table 4.1: Shapiro- Wilk Test of normality for 100 NTU and 1000 NTU formazin solution in stray light conditions.

Probe	1	2	3	4	5	6
100 NTU						
Statistic	0.856	0.763	0.744	0.992	0.764	0.358
df	41	41	41	41	41	41
Sig.	0	0	0	0.989	0	0
1000 NTU						
Statistic	0.920	0.954	0.960	0.930	0.571	0.704
df	36	36	36	36	36	36
Sig.	0.024	0.159	0.234	0.226	0	0

Normality is one of the most common assumptions made in the development and use of statistical procedures. The normality of an underlying data distribution can have a considerable effect on the properties of estimation or inferential procedures used in the analysis of the data (Kim, 2011). A normality quantile-quantile (Q-Q) plot was made in order to test whether light sources have an effect on the sensor readings.

The Q-Q plot is a plot of the percentiles (or quintiles) of a standard normal distribution against the corresponding percentiles of the observed data. If the observations follow approximately a normal distribution, the resulting plot should be an approximate straight line with a positive slope. **Figure 4.2** showed that the data in ambient light were not normally distributed for 100 NTU, while the correlation for 1000 NTU is linear. This was also confirmed by the Shapiro-Wilk test (**Table 4.1**). This test is based on regression and correlation. Indeed, a normal probability plot, such as a Q-Q plot which examines the fit of a sample data set to the normal is rather like a linear regression. By analysing the scale of this variation (analysis of variance) the quality of the fit can be examined.

The test statistic is:

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\sum_{i=1}^{n}(x_i - \overline{x})^2} \qquad \textbf{Eq.4.2}$$

where $x_{(i)}$ is the smallest number in the sample, $\overline{x}$ is the sample mean and a_i is a constant given by:

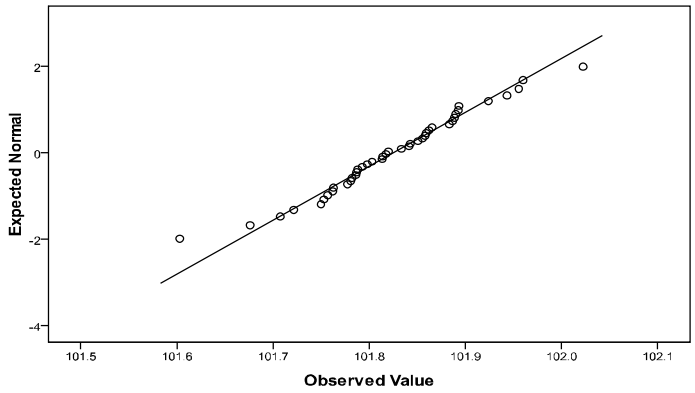
$$(a_1, \ldots, a_n) = \frac{m^{\top} V^{-1}}{(m^{\top} V^{-1} V^{-1} m)^{1/2}} \qquad \textbf{Eq.4.3}$$

where the vector *m* represents the expected values of the elements of x, i.e. the mean values of the order statistics for the normal distribution, and *V* is the covariance matrix of those order statistics. If the significance value (Sig.) is lower than 0.05, the data is not normal distributed.

The effect of light is more evident on the low turbidity solution than on the higher one. This may be due to the fact that low turbidity solutions, having fewer particles suspended, are more sensitive to reflection and refraction phenomena due to the light that reaches the detector, causing a positive error.

In darkened conditions, the normally distribution of the data greatly improved, confirming that the sensors are indeed sensitive to external sources of light (**Figure 4.3** and **Table 4.2**), especially for low turbidity values. Thus, in these conditions, the reading is more accurate and precise. For these reasons, further experiments were performed under dark conditions.

a)



b)

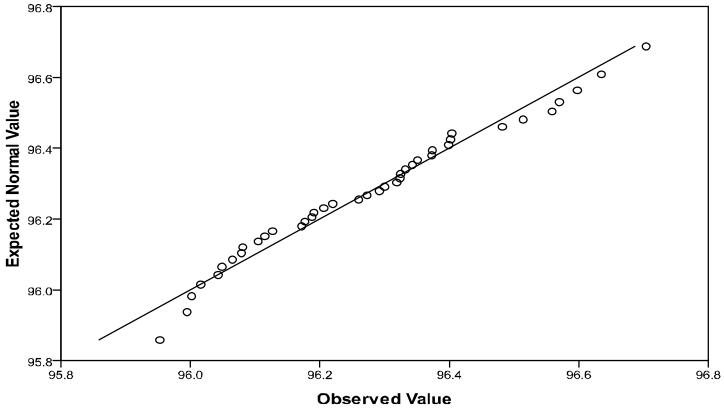


Figure 4.3: Normality Q-Q plot for 100 NTU (a) and 1000 NTU (b) formazin suspensions in darkened conditions.

The suspension were mixed at 300 rpm and recorded for 2 min.

Table 4.2: Shapiro- Wilk Test of normality for 100 NTU and 1000 NTU formazin solution in darkened conditions.

Probe	1	2	3	4	5	6
100 NTU						
Statistic	0.948	0.964	0.972	0.955	0.967	0.986
df	42	42	42	42	42	42
Sig.	0.056	0.204	0.385	0.094	0.262	0.884
1000 NTU						
Statistic	0.972	0.965	0.965	0.978	0.955	0.963
df	36	36	36	36	36	36
Sig.	0.332	0.272	0.168	0.256	0.155	0.155

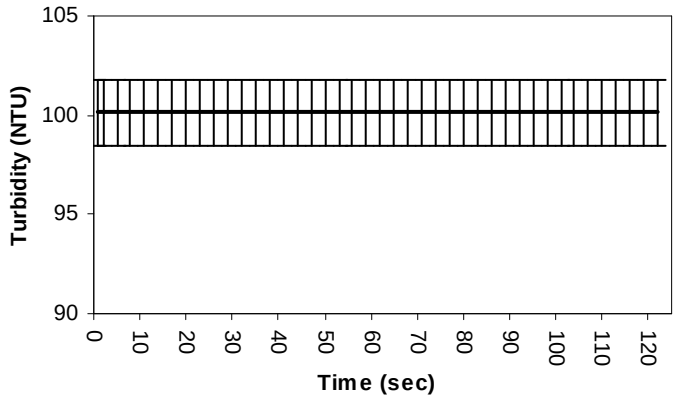
4.3.2 Reproducibility test

If a measurement is repeated many times under essentially identical conditions, the results of each measurement will be distributed randomly about a mean value. In this case, it is important to guarantee that the reading of each sensor was reproducible and all six probes gave similar outputs for a sample. Formazin suspensions containing 100 and 1000 NTU were run three times in darkened conditions at high rpm to avoid fluctuations in the suspensions during the recording.

The mean and standard deviation of each probe was calculated and the confidence interval was set at 95% (α= 0.05). As shown in **Table 4.4,** the probes read within the 95% of confidence interval with a small error indicated by the standard deviations.

The overall measurement variability was then analysed for all the six probes and runs (**Figure 4.4**) to obtain the range of the dataset. The mean of the six probes run three times for the low turbidity standard was **100.13±1.6**, while the mean of high turbidity standard was **1003±3.09**. These results validated the instrument accuracy for NTU determination.

a)



b)

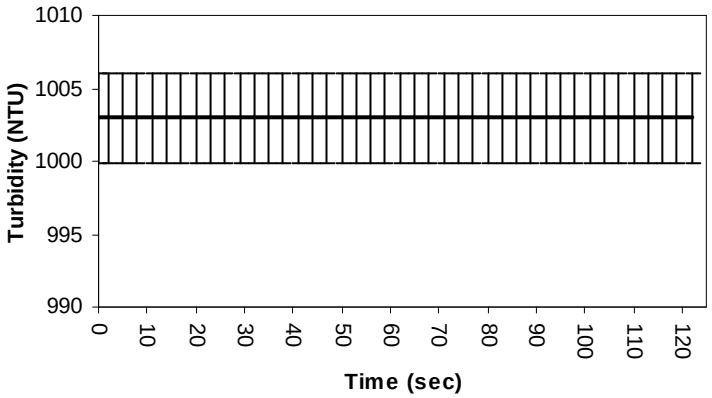


Figure 4.4: Overall measurement variability for 100 (a) and 1000 NTU (b) for all the probes and runs.

Error bars indicates the standard deviation.

Table 4.3: Descriptive statistics for measurement variability using 100 and 1000 NTU.

Test repeated three times in darkened conditions at 200 rpm.

	100 NTU						1000 NTU					
Probe	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Mean	94.43	96.36	99.91	96.06	103.72	97.12	1002.89	1004.62	1007.77	998.24	1003.31	1001.10
Confidence level, 95%	0.36	0.10	0.05	0.52	0.12	0.25	0.29	0.15	0.13	0.12	0.09	0.11
STDEV	2.02	0.56	0.30	0.09	0.67	1.40	1.71	0.88	0.79	0.75	0.56	0.66
Deviation from the Pop. Mean (%)	3.6	1.6	2.0	1.9	5.9	0.8	0.16	0.01	0.47	0.48	0.19	0.03

4.3.3 Limit of Detection (LOD)

Kaolin is one of the most widely used turbidity standard solutions used in water treatment facilities (Cheng et al., 2011, Gregory and Barany, 2011, Tse et al., 2011). Therefore, it is important to precisely measure its value in NTU each time that an experiment is carried out. By definition, the limit of detection (LOD), expressed as a concentration c_l (or amount, q_l), ñis derived from the smallest measure, x_l, that can be detected with reasonable certainty for a given analytical procedureò (IUPAC, 1976a). After calibration with formazin suspensions, the jar test LOD was obtained by reading a set of kaolin suspensions from 0 g/L to 2 g/L in 0.05 g/L intervals, data was collected for 2 min and plotted as g/L versus NTU as shown in **Figure 4.5** to provide a calibration graph.

The LOD calculated from the graph is **1.9 NTU** and the instrument sensitivity was calculated through the slope of the linear regression giving a result of **733.39 NTU** per g/L. Using these data, a 5 point calibration with kaolin solutions (from 0 to 2 g/L) was applied to subsequent flocculation tests, **Figure 4.5**.

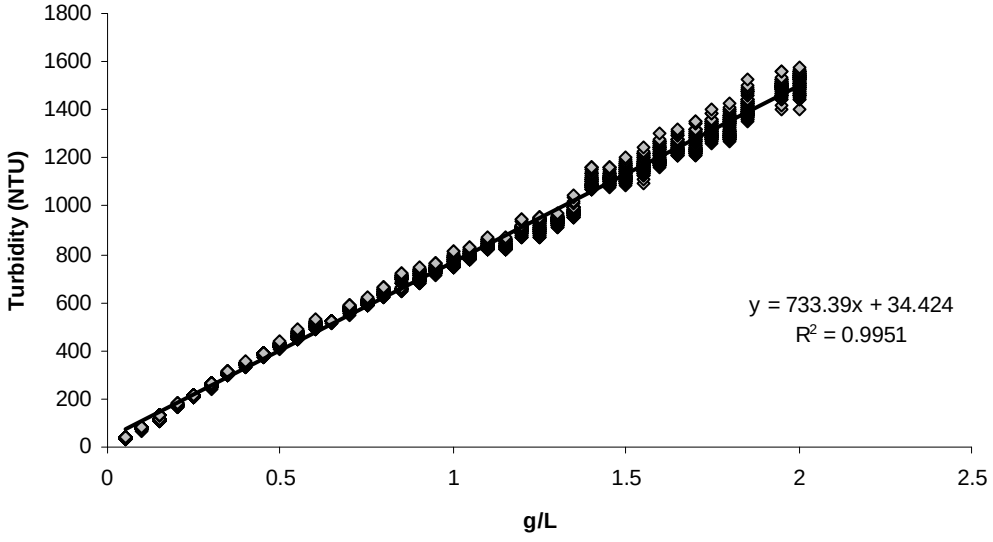


Figure 4.5: Calibration curve from 0 to 2 g/L in 0.05 g/L of kaolin powder.

The amount of 0.05 g of kaolin was added to 1L of DI water up to 2 g/L. Each point was recorded for 2 minutes and repeated 3 times in dark conditions.

As previously mentioned, kaolin is one of the most turbid synthetic water types used in the laboratories, in Japanese water treatment is used as turbidity standard (Sadar, 1998) and it might be more accurate to calibrate the instrument with kaolin solutions than formazin. Moreover, kaolin solutions have the great advantage of being more stable (especially at low NTU) and less expensive than formazin.

4.3.4 Kaolin calibration and reproducibility test

A reproducibility test, using 0.125, 0.250, 0.5, 1 and 2 g/L kaolin, was performed as previously done for formazin. Results showed that, with increased turbidity, the absolute error within the probes (indicated by the deviation from the population mean) decreased, indicating that low turbidity samples had a higher level of interference due to stray light; this suggests that although external sources of light were eliminated by the means of the screen, reflections due to the sensor may still occur. This caused low kaolin concentrations to have a greater error than high turbidity samples; however the relative error for each probe (indicated by the standard deviation) increased with high turbidity solutions (**Table 4.4**).

Table 4.4: Descriptive statistics for measurement variability using 0.125 to 2 g/L.

Test repeated three times in darkened conditions.

g/L		Probe 1	Probe 2	Probe 3	Probe 4	Probe 5	Probe 6
0.125	**Mean**	130.73	213.84	156.24	123.65	102.16	264.33
	Confidence, 95%	0.51	1.05	1.89	1.38	0.22	6.60
	STDEV	2.91	5.93	10.67	7.79	1.24	37.33
	Deviation from pop. Mean (%)	20.85	29.48	5.40	25.13	38.15	60.05
0.25	**Mean**	217.15	351.79	224.79	204.67	286.68	314.83
	Confidence, 95%	0.84	0.39	18.90	0.53	0.62	0.69
	STDEV	4.75	2.19	106.97	2.97	3.52	3.88
	Deviation from pop. Mean (%)	22.19	26.06	19.45	26.66	2.72	12.81
0.5	**Mean**	474.38	604.26	444.00	646.77	536.28	605.29
	Confidence, 95%	1.72	0.87	2.43	2.43	2.35	2.92
	STDEV	9.75	4.93	13.77	13.73	13.32	16.52
	Deviation from pop. Mean (%)	14.03	9.50	19.54	17.20	2.82	9.69
1	**Mean**	889.07	1003.87	975.37	1098.97	1001.52	1094.00
	Confidence, 95%	1.89	5.62	3.64	3.24	3.63	3.51
	STDEV	10.69	31.77	20.60	18.32	20.56	19.86
	Deviation from pop. Mean (%)	16.15	5.32	8.01	3.65	5.54	3.18
2	**Mean**	1681.43	1893.03	1863.24	1981.51	1891.17	1890.87
	Confidence, 95%	4.06	2.32	7.07	5.25	4.53	6.97
	STDEV	23.00	13.13	39.98	29.70	25.66	39.47
	Deviation from pop. Mean (%)	5.32	6.59	4.91	11.57	6.49	6.47

This result is not surprising since it was predicted from the calibration curve and formazin studies. Higher kaolin concentrations mean that more particles can scatter the light, giving higher NTU values and more variability.

The reproducibility tests for kaolin gave results similar to the reproducibility test with formazin: at more concentrated kaolin solutions, and consequently more turbid suspensions, the error indicated by the standard deviation is higher than the less concentrated ones.

These results confirmed that all 6 of the sensors can be calibrated with kaolin solutions in a reproducible manner such that the results from each probe are comparable.

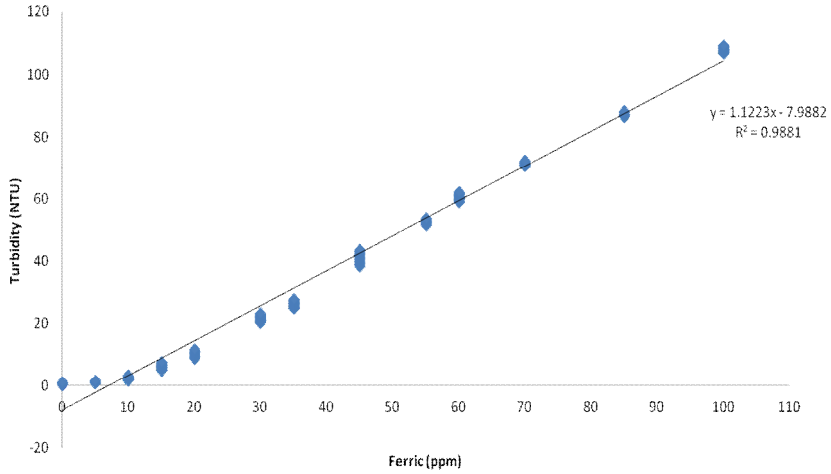
All together these results proved that the probes can be calibrated with kaolin solutions for the study of flocculation and sedimentation.

4.3.5 Effect of flocculantôs colour on reading

Ferric has a typical rusty colour that is often a problem in drinking water processes: it stains water and pipe systems and requires specific treatment to be removed (EPA, 2002). Moreover, the presence of dissolved, colour-causing substances that absorb light may cause a negative interference to a turbidity sensor: a coloured substance absorbs light energy in certain bands of the visible spectrum, changing the character of both transmitted light and scattered light and preventing a certain portion of the scattered light from reaching the detection system (Sadar, 1998). This aspect is usually underestimated in other reported studies; however, in order to obtain precise measurements of turbidity removal, particularly at low turbidities, it is important to test whether the colour of the coagulant influences measurements. To address this problem, the colour effect of the two flocculants was examined in DI water and kaolin suspensions.

As shown in **Figure 4.6**, alum did not increase the turbidity at high concentrations, while ferric deeply affected the turbidity in a proportional way.

a)



b)

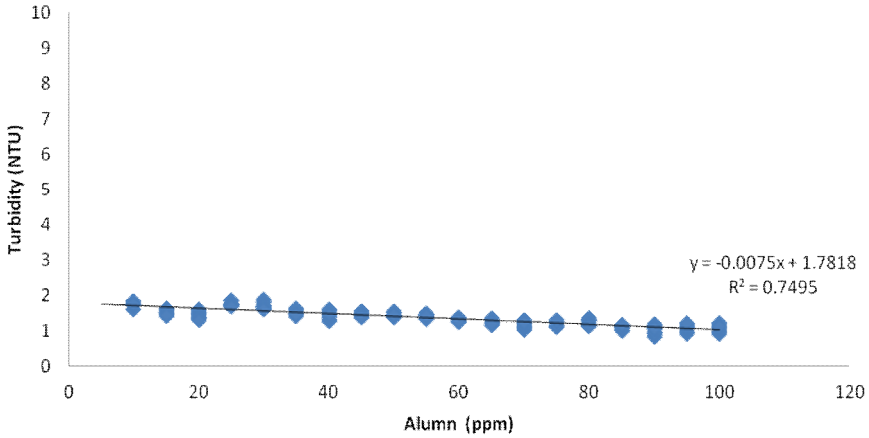


Figure 4.6: Turbidity measurements in DI water of ferric a) and alum b) from 0 to 100 ppm in 5 ppm intervals.

Since kaolin is the turbidity solution used in the present study, the colour effect of the two flocculants was examined under low and high kaolin solutions at high rpm in order to avoid formation of flocs during the reading. Then, a response surface graph was created.

The response surface methodology (RSM) consists of a group of mathematical and statistical techniques that can be used to define the relationships between the response and the independent variables. RSM defines the effect of the independent variables, alone or in combination, in the processes. In this case, it can be a suitable method to investigate whether the different concentrations of flocculants really influence the turbidity reading when increasing the kaolin concentration.

The response surface graph (**Figure 4.7**) shows that ferric had a significant effect on turbidity values at low kaolin concentrations but less at high kaolin concentrations, confirming the data obtained in DI water.

This may be due to the presence of iron precipitates that interfere with the measurement of the kaolin particles which are more numerous at high concentrations.

Interestingly, **Figure 4.7** shows that high alum concentrations only had an effect at high kaolin concentrations (2 g/L). Once again this might be due to possible aggregates of alum forming at high concentrations.

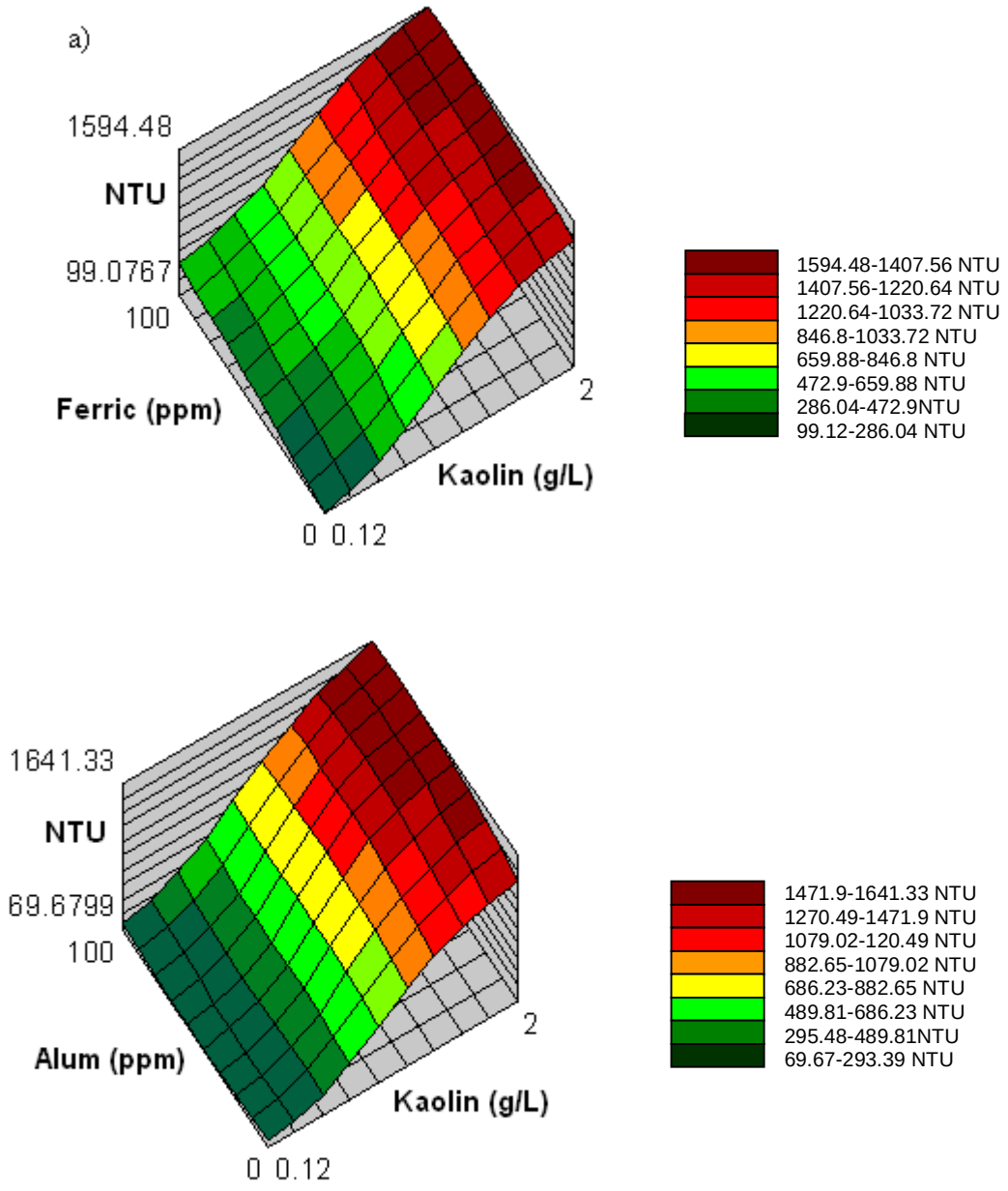


Figure 4.7: Response surface graphs for measured turbidity with varying ferric a) or alum b) and kaolin concentrations.

These results were further confirmed by using a two way ANOVA statistics and the correlated mean plot (**Figure 4.8**). The two way ANOVA tests whether or not there is an interaction between two factors (i.e. flocculant concentration and kaolin concentration) on the process.

The null hypothesis (H_0) postulated was that the different amount of flocculant would not affect the turbidity of kaolin suspensions. The results confirmed that ferric sulphate has a significant effect on the variance in turbidity values, since the p-values were small (less than 0.05) so the H_0 can be rejected, while alum effects the turbidity probes reading only at 2 g/L since for this concentration the p-values were higher than 0.05. These results are showed in the means plot for 0, 1 and 2 g/L taken as an example (**Figure 4.8**). The mean plot is an informative way to gain information on how the mean response changes if the factors are varied in a two way ANOVA model.

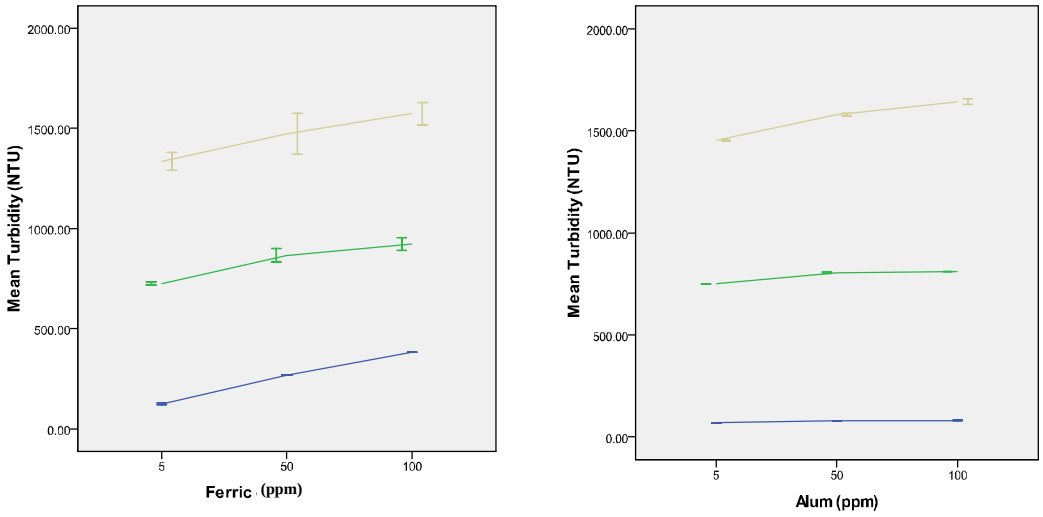


Figure 4.8: Mean plots for 0, 1 and 2 g/L of kaolin suspensions for ferric and alum.

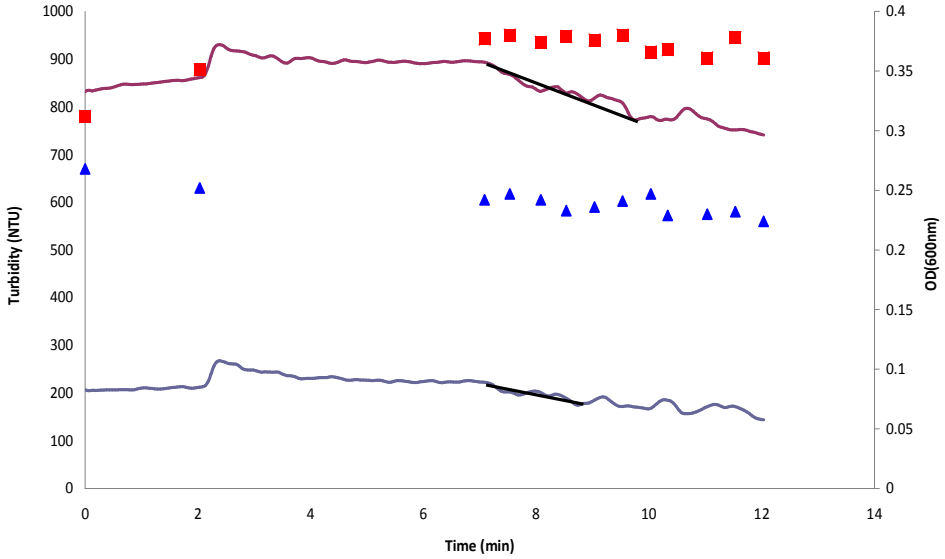
In a mean plot, the average response values are plotted for all the possible factor level combinations. Flocculant concentration is plotted on the *x*-axis and kaolin concentrations are plotted as different coloured curves: blue: 0 g/L; green: 1 g/L and yellow: 2 g/L.

These results demonstrated that even in dark conditions the use of ferric may affect the NTU values, especially at low turbidity levels, and must be taken into account during a flocculation assay.

4.3.6 Measurements of flocculation activity and comparison with the spectrophotometer method

The main aim of this study was to develop a continuous, on-line quantitative method to measure flocculation activity using commercially available turbidity meters and jar test apparatus. So far the standard spectrophotometric method used involves the collection of samples every 30 seconds subsequently read at 600 nm (**Chapter 2, Section 2.2.15**). In this section, a comparison between the data obtained with the turbidity meters and spectrophotometer to confirm the full operation of the probes was proposed. Four concentrations of aluminium sulphate and ferric sulphate (20, 40, 60, 80 ppm) were added to five different concentrations of kaolin (0.125, 0.25, 0.5 1 and 2 g/L), a sample from 40 and 80 ppm was taken every 30 sec for the reading at the spectrophotometer (**Figure 4.9**). The main steps of a typical water treatment process were carried out: 2 minutes at high rpm to mix the flocculant with the kaolin solutions; 5 minutes at low mixing to allow flocculant-particle interactions and 5 minutes of sedimentation. **Figure 4.9** shows the correlation between the spectrophotometer assay (OD) and jar test (NTU) for 40 ppm of alum and ferric at 0.25 and 2 g/L of kaolin as an example; however, the flocculation plots for other concentrations are shown in **Appendix D**. The trend of the graphs was similar for the two flocculants at high and low kaolin concentration. These results further validate the use of turbidity probes as methods for measuring flocculation assays. For the analysis of flocculation assay, the present work has focused principally on the kinetics of flocculation rather than floc structure and size, although it should be possible to get this data.

a)



b)

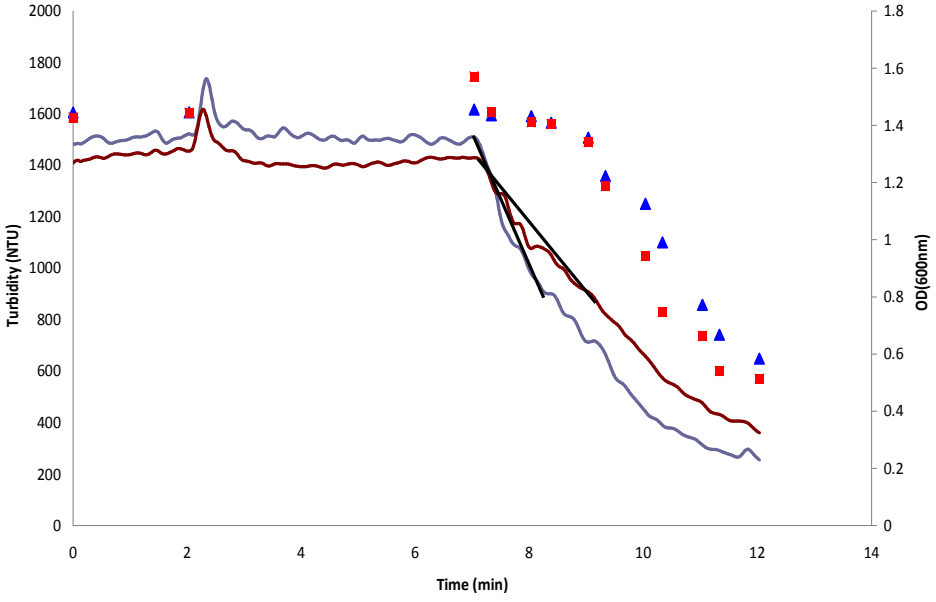


Figure 4.9: Comparison of flocculation assay using the turbidity probes and spectrophotometer in 0.25 g/L a) and 2 g/L b) of kaolin suspension.

Red line 40 ppm ferric sulphate and Blue line 40 ppm aluminium sulphate at turbidity meters. Red squares 40 ppm ferric sulphate and blue triangles 40 ppm aluminium sulphate at the spectrophotometer. Black line indicates the initial slope.

In flocculation assays it is usually only the residual NTU or turbidity removal obtained after the settling time that is considered, together with the time taken to achieve this, or the level of turbidity after a pre-determined time period (Huang and Chen, 1996, Rossini et al., 1999, Franceschi et al., 2002, Pritchard et al., 2010a). Further studies were mainly preoccupied with floc size as selective parameter for the appropriate coagulant. In this work, an alternative method to classify the turbidity has been developed which allows the sedimentation rate, defined by the initial slope of turbidity as a function of time. This should provide a better understanding of the kinetics of flocculation and allow a better description and comparison of flocculants in terms of rate and efficiency: the more negative the slope, the faster the rate of floc formation and consequent sedimentation. This is clearly demonstrated in **Table 4.5**, where the sedimentation rates had different values for alum and ferric. From the comparison of these rates, ferric showed a higher flocculation activity at low kaolin concentration than alum, while the latter worked better at concentrations >0.5 g/L. In fact, the sedimentation rates for ferric at 0.125 and 0.250 g/L were more negative and higher than for alum (for example, -33 NTU/min vs. -11.50 NTU/min at the dosage of 20 ppm of ferric and alum, respectively), as confirmation that the flocculation is faster and better. By contrast, for higher kaolin concentrations the alum values were higher and more negative. For example, at 1 g/L of kaolin suspension the sedimentation rates were between -114.51/-126.04 NTU/min for alum, while between -76.52/-84.40 NTU/min for ferric. Moreover, these values increased with increasing kaolin concentration: indeed, it is well known that at low colloid concentrations there is very little opportunity colloid-colloid interactions, therefore some particles will remain stable even though the coagulant is introduced (Bratby, 2006). This was further confirmed by plotting the standard deviation of turbidity versus time (**Figure 4.10**). As previously demonstrated by Cheng et al. (2008), during the high mixing time the colloidal suspension is uniform

and the reflected light intensity shows little variation with time. As a consequence the turbidity during the first two minutes is relatively constant. During the slow mixing time and period of sedimentation, the flocs are allowed to form resulting in fluctuations in turbidity represented by the standard deviation which is directly related to floc size. The results (**Figure 4.10**) showed that in the system developed here the flocs formed at higher kaolin concentrations were significantly larger than those at low concentrations for both flocculants.

Moreover, **Table 4.5** shows that while turbidity removal may be affected by the colour of the coagulant and gave negative results, as for low kaolin concentrations, the sedimentation rates were not affected.

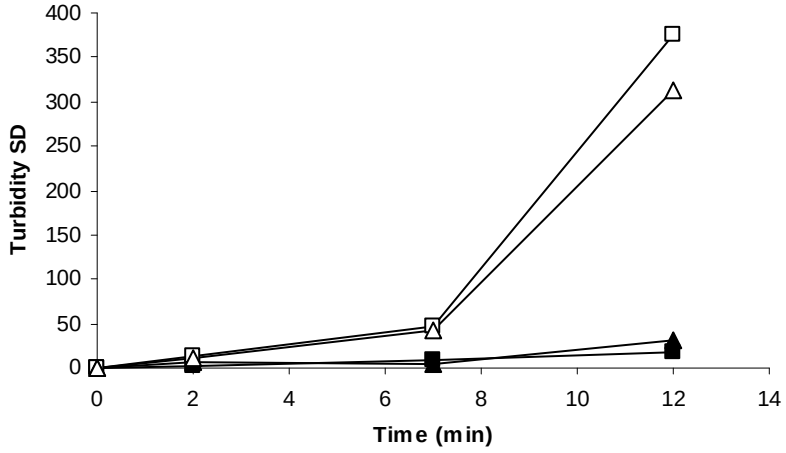


Figure 4.10: Plot of the SD of turbidity at various times for alum and ferric.

The data between minutes 0 and 2, 2 and 7 and 7-12 were grouped together and the SD calculated and plotted Cheng et al. (2008). Squares: 40 ppm of alum in 0.25 g/L kaolin suspension; Open squares: 40 ppm of alum in 2 g/L kaolin suspension; Triangles: 40 ppm of ferric in 0.25 g/L kaolin suspension; Open Triangles: 40 ppm of ferric in 2 g/L kaolin suspension

Table 4.5: Flocculation assay results for different concentration of aluminium sulphate and ferric sulphate in five kaolin suspensions (from 0.125 to 2 g/L).

Slope indicates the mean of the sedimentation rates over three runs for each concentration of flocculant. Turbidity removal represents the mean of turbidity removal over three runs.

		Aluminium sulphate (ppm)				Ferric sulphate (ppm)			
Kaolin (g/L)		**20**	**40**	**60**	**80**	**20**	**40**	**60**	**80**
0.125	**Sedimentation rate (NTU/min)**	-11.50	-11.78	-11.65	-12.56	-33.00	-27.05	-16.63	-15.41
	STDEV(Sedim. rates)	0.7	0.9	0.9	1.7	4.3	3.6	4.2	8.4
	Turbidity removal (%)	39.38± 7.6	34.75±7.6	33.85±11.1	34.54±6.8	27.40±12.5	10.20±77.7	-16.54±39.3	-42.51±5.7
0.25	**Sedimentation rate (NTU/min)**	-19.39	-19.80	-24.26	-25.37	-44.24	-40.72	-29.12	-31.40
	STDEV(Sedim. rates)	6.6	4.7	2.8	2.2	7.5	2.1	6.1	6.7
	Turbidity removal (%)	40.97±5.7	48.31±12.6	32.38±4.6	38.11±1.4	34.11±7.8	24.39±3.1	19.42±2.8	27.16±5.6
0.5	**Sedimentation rate (NTU/min)**	-80.00	-67.48	-75.37	-83.27	-90.85	-55.21	-72.18	-55.72
	STDEV(Sedim. rates)	61.1	4.1	57.3	50.5	28.7	0.7	19.7	1.0
	Turbidity removal (%)	63.30±15	51.30±3.7	61.21±18.5	65.78±18.4	42.12±8.3	43.17±0.6	45.10±5.1	38.16±4.6
1	**Sedimentation rate (NTU/min)**	-125.46	-114.51	-115.81	-126.04	-76.52	-78.40	-80.59	-84.40
	STDEV(Sedim. rates)	2.1	9.1	8.4	11.4	6.1	8.2	0	14.2
	Turbidity removal (%)	66.26±3.3	72.24±0.9	64.86±3.9	71.22±7.9	55.11±3.6	57.70±4.4	66.46	65.71±11.5
2	**Sedimentation rate (NTU/min)**	-323.56	-305.50	-311.31	-381.12	-287.17	-238.44	-247.18	-255.96
	STDEV(Sedim. rates)	5.9	15.3	41.2	32.7	6.6	7.7	6.1	32.1
	Turbidity removal (%)	83.34±6.8	92.73±9.2	78.83±2.7	84.65±2.6	86.19±2.3	86.57±1.8	87.05±3.3	85.91±1.0

4.3.7 Conclusions

In this study, a continuous, online, quantitative method for flocculation assays was developed. This was completed by adapting a standard jar test apparatus through the addition of six commercial turbidity probes connected to a data acquisition system. This allows six simultaneous, online measurements of flocculation activity under identical conditions. As the samples in a standard spectrophotometric assay are taken off line, while the measurements using turbidity probes are taken online, it should be possible to determine the kinetics of flocculation more accurately and more quickly using multiple measurements simultaneously.

Although Cheng et al. (2008) first connected turbidity probes to a jar test vessel, they investigated the floc size during the flocculation process using only one beaker. Our study delves more into the use and characteristics of a jar test apparatus with six vessels connected to six probes and the analysis of the data gained.

The validation studies proved that the data collected from the six turbidity probes are affected by ambient light, thus the experiments needs to be carried out in darkened conditions; however, the results are repeatable between probes and for each probe itself. The slope of the NTU as a function of time is defined as the sedimentation rate which, combined with the total difference in NTU during the flocculation experiment, are measurements of the efficiency of the flocculation. These parameters may play an important role in the evaluation and control of the flocculation activity of the different flocculants and types of water in order to optimise parameters such as dosage and pH. This should greatly facilitate research into water treatment, such as comparing the efficiency of different flocculating agents, while providing key information to water treatment facilities on variations in water turbidity and the optimum conditions for the treatment of this water.

CHAPTER 5

Characterisation of flocculation activity of a range of purified proteins compared to soluble protein extracts from press-cakes of different oleaginous plants

Abstract:

Coagulation and flocculation are very important steps in drinking water treatment processes. Currently, inorganic salts of iron and aluminium are used in association with polymers such as acrylamide derivatives which may be associated with health risks and the resulting sludge may require specialised disposal.

Attempts have been made to find alternatives, particularly for use in developing countries, which are cheap, sustainable and ideally biodegradable. To this end, extracts of the seeds and press-cakes of *Moringa oleifera* have been widely reported, although such plants are limited to sub-tropical regions. Since it has been suggested that the proteins found in soluble extracts of moringa are the active components of these oleaginous seeds, the aim of this work was to determine whether proteins from other oleaginous plant seeds and commercially available proteins also exhibited flocculation activity. In this study, the flocculation activity of extracts from seed press-cakes of hemp, sunflower and rapeseed have been investigated using a modified on-line jar test apparatus. The activity of these extracts, together with a range of commercially available proteins exhibiting a range of molecular size and pI, has been compared to the activity obtained with aluminium and ferric sulphates.

5.1 Introduction

Aluminium and iron salts are the most commonly used coagulants in water treatment. However, the cost and the environmental side effects of these compounds increased the interest in the use of organic coagulants derived from plants (Ghebremichael et al., 2005).

The most extensively studied as alternative water treatment agents are seed extracts of the oleaginous plant *Moringa oleifera*.

It has been reported that the active components of *M. oleifera* are cationic peptides of relatively low molecular weight, 6.5 and 13 kDa (Gassenschmidt et al., 1995, Ndabigengesere et al., 1995), and adsorption and neutralisation of the charges are the main mechanisms of coagulation (Ndabigengesere et al., 1995).

M. oleifera extracts remove between 80 and 99% of the turbidity, have negligible effect on the pH and conductivity of the water after treatment, also produce sludge with five times less volume than alum (Ndabigengesere et al., 1995). Moreover, in 2005 Ghebremicheal showed that cationic peptides have also an anti-bacterial activity, a characteristic that makes these flocculating agents unique.

For these reasons, it has been suggested that these extracts may be considered non-toxic and biodegradable coagulants for the treatment of drinking water especially in developing countries where moringa grows.

However, the major disadvantage of using moringa extracts as bioflocculants here is that this oleaginous plant does not grow well in Europe as it is typical of sub-tropical countries, like Sudan and India. As a consequence, the importing and storage costs would be prohibitive to European countries, while the variability in flocculation activity resulting from storage and spoilage would be difficult to control. Therefore, it would be important to determine whether flocculants may also be extracted from plant seeds that are more common in Europe.

In **Chapter 3**, it was shown that NSC extracted from rapeseed have a very high flocculation activity. Nevertheless, the aqueous layer obtained through UREA treatment showed also a high flocculation activity due to the presence of soluble proteins.

Press-cakes (PC) represent the by-product of the seeds after oil extraction and are usually considered a waste product, although they are very rich in proteins. In recent years their application in water treatment as bio-sorbent has become an expanding field (Boucher et al., 2007, Boucher et al., 2008b)

It has been shown that moringa PC extracts still contain the active components effecting the flocculation (Sutherland et al., 1994, Doerries, 2005), clearly indicating that a scale-up might be feasible using PC as source (Doerries, 2005). The extraction of flocculating proteins from PC may have a great advantage since, being considered waste products, they would be a very cheap raw material.

Rapeseed is one of the most cultivated crops for oil and animal feed in Europe with production of 21.5 million tonnes a year, while annual sunflower seed production is equivalent to 6.9 million tonnes (Fediol). Hemp production has also dramatically increased in recent years (Karus and Vogt, 2004). Thus, PC extracted from these seeds would be a readily available source.

In this chapter, flocculation activity of extracts from hemp, rapeseed, sunflower and moringa PC were tested using the modified on-line jar test apparatus described in **Chapter 4** and compared to the activity of aluminium sulphate (alum) and ferric sulphate (ferric), two flocculants currently used in water treatment

Moreover, to investigate whether flocculation activity is a unique property of oleaginous seeds or it is detectable in other kind of proteins, different commercial purified proteins from animal sources were tested for flocculation and their activity has been compared to PC extracts. The purified proteins analysed were γ-globulin, BSA, casein and α-lactalbumin which have been chosen for their differing molecular weights

and pIs. The activity of two well-known animal proteins used in the food industry, fish gelatin and isinglass, have also been tested and compared with the flocculation activity of PC extracts.

5.2 Materials and Methods

5.2.1 Preparation of commercial purified protein solutions

BSA, casein, γ-globulin and fish gelatin solutions were made in DI water at concentrations of 20 mg/mL, while a α-lactalbumin solution was prepared at 10 mg/mL. All solutions were stored at 4°C until use. Isinglass was obtained as a solution from Homebrewery (Ireland).

For all the six solutions a BCA protein assay was carried out to determine the exact concentration of proteins before flocculation test.

The commercially available proteins were chosen according to their molecular weight and pI (**Table 5.1**).

Table 5.1: **Characteristics of the selected commercial purified proteins**

Protein	Molecular Weight (kDa)	Isoelectric point (pI)
γ-globulin*	150	6.5
BSA*	66	4.7
Casein*		
α-s1	22-23.7	10.0
α- s2	25	--
β	24	4.6-5.1
κ	19	4.1-5.8
α -lactalbumin*	14	4.2-4.5
Fish Gelatin*	60	6
Isinglass**	140	5.5

***** Information available from Sigma website (http://www.sigmaaldrich.com)
**(Hornsey, 2007)

5.2.2 BLAST

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Protein BLAST was used to determine if amino acid sequences similar to *MO 2.1* (Gassenschmidt et al., 1995) (accession number: P24303.1) were observed in proteins from rapeseed (*Brassica napus*), hemp (*Cannabis sativa*) and sunflower (*Helianthus annuus*). All the sequence alignments were performed using default parameters in each specific organism, using Non- Redundant protein sequences.

5.3 Results and Discussion

5.3.1 Flocculation activity of soluble proteins extracted from different Press-cakes

The use of seeds from different plant sources in water treatment has been investigated for many years (Ndabigengesere et al., 1995, Babu and Chaudhuri, 2005, Pritchard et al., 2009). However, although natural flocculants provide benefits not only to the environment but also to human health, it might be questionable whether the scale-up system can sustain similar treatment performance at comparable (or reduced) cost with the natural coagulants when compared with established chemical coagulants (Yin, 2010). In the case of oleaginous seeds, the use of the by-product of vegetable oil production, namely PC, might be a viable alternative for the extraction of natural flocculants. Since the use of moringa extracts is difficult due to limited global distribution of the plant, in this study the flocculation activity of extracts from plants

that commonly grow in Ireland and across Europe (hemp, rapeseed, sunflower) was determined using a quantitative modified jar test apparatus, as described in **Chapter 4**.

Previous studies demonstrated that the agent responsible for the flocculation activity in moringa seeds and PC extracted under high ionic conditions (Okuda et al., 1999, Okuda et al., 2001a, Okuda et al., 2001b, Doerries, 2005) shows better coagulation activity than components extracted by distilled water in the removal of kaolinite turbidity. The author attributed this enhanced effect to the salting-in mechanism in proteins wherein a salt increases protein-protein dissociations and protein solubility as the salt ionic strength increases (Okuda et al., 1999). For this reason, a similar extraction procedure was employed for all the PC under investigation.

PC protein content from each plant was analysed using the BCA assay as shown in **Table 5.2**.

Table 5.2: **Total protein concentration of the PC extracts measured using BCA method**

Press-cakes	Total protein (mg/mL)
Moringa	50.12
Hemp	16.50
Rapeseed	27.43
Sunflower	39.29

Results in **Table 5.2** showed that, using a salt extraction method, Moringa extracts contained the highest amount of proteins (50.12 mg/mL) followed by sunflower (39.29 mg/mL), rapeseed and hemp (27.43 and 16.50 mg/mL, respectively).

In order to standardise the flocculation assays and allow direct comparison, the amount of the soluble PC extracts added to the flocculation assay was kept constant at a level of 10 and 40 ppm and subsequently tested for flocculation activity using the modified jar test apparatus with kaolin as model particles at three pH values (pH 5, 7 and 9) and two ionic strengths (10 and 100 mM NaCl). The data obtained from the continuous, on-line

data acquisition system operating with LabView, were plotted as the average of the three runs. Activities were compared to those of the reference industrial flocculants alum and ferric. As explained in **Chapter 2, Section 2.2.16**, the sedimentation rate was calculated by determining the initial slope of turbidity as a function of time. **Figure 5.1** shows an example of the procedure for alum at pH 5 under high ionic strength conditions.

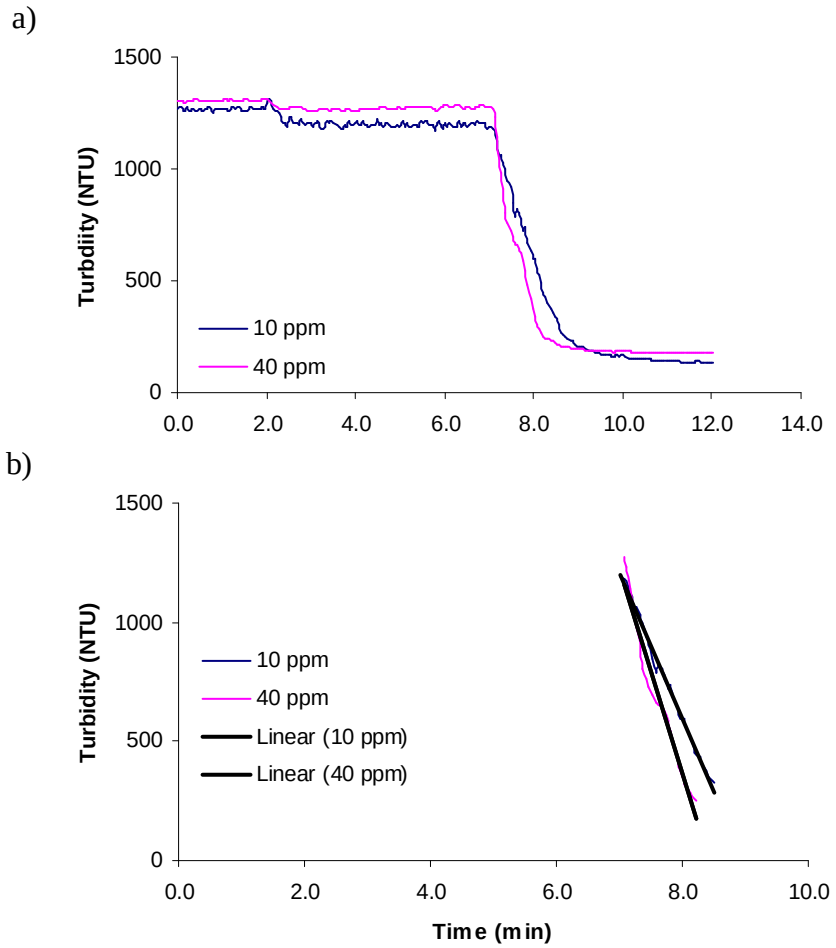


Figure 5.1: **Flocculation activity of alum (10 and 40 ppm) at pH 5 under high ionic strength conditions.**

The solution is agitated at a high rate for 2 min, agitated at a low rate for 5 min and no agitation, to enable sedimentation for 5 min (from min 7 to 12). The data were plotted in function of time (a) and the sedimentation rates were determined from the initial linear slopes from min 7 to min 8.5 for 10 ppm and 7 to 8.2 min for 40 ppm (b). R^2 values were 0.963 and 0.9931 for 10 and 40 ppm, respectively.

After plotting the NTU values as function of time (**Figure 5.1a**), the linear slope was identified and a second graph was plotted from min 7 to the end of the linear slope (**Figure 5.1b**), in this specific case to min 8.5 and 8.2 for 10 and 40 ppm, respectively. However, this was adapted to each sample and the sedimentation rate calculated according to **Equation 2.2**.

5.3.2 Effect of pH and ionic strength on the flocculation of soluble proteins extracted from PCs

5.3.2.1 Effect of high ionic strength

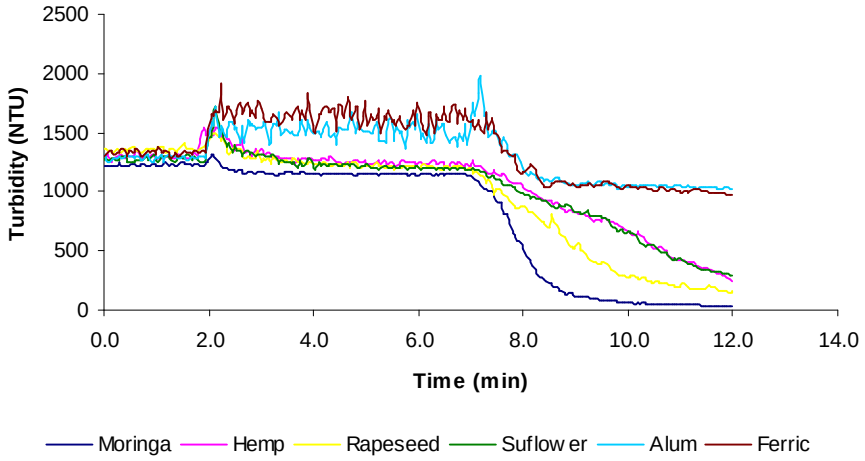
The sedimentation rates and total turbidity removal by the PC were analysed at pH 5, 7 and 9 as shown in **Table 5.3**. The results for pH 7 under high ionic strength are shown in **Figure 5.2**, while the results for pH 5 and 9 are shown in **Appendix E**.

At pH 7 all seed extracts showed a higher flocculation activity than the reference standards (alum and ferric, **Table 5.4)**. Among them moringa extracts at 10 and 40 ppm showed the highest activity with 97.24% and 89.26% of turbidity removal that is 70% and 47% higher than alum and ferric, respectively. Rapeseed extracts showed a turbidity removal >82% at pH 7, while extracts from hemp and sunflower presented a 70-80% turbidity removal. These values indicated that flocculation activity in PC extracts was higher than the two standard ones by 30-40%. Indeed, although alum and ferric have very negative sedimentation slopes at pH 7 (-334.18 and -480.15 NTU/min for 10 and 40 ppm of alum; -327.80 and -725.11 NTU/min for 10 and 40 ppm of ferric), the overall turbidity removal is less than 45%, indicating that the flocs formed are not dense enough to sediment.

Interestingly, the highest flocculation activity was observed with 10 ppm of all extracts tested with the exception of sunflower, whose highest activity was detected for 40 ppm (**Table 5.3**).

It may be postulated that rapeseed and hemp extracts have a polymer bridging mechanism, in a similar way to moringa proteins, since large doses of moringa extracts lead to overdosing that saturates the polymer bridge sites. This result suggests that the re-stabilization of the de-stabilized particles is due to an insufficient number of particles necessary to form further inter-particle bridges (Pise et al., Alfrey, 1993, Muyibi and Evison, 1995a, Majithiya et al., 2013). On the other hand, sunflower extracts may contain proteins that work through a sweep flocculation mechanism, since higher concentrations are required to give optimum activity. It appears that these two mechanisms provide underlying principles to the plant-based coagulants (Yin, 2010).

a)



b)

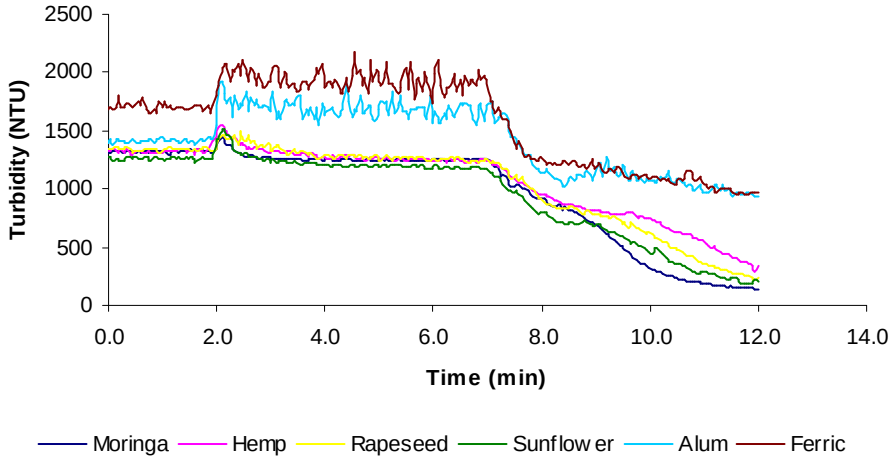


Figure 5.2: **Flocculation activity of soluble proteins extracted from different PC measured at pH 7 under high ionic strength conditions using 10 ppm (a) and 40 ppm (b) of protein extracts.**

At pH 5, all extracts showed an activity comparable or higher to those of alum and ferric, although their flocculation activity was higher than at pH 7, especially for alum where the turbidity removal is 89.56% and 86.61% with a sedimentation slope of -574.22 and -888.64 NTU/min. Also ferric showed an increase in activity, but only for the lowest concentration (76% of turbidity removal).

Moringa extracts at pH 5 displayed a decrease of 3% in turbidity removal compared to pH 7, although it still had the highest values among all extracts. Hemp and rapeseed extracts had a very high flocculation activity, with over 92% turbidity removal, while sunflower extracts had more than 86%. The sedimentation rate for these three extracts was slower than alum, though it was faster than ferric and the overall turbidity removal was improved by 5% for hemp and rapeseed compared to alum and 15-60% compared to ferric (for 10 and 40 ppm).

At pH 9, all PC extracts presented a flocculation activity comparable to the two standards. Alum showed a high flocculation activity only at 40 ppm (96.47% turbidity removal compared with 50.62% at 10 ppm), while ferric showed a 62.18% and 82.06% turbidity removal at 40 and 10 ppm, respectively. The initial sedimentation rates were slower than at pH 7: -142.33 and -457.41 NTU/min for 10 and 40 ppm of alum; -165.44 and -255.83 NTU/min for 10 and 40 ppm of ferric.

Moringa extracts showed the best flocculation activity among the three pH: for 10 ppm, -783.33 NTU/min for the sedimentation rate and 97.71% of turbidity removal; for 40 ppm -298.91 NTU/min for the sedimentation rate and 90.56% of turbidity removal. It has been demonstrated by several studies (Ndabigengesere et al., 1995, Mandloi et al., 2004) that the optimum pH of moringa flocculants is slightly basic since at pH higher than 7, kaolin and clay particles are predominately negatively charged. This enables adsorption to occur between kaolin particles and cationic polyelectrolytes from moringa which destabilised the former, causing charge neutralisation to occur.

Rapeseed extracts showed a significant flocculation activity having a turbidity removal higher than 87% and sedimentation rates higher than -315 NTU/min makes this extract better than ferric. Sunflower extracts have still a good activity with turbidity removal of 78 and 85%, while hemp extracts displayed decreasing activity under this condition (72.91% and 69. 98% of turbidity removal), indicating that, despite the high ionic

strength, the negative charged kaolin particles at pH 9 may have affected the flocculation mechanisms of this extract.

Table 5.3: **Sedimentation rate values and total turbidity removal (Δt) for PC extracts at high ionic strength**

		Moringa		Hemp		Rapeseed		Sunflower	
	ppm	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-563.70	-252.37	-501.14	-282.26	-370.88	-311.89	-299.62	-318.68
	r^2	0.97	0.99	0.96	0.98	0.98	0.99	0.99	0.99
	Turbid. Removal (%)	95.99±0.5	87.37±1.7	93.12±1.3	93.19±0.6	92.05± 1	92.35±3.2	86.61±1.1	87.42±0.7
pH 7	**Sediment. Rate (NTU/min)**	-556.24	-284.42	-195.29	-180.03	-312.09	-203.35	-174.52	-206.76
	r^2	0.98	0.99	0.99	0.97	0.99	0.98	0.99	0.98
	Turbid. Removal (%)	97.24±0.3	89.26±2.6	80.77±5.0	74.44±2.7	88.32±2.1	82.12±2.4	76.48±3.0	83.88±7.7
pH 9	**Sediment. Rate (NTU/min)**	-783.33	-298.81	-188.40	-176.78	-327.04	-318.69	-193.50	-225.11
	r^2	0.99	0.99	0.98	0.97	0.99	0.99	0.98	0.99
	Turbid. Removal (%)	97.71±0.98	90.56±0.93	72.91±5.6	69.98±5.9	87.34±1	88.71±1.9	78.33±6.5	85.90±3.7

Hemp, rapeseed and sunflower extracts exhibit a higher activity (<80% of turbidity removal and sedimentation rates > than -310 NTU/min) at pH 5 which is in contrast to moringa extracts. This may suggest that in these PC the flocculating agents may have a lower pI than Moringa and/or in the flocculating mechanism they are more dependent on kaolinôs positive charge.

However, as shown so far, the flocculation activity of PC extracts is comparable to that of alum and ferric, indicating that these PC extracts have potential as bioflocculants.

Table 5.4: Sedimentation rates and total turbidity removal (Δt) for alum and ferric at high ionic strength

		Alum		**Ferric**	
	ppm	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-574.22	-888.64	-251.52	-209.91
	r^2	0.99	0.96	1.00	0.96
	Turbid. Removal (%)	89.57±2.7	86.61±1.3	76.00±4.1	25.51±0.1
pH 7	**Sediment. Rate (NTU/min)**	-334.18	-480.15	-327.80	-725.11
	r^2	0.87	0.95	0.91	0.94
	Turbid. Removal (%)	22.28±3.4	32.28±5.3	25.25±5.0	42.31±2.8
pH 9	**Sediment. Rate (NTU/min)**	-142.33	-457.41	-165.44	-255.83
	r^2	0.92	0.99	0.97	0.99
	Turbid. Removal (%)	50.62±5.2	96.47±1.5	62.18±6.2	82.06±2.0

5.3.2.2 Effect of Low Ionic Strength

The results for pH 7 under low ionic strength are described here and shown in **Figure 5.3**, **Tables 5.5** and **5.6**, while the results for pH 5 and 9 are shown in **Appendix F**. Under low ionic strength conditions at pH 7, moringa showed the highest activity (96.16% and 91.61% of turbidity removal for 10 and 40 ppm extracts, respectively) which is 75% and 81% higher than alum and ferric, respectively. The sedimentation rates are -726.70 NTU/min and -345.66 NTU/min for moringa extracts, -293.77 and -302.62 NTU/min for alum and -455.38 and -188.58 NTU/min for ferric depending on the concentration of flocculant added. Thus, moringa protein extracts had a faster sedimentation of flocs than the two standards. All the other PC extracts showed higher flocculation activity than the reference standards with turbidity removal > 70% in all cases. However, the rates of sedimentation were slower than for moringa extracts, alum and ferric as shown in **Figure 5.3** and **Table 5.5**.

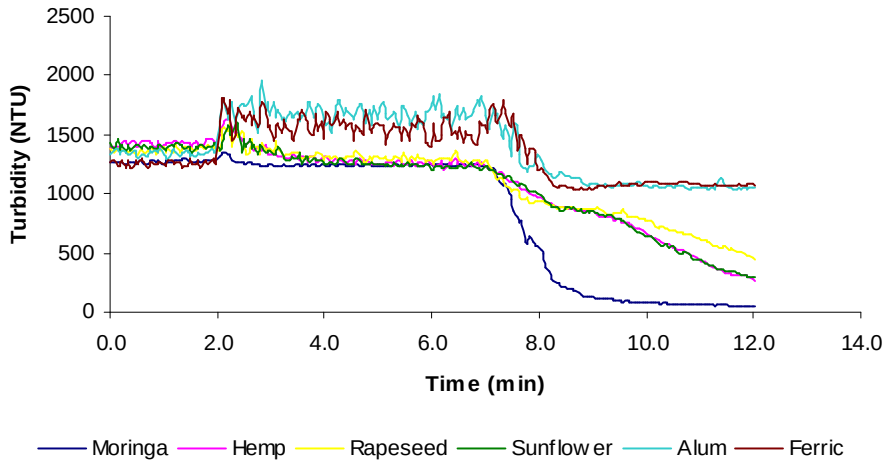
Interestingly, at pH 5 rapeseed and hemp show an activity which is comparable to moringa and alum, but up to 60% higher than ferric. For example for hemp, the rate of sedimentation (-572.68 and -491.94 for 10 and 40 ppm, respectively) is 53-83% slower than alum and 77-144% slower than moringa extracts. However, the total turbidity removal using hemp proteins (95.56-99.04%) was actually higher than Moringa (91.54-93.62%), alum (86.23-95.39%) and ferric (39.97-73.95%) (**Table 5.5** and **5.6**).

As previously shown under high ionic strength condition, at pH 9 moringa retained a very high flocculation activity (over 94% turbidity removal) with sedimentation of flocs faster than 160% of the two standards. On the other hand, the other three PC extracts had a very low activity (> 50% of turbidity removal with the exception of 40 ppm rapeseed). For example, hemp extracts had a turbidity removal < 45% and sedimentation slope around -125 NTU/min, indicating that floc formation occurred at a lower density.

Generally the PC extracts showed high turbidity removal under the three pH values analysed, which is in contrast with data from other plant extracts, such as cactus, moringa, common bean and *Jatropha carcas*, that exhibited high turbidity removal at pH 9/10 (Okuda et al., 2001b, Ġíban et al., 2005, Miller et al., 2008, Antov et al., 2010, Abidin et al., 2013). Thus, for these extracts it might be likely that the flocculating agent(s) has a common mechanism such as charge neutralisation. However, it can be hypothesized that the flocculating compounds present in PC extracts have different mechanisms which allow them to have high flocculation at different pH values.

Interestingly, while hemp, rapeseed and sunflower extracts did possess total turbidity removal of 70-90% at pH 5 and 7 under high and low ionic strength conditions and pH 9 at high ionic strength, at pH 9 under low ionic strength conditions, there was a dramatic reduction in turbidity removal of 30-40% which may indicate that neutralisation of charges plays an important role in flocculation mechanism.

a)



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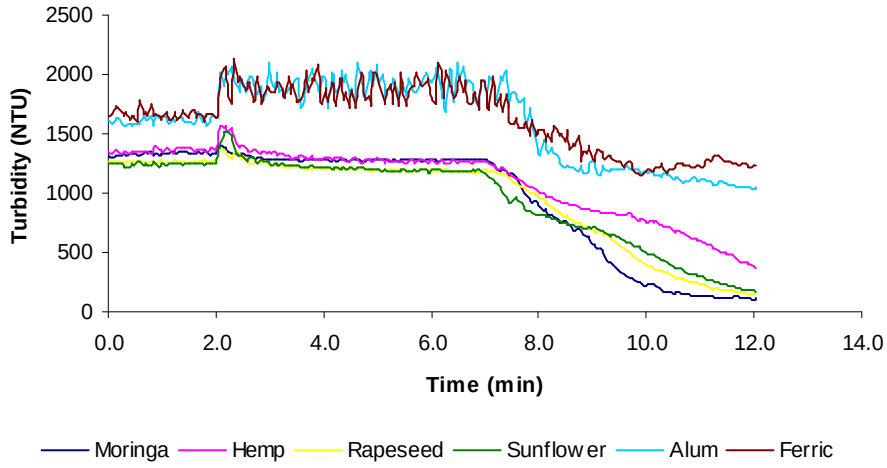


Figure 5.3: **Flocculation activity of soluble proteins extracted from different PCs at pH 7 under low ionic strength conditions at 10 ppm (a) and 40 ppm (b)**

Table 5.5: **Sedimentation rates and total turbidity removal (Δt) for each PC extract at low ionic strength.**

		Moringa		Hemp		Rapeseed		Sunflower	
	ppm	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-743.81	-341.56	-572.68	-491.94	-350.18	-699.75	-314.27	-374.57
	r^2	0.96	0.98	0.99	0.98	0.98	0.99	1.00	0.99
	Turbid. Removal (%)	93.62±2.9	91.54±1.7	95.56±0.9	99.04±0.1	89.57±1.3	96.5±1.8	85.61±3.3	83.28±1.9
pH 7	**Sediment. Rate (NTU/min)**	-726.70	-345.66	-195.70	-178.25	-162.75	-255.97	-184.95	-196.22
	r^2	0.98	0.99	0.99	0.98	0.95	1.00	0.99	0.98
	Turbid. Removal (%)	96.16±2.0	91.61±1.8	80.39±2.2	72.50±2.6	67.13±3.7	88.45±1.5	79.09±1.7	86.07±2.3
pH 9	**Sediment. Rate (NTU/min)**	-687.47	-575.87	-126.64	-125.37	-519.88	-204.91	-124.34	-112.66
	r^2	0.99	0.99	0.91	0.84	0.95	0.99	0.91	0.84
	Turbid. Removal (%)	95.59±0.7	93.99±0.5	45.33±4.6	40.88±8.9	35.40±2.2	75.60±6.2	35.11±2.55	48.71±9.7

These results clearly show that it is possible to produce aqueous extracts from a range of press-cakes and that these extracts have a rate of flocculation under certain conditions of pH, ionic strength and dosage, which are comparable to and, in some cases better than, the activity of the reference compounds alum and ferric. The active component(s) within the extracts would appear to be protein, since treatment with proteases (proteinase K) and reducing agents (DTT) resulted in complete loss of flocculation activity (Conaghan, 2013).

Since it has been reported that moringa extracts have high flocculation activity due to the presence of a heterodimeric protein having a pI of >10.5 (Ndabigengesere et al., 1995), this would explain why moringa extracts exhibited a high flocculation activity under all pH conditions (the net charge on these highly cationic proteins would always be positive at pH 5, 7 and 9).

In a water treatment process combining extracts from a range of PC, could potentially be used to ensure high flocculation rates over a broad range of conditions (pH, ionic strength and dosages).

Table 5.6: Sedimentation rates and total turbidity removal (Δt) for alum and ferric at low ionic strength

		Alum		**Ferric**	
	ppm	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-1081.88	-597.36	-228.43	-122.04
	r^2	0.98	0.99	0.98	0.96
	Turbid. Removal (%)	95.39±0.4	86.23±0.8	73.95±3.1	39.97±11.0
pH 7	**Sediment. Rate (NTU/min)**	-293.77	-302.62	-455.38	-188.58
	r^2	0.81	0.88	0.92	0.90
	Turbid. Removal (%)	21.13±5.1	35.47±1.6	14.98±5.1	25.23±3.4
pH 9	**Sediment. Rate (NTU/min)**	-149.50	-353.04	-229.18	-249.43
	r^2	0.98	0.98	0.99	0.99
	Turbid. Removal (%)	54.34±15.9	94.73±1.4	83.83±8.8	91.22±5.4

Suarez et al. (2005) analyzing the amino acid sequence of Flo, the recombinant protein of MO 2.1 (Broin et al., 2002), found an high degree of similarity with several polypeptides belonging to the family of 2S albumin seed storage proteins from various plant species. These proteins, which are compact globular proteins with conserved cysteine residues, are synthesized as a precursor polypeptide that is cleaved to form the A and B chains of the mature protein (Shewry et al., 1995).

A database and literature search was undertaken to identify possible proteins within the seeds and press-cakes of the studied plant species that are similar to the moringa proteins MO 2.1 and MO 2.2 reported by Gassenschmidt et al. (1995).

For hemp no sequences for seed proteins were found whereas for rapeseed and sunflower a match was found with the seed storage proteins, napin and albumin 8 (**Figure 5.4**).

```
Rapeseed:
MO 2.1   CPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST
         CP+L+QA + + + QGQ GP Q  ++Y++A N+P+
Napin    CPTLKQAAK-SVRVQGQHGPFQSTRIYQIAKNLPNV

Sunflower:

MO 2.1       RQPDF-QRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQVGPQQVR---QMYRVASNIP
             R+ D  Q C  QL+N+     CP++   +           P  +R   Q+  +A N+P
Albumin 8    REEDHKQLCCMQLKNLDEKCMCPAIMMMLN---------EPMWIRMRDQVMSMAHNLP
```

Figure 5.4: **Primary sequences of flocculating protein published by Gassenschmidt et al. (1995) and matches found in rapeseed and sunflower seeds.**

The sequences correspond to napin nIa (large chain) in rapeseed (accession number: AAB21100.1) and albumin 8 in sunflower (accession number: P23110). The ñ+ò symbol represents equivalent amino acids.

As shown in **Figure 5.4**, homologies with the large chain of napin nIa in rapeseed seeds and MO 2.1 were identified. Napin belongs to the 2S seed storage albumin family and has a low molecular weight (14.5 kDa), however, a minor component is represented by

nIa and nIb which are 12.5 kDa and are typical of *B. napus* (Monsalve et al., 1991). Both proteins are composed of 2 different chains, small and large linked by disulphide bonds. This is consistent with the homology indicated by Suarez et al. (2005), who suggested that Flo is homologous to a part of the large chain of napin, implying that it corresponds to the B chain of the moringa 2S seed protein. **Figure 5.4** shows the results obtained for sunflower: albumin 8, a methionine rich protein belonging to the 2S albumin family. Data in the literature report that this protein has a molecular weight of around 12 kDa and it is composed of two polypeptide chains (Kortt et al., 1991).

The pI of these two proteins is 11 for napin (Ericson et al., 1986) and 5.9 for albumin 8 (Kelly et al., 2000).

The characteristics of low molecular weight and pI, especially the high pI for napin, might explain why extracts from these plant species exhibit high flocculation activity and thereby make these two proteins potential candidates for bio-flocculating agents. Moreover, these results are in agreement with the report that the moringa seed protein MO 2.1 belongs to 2S family of proteins (Doerries, 2005, Suarez et al., 2005) and would explain why no flocculation activity was detected after treatment with reducing agents such as DTT (Conaghan, 2013).

Further studies would be required to isolate and characterise these proteins from the different PC extracts to determine the relative proportion of the cationic proteins and whether these are principally responsible for the measured activity.

5.3.3 Flocculation activity of commercial purified proteins

The results with PC protein extracts raises the question as to whether a family of proteins commonly found in oleaginous plant seeds is responsible for the flocculation activity or whether it is a general property of proteins. In order to test this the flocculation activity of 5 commercially available purified proteins including BSA, α-lactalbumin, casein, γ-globulin, isinglass and fish gelatin were determined and compared to the activity of PC extracts. These purified proteins were selected to obtain a range of molecular weights and pIs (**Table 5.1**).

All proteins were tested under identical conditions to those used for PC extracts and compared to the reference flocculants alum and ferric.

5.3.3.1 Effect of high ionic strength on purified proteins

The results for pH 7 and high ionic strength are discussed here (**Figure 5.5**), while results for pH 5 and 9 are shown in **Appendix G**. At pH 7, at dosages of 10 and 40 ppm, all proteins exhibited sedimentation rates (**Table 5.7**) as good as or better than those which were observed with PC extracts and the reference compounds alum and ferric. However, the turbidity removal of γ-globulin, BSA, casein and lactalbumin was 30% less than the PC extracts. Fish gelatin and isinglass showed extremely high flocculation activity with a total turbidity removal of approximately 92% for fish gelatin and 89-97% for isinglass which is comparable to moringa extracts (97%; **Table 5.3**), it was also over 54-64% higher than for ferric and alum (**Table 5.4**). Indeed isinglass had an extremely fast sedimentation rate (-1705.91/-992.44 NTU/min) for both 10 ppm and 40 ppm doses which is 306-348% higher than moringa, 510-206% higher than alum and 521-137% higher than ferric (**Tables 5.4 and 5.7**).

At pH 5, fish gelatin and isinglass showed a very high flocculation activity with a total turbidity removal >80% and very fast sedimentation rates (-683.56/-738.59 NTU/min

for fish gelatin and -907.31/-782.03 NTU/min for isinglass). These values were higher than those for alum and ferric, although alum at 40 ppm has a sedimentation rate of -888.64 NTU, but a turbidity removal of only 86.61%. Though the two proteins possessed turbidity removal which was in the range of the PC extracts (equal or even higher than 86%), they displayed a ~300% faster sedimentation rate (**Table 5.7** and **Table 5.3**).

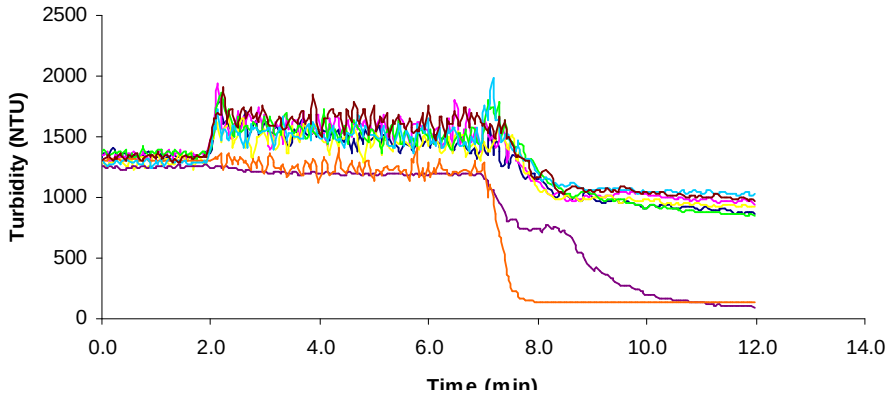
Interestingly, under this pH and ionic strength conditions, γ-globulin, BSA, casein and α-lactalbumin have a high flocculation activity with turbidity removal between 58-93%. γ-globulin and also BSA has sedimentation rates (-286.27/-368.62 NTU/min and -398.80/500.51 NTU/min, respectively) comparable to those of the two standards and the PC extracts.

At pH 9 and a dosage of 10 ppm, isinglass and fish gelatin show turbidity removal of 96.6% and 91.90% which is very close to moringa extracts (97.71%), and at least 30% less than alum and ferric. Surprisingly the sedimentation rate of isinglass is also 170% higher than moringa. However, at 40 ppm these two proteins and α-lactalbumin have activities comparable to the two standards and PC extracts (with the exception of hemp). In fact, all of them have a turbidity removal higher than 80%, with fish gelatin having a sedimentation rate very close to that of alum (-453.94 NTU/min vs. -457.41 NTU/min) and isinglass showing the highest value (-659.16 NTU/min) of all of proteins tested (**Table 5.7**).

The flocculation activity of isinglass and fish gelatin also appears to be independent of pH and ionic strength. This result is particularly interesting since isinglass is a protein extract from the swim bladders of fish (usually cod) and is commonly used as a clarification agent for the removal of yeast in brewing and the manufacture of wine, cider mead and certain juices (Weber et al., 2010). Isinglass is essentially composed of type 1 collagen with a molecular weight of 140 kDa and a pI of 5.5 (**Table 5.1**). Fish

gelatin is hydrolysed collagen (molecular weight 60 kDa) obtained from various parts of fish species and therefore it would be expected that isinglass and fish gelatin exhibit similar properties, including pI of 6 (Weber et al., 2010). Surprisingly, these proteins, despite the higher molecular weight and lower pI than moringa PC extracts, exhibited very high flocculation activities. In particular, high flocculation activity was reported at pH 7 where the net charge on these proteins is expected to be negative, whereas moringa proteins would be expected to be positively charge. Indeed, the flocculation activity and turbidity removal appear to be highest at pH 9 when the proteins have a high net negative charge, although high activity is also shown at pH 5 (**Table 5.7**). It is therefore clear that flocculation activity is not confined to highly cationic proteins, such as those widely reported for moringa, and that many proteins exhibit flocculation activity albeit at varying rates.

a)



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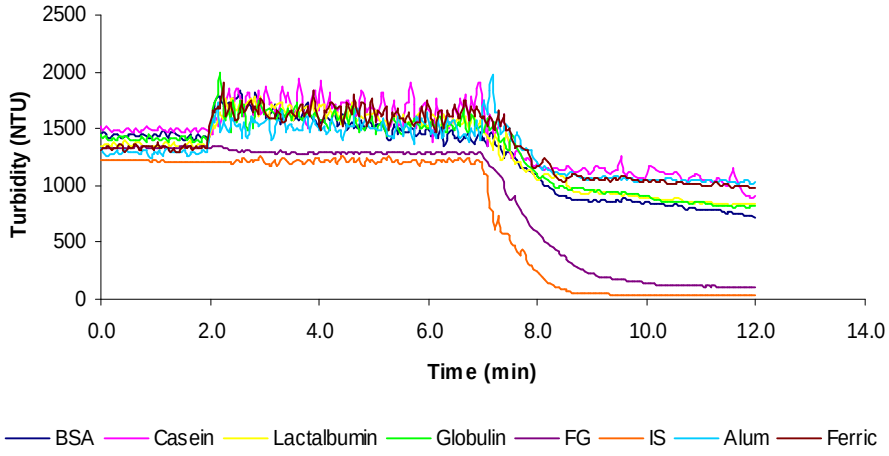


Figure 5.5: Flocculation activity of commercially available proteins at pH 7 under high ionic strength conditions and dosages of 10 ppm (a) and 40 ppm (b).

Despite fish gelatin and isinglass showing the highest activity of all of the proteins tested, it was seen that at pH 5 γ-globulin, BSA and casein also had flocculation activities and turbidity removal of 58-92%, values which are comparable to those of alum and ferric (**Table 5.7**). Once again there is no clear correlation between the molecular weight or pI and the flocculation activity. However, these results are in agreement with Piazza and Garcia (2010) where proteins extracted from meat and bone meal enhanced flocculation activity around pH 5. The authors proposed that this effect

is due to protonation of carboxylic acid groups at lower pH values giving the peptide/protein a net positive charge. This would encourage binding to the negatively charged silica groups residing at the surface of clay and other particles present in drinking water. The main amino acids found in collagen are glycine, proline and hydroxyproline, which impart an amphoteric nature to proteins (Pearse, 2003) and are believed to impart a polymer bridging function to these proteins resulting in the formation of dense flocs (Gregory, 1983).

Table 5.7: Sedimentation rates and total turbidity removal (Δt) for purified proteins at high ionic strength.

		γ-globulin		BSA		Casein		α–lactalbumin		Fish Gelatin		Isinglass	
	ppm	10	40	10	40	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-286.27	-368.62	-398.80	-500.51	-195.90	-163.53	-154.26	-154.70	-683.56	-738.59	-907.31	-782.03
	r^2	0.99	0.99	0.99	1.00	0.99	0.98	0.99	0.96	0.98	0.98	0.96	0.95
	Turbid. Removal (%)	85.72±1.2	90.25±0.7	91.05±2.7	87.82±0.5	81.56±0.5	70.30±2.4	58.56±7.7	62.29±0.5	92.95±2.0	83.27±1.1	84.63±1.8	85.77±3.5
pH 7	**Sediment. Rate (NTU/min)**	-526.06	-457.99	-285.52	-342.29	-224.67	-594.19	-290.62	-293.83	-356.45	-646.48	-1705.91	-992.44
	r^2	0.95	0.95	0.88	0.98	0.90	0.80	0.86	0.84	0.89	0.99	0.97	0.88
	Turbid. Removal (%)	37.10±1.7	43.42±3.8	34.30±6.9	49.69±5.5	30.16±1.3	40.75±6.3	28.86±6.2	37.62±2.4	92.28±0.3	91.74±2.6	89.31±5.2	96.58±5.3
pH 9	**Sediment. Rate (NTU/min)**	-321.14	-374.23	-158.83	-387.04	-124.82	-484.10	-184.61	-215.21	-337.66	-453.94	-1335.11	-659.16
	r^2	0.90	0.87	0.91	0.93	0.84	0.89	0.97	0.98	0.98	0.91	0.93	0.96
	Turbid. Removal (%)	37.76±1.9	35.02±4.4	53.46±2.8	41.99±4.0	29.35±0.8	34.43±3.5	69.01±4.1	83.48±6.3	91.90±0.8	92.83±2.5	96.66±2.4	90.31±3.8

5.3.3.2 Effect of low ionic strength

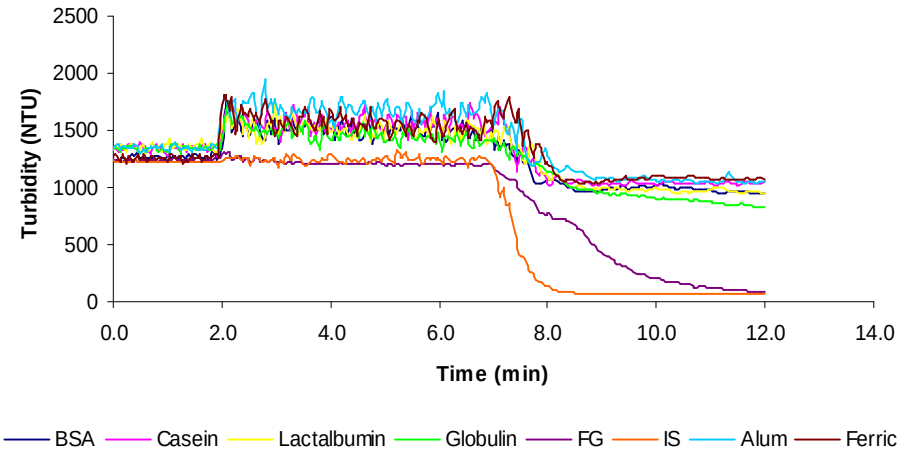
Under low ionic strength conditions similar results to those at high ionic strength were obtained for the different proteins (**Table 5.8**). The results at pH 7 under low ionic strength are described here, while the results for pH 5 and 9 are shown in **Appendix H**. At this pH, only fish gelatin and isinglass showed a high flocculation activity with rates comparable to those under high ionic strength conditions, while the two reference standards show a relatively poor activity with generally <30% turbidity removal (**Figure 5.6**).

However, this activity is comparable to that of moringa extracts since these turbidity removals are higher than 90%, but the sedimentation is improved in the two animal proteins where the values are extremely negative (for example isinglass sedimentation slope is -1343 NTU/min).

At pH 5 and 9, similar results to the high ionic strength were obtained. Thus, at pH 5 γ-globulin, BSA, casein and lactalbumin showed a flocculation activity comparable to alum and ferric with turbidity removal between 70 and 90%. However, at pH 9 their activity dramatically decreased to 20-50%.

These results indicate that the ionic strength had little effect on either the rate of flocculation or turbidity removal of purified proteins. Moreover, among the 6 purified proteins tested, only fish gelatin and isinglass had, through all conditions, a flocculation activity comparable to PC extracts, especially extracts from moringa. This shows that many proteins exhibit flocculating properties and that this activity is not only directly correlated with pI and molecular weight.

a)



b)

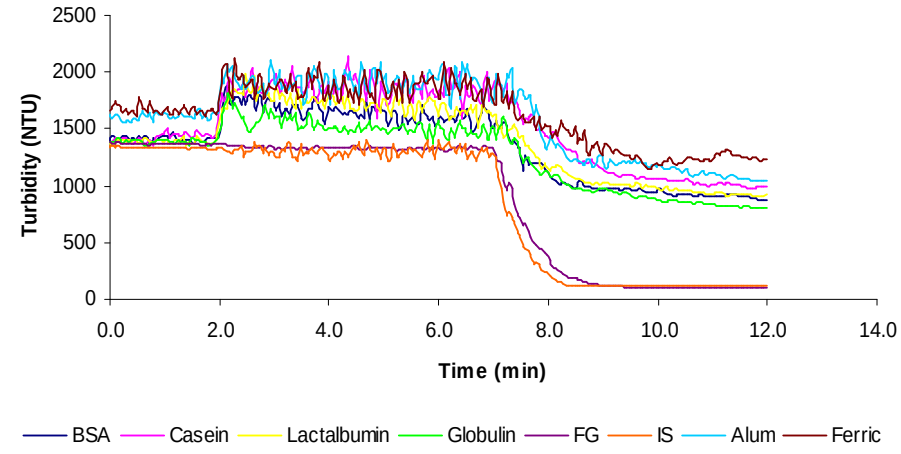


Figure 5.6: **Flocculation activity of purified commercial proteins at pH 7 under low ionic strength conditions at 10 ppm (a) and 40 ppm (b).**

Table 5.8: Sedimentation rate values and total turbidity removal (Δt) for purified proteins at low ionic strength.

		γ-globulin		BSA		Casein		α -lactalbumin		Fish Gelatin		Isinglass	
	ppm	10	40	10	40	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-402.70	-585.00	-513.86	-701.71	-219.35	-144.24	-195.90	-170.84	-670.11	-860.03	-658.00	-994.40
	r^2	0.99	0.98	0.98	0.98	0.99	0.97	0.99	0.99	0.99	0.99	0.99	0.89
	Turbid. Removal (%)	90.82±1.5	95.20±2.2	92.09±2.3	91.26±3.2	83.83±2.0	56.84±5.9	70.73±11.1	67.1±11	92.74±0.66	85.24±3.4	90.11±1.6	93.24±0.4
pH 7	**Sediment. Rate (NTU/min)**	-246.23	-342.05	-327.54	-411.02	-574.46	-440.29	-274.22	-365.83	-267.63	-983.57	-1343.07	-1210.55
	r^2	0.97	0.91	0.86	0.92	0.84	0.96	0.91	0.94	0.99	0.98	0.97	0.94
	Turbid. Removal (%)	38.12±4.0	42.07±4.3	23.16±3.9	37.27±1.3	21.28±6.0	28.07±5.4	28.12±1.5	33.27±4.1	92.73±1.7	92.89±0.8	94.05±1.9	91.38±4.8
pH 9	**Sediment. Rate (NTU/min)**	-292.82	-378.92	-263.40	-314.30	-106.96	-536.28	-426.86	-250.58	-220.75	-550.22	-257.31	-502.55
	r^2	0.94	0.84	0.87	0.81	0.85	0.90	0.93	0.95	0.97	0.99	0.95	0.99
	Turbid. Removal (%)	34.20±1.3	33.44±2.3	35.44±2.4	29.704±4.6	30.56±4.4	37.43±3.2	38.28±0.5	35.43±2.0	75.82±10.7	94.47±0.7	78.79±2.9	97.42±1.3

5.4 Conclusions

In this study, it has been clearly demonstrated that water soluble extracts from the presscakes (PC) of *Moringa oleifera* have a high flocculation activity which is greater with respect to both the rate of flocculation and ability to remove turbidity compared to the standard aluminium and ferric sulphates (alum and ferric). For many years this has been reportedly due to the presence of highly cationic proteins/peptides in the extracts, which resulted in *Moringa* sp. being considered to be unique. In this work it has been clearly shown that soluble extracts from other oleaginous plants such as rapeseed, hemp and sunflower also possess very high flocculation activities over a range of pH and ionic strengths.

Further analysis of a range of commercially available proteins, demonstrated very high flocculation activity by collagen and hydrolysed collagen (fish gelatin) and that the activity was not due to the cationic nature of these proteins. All proteins tested, regardless of molecular weight, pI and ionic strength of the water, showed some flocculation activity.

These results lead to interesting potential application for proteins, particularly those from both oleaginous plants and collagen-based sources, as bioflocculants in the treatment of drinking water. The ability to flocculate and form dense flocs which sediment rapidly, suggests that the sludge from such a treatment would be biodegradable and not require incineration. In addition many sources of these proteins are waste products of other industries, such as the press-cakes from oleaginous seeds after the vegetable oil has been removed, and the fish proteins extracted from the residues of most fish species. The extraction method requires a simple salt solution consequently this makes them both very cheap and completely sustainable while the

biodegradability would reduce the environmental impact of the currently used inorganic salts (alum and ferric) coupled with polymers such as acrylamide derivatives.

CHAPTER 6

A comparison of flocculation activity of natural submicron-capsules from oleaginous seeds and soluble protein extracts from press-cakes of different oleaginous plants

Abstract:

In **Chapter 3**, it has been shown that rapeseed NSC have high flocculation activity under high ionic strength condition at pH 5, 7 and 9. It was also demonstrated that the activity is due to the intact NSC structure as denaturising treatments resulted in a loss of activity.

In this chapter it is investigated whether flocculation is a characteristic of other NSC from other plant seeds. NSC extracted from hemp, sunflower soybean and rapeseeds are tested using the on-line jar test apparatus. It is demonstrated that all NSC have a flocculation activity higher than the two reference standards, aluminium sulphate and ferric sulphate, and hemp, sunflower and soybean NSC are less affected by ionic strength than rapeseed NSC.

A comparison between rapeseed, hemp and sunflower PC extracts and NSC was done in order to identify differences and characteristic between these two types of flocculating agents. Although PC extracts do show a flocculation activity higher than the two standards (**Chapter 4**), NSC generally showed a higher and faster activity.

6.1 Introduction

Seeds represent a valuable source of natural flocculants as has been proved here and in literature (Ndabigengesere and Narasiah, 1998b, Raghuwanshi et al., 2002, Paterniani et al., 2010). In **Chapter 3**, the flocculation activity of submicron capsules, named oil-bodies, from rapeseed seeds was demonstrated. NSC were extracted using an aqueous based flotation-centrifugation method, where a hexane step was added in order to remove any broken NSC. The experimental section indicated that the observed flocculation was due to the intact NSC and was higher than the two standards, alum and ferric. In order to exploit oleaginous seeds and search for other possible flocculating agents, in **Chapter 4,** extracts from PC have been tested for flocculation activity. It has been shown that extracts from hemp, sunflower and rapeseed PC indeed exhibit flocculation activity and that this activity may be comparable to moringa extracts as well as alum and ferric.

The aim of this chapter is to investigate if NSC extracted from hemp, sunflower and soybean along with rapeseed show a flocculation activity. Moreover, a comparison between rapeseed, hemp and sunflower NSC and soluble protein extracts from PC was carried out in order to compare the efficiency of the different groups flocculating agents.

6.2 Materials and Methods

6.2.1 SDSPAGE

A sample of the final cream layer was collected and extracted with diethyl ether and the total amount of protein determined.

The samples were precipitated with methanol/chloroform/water precipitation (Wessel and Flugge, 1984) with the following modifications: 100 µg of protein was dissolved in 400 µl of methanol, 200 µl of chloroform and 400 µl of water and centrifuged for 5 min at 8000 rpm (Centrifuge 5415 R, Eppendorf, Germany). The upper layer was carefully removed and discarded, 600 µl of methanol was added and centrifugation repeated at 13000 rpm for 30 min. The supernatant was removed and the pellet air dried for 10 min. Finally, the pellet was resuspended in Leammli buffer (BioRad, UK) and boiled for 10 min at 90ºC.

A total of 15 µg of protein was loaded onto a Novex® NuPAGE® 4-12% Bis-Tris Precast Gel (Novex®, UK) and run for 180 min at 120 Volts. After electrophoresis, the gel was stained with SimplyBlueÊ SafeStain following manufacturerõs instructions (Novex®, UK)

6.2.2 Staining of NSC before flocculation assay

To a sample of 3 mL of rapeseed NSC a 1 µg/mL Nile Red solution was added and the preparation incubated for 5 min (Greenspan et al., 1985). After incubation, the preparation was centrifuged, the lower aqueous layer removed carefully using a syringe and 5 mL of 10 mM Tris-HCl pH 8.6 was added. The centrifuge, the removal of the lower layer and resuspension in Tris buffer represent a wash cycle. This procedure was repeated twice.

After the staining, 100 µl of the stained sample was used as flocculant in a 50 ml Falcon tube containing a solution of 2 g/L kaolin (sodium phosphate buffer pH 7, 100 mM

NaCl). The suspension was mixed for 2 min and allowed to sediment for 1 h. After sedimentation, samples of flocs were carefully taken and observed at 1000x focus through TRITC filter (for fluorescence) using a Nikon Eclipse TI inverted microscope equipped with 100x oil immersion objective. Images were recorded with NIS-elements AR software. The length size of the flocs was estimated by comparison with a calibrated scale.

6.3 Results and Discussion

In **Chapter 3** the flocculation activity of rapeseed NSC was studied using a spectrophotometric flocculation assay. The promising results obtained with rapeseed, raised the question as to whether NSC extracted from other oleaginous sources show flocculation activity?

In order to verify this hypothesis NSC from hemp, sunflower and soybean seeds were extracted using an aqueous-based flotation-centrifugation method as used for rapeseed seeds (**Chapter 3**). Subsequently, in order to standardise the amount of NSC to add to the kaolin suspensions and to compare the flocculation activity of the different NSC, the total protein content was determined using the Peterson's modification of Lowry method (Peterson, 1977) and the results shown in **Table 6.1**.

Table 6.1: **Total protein concentration of NSC suspensions measured using Peterson's modification of Lowry method**

NSC suspension	Total protein (mg/ml)
Rapeseed	34.99
Hemp	18.32
Sunflower	17.85
Soybean	8.5

The total amount of protein within each NSC suspension is extremely variable with rapeseed NSC showing the highest amount (34.99 mg/mL) and soybean the lowest (8.5 mg/mL). The SDS-PAGE showed very different protein patterns (**Figure 6.1**), therefore it is unlikely that any flocculation measured would be due to the same proteins. In addition, the presence of these associated proteins to the wall of NSCs may affect the charges available for the flocculation. This aspect would be further discussed in **Section 6.3.2.**

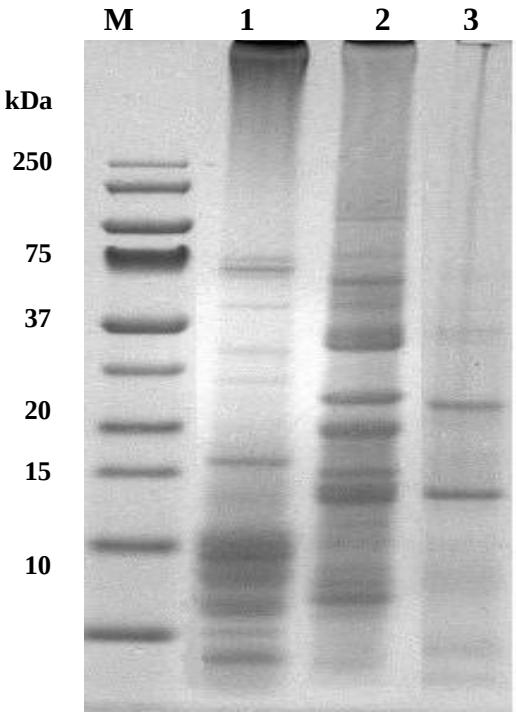


Figure 6.1: NuPAGE® Bis-Tris Gel (4ï 12%) of NSC extracts from various plant seeds species.

M: Novex® Sharp Pre-Stained Protein Standard (Novex, UK); **1**: Rapeseeds; **2**: Soybean; **3**: Hemp, 15µg of proteins was loaded in each well.

In **Chapter 3** it was shown that the activity of rapeseed NSC was directly proportional to the concentration of NSC added to the kaolin solution (**Figure 3.3** and **Section 3.2.2**), consequently tests were made on NSC extracted from other sources. In order to compare the NSC from different seeds with aqueous extracts from PC all assays were carried out with both 10 and 40 ppm of NSC (with respect to protein content) and the flocculation activity and sedimentation rates compared to those of alum and ferric under high and low ionic strength at three pH values (5, 7 and 9) using the on-line jar test apparatus. It is expected that these two parameters would affect the flocculation of NSCs due to the charges present on NSC surface and kaolin, as shown for rapeseed NSC.

6.3.1 Effect of pH and high ionic strength on flocculation activity of NSC extracted from hemp, sunflower and soybean seeds

Figure 6.2 shows the results for pH 7 for 10 and 40 ppm of NSC, while in **Appendix I** are reported the results for pH 5 and 9.

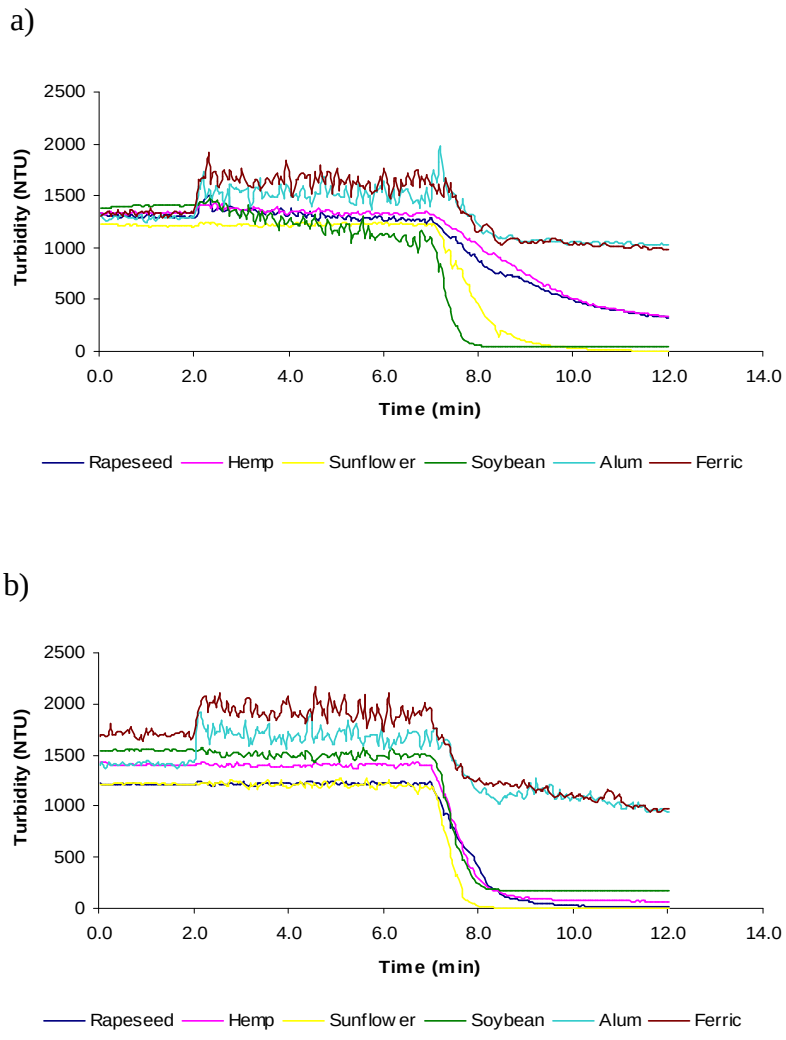


Figure 6.2: Effect of high ionic strength at pH 7 on 10 (a) and 40 (b) ppm.

At pH 7, NSC from hemp, soybean, sunflower and rapeseed showed a higher flocculation activity than the two reference standards tested (alum and ferric, **Table 6.2** and **6.3**). The microscopic investigation performed on flocs formed after 1 h incubation between kaolin and rapeseed NSC showed agglomeration with the elimination of

charges due to the presence of NaCl, since NSC would not be able to agglomerate due to the negative charge of oleosins (**Figure 6.3**) (Tzen and Huang, 1992a, Tzen et al., 1992b). This showed that NSCs behave as both coagulants and flocculants. In fact, the presence of cations such Na^+ stimulates the aggregation by decreasing the electrostatic repulsion caused by oleosin proteins and kaolin particles. By removing the electrostatic repulsion, the Van der Waals forces become more prominent and agglomeration occurs (**Figure 6.4**).

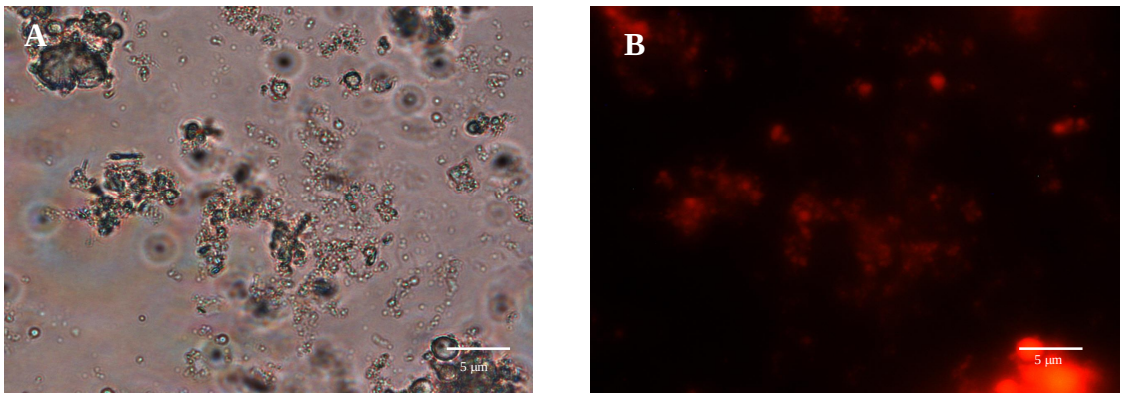


Figure 6.3: Microscopic investigations of flocs formed after 1 h incubation with kaolin suspensions and NCS extracted from rapeseed seeds.
A) Light and B) Fluorescence microscopy, NSC stained with Nile Red shows the oil core. NSC stained with Nile Red shows the oil core. Size of flocs 3.48 µm ± 1.08.

This is in agreement with mechanism proposed for anionic polymers. Anionic polymer adsorption to clays is based mainly on electrostatic linkages, hydrogen bonding or ionic bonding (Bratby, 2006). However, electrical repulsions may prevent adsorption in some cases. Nevertheless, it is often found that a certain salt concentration is needed to promote adsorption (and flocculation) since the ionic strength would help the destabilization by 1) reducing electrostatic repulsion between particles of similar charge (Bratby, 2006), and 2) complexing with polymers functional groups, thereby aiding the adsorption of negative particles, such as kaolin, by a ion binding effect (Lee et al.,

1991a, Peng and Di, 1994, Gregory and Barany, 2011). This was also demonstrated for the natural anionic polymer extracted from Nirmali seed (Muthuraman et al., 2013).

At 10 ppm, rapeseed and hemp NSC showed similar turbidity removal (around 75%), although the sedimentation rates of rapeseed is 132% faster (**Table 6.3**). Sunflower NSC showed a turbidity removal of 99% which is the highest recorded, although soybean NSC showed also a very high removal (96%) with the sedimentation achieved in less than 8 min, representing the fastest measured (-1394 NTU/min). Under the same conditions the two standards, alum and ferric, have a very poor turbidity removal (22-25%) which is 50-70% less than NSC.

At the dosage of 40 ppm, an increase in flocculation activity was detected for all NSCs which showed a turbidity removal between 89 and 99%, confirming that concentrations > 10 ppm result in more sites available to neutralise and destabilize the colloidal particles, promoting a bridging-type flocculation (**Chapter 3**). It is interesting to note that the sedimentation is very fast for all four NSC (less than 9 min) and that the rates increased dramatically compared to 10 ppm (**Table 6.3**). For example, the rate of sedimentation of sunflower NSC at the dosage of 40 ppm was -1725.81 NTU/min: this was 229% higher than 10 ppm, although the final turbidity removal was the same between the two concentrations (99%). These values are significantly higher than for alum and ferric which showed a turbidity removal of 32-42%.

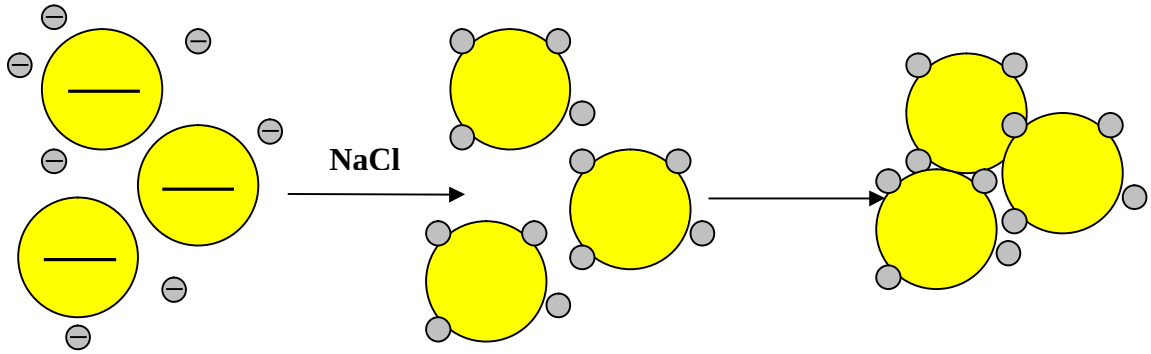


Figure 6.4: Schematic representation of hypothesized flocculation mechanism for NSC flocculation activity.

Grey circles: kaolin particles; yellow circles: NSC.

Since kaolin particles and NSC have the same charge under the pH examined, it is supposed that the ionic strength (NaCl) plays an important role by suppressing the electrical double layer of both kaolin and NSC and, consequently, resulting in the agglomeration between kaolin particles and NSC. Subsequently, the agglomerates form a larger flocs and settle down.

At pH 5, NSC showed a flocculation activity comparable or higher than the two standards (**Table 6.2**). For example, 10 ppm of soybean NSC had a turbidity value and sedimentation rate higher than alum (92.5% vs. 89.5%, and -586.4 NTU/min vs. -574.22 NTU/min, respectively). On the other end, rapeseed, sunflower and hemp NSC showed a higher activity than ferric with turbidity removal between 77-86% (**Table 6.3**). At 40 ppm, the activity of the two standards decreased (86 and 25% of turbidity removal), while those of NSC increased as observed for pH 7. Interestingly, rapeseed and soybean NSC remove 99-100% turbidity in less than 8 min (sedimentation rates of -1366.88 NTU/min and -2402.41 NTU/min for rapeseed and soybean, respectively; **Table 6.3**).

Table 6.2: Sedimentation rates and total turbidity removal (Δt) for alum and ferric at high ionic strength

		Alum		Ferric	
	ppm	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-574.22	-888.64	-251.52	-209.91
	r^2	0.99	0.96	1.00	0.96
	Turbid. Removal (%)	89.57±2.7	86.61±1.3	76.00±4.1	25.51±0.1
pH 7	**Sediment. Rate (NTU/min)**	-334.18	-480.15	-327.80	-725.11
	r^2	0.87	0.95	0.91	0.94
	Turbid. Removal (%)	22.28±3.4	32.28±5.3	25.25±5.0	42.31±2.8
pH 9	**Sediment. Rate (NTU/min)**	-142.33	-457.41	-165.44	-255.83
	r^2	0.92	0.99	0.97	0.99
	Turbid. Removal (%)	50.62±5.2	96.47±1.5	62.18±6.2	82.06±2.0

At pH 9 and dosage of 10 ppm, alum and ferric showed a low flocculation activity compared to those of NSC. Indeed, the turbidity removal of the two standards was 50-40% less than those of NSC, which displayed a turbidity removal < 97%. The sedimentation was reached in 8 min (or even less for soybean) as shown by high sedimentation rates (between -815 and -1452 NTU/min). This is in complete contrast to the rates of the two standards (-142 and -165 NTU/min for alum and ferric) which showed a poor sedimentation. At the highest concentration of flocculants, although alum and ferric had an improved activity (96% and 82 % of turbidity removal and -452 and -255 NTU/min as sedimentation rates), higher flocculation activity was recorded with all three NSC suspensions. For example, rapeseed NSC were able to sediment all kaolin particles in suspension in less than 8 min (-1184.73 NTU/min). This sedimentation is 262% and 464% higher than those of the two standards under the same conditions (**Table 6.3**).

Thus, under high ionic strength conditions and at a dosage of 10 ppm, the turbidity removal of all NSC was comparable to those of the two standards, in particularly rapeseed and hemp NSC at pH 5 and 7, but in some cases even higher (for example at pH 9 all NSC showed a turbidity removal which is 30% higher than those of the two standards). As expected, at the higher dosage of 40 ppm of NSC an increase of the activity to 80-100% of turbidity removal and higher than the two standards was observed. This is indicated by the sedimentation rates that were highly negative and higher than alum and ferric. It is noteworthy that at pH 9, the flocs sediment in 8 min or less and at a range between -929 and -1527 NTU/min.

The high flocculation activity recorded at all three pH values, especially at pH 9, is in contrast with result reported for anionic polymer which, actually, showed a decrease in the activity, due to the increasing negative charge of kaolin (Peng and Di, 1994, Hocking et al., 1999, Muthuraman et al., 2013). However, these results are in agreement with the observation that higher pH values are optimal for other coagulants extracted from plants e.g. from M. oleifera (Okuda et al., 2001b), from *P. juliflora* and *C.latifaria* (Diaz et al., 1999), from *C. angustifolia* (Sanghi et al., 2002) and seed extracts from *C. sativa* and *Ae. Hyppocastanum* (Sciban et al., 2009).

Table 6.3: **Sedimentation rate values and total turbidity removal (Δt) for NSC flocculation activity at high ionic strength**

		Rapeseed		Hemp		Sunflower		Soybean	
	ppm	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-224.53	-1366.88	-246.04	-487.64	-273.12	-581.02	-586.44	-2402.41
	r^2	0.98	0.97	0.99	0.99	0.98	0.97	0.98	0.98
	Turbid. Removal (%)	81.12 ± 2.9	100.00	77.5±1.6	82.21±1.6	86.58±0.8	93.82±2.5	92.50±2.7	99.58±0.7
pH 7	**Sediment. Rate (NTU/min)**	-362.61	-799.78	-273.95	-1015.32	-753.94	-1725.81	-1394.05	-1439.79
	r^2	0.99	0.99	0.99	0.99	0.98	0.98	0.97	0.98
	Turbid. Removal (%)	75.97±1.2	99.184±1.2	74.86±6.0	95.14±1.2	99.76±0.2	99.70±0.3	96.78±1.9	89.15±0.44
pH 9	**Sediment. Rate (NTU/min)**	-1070.77	-1184.73	-814.92	-929.13	-1127.80	-1527.38	-1452.36	-1240.58
	r^2	0.98	0.97	0.99	0.98	0.98	0.97	0.97	0.98
	Turbid. Removal (%)	100.00	100.00	97.523±0.7	99.99	100.00	99.79±0.4	98.79±0.8	94.27±0.7

6.3.2 Effect of pH and low ionic strength on flocculation activity of NSC extracted from hemp, sunflower and soybean seeds

The results under low ionic strength at pH 7 are shown in **Figure 6.5**, while those for pH 5 and 9 are shown in **Appendix J.**

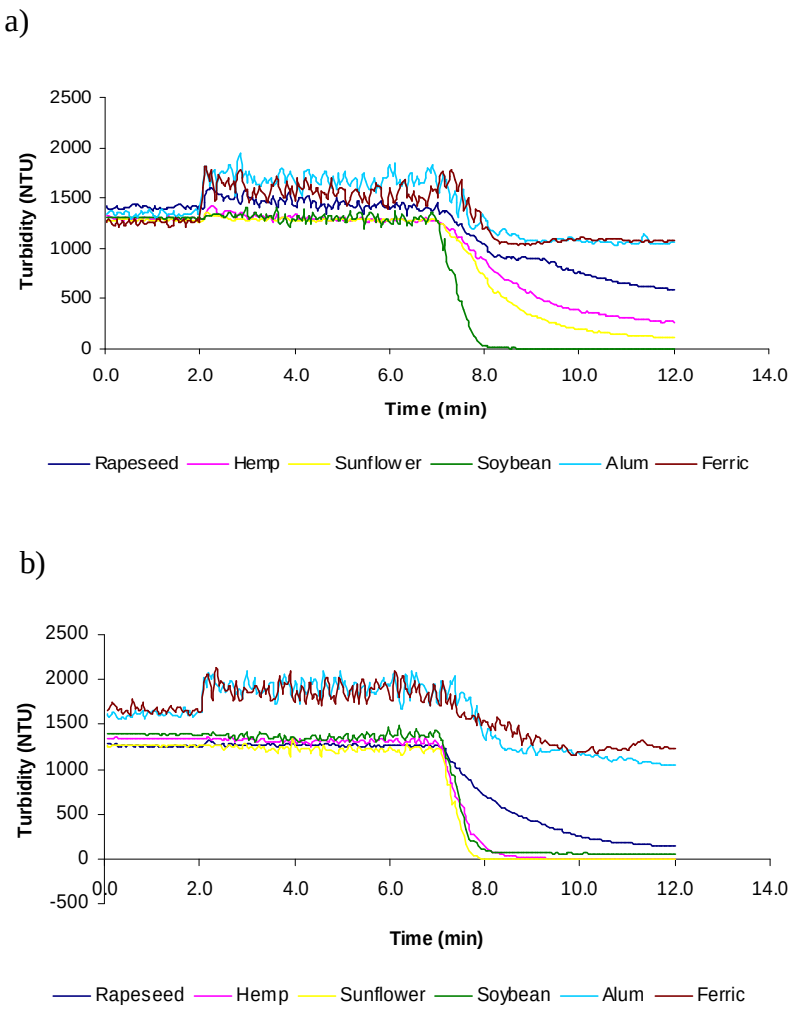


Figure 6.5: Effect of low ionic strength at pH 7 on 10 (a) and 40 (b) ppm.

Under these conditions, both concentrations (10 and 40 ppm) of NSC from different sources showed a higher activity than the two standards, **Table 6.4** and **Table 6.5**.

Indeed, alum and ferric had turbidity removal between 14 and 35%, indicating that the density of the flocs was not enough to allow sufficient sedimentation, whereas the turbidity removal of NSC was 65-85% higher. For example, soybean NSC showed a good activity at 10 and 40 ppm dosages with sedimentation reached in less than 8 min and a turbidity removal of 99 and 95% for 10 and 40 ppm, respectively. At 40 ppm a higher activity was observed, for example sunflower and hemp NSC showed 100% turbidity removal in less than 8 min (sedimentation rates of -1702.20 and -1265.43 NTU/min). On the other hand, rapeseed NSC were more affected by ionic strength at this pH, as previously described in **Chapter 3**, since turbidity removal was 58.91 and 89.05% for 10 and 40 ppm respectively, which is 20-10% less than the other NSC. The sedimentation rates was also slower than for the other seed (-221.06 and -498.94 NTU/min compared to -1442.43 and -1554.27 NTU/min of soybean NSC fot10 and 40 ppm, respectively).

Table 6.4: Sedimentation rates and total turbidity removal (Δt) for alum and ferric at low ionic strength

		Alum		**Ferric**	
	ppm	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-1081.88	-597.36	-228.43	-122.04
	r^2	0.98	0.99	0.98	0.96
	Turbid. Removal (%)	95.39±0.4	86.23±0.8	73.95±3.1	39.97±11.0
pH 7	**Sediment. Rate (NTU/min)**	-293.77	-302.62	-455.38	-188.58
	r^2	0.81	0.88	0.92	0.90
	Turbid. Removal (%)	21.13±5.1	35.47±1.6	14.98±5.1	25.23±3.4
pH 9	**Sediment. Rate (NTU/min)**	-149.50	-353.04	-229.18	-249.43
	r^2	0.98	0.98	0.99	0.99
	Turbid. Removal (%)	54.34±15.9	94.73±1.4	83.83±8.8	91.22±5.4

At pH 5, 10 ppm of alum showed the best activity with over 95% turbidity removal that in less than 8 min (-1081.88 NTU/min), while NSC from all sources showed a similar activity with turbidity removal between 86-89% and a sedimentation rates up to 327% slower than for alum. Nevertheless, these values are higher than those of ferric which showed a sedimentation rate of -228.4 NTU/min and turbidity removal of 73.95%.

At a dosage of 40 ppm, NSC from all seeds showed and improvement of 10% turbidity removal compared to 10 ppm. For example, soybean NSC had a very fast sedimentation rate which achieved 100% turbidity removal in less than 8 min (the rate is -1629.38 NTU/min), **Table 6.5**. Under these conditions, NSC exhibited a significantly higher activity than alum and ferric which, conversely, showed a decreasing in their turbidity removal (86.23 and 39.97% of turbidity removal for alum and ferric, respectively) and sedimentation rates halved (-597.36 and -122.04 NTU/min for alum and ferric, respectively, **Table 6.4**).

At pH 9 and 10 ppm, the two standards removed 54 and 83% of the turbidity which is comparable or lower to NSC. For example, sunflower and soybean NSC displayed a very high turbidity removal compared to the reference compound (98-99%), and a sedimentation 116-316% faster (**Table 6.5**). On the other hand, rapeseed and hemp NSC had an activity similar to that of ferric (78-80% turbidity removal), but faster sedimentation rates (-538.80 NTU/min and -765.10 NTU/min for rapeseed and hemp, respectively, compared to -229.18 NTU/min for ferric). For the higher dosages (40 ppm), the flocculation activity increased for both standards and NSC, with the exception of soybean NSC. Both the two standards and NSC were able to remove 87-99% of kaolin, however, NSC were over 4 fold more efficient in terms of sedimentation rates (Ó -988 NTU/min) compared to alum and ferric (**Table 6.5**).

Thus, under low ionic strength, similar conclusions to those for high ionic strength can be drawn namely that the activity is comparable or in some cases even higher than for the two reference standards (for example at pH 7).

These results showed that, while for rapeseed NSC under low ionic strength there is a clear decrease in flocculation activity compared to high ionic strength, this correlation can not be made for the other NSC (such as hemp and soybean) where in some cases it was recorded an increase of activity. This is completely in contrast with results of anionic polymer were the ionic strength would increase the flocculation activity (Gregory and O'Melia, 1989, Muthuraman et al., 2013) and suggests that the composition of NSC from different seed sources may have an effect on flocculation activity. Tzen et al. reported that although oleosin is the major protein component in NSC, there are other two classes of NSC integral proteins: caleosin (Frandsen et al., 2001) and steroleosin (Lin et al., 2002, Lin and Tzen, 2004). These three proteins are characterized by a very hydrophobic domain which allowed them to anchor the phospholipid membrane of NSC, like oleosin. However, the main difference between them is represented by the N- and C-terminal (Tzen, 2012). Caleosin (27 kDa) comprises a calcium-binding motif and several potential phosphorylation sites, that are well-known candidates involved in signal transduction, and thus it may possess biological function(s) in addition to its structural role for the stability of NSC (Tzen, 2012). On the other hand, steroleosin (39 kDa) possesses a sterol-regulatory dehydrogenase domain that belongs to a superfamily of presignal proteins involved in signal transduction via activation of its partner receptor after binding to a regulatory sterol (Tzen, 2012).

Table 6.5: **Sedimentation rate values and total turbidity removal (Δt) for NSC flocculation activity at low ionic strength**

		Rapeseed		Hemp		Sunflower		Soybean	
	ppm	10	40	10	40	10	40	10	40
pH 5	**Sediment. Rate (NTU/min)**	-332.37	-820.45	-765.10	-1009.07	-335.70	-602.77	-329.64	-1629.38
	r^2	0.99	0.98	0.98	0.98	0.99	0.99	0.97	0.95
	Turbid. Removal (%)	89.48± 2.0	97.45 ± 3.2	86.31±2.5	93.90 ±1.95	88.13±4.5	99.79±0.4	89.89±1.9	100.00
pH 7	**Sediment. Rate (NTU/min)**	-221.06	-498.94	-368.40	-1265.43	-531.69	-1702.20	-1442.43	-1554.27
	r^2	0.98	0.98	0.99	0.99	0.98	0.98	0.97	0.98
	Turbid. Removal (%)	58.91±2.4	89.05±1.6	79.61±6.8	100.00	91.50±5.8	100.00	99.50±0.7	95.62±5.9
pH 9	**Sediment. Rate (NTU/min)**	-538.80	-1345.04	-251.82	-684.23	-765.94	-1165.42	-1598.08	-988.93
	r^2	0.98	0.98	0.99	0.99	0.98	0.98	0.91	0.98
	Turbid. Removal (%)	80.32±5.4	97.40±0.6	78.74±1.5	88.98±1.6	98.06±1.7	99.83±0.2	99.89±0.1	87.62±2.8

In addition to the three known integral NSC proteins, increasing numbers of studies report new proteins as potential NSC proteins. For example, in rapeseed NSC suspensions 11- beta-hydroxysteroid dehydrogenase-like protein, ATS1, short chain dehydrogenase/reductase enzyme, myrosinase and myrosinase-binding proteins were identified (Katavic et al., 2006), while in NSC extracted from soybean P34, a monomeric insoluble glycoprotein, has been reported (Wilson et al., 2005). In sunflower seeds, Thoyts et al. (1996) discovered a 22.5 kDa protein associated with NSC which belongs to the 2S albumin family. Thus, the presence of so many different proteins, depending on the seeds species, can greatly influence the functional groups present on NSC surface and available for adsorption and, consequently, affect the flocculation activity. In case of NSC, it might be hypothesized that the adsorption which is at the base of bridging mechanism mainly occurs through hydrogen bonding between the hydroxyls groups of kaolin and amides groups of proteins (Bolto and Gregory, 2007). This would also explain the reason why higher NSC concentrations had higher activity. However, the ion binding previously mentioned can not be excluded.

This study, however, proved that NSC extracted from a range of plant species do have flocculation activity which in many case is 80% higher and faster than alum and ferric. Moreover, NSC may be used over a range of conditions since flocculation was recorded from pH 5 to 9 at high and low ionic strength.

6.3.3 Comparison of the flocculation activity between NSC from oleaginous and soluble protein extracts from PCs

In the last section, it has been demonstrated that NSC extracted from different oleaginous seeds had high flocculation activity under several buffer conditions. As described in **Chapter 3**, this activity is due to the intact NSC, since treatment with proteinase K and diethylether, showed loss of activity. The SDS-PAGE of rapeseed NSC during the five steps of extraction showed an enrichment of the oleosin band (Tzen et al., 1993), although extracts still contained other types of proteins associated with NSCôs surface. Removal of these proteins actually resulted in an increased flocculation activity, indicating that their presence partially block particle removal (**Chapter 3, Section 3.2.4**). Nevertheless, the aqueous layer obtained from treatment of NSC with urea, which contains aqueous-soluble proteins, exhibited also a high flocculation activity (**Chapter 3, Figure 3.9**), suggesting that there are multiple proteins with flocculating activity present in rapeseed seeds. Thus, a salt extraction method was applied to extract proteins from press-cakes (**Chapter 5**). The extracts obtained were not NSC, but soluble protein extracts, since treatments with denaturing agents (Conaghan, 2013) resulted in loss of activity. It seems unlikely that oleosins, which are still present in PC (Ramlan et al., 2002), can be considered possible flocculating agents in these extracts, since in **Chapter 3** it was proved that de-fatted NSC were unable to remove kaolin particles. Thus, in light of the homologies between the sequences of MO 2.1 and proteins belonging to 2S albumins it is more logical to link the flocculation activity of soluble PC extracts with these proteins.

In this section, a comparison of flocculation activity from soluble protein extracts and NSC extracted from rapeseed, sunflower and hemp is presented, in order to identify the different characteristics of the two flocculating agents.

Figure 6.6 and **6.7** shows a direct comparison between turbidity removal and sedimentation rates of rapeseed, hemp and sunflower NSC and soluble protein extracted from PC at 40 ppm under high and low ionic strength, while the data for 10 ppm under high and low ionic strength are given in **Appendix K**.

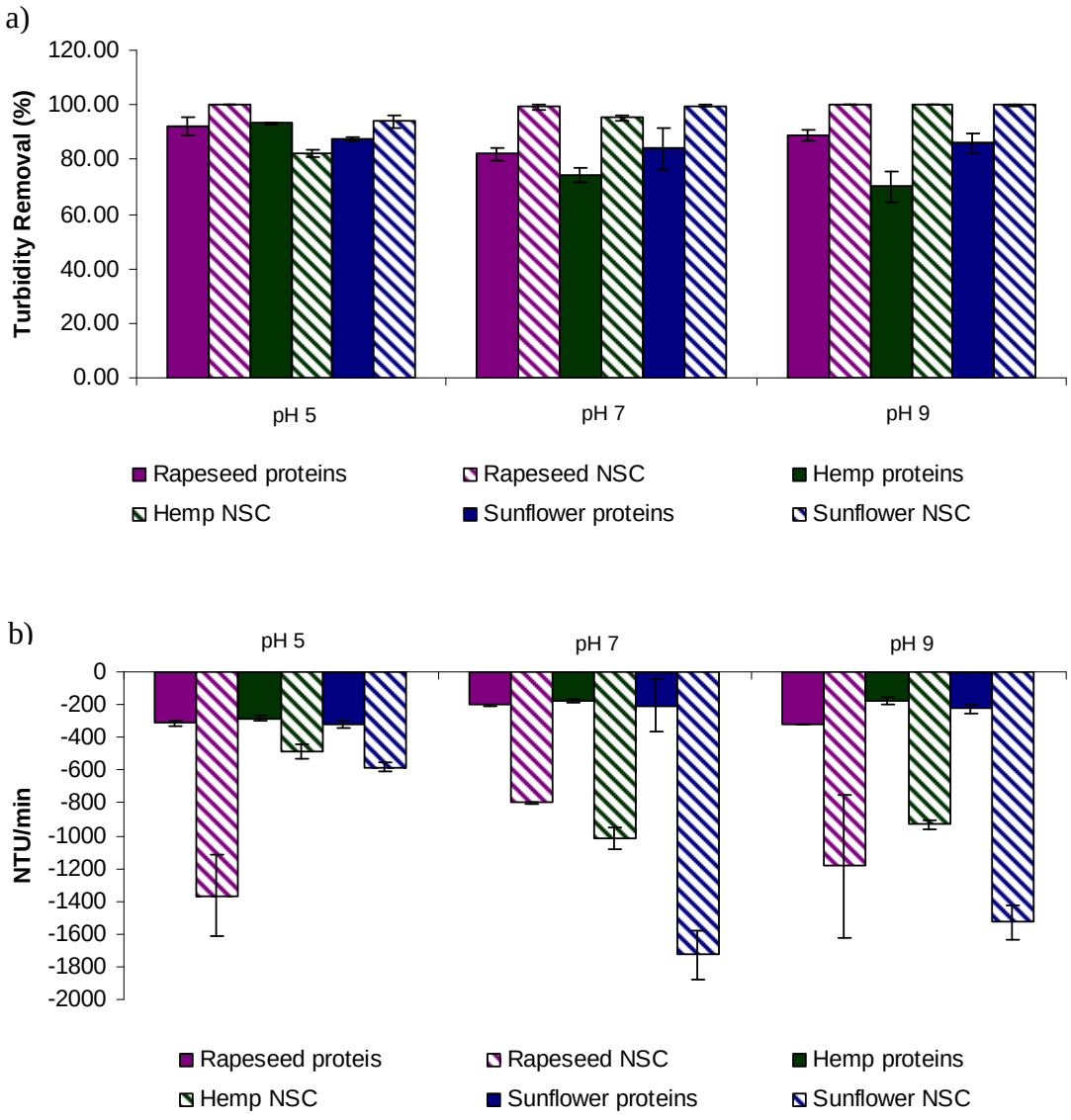


Figure 6.6: Flocculation activity of 40 ppm of protein soluble extracts compared to 40 ppm of NSC suspension from rapeseed, hemp and sunflower under high ionic strength.

a) Turbidity removal and b) Sedimentation rates. The bars indicates the standard deviations of three runs.

Although protein extracts and NSC had high turbidity removal (<70%), NSC showed a higher activity, especially at pH > 7, where the turbidity removal is close to 100% (**Figure 6.6**). This is shown by the extremely fast sedimentation rates achieved by NSC which are 300-850% higher than for soluble protein extracts at pH 7 and 9.

At the dosage of 10 ppm (**Appendix K, Figure 1**) a more detailed comparison has to be done. At pH 5, PC extracts showed a higher or comparable flocculation activity to those of NSC, with a turbidity removal between 77-93% and sedimentation rates also very close between each other, with the exception of rapeseed and hemp extracts which showed a faster sedimentation (120-200% faster). Similar results were obtained at pH 7 with the exception of sunflower NSC which removed 97% of kaolin particles, which is 10-20% higher than the other samples (the sedimentation rate is also higher than the others: -753.94 NTU/min compared to -174.52 NTU/min of sunflower proteins). A different scenario it is proposed at pH 9 where NSC showed the highest flocculation activity with turbidity removal Ò 100% and sedimentation rates between -800/-1030 NTU/min, which is 535% higher than PC extracts.

Interestingly, compared to data of moringa extracts (**Chapter 5, Table 5.3**), while at a dosage of 10 ppm NSC and moringa showed a similar flocculation activity, at a dosage of 40 ppm NSC showed the best activity. This was reinforced by the very fast sedimentation rates achieved by NSC. Indeed, it is reported widely that flocculating agents from moringa may work through a polymer bridge mechanism, hence large doses of flocculant lead to overdosing resulting in saturation of polymer bridges and subsequently restabilization of destabilized particles (Pise et al., 2009). On the other hand, the activity of NSC increased with increasing concentration, although a bridging-type mechanism was also suggested. In this case, it has to be taken into account the different nature of the two flocculants: moringa extracts contains cationic proteins of

low molecular weight and high pI (Ndabigengesere et al., 1995), therefore the flocculation mechanism would be as a cationic polymer where charge neutralization occurs under the three pH values examined. However, being organelles of high mass and with same charge of kaolin suspension, NSC would base the bridging mechanism through H bonding as previously explained (**Section 6.3.2**); similar explanation was given for *Opuntia* spp extracts which having a pI of 2, possess a very high anionic nature (Miller et al., 2008).

Under low ionic strength at 40 ppm (**Figure 6.7**) and pH 5 similar turbidity removal between rapeseed and hemp NSC and PC extracts was recorded, while for sunflower NSC it was higher than soluble proteins (99.79% vs. 83.3%). At pH 7, hemp and sunflower NSC were able to remove 100% of kaolin particles, at least 12% more than the extracts, while similar removal values were obtained for rapeseed NSC and extracts. Again, at pH 9, NSC from all seeds showed significantly higher turbidity removal than PC extracts with values between 88-99%. The sedimentation rates for NSC were higher than those for PC for all pH values studied. This difference is more accentuated at pH 9 where the sedimentation rates and turbidity removal of PC extracts were the lowest recorded.

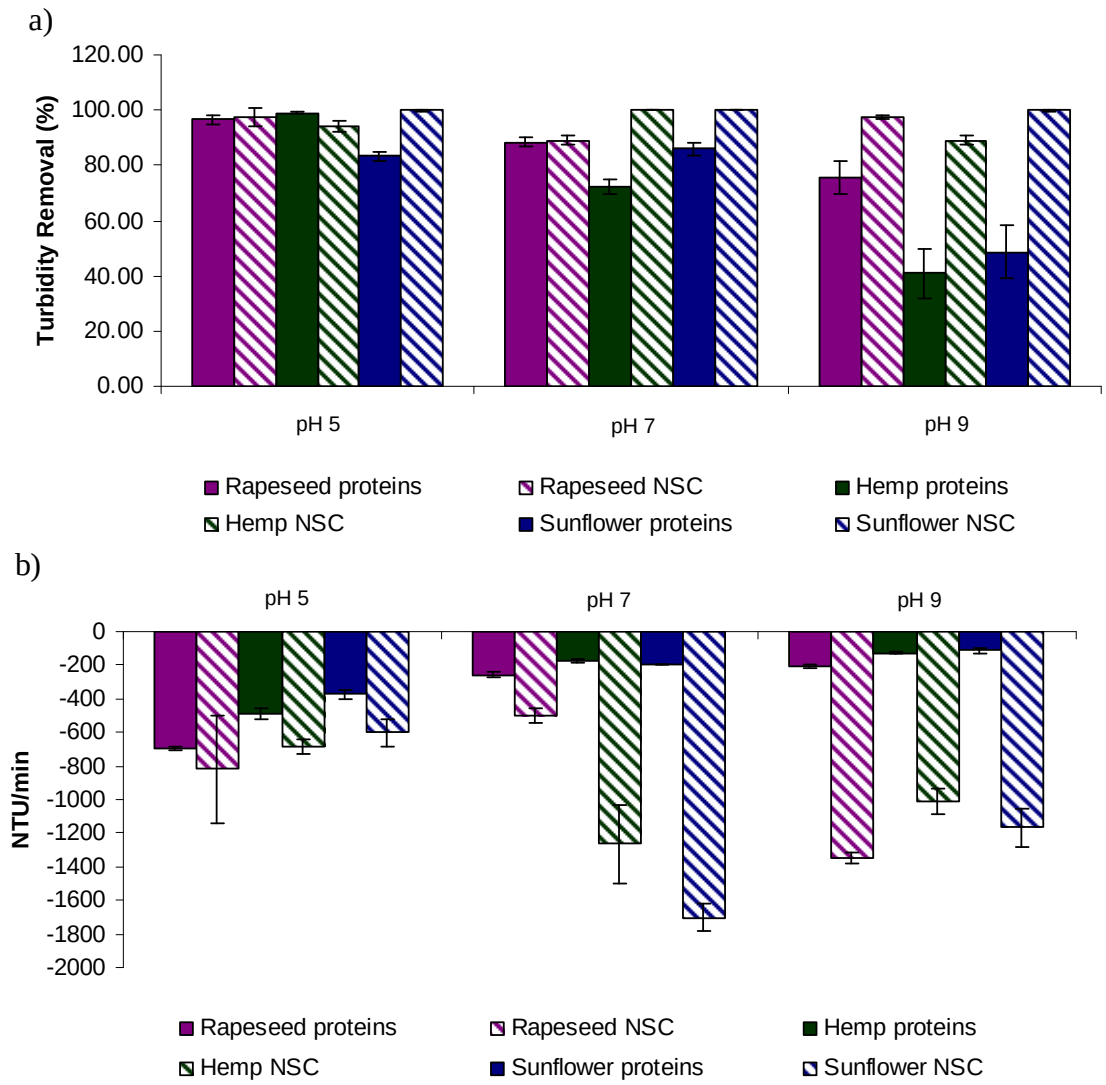


Figure 6.7: Flocculation activity of 40 ppm of protein soluble extracts and NSC under low ionic strength.

a) Turbidity removal and b) sedimentation rates. The bars indicates the standard deviations of three runs.

At 10 ppm (**Appendix K, Figure 2**) and pH 5, only hemp extracts showed a turbidity removal higher than the other samples (95% vs. 85%) along with the highest sedimentation rate (-572.62 NTU/min), while rapeseed and sunflower NSC and extracts showed comparable flocculation activity.

At pH 7, while hemp showed similar results for both NSC and proteins, rapeseed extracts showed a turbidity removal 10% higher than NSC and sunflower NSC showed the highest activity of all (91.5% of turbidity removal and -531.69 NTU/min).

Turbidity removals of NSC for pH 9 were at least 30% higher than for PC extracts in these conditions. Rapeseed NSC and extracts displayed a comparable sedimentation (-519 NTU/min vs. -538 NTU/min), while hemp and sunflower NSC showed a sedimentation rate 600% higher than for PC extracts.

When compared to moringa extracts under low ionic strength (**Chapter 5, Table 5.5**), NSC activity at the dosage of 10 ppm was generally lower, but at 40 ppm increased dramatically up to 493% higher than for moringa.

Thus, NSC extracted from oleaginous seeds represents a valid alternative to moringa extracts for use as bioflocculants especially in developed western countries.

6.4 Conclusions

In recent years, NSC from oleaginous seeds have been exploited for different applications in biotechnology such as in the production and purification of recombinant proteins (Bhatla et al., 2010). While the use of plant-based materials to clean and clarify turbid raw water is not in itself a novel idea, since soluble protein extracts and even ground seeds have been used for many years, in this study it was demonstrated that NSC from a range of oleaginous plants had a very high flocculation activity capable of being used alone, in the absence of inorganic compounds or artificial polymers. The flocculation activity is rapid and results in complete removal of turbidity in the on-line jar test assay. Moreover, it has been demonstrated that the flocculation activity varies between seeds sources and buffer conditions, making possible the selection of the NSC source according to the type of water to treat.

The activity of these NSC were compared to those of PC extracts investigated in **Chapter 5**, which have been demonstrated to have a flocculation activity higher than alum and ferric. These extracts from rapeseed, sunflower and hemp may represent an important, new alternative to Moringa extracts, since these plants are commonly found in European countries for the production of vegetable oils. Thus, the extraction of flocculants from PC would be an economical solution.

The comparison demonstrated a very interesting result: the flocculation activity of NSC was generally higher than PC protein extracts, indicating that flocs formed through NSC might be denser to sediment quickly.

Altogether these results proved that oleaginous seeds have two different types of flocculants: NSC extracted from seeds and proteins extracted from PC as a cheaper alternative. Thus, they may be considered as natural, sustainable, environmentally friendly and biodegradable flocculant candidates for water treatment.

CHAPTER 7

Recovery of pharmaceutical from water using natural submicron capsules

Related publication

Tassinari, B., Demont, A.., and Marison, I. W. "Removal of pharmaceuticals from water using natural submicron-capsules". Submitted to Journal of Environmental Management (*under review*).

Abstract:

In recent years increasing amounts of pharmaceutical compounds are being detected in the aquatic environments and, in some cases, they have been discovered in drinking water. This raises concerns about the potential effect of these pharmaceuticals on the environment and on human health. Their presence is attributed mainly to the inability of sewage treatment plants (STPs) to adequately remove these compounds from sewage influent. The aim of this study was to investigate the feasibility and kinetics of using natural submicron capsules (NSC), as an alternative to liquid-liquid extraction for the removal of a range of common pharmaceutical compounds. NSC are small individual plant organelles consisting of an oil core surrounded by a phospholipid monolayer with integrated proteins. NSC have been isolated from hemp, rapeseed, sunflower, linseed and soybean seeds. They have been shown to be capable of the extraction of hydrophobic pharmaceuticals from water in a technique termed capsular perstraction.

7.1 Introduction

Residues of pharmaceutically active compounds (PhACs) entering the environment represents an emerging environmental issue and was selected as a research priority in the European Union 5th Framework Programme for Research under the POSEIDON project (Ternes and Joss, 2006).

PhACs are complex molecules with different functionalities and physico-chemical and biological properties. They are developed and used because of their more or less specific biological activity. Most of them are lipophilic in order to be able to pass membranes (Halling-Sorensen et al., 1998). The molecular weights of the chemical molecules range typically from 200 to 500/1000 Da. Such PhACs are often called ñsmall moleculesò and are part of a group of compounds called ñmicro-pollutantsò because of their presence in the aquatic environment in the μg/L or ng/L range (Kümmerer, 2009).

Pharmaceuticals are excreted by humans or animals after treatment in their native form or as metabolites and enter aquatic systems by different routes. The main pathway from humans is ingestion following excretion and disposal via wastewater (Fent et al., 2006). In fact, the observed concentrations of drugs in raw wastewater confirm that municipal wastewater represents the main disposal pathway for households, hospitals and industry (**Figure 7.1**). Veterinary pharmaceuticals should only reach municipal wastewater to a limited extend (Ternes and Joss, 2006). In sewage treatment plant (STPs) effluents most pharmaceuticals are still present (generally in the range of ng/L to μg/L), because many of these polar and persistent compounds are only partially removed, or in some cases, are not removed at all. This results in the contaminations of rivers, lakes and estuaries, where the range is in ng/L (Buser et al., 1998, Weigel et al., 2002, Ashton et al., 2004, Thomas and Hilton, 2004). Where sewage sludge is applied to agricultural fields, contamination of soil, runoff into surface water but also drainage may occur

(Fent et al., 2006). Moreover, the treated wastewater might percolate or infiltrate into groundwater or bank filtrates. If drinking water is produced using raw water containing a substantial proportion of treated wastewater (e.g. from river water downstream of communities) the water cycle is closed and indirect potable reuse occurs (**Figure 7.1**) (Ternes and Joss, 2006).

Of environmental concern is the persistence of PhACs and their toxicity at trace levels (ng/L) for aquatic flora (Fent, 2008, Nentwig, 2008), fauna and/or human beings. Pharmaceuticals like erythromycin cyclophosphamide, naproxen, sulphamethoxazole, sulfasalazine or metabolites such as clofibric acid are known to persist for over a year in all natural water (Zuccato et al., 2000). Furthermore, many drugs are candidates to produce acute or chronic adverse effects on ecosystems and humans. For example, hormones like estrogens are recognised as endocrine disruptors or modulators because they may cause adverse effects on reproductive and sexual development like feminization of male fish when present at ng/L (Ingerslev et al., 2003, Fent et al., 2006, Robinson et al., 2007).

A few cases were reported where pharmaceuticals were detected in drinking water (Heberer and Stan, 1996, Vieno et al., 2007, Kozisek et al., 2013). This happens because techniques such as flocculation and sand filtration, normally used in turbidity removal, have been found to be inefficient in the elimination of PhACs (Vieno et al., 2007).

Concern has been raised, however, because exposure to pharmaceuticals through drinking water is an unintended and involuntary exposure, over potentially long periods of time.

The removal of pharmaceuticals can only be assured using ozonation, activated carbon or membrane filtration (Ternes et al., 2002). However, the economic consequences have

to be evaluated carefully before investing into these advanced treatment technologies on a large scale (Castiglioni et al., 2006).

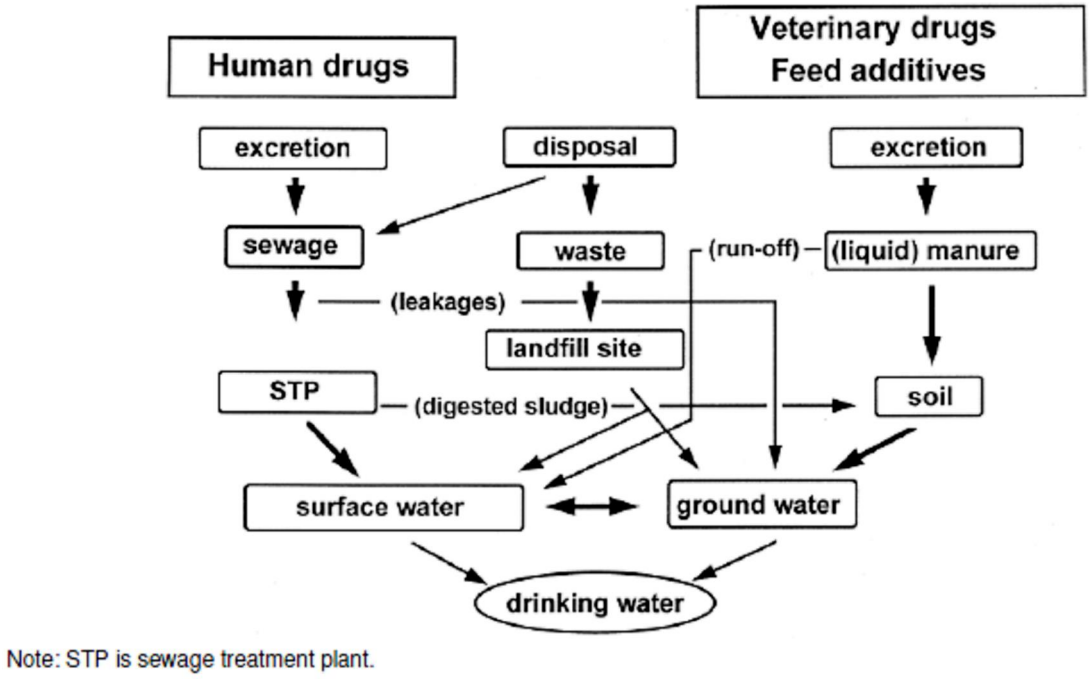


Figure 7.1: **Fate and transport of pharmaceuticals in the environment (Ternes, 1998)**

7.1.1 Liquid-liquid extraction (LLE)

Liquidï liquid extraction (LLE) is the transfer of certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. The desired product can be extracted selectively out of the water phase into the solvent phase by choosing the suitable solvent (Wells, 2003). The effectiveness of a solvent can be measured by the partition coefficient K, which is defined as the ratio of the desired product in the solvent phase to the concentration in the aqueous phase at equilibrium. A system with a large K value requires less solvent and produces a more concentrated extract phase. This process is widely employed in the chemical industry owing to its simplicity and low costs (Mazzola et al., 2008). LLE is traditionally used

for hydrophobic non-volatile trace analytes such as pesticides, phenols, polyaromatic hydrocarbons and has applications in water treatment also (Woller et al., 1996, Stackelberg et al., 2004).

Unfortunately this process is susceptible to several problems: the main one is the maximization of the mass transfer by producing as much interfacial material as possible between the two phases that makes the application to large volumes unfeasible. However, this obstacle can be overcome by increasing the agitation speed and/or the volume of the solvent used, leading to elevated costs or the formation of a stable emulsion (Boucher, 2006, Whelehan et al., 2010).

In order to overcome these disadvantages, several alternative techniques have been proposed such as membrane solvent extraction (MSE) or perstraction (Baudot et al., 2004), where the two phases are physically separated by immobilizing the interface between the aqueous feed and the solvent inside a porous membrane. Another approach consists of trapping oil droplets inside a membrane, thus creating oil core microcapsules (Stark et al., 2003, Wyss et al., 2004). This membrane prevents direct contact between the two phases which stops the formation of stable emulsions and also enables rapid extraction of compounds due to the large interfacial contact area provided by microcapsules. This novel methodology of encapsulating a hydrophobic organic solvent within hydrogel has been successfully and efficiently demonstrated for the extraction of a range of pesticides and herbicides from aqueous solutions (Wyss et al., 2004).

7.1.2 LLE of PhACs using natural submicron-capsules

Natural submicron-capsules (NSC), also named oil-bodies, are organelles involved in oil storage in oleaginous plant seeds. They have a typical structure made of a triacylglycerol matrix surrounded by a lipoproteinaceous membrane, whose main protein is called oleosin (Huang, 1994). NSC are stable and do not coalesce thanks to

this abundant protein which provides the steric hindrance and surface charges (electrostatic repulsion), keeping them individual entities in the range of micro/nanometres (Huang, 1996). The physiological significance of maintaining NSC as small, individual and stable organelles is to provide ample surface area for attachment of lipase in order to get energy during germination (Tzen and Huang, 1992a).

Since NSC are natural oil core submicron-capsules, the present chapter investigated the possibility of using them as an alternative LLE for the removal of drugs. It has been shown that NSC from rapeseed quickly absorb compounds with increasing hydrophobicity from aqueous solutions into the oil core (Boucher et al., 2008a). It is anticipated that the use of NSC may greatly improve water treatment since they combine flocculation and absorption of contaminants in only one step.

NSC extracted from hemp, rapeseed, sunflower, linseed and soybean were tested for the sorption of seven drugs characterised by different hydrophobicities.

7.2 Materials and Methods

7.2.1 Glassware preparation

Prior to use, all glassware was rinsed twice with deionised water and once with methanol.

7.2.2 Reverse phase high performance liquid chromatography (RH-HPLC)

RP-HPLC was used throughout this work using an Agilent 1100 series unit (Agilent Technologies, USA). The analytes were separated on a reverse-phase C18 Luna (2) Column, dimensions 150mm X 4.6 mm X 5m with an associated security guard cartridge system (Phenomenex, Cheshire, UK). The mobile phase was a binary gradient mixture of 0.1% (v/v) ammonium acetate, adjusted to pH 5.5 by the addition of formic acid and methanol. The gradient started at 10% (v/v) methanol, maintained isocratically for the first 5 min, thereafter the methanol content was raised linearly to 90% (v/v) within 13 min and was maintained isocratically for 20 min. This enabled complete and separate elution of all pharmaceuticals. The total run time was 30 min and retention time was used for peak identification. Samples (20 μL) were injected onto the column, which was maintained at 25ºC, using a mobile phase flow rate of 0.8 mL/min and all pharmaceuticals were detected at a fixed wavelength of 270 nm. All samples were diluted in methanol and filtered using 0.2 μm PTFE filters (VWR, Dublin, Ireland) before analysis. Internal and external standards were run to ensure accurate results. The quantification of compounds was based on the external standard method using chromatogram peaks areas (Whelehan et al., 2010), **Appendix L**.

7.2.3 Extraction of pharmaceuticals using NSC

7.2.3.1 Aqueous solutions containing pharmaceuticals

Stock solutions of the pharmaceuticals were prepared in water using methanol as a co-solvent (Sangster, 1997) to facilitate the solubility of each drug in the aqueous (water) phase. Initially the drugs were dissolved in methanol and were then added to the water, with a final methanol concentration of 5% (v/v), to obtain the desired concentration of the pharmaceuticals. The compounds were present in the aqueous phase at a concentration of 10 mg/L for all experiments. The presence of methanol at the employed concentration had a negligible effect on the mass transfer. Stocks were filtered under vacuum using 0.2 μm nylon membranes (Millipore, Ireland). Standard solutions for calibration were prepared in 50:50 methanol:water. Three samples of 0.5 ml were removed from the solution and diluted in 0.5 mL of methanol to determine the initial concentration of the pharmaceuticals.

7.2.3.2 Sorption experiments and LLE

A defined amount of NSC suspension or oil (V_{org}) equal to 0.1 ml of oil were added to 5 ml pharmaceutical solutions in 15 ml glass test tubes and agitated (IKA platform shaker, Ireland) overnight at 250 rpm under dark conditions due to the light-sensitivity of some compounds. From the solutions containing NSC 1.5 ml was removed and centrifuged (Eppendorf Centrifuge 5415 R, Eppendorf, Germany) at 10,000 rpm for 30 min and 0.5 ml of the aqueous phase was removed with a syringe and placed in an Eppendorf tube to which 0.5 ml of methanol was added. After filtration through 0.2 μm PTFE filters, samples were transferred into HPLC vials for analysis.

From the initial aqueous concentration of PhACs (C_{aq}^{0}) and the aqueous equilibrium concentration (C_{aq}^{e}), the corresponding equilibrium concentration in the NSC (C_{org}^{e}), may be calculated from the following mass balance in **Equation 7.1**:

$$V_{aq}(C_{aq}^{0} - C_{aq}^{e}) = V_{org}(C_{org}^{e} - C_{org}^{0}) \qquad \textbf{Eq.7.1}$$

where C_{aq}^{0} is the initial concentration of the pharmaceutical in the aqueous phase, V_{org} is the volume of the organic phase (0.1 mL oil inside NSC) and C_{org}^{0} is the initial concentration of the pharmaceutical in the organic phase, which will be taken to be 0 and V_{aq} is the volume of the aqueous phase. The volume of the aqueous phase was calculated as $V_{aq} = V_{phac} + V_{nsc}$, where V_{phac} is the pharmaceutical solution (5 ml), V_{nsc} is the water content within NSC.

Thus the C_{org}^{e} is given by

$$C_{org}^{e} = \frac{Vaq}{Vorg} \cdot \left(C_{aq}^{0} - C_{aq}^{e}\right) \qquad \textbf{Eq.7.2}$$

The partition coefficient *K* is defined as the ratio of the pharmaceutical compound in the organic phase (oil or oil into NSC) to the concentration in the aqueous phase at equilibrium and is obtained from the following equation (**Equation 7.3)**:

$$K = \frac{C_{org}^{e}}{C_{aq}^{e}} \qquad \textbf{Eq.7.3}$$

Controls, where no NSC solution or oil was added, were run in order to check the influence of filter retention, adsorption of tube walls, light degradation and volatilisation. All the experiments were repeated three times.

7.2.3.3 Kinetic of pharmaceutical extraction with oil and NSC

All kinetic experiments were performed at 21°C in 25 mL glass tubes each one containing 5 mL of the 10 mg/L of pharmaceutical solution. A sample from the aqueous

phase was taken to determine the initial concentration (C_{aq}^{0}) of the pharmaceutical(s). NSC or oil were added to the pharmaceutical solution and placed on an orbital shaker at 100 or 200 rpm. Sampling from the aqueous phase was performed at defined time intervals, using a syringe and needle for the oil and filtration with 0.2 membrane (Millipore, Ireland) filters for the NSC. The samples were analysed using RP-HPLC to determine the extraction kinetics of the pharmaceuticals into the organic phase. Control experiments containing only aqueous phase and the pharmaceutical(s) were run for all extraction experiments to ensure that any decline in pharmaceutical concentration was not due to degradation. All the experiments were repeated three times and the error bars show the standard deviation.

7.2.4 Defatted NSC

A defined amount of hemp NSC were defatted by means of diethylether extraction (Tzen et al., 1997) in a ratio 1:6. After vortexing, the suspension was centrifuged for 20 min at 7000 rpm (Thermo Electron FL 40R centrifuge, Thermo Scientific, France), the upper diethylether layer was carefully removed and the lower layer used for the kinetic studies.

7.3 Results and discussion

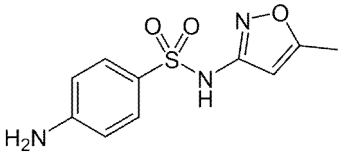
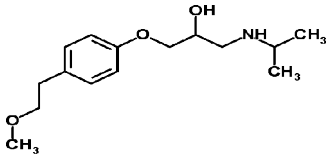
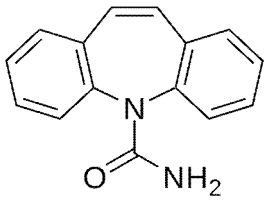
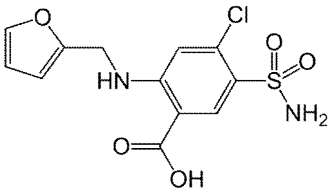
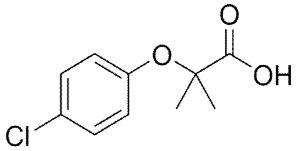
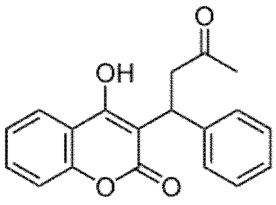
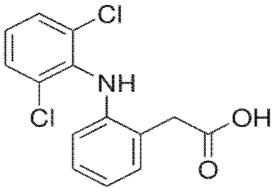
7.3.1 Analysis of PhACs and NSC

To examine the sorption of PhACs in NSC for this study, seven different compounds (**Table 7.1**) were chosen for extraction and selection was based on their varying $LogP_{oct}$ values. Drugs were also pre-selected on their ability to match the following criteria:

1) presence in the top 100 most commonly prescribed medicinal products in Ireland
2) specific mode of action (different therapeutic classes)
3) inability to be adequately/fully removed by STPs
4) presence in the aquatic environment

Table 7.1: **Characteristic of PhACs which were used to help chose the compounds for the work undertaken in this study.**

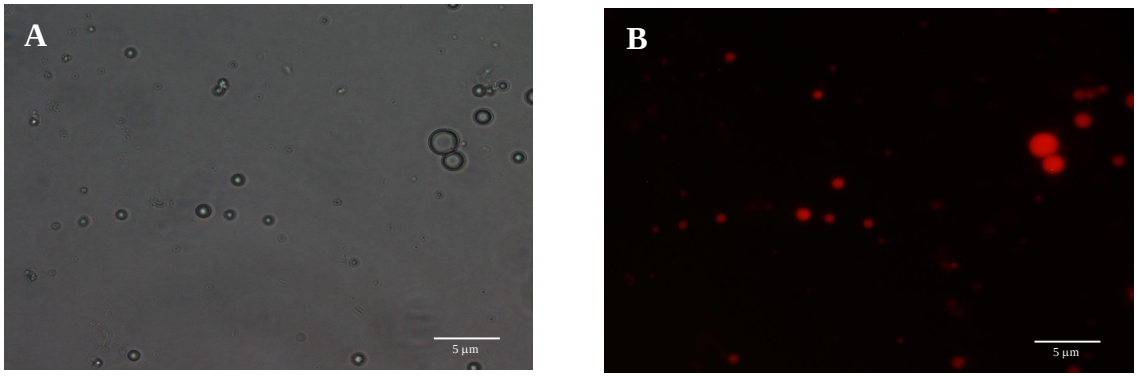
Drugs are listed according to increasing hydrophobicity ($LogP_{oct}$).

Compound (CAS No.)	Therapeutic class	Chemical structure	*$LogP_{oct}$	Conc. in environmental water (ng L^{-1})
Sulfamethoxazole (723-46-6)	Antibiotic		0.66	410 [a]
Metoprolol (37350-58-6)	Beta-blocker		1.63	2200 [b]
Carbamazepine (298-46-4)	Anticonvulsant		1.89	1075 [a]
Furosemide (54-31-9)	Loop diuretic		2.30	585 [c]
Clofibric Acid (882-09-7)	Metabolite of three lipid regulators		2.42	270 [a]
Warfarin (81-81-2)	Anticoagulant		3.13	1.0 [d]
Diclofenac (15307-86-5)	Analgesic/anti-inflammatory		4.55	990 [e]

*Sigma data; [a] (Heberer, 2002); [b] (Owen et al., 2007); [c] (Zuccato et al., 2005); [d] (Kolpin et al., 2002); [e] (Tixier et al., 2003).

The oleaginous seeds were chosen based on ease of availability in Ireland and across Europe and were used to replace moringa seeds, usually used to treat and clarify drinking water in African rural villages (Jahn and Dirar, 1979, Doerries, 2005, Ghebremichael et al., 2005). Moringa plants are usually found only in sub-saharan countries as the plant only grows in very hot and dry conditions. Use of this plant to treat water in colder northern countries would result in higher costs due to transportation (Doerries, 2005). For this reason, it is important to find feasible alternatives in Europe.

NSC were extracted from hemp, rapeseed, sunflower, linseed and soybean seeds through an aqueous flotation-centrifugation method, where hexane steps were added, in order to remove defective NSC (Tzen et al., 1997). Microscopic investigations showed the presence of NSC, the spherical shape and ability to remain as individual organelles due to the structural oleosin proteins (**Chapter 3**). This was confirmed by, staining with Methylene Blue and Nile Red which showed the presence of a proteinaceous external layer and an oil core of NSC with an average diameter of 500-900 nm depending on the origin of the NSC (**Figure 7.2**)



7Figure 7.2: Microscopic investigations of NCS extracted from hemp seeds.
A) Light microscopy, NSC stained with Methylene Blue shows the protein coat and B) Fluorescence microscopy, NSC stained with Nile Red shows the oil core.

NSC were further analysed with respect to total oil and water content (**Table 7.2**), since these parameters are required to calculate the oil volume and correct for water content in subsequent LLE experiments.

Table 7.2: **Characterisation of NSC preparations.**
Water content measured gravimetrically by evaporation. Oil content measured gravimetrically by diethyl ether extraction.

	Water content (mg/mL)	**Oil content (mg/mL)**
Hemp	810	217.15
Rapeseed	844.5	136.8
Sunflower	798.5	186.5
Linseed	803.5	175.5
Soybean	845	115.1

7.3.2 Partitioning of pharmaceuticals into NSC

The partition coefficient for NSC extracted from each seed was calculated after 24h incubation under dark conditions because of the light-sensitivity of some compounds. The extraction was performed using the equivalent of 0.1 mL oil inside NSC. This volume was calculated from the measured oil content of each NSC suspension in order to maintain constant oil content for all extractions.

It is expected that the *K* would increase with increasing the $LogP_{oct}$ of the compounds and therefore be higher for warfarin and diclofenac, since the very hydrophobic nature and limited solubility in water would favour mass transfer into the hydrophobic core of NSC (Whelehan et al., 2010).

As shown in **Table 7.3**, there was no direct correlation between $LogP_{oct}$ and the equilibrium partition coefficient with each PhAC removed to a different degree depending on the source of the NSC used, suggesting that extraction is affected by the composition of the NSC which varies with seed source. For example, only hemp NSC were able to extract all seven pharmaceutical compounds, while rapeseed, linseed, sunflower and soybean NSC failed to extract at least one of the compounds. It interesting to note that soybean NSC were less successful in removing PhACs with lower $LogP_{oct}$, but showed the highest partition coefficient for diclofenac and warfarin of all NSC tested. These results suggest that the composition of the oil core and/or membrane of NSC plays a role in the sorption mechanism.

Pharmaceuticals with $LogP_{oct} < 2.5$ (sulfamethoxazole, metoprolol, furosemide and carbamazepine) have been generally removed from water by NSC extracted from all five seeds. Moreover, for these compounds, the removal seems to be correlated with the hydrophobicity. However, drugs with $LogP_{oct} > 2.5$ showed a dramatic decrease in the level of extraction with the exception of diclofenac, which had a high *K* value in all NSC. Thus, in agreement with Whelehan et al. (2010), a direct correlation between the

amount of these PhACs extracted into the organic phase with their $LogP_{oct}$ cannot be made. A closer analysis of the compounds shows that sulfamethoxazole, metoprolol, furosemide, carbamazepine and diclofenac have at least one amine group, which may play an important role in the sorption, by reducing the repulsions between drugs and the highly negatively charged membrane of NSC. It has been reported that NSC are maintained as individual organelles by the presence of the structural protein oleosin that has a net negative charge at pH 7 (Huang, 1996). This may interfere with the sorption of drugs that have mainly negative functional groups as clofibric acid and warfarin. Therefore, the chemical properties of each drug have to be taken into account in the mechanism of sorption.

Taken together these results indicate that while the partitioning of the PhACs in NSC is dependent on hydrophobicity for many compounds, functional charged groups, steric effects and size of the compound may have significantly greater effects by increasing the mass transfer resistance (Dimitrov et al., 2002, Boucher et al., 2008a, Whelehan et al., 2010). Since the oleosin composition, phospholipid and triglyceride profiles of NSC from different sources will differ, there is likely to be an influence on the extraction of compounds depending on the composition of NSC as well.

Since hemp and soybean showed the highest and lowest *K*, it was decided to use them for further analysis.

Table 7.3: **Partition Coefficient (*K*) of the different pharmaceuticals for each seeds NSC determined at the equilibrium.**

For some drug no sorption was found (-).

	Hemp	Rapeseed	Sunflower	Linseed	Soybean
Sulfamethoxazole	4.695 ± 0.095	5.215 ± 0.094	4.907 ± 0.095	3.140 ± 0.100	0.247 ± 0.527
Metoprolol	8.842 ± 0.180	9.250 ± 0.180	2.427 ± 0.207	2.208 ± 0.212	0.346 ± 0.749
Furosemide	13.466 ± 0.061	19.926 ± 0.061	2.738 ± 0.068	4.715 ± 0.063	-
Carbamazepine	30.534 ± 0.045	23.265 ± 0.046	9.928 ± 0.046	10.383 ± 0.046	6.733 ± 0.047
Clofibric acid	3.911 ± 0.056	2.456 ± 0.061	-	1.110 ± 0.086	3.313 ± 0.057
Warfarin	1.217 ± 0.064	-	3.462 ± 0.045	-	65.300 ± 0.042
Diclofenac	11.323 ± 0.038	11.651 ± 0.016	22.231 ± 0.010	7.268 ± 0.083	27.596 ± 0.030

In order to understand further the role of the organic core of the NSC in the extraction process, standard LLE was carried out by contacting hemp and soybean oil with drugs solutions and the partition coefficients of the seven drugs between the aqueous phase and oil at the equilibrium determined (**Table 7.4**).

It is expected that the coefficient partition would increase increasing the $LogP_{oct}$ and it may vary taking into account the composition of the two oils.

Table 7.4: **Partition coefficient (*K*) of the different pharmaceuticals for hemp and soybean oil, respectively, determined at the equilibrium.**
For some drug no sorption was found (-).

	Hemp oil	**Soybean oil**
Sulfamethoxazole	1.073±0.151	4.664±0.095
Metoprolol	0.307±0.840	0.943±0.322
Furosemide	-	1.365±0.088
Carbamazepine	5.968±0.047	4.471±0.048
Clofibric acid	2.937±0.059	3.373±0.057
Warfarin	66.323±0.041	87.115±0.042
Diclofenac	53.362±0.339	63.415±0.339

According to **Table 7.4**, although hemp NSC extracted all seven pharmaceutical compounds with those with a $LogP_{oct}$ <2.5 exhibiting high equilibrium coefficients, this changed considerably when the extraction was carried out using the free, purified oil, with compounds with $LogP_{oct}$ <2.5 being poorly extracted, while warfarin and diclofenac exhibited very high *K* values. The latter results show the expected correlation between *K* and $LogP_{oct}$. These data clearly indicate that the chemical properties of each compound affect the extraction: because of ionised functional groups and low $LogP_{oct}$ sulfamethoxazole, metoprolol, furosemide and clofibric acid do not partition easily into free oil (Waller et al., 2009). The results confirm the hypothesis that the protein coat

around the NSC plays an important role in controlling the mass transfer resistance between the bulk fluid and the oil core of the NSC.

These results are in contrast with those of Boucher et al. (2008a) who observed similar partition coefficients for pesticides and herbicides for NSC and oil. This is almost certainly due to the difference in physico-chemical properties of the pharmaceutical compounds compared to pesticides and herbicides.

7.3.2.1 Partition coefficient of NSC treated with Proteinase K

The surface of NSC is reported to be a coat formed from oleosin proteins in which the central hydrophobic domain penetrates into the TAG matrix and have been demonstrated to protect NSC from proteolytic digestion and coalescence (Tzen and Huang, 1992a, Tzen et al., 1992b, Frandsen et al., 2001). In order to test whether the proteinaceous coat provided by the oleosins influences the sorption of the pharmaceutical compounds, hemp NSC were treated with Proteinase K, to remove the surface protein domains.

The partition coefficient for each drug at equilibrium was then calculated and compared to the *K* of un-treated NSC (**Table 7.5**).

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Table 7.5: **Partition coefficient (*K*) of the different pharmaceuticals for untreated hemp NSC and hemp NSC treated with 8 U of Proteinase K, respectively, determined at the equilibrium.**

For some drug no sorption was found (-).

	Control	Proteinase K treated
Sulfamethoxazole	4.695 ± 0.095	8.634 ± 0.074
Metoprolol	8.842 ± 0.180	9.356 ±0.180
Furosemide	13.466 ± 0.061	0.376 ± 0.236
Carbamazepine	30.534 ± 0.045	21.856 ± 0.046
Clofibric acid	3.911 ± 0.056	-
Warfarin	1.217 ± 0.064	6.984 ± 0.042
Diclofenac	11.323 ± 0.038	13.340 ± 0.034

The results indicate that removal of the surface protein layer of the NSC had a relatively minor effect on sorption of sulfamethoxazole, metoprolol, diclofenac and carbamazepine. However, for clofibric acid no sorption was detected after Proteinase K treatment, whereas for furosemide a 35-fold decrease in sorption was detected. These results suggest that for these two compounds the removal by NSC was actually due to an adsorption process to the proteins surrounding the NSC. On the other hand the partition coefficient for warfarin actually increased by 5.74- fold indicating that for this highly hydrophobic compound the protein coat of the NSC actually increased the mass transfer resistance. Removal of the protein coat using Proteinase K, would expose the phospholipid monolayer surrounding the oil core of the NSC (Tzen & Huang, 1992a), the nature of the groups associated with the phosphate moiety of the phospholipid would also be expected to play a role in the overall sorption/adsorption and mass transfer resistance.

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7.3.3 Kinetics of sorption into hemp and soybean NSC and oil

To assess the efficiency of sorption using NSC to recover pharmaceuticals from aqueous solutions, NSC extracted from hemp seeds were incubated in water containing metoprolol or warfarin agitated at 100 and 200 rpm. The kinetics of partitioning of metoprolol into free oil and NSC has been compared. The concentrations in the aqueous phase have been normalised to the concentration at a defined time by the initial concentration (C_t/C_0) in order to compare the kinetics, since the initial concentration of the two drugs may vary between experiments.

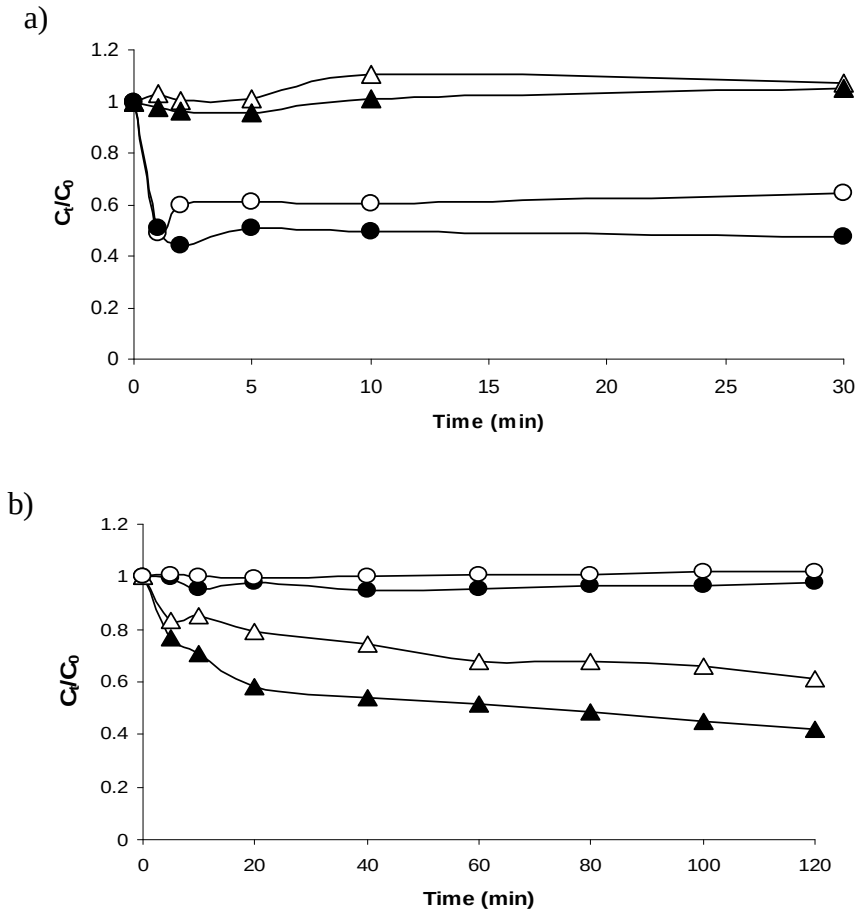


Figure 7.3: Effect of agitation speed on the removal of metoprolol and warfarin from aqueous solutions using hemp NSC (a) and oil (b).

Open triangle: warfarin removal at 100 rpm; Triangle: warfarin removal at 200 rpm; Open circles: metoprolol removal at 100 rpm; Circles: metoprolol removal at 200 rpm. C_t/C_0 represents the ratio of the concentration at time t to the initial concentration C_0.

Figure 7.2 shows the sorption kinetics of metoprolol and warfarin into hemp NSC and free oil respectively. Two agitation speeds (100 and 200 rpm) were tested in order to determine whether the rate of extraction was mass transfer limited in the bulk solution. The results show that the rate of partitioning of the two pharmaceutical compounds using NSC was extremely fast (< 2min) and independent of agitation speed, whereas for free oil between 20-60 min were required, with the extraction level higher at the higher agitation speed. The rapid partitioning using NSC is due to the very high interfacial surface area for mass transfer (Boucher et al., 2008a) and the presence of a phospholipid monolayer instead of the typical bilayer present in the cell membrane (Baláģ, 2000, Lukacova et al., 2007). These experiments further confirm the results obtained for the partitioning into NSC and free oil with hemp NSC extracting higher levels of metoprolol than warfarin, whereas the opposite situation occurs when hemp oil was used (**Table 7.3** and **7.4**).

The very rapid sorption kinetics for certain pharmaceutical compounds are much higher than those observed using microcapsules where equilibrium was attained after a minimum of 60 min (Wyss et al., 2004, Whelehan et al., 2010).

Further tests were performed on soybean free oil and NSC to confirm these data (**Figure 7.3**). As expected, the partitioning of the drugs confirmed the results previously obtained: the equilibrium in NSC is achieved in less than 2 minutes while in oil in 60 minutes. Moreover for soybean, as shown in the equilibrium data, the sorption occurred only for warfarin.

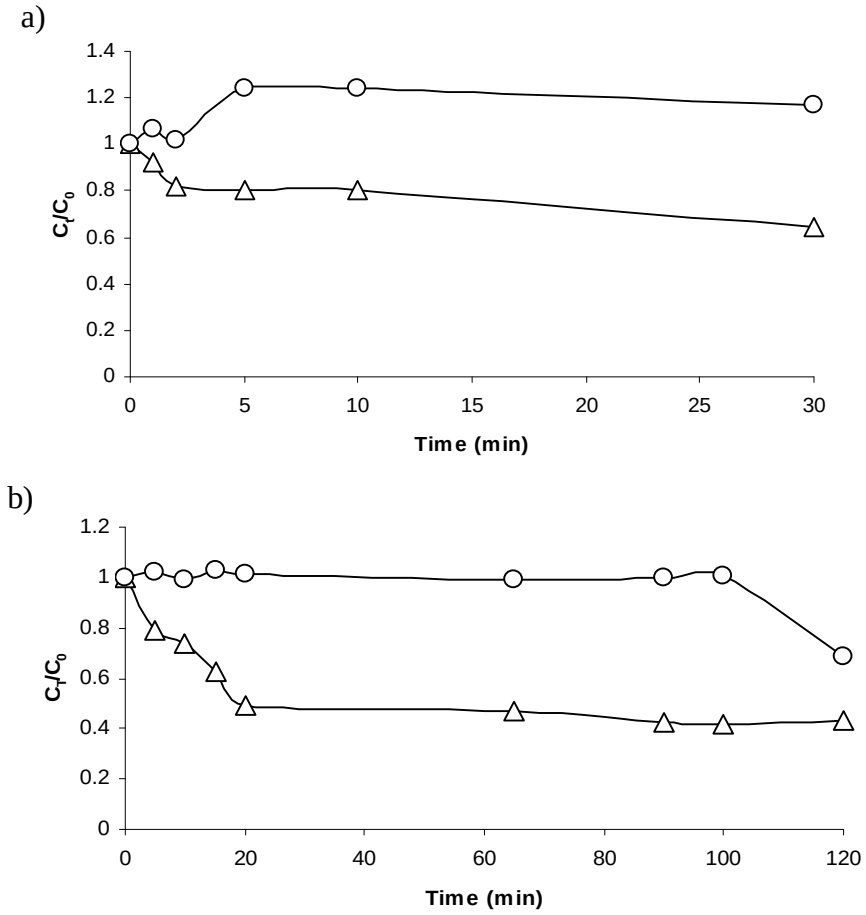


Figure 7.4: Removal of metoprolol and warfarin from aqueous solutions using soybean NSC (a) and oil (b) at 100 rpm.

Open circles: metoprolol removal at 100 rpm; Open triangles: warfarin removal at 100 rpm. C_t/C_0 represents the ratio of the concentration at time t to the initial concentration C_0.

7.3.4 Partitioning of metoprolol in defatted NSC

To further test if the sorption of pharmaceutical compounds was mainly due to the oil or the interaction with the NSC protein surface, hemp NSC were treated with diethyl ether to remove the triacylglycerol core (Tzen et al., 1997) and the suspension of defatted NSC incubated with metoprolol (**Figure 7.4**).

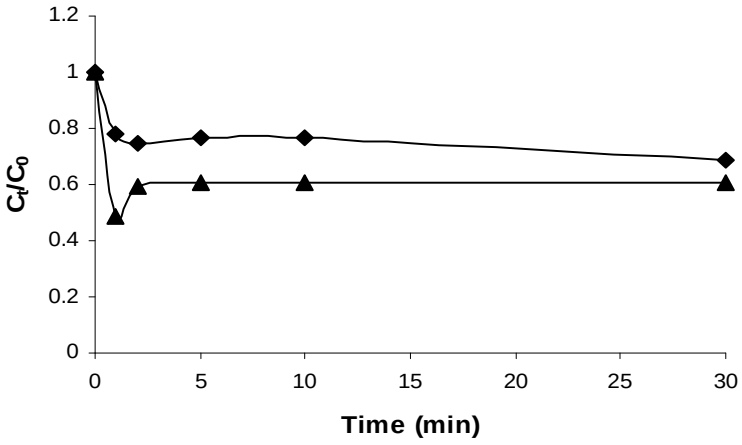


Figure 7.5: Partitioning of metoprolol into defatted NSC extracted from hemp. Diamonds: defatted NSC; Triangle: control. C_t/C_0 represents the ratio of the concentration at time t to the initial concentration C_0.

The results show that the partitioning of the drug into hemp NSC decreased by approximately 30% presented-fatting. This suggests that there are indeed some interactions between metoprolol and the surface of NSC. In this case however, the level of interaction may be greater than that expected for completely de-fatted NSC since the oleosins possess a large, extremely hydrophobic domain which would still be available for interaction with metoprolol (Tzen et al., 1997).

7.3.5 Partitioning of drugs in the same aqueous phase into hemp NSC

Pharmaceuticals do not occur as isolated, pure substances in an environmental compartment. They are present as multi-component mixtures in the environment. Most pharmaceuticals can either be transformed by physical and chemical processes in the environment and/or taken up by some organism and subsequently bio-transformed. In general, knowledge about the toxicity of mixtures of compounds is limited. This new field of ecotoxicology is just beginning and much remains to be learned (Kümmerer, 2009).

To test the partitioning of NSC when all the drugs are present in the same solutions, an equilibrium test with hemp NSC and hemp oil was performed and the *K* was calculated and compared to the *K*s of the single compounds for NSC and oil extraction.

Table 7.6: **Partition coefficient (*K*) of a mixed solution containing the pharmaceuticals (10 mg/L) for hemp NSC and hemp oil at equilibrium.**
Clofibric acid had to be removed because of interference with carbamazepine.

	NSC	Oil
Sulfamethoxazole	-	0.816
Metoprolol	29.523	0.526
Furosemide	-	0.265
Carbamazepine	20.875	4.319
Warfarin	1.186	52.925
Diclofenac	0.226	204.174

The results show that in the presence of a mixture of pharmaceuticals both NSC and oil decreased their sorption ability compared to the solutions of containing single

PhACs. This may be due to interactions between the drugs that make sorption more difficult.

7.4 Conclusion

The experiments show the possibility of extracting PhACs from water using NSC extracted from different seeds. The extraction is very fast compared to liquid-liquid extraction and capsular perstraction with compounds removed at different levels depending on the source of NSC. Higher levels of extraction/adsorption would be achieved by simply increasing the amount of NSC added to the water. In order to facilitate the analysis, in all experiments higher PhAC concentrations than those routinely found in STPs, consequently it is unlikely that higher concentrations of NSC would be required in practice.

The sorption mechanism was shown to depend on the oil core of NSC, as well as interactions between functional groups of both the pharmaceutical compounds and NSC surface. This leads to the possibility of combining NSC from a range of plant origins to enable the removal of a broad range of pharmaceutical compounds and other hydrophobic organic pollutants.

NSC have many advantages over more traditionally used methods: they are natural products that can be easily extracted from seeds commonly grown in Europe; the ability to perform a very rapid extraction at low agitation speed makes them suitable for the extraction of highly volatile compounds and the simple recovery of submicron-capsules from the water by inducing their aggregation using divalent cations such as calcium (Boucher et al., 2008a).

Since, in **Chapter 3** and **6**, NSC were shown to have a high flocculation activity, there is the unique potential of combining sorption of pollutants and flocculation in a single step.

CHAPTER 8

Conclusions and future work

8.1 Conclusions

Drinking water treatment is an expanding field of research due to the increasing importance of potable drinking water scarcity in the world. The currently used treatment methods have been shown to exhibit some disadvantages and potential problems with the result that considerable importance is being given to finding environmentally friend alternatives.

As previously mentioned, coagulants such as alum and ferric salts, may be hazardous for the environment and human health (Nayak, 2002, Gillette-Guyonnet et al., 2007), while synthetic organic polymers, such as acryl amide, have neurotoxic and carcinogenic effects (Friedman et al., 1995, Rice, 2005) with the result that EU legislation prevents the disposal of sludge through spreading. This gave rise to a plethora of studies focused on developing biological and safe flocculants, mainly from plants (Yin, 2010, Muthuraman et al., 2013).

This project is focused on the application of novel flocculating agents extracted from oleaginous seeds and PC for drinking water treatment.

In this study quantitative flocculation assays were performed using kaolin as turbidity model and an on-line jar test method to investigate whether oil-bodies (OBs) extracted from seeds and proteins extracted from PC exhibit flocculation activity. Since the currently used lab-bench methods may be inaccurate and time consuming, a continuous, on-line and quantitative method was developed where a typical jar test device was adapted by the installation of six commercially available turbidity probes connected to a PC. This allows the study of flocculation kinetics of such as turbidity removal and sedimentation rates for six water samples simultaneously.

OBs from a range of different plants seeds, such as rapeseed, hemp, sunflower and soybean, were demonstrated to have flocculation activity which was considerably

higher than the two standards, aluminium sulphate and ferric sulphate, without the addition of inorganic compounds or polymers. Thus, although OBs in have been used in food technology and the production of recombinant proteins, application as flocculants represents a new alternative to currently used methods and has not been previously reported.

We also suggested that since OBs are small organelles with an oil core surrounded by a phospholipid monolayer and proteins (Huang, 1994), a more descriptive and appropriate term would be natural submicron capsules (NSC).

Experiments performed on rapeseed NSC showed that flocculation activity is dependent on the intact structure of NSC, since treatments aimed at removing the oil core or the surrounding proteins, dramatically decreased the flocculation activity. Moreover, the activity increases with dosage, even though low dosages, such as 5 and 10 ppm are already effective. We hypothesize that the mechanism behind NSC flocculation activity would be a bridging-type where the adsorption to particles occurs mainly through H-bonding and ion-bonding, as suggested for other natural and synthetic polymers (Gregory and O'Melia, 1989, Miller et al., 2008). This study also showed that the total turbidity removal achieved with NSC from different seeds is very high with different turbidity buffers, generally being between 80-100%, indicating that it would be possible to use NSC from different sources according the type of water to be treated.

Moreover, we showed that aqueous soluble proteins from rapeseed, sunflower, hemp PC also have a high flocculation activity indeed, which is higher than the standards over a range of pH and ionic strength. In flocculation tests, proteins of animal origin were also tested and it was shown that proteins such as isinglass and fish gelatin, commonly used in the food industry, also showed a very high flocculation activity, indicating that many proteins and protein- structures, may be potential flocculating agents. However, the possibility of allergenic reactions to fish proteins in treated water has to be taken

into account (Walker et al., 2007, Weber et al., 2010) and preferably avoided even though the dosages used are very small. On the other hand, the extraction of flocculant proteins from PC might be more advantageous since it is an economical by-product of the vegetable oil industry and, as shown for Moringa extracts (Folkard et al., 1995, Doerries, 2005), large scale production would be easy. Unfortunately, flocculation performed with current flocculants does not remove pharmaceuticals, whose presence in water is now raising concerns, in particular due to their effect on environment and human health (Ternes and Joss, 2006). Since NSC have an oil-core and many drugs are lipophilic in order to pass through biological membranes, the ability of NSC to extract drugs from water was investigated. The experiments showed that NSC from rapeseed, hemp, sunflower, soybean and linseed seeds can quickly and efficiently remove pharmaceuticals from water. Extraction is dependent on the oil core of NSC, as well as interactions between functional groups of both the pharmaceutical compounds and NSC surface. Thus, combining NSC from a range of plant origins would enable the removal of a broad range of pharmaceutical compounds and other hydrophobic organic pollutants.

In conclusion, this study demonstrated that NSC represent a very interesting and potentially useful material in drinking water treatment. NSC not only show flocculation activity, but also sorption activity for a range of pharmaceuticals, which makes them very versatile and unique. Both activities are very fast, thus NSC might be used when rapid kinetics is necessary. Moreover, aqueous based extraction is both cheap and readily scalable compared to other reported methods (Tzen et al., 1997, Jolivet et al., 2004, White et al., 2006, Chen and Ono, 2010, Lu et al., 2010) and would make the extraction of NSC easy and quicker.

On the other hand, the extraction of flocculant proteins from PCs provides a new insight into the applications of PC that have been extensively studied as biosorbent (Boucher et al., 2007, Boucher et al., 2008b, Breguet et al., 2008).

Thus, oleaginous seeds and PC may be considered as **natural**, **sustainable**, **environmentally friendly** and **biodegradable** flocculant candidates for drinking water treatment.

8.2 Future work

Future work could focus on the study of flocculating activity of proteins extracted from PC. As shown in **Chapter 4**, rapeseed, hemp, sunflower PC extracts have flocculant proteins which need to be characterised. A preliminary bioinformatic analysis identified possible candidates in rapeseed and sunflower seeds as flocculating agents. In order to properly characterise flocculating agents in PC extracts, size exclusion chromatography could be performed to obtained fractions of proteins in different molecular weight ranges. Subsequently, each fraction could be tested for flocculation activity using the jar test under different pH values and ionic strengths.

The fractions which exhibit flocculation activity could be further analysed using LC-MS for total protein identification and through capillary electrophoresis and 2D-PAGE in order to characterise their pI and potentially identify range for proteins present.

These experiments would clarify the molecular weight and pI of rapeseed, sunflower ad hemp protein extracts and, consequently, the flocculation mechanism.

In **Chapter 6**, the feasibility of using NSC to remove pharmaceuticals from water has been explored, however, the question is then how to dispose of NSCs after the sorption process is complete. One possibility is to test whether bacteria are able to degrade the pharmaceuticals extracted from NSC, in a similar approach proposed by Wyss et al.

(2006). After the back extraction of pharmaceuticals, NSC might be recycled for flocculation/sorption.

Since it has been reported that Moringa flocculating proteins have also antimicrobial activity, it still remain to understand whether NSC and PC extracts from rapeseed, sunflower and hemp also possess such an activity. An antimicrobial test might be performed using a suspension containing *E.coli* and/or *P.aeruginosa* and PC extracts or NSC for 24h at 37°C. Subsequently, diluted samples of these suspensions might be spread on nutrient agar plates and incubated for an additional 24h, following colony counting.

A necessary further test to corroborate the data obtained with synthetic turbid water would be the flocculation of NSC and proteins extracted from PC using natural raw water collected from rivers and/or lakes as well as the drugs removal.

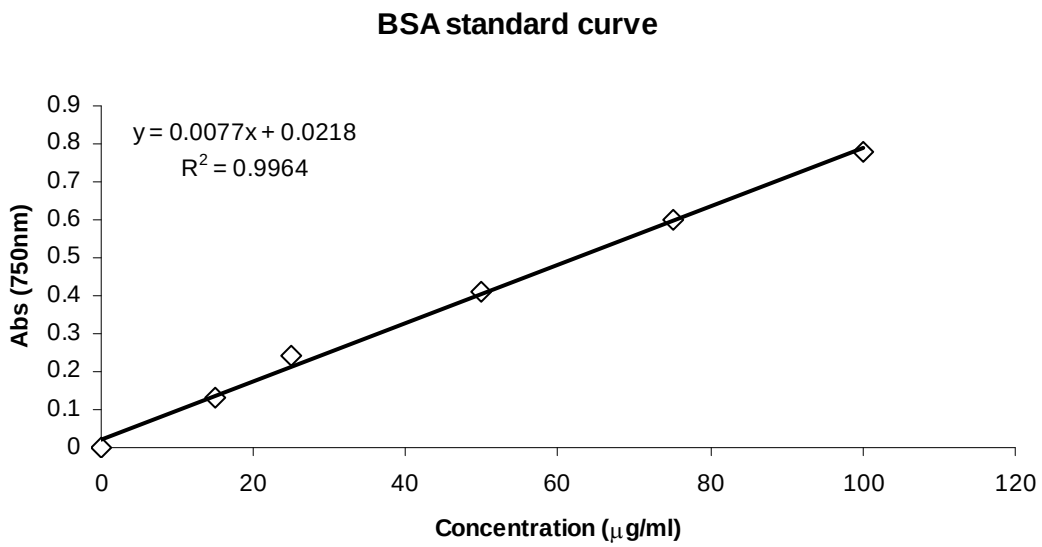
This project focused on oleaginous seeds; however, the study of non oleaginous seed extracts might clarify whether or not they also show high flocculation activity and whether the flocculating components have the same characteristics of oleaginous ones.

This would help to identify possible families of flocculating agents in a wide range of plant seeds, which can be used as cheap, natural agents in drinking water treatment over a wide range of climatic and geographic locations using locally available crop plants.

Appendices

Appendix A

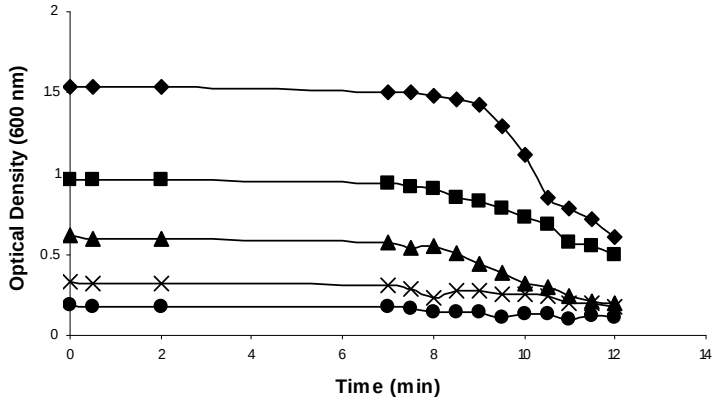
Example of a standard curve for the quantification of proteins in NSC samples using BSA as standard and Peterson's modification of Lowry method (**Chapter 2**, **Section 2.2.6**).



Appendix B

Flocculation activity of 5 ppm of NSC total proteins suspension at different kaolin concentration suspensions at pH 5 and 9 (**Chapter 3**, **Section 3.2.2**).

a)



b)

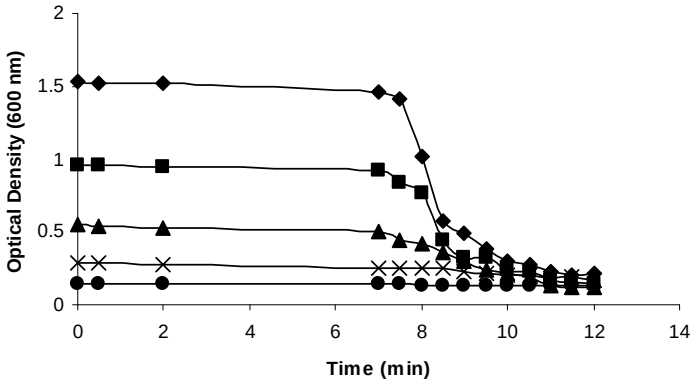


Figure 1: Flocculation activity of 5 ppm NSC at different kaolin concentrations.
The dosage of 5 ppm of NSC was added to the kaolin suspensions, mixed for 2 min at high rpm, 5 min at slow rpm and let sediment for 5 min. a) pH 5 and b) pH 9. Diamonds: 2 g/L; Squares: 1 g/L, Triangles: 0.5 g/L; Crosses: 0.250 g/L and Circles: 0.125 g/L.

Appendix C

Flocculation activity of 10 ppm of NSC, alum and ferric at pH 5 and 9 under high and low ionic strength (**Chapter 3**, **Section 3.2.3**)

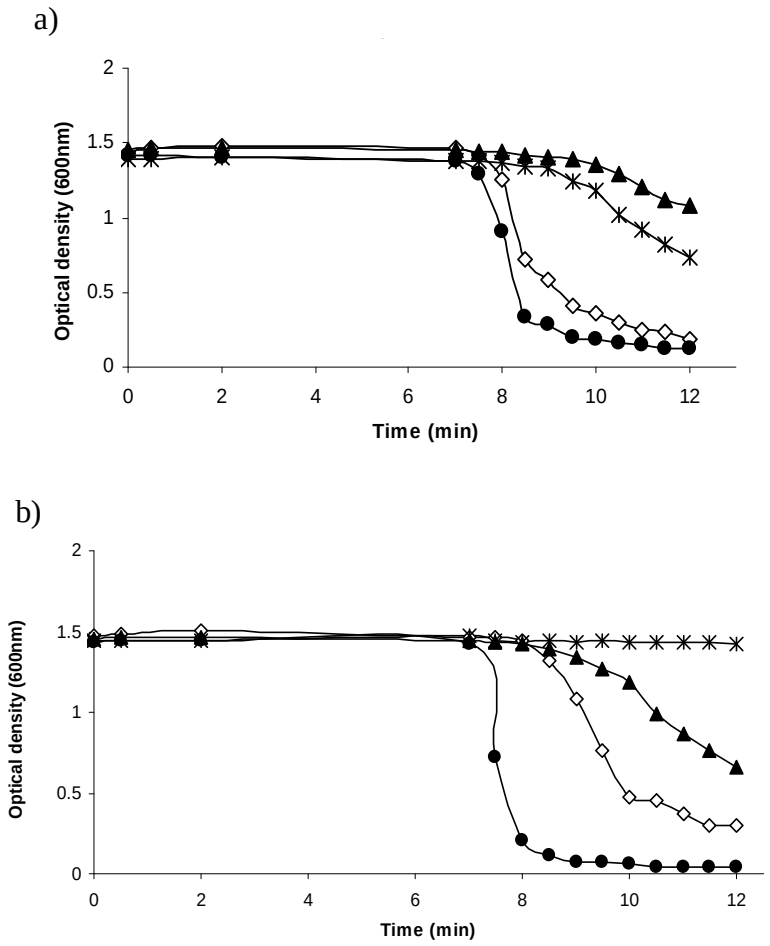


Figure 1: Effect of ionic strength and pH on FA at pH 5.

10 ppm of NSCs is compared to 10 ppm of alum sulphate and 10 ppm of ferric sulphate at pH 5. a) High ionic strength (100mM NaCl). b) Low ionic strength (10 mM NaCl). The mixing was stopped at min 7. Open diamonds: NSCs; Circles: aluminium sulphate; Triangles: ferric sulphate; Cross: control.

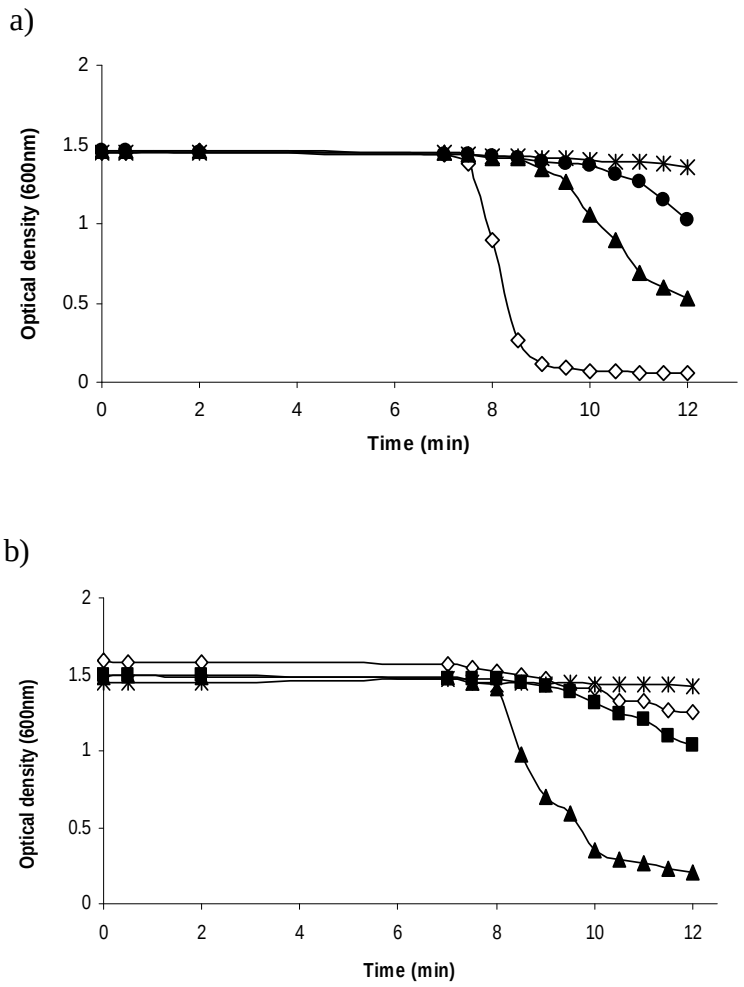


Figure 2: Effect of ionic strength and pH on FA at pH 9.

10 ppm of NSCs is compared to 10 ppm of alum sulphate and 10 ppm of ferric sulphate at pH 9. a) High ionic strength (100mM NaCl). b) Low ionic strength (10 mM NaCl). The mixing was stopped at min 7. Open diamonds: NSCs; Circles: aluminium sulphate; Triangles: ferric sulphate; Cross: control.

Appendix D

Flocculation activity of Alum and Ferric (20, 40, 60 and 80 ppm) at different kaolin concentrations (0.125, 0.250, 0.5, 1 and 2 g/L) (**Chapter 4**, **Section 4.3.6**).

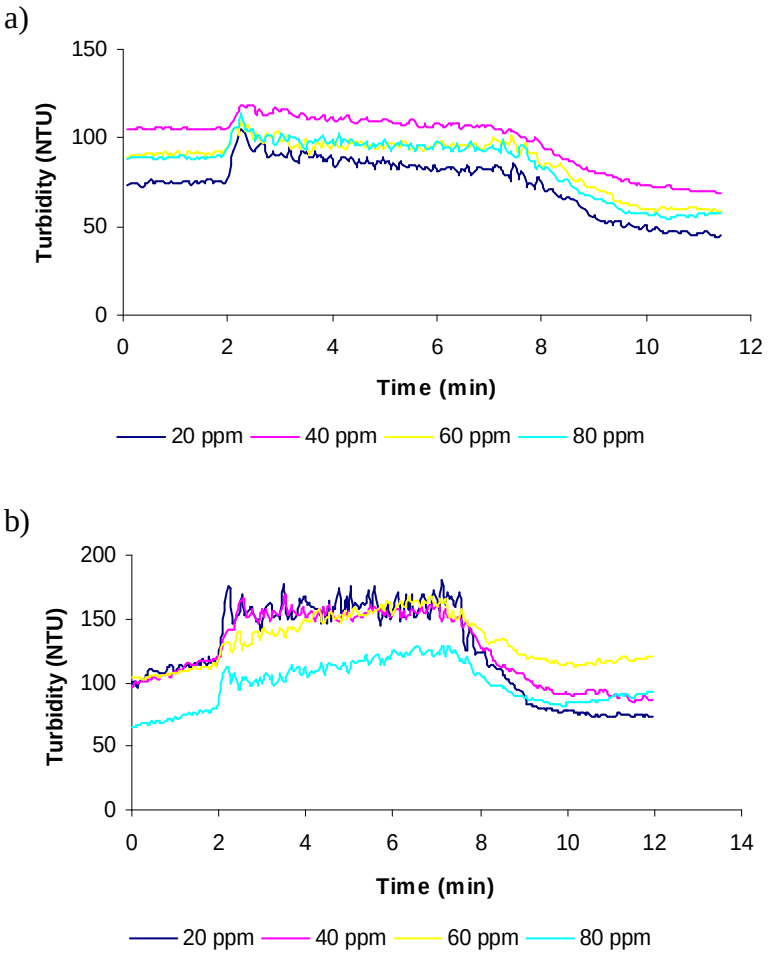


Figure 1: Flocculation activity of alum (a) and ferric (b) at 0.125 g/L of kaolin solution.

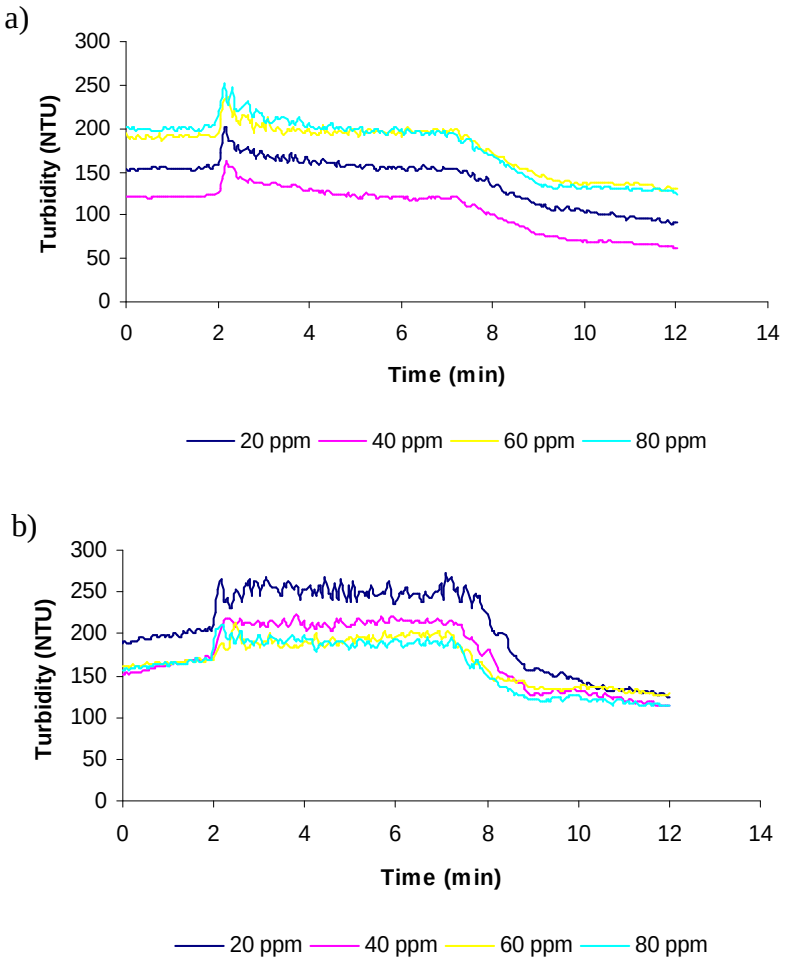


Figure 2: Flocculation activity of alum (a) and ferric (b) at 0.250 g/L of kaolin solution.

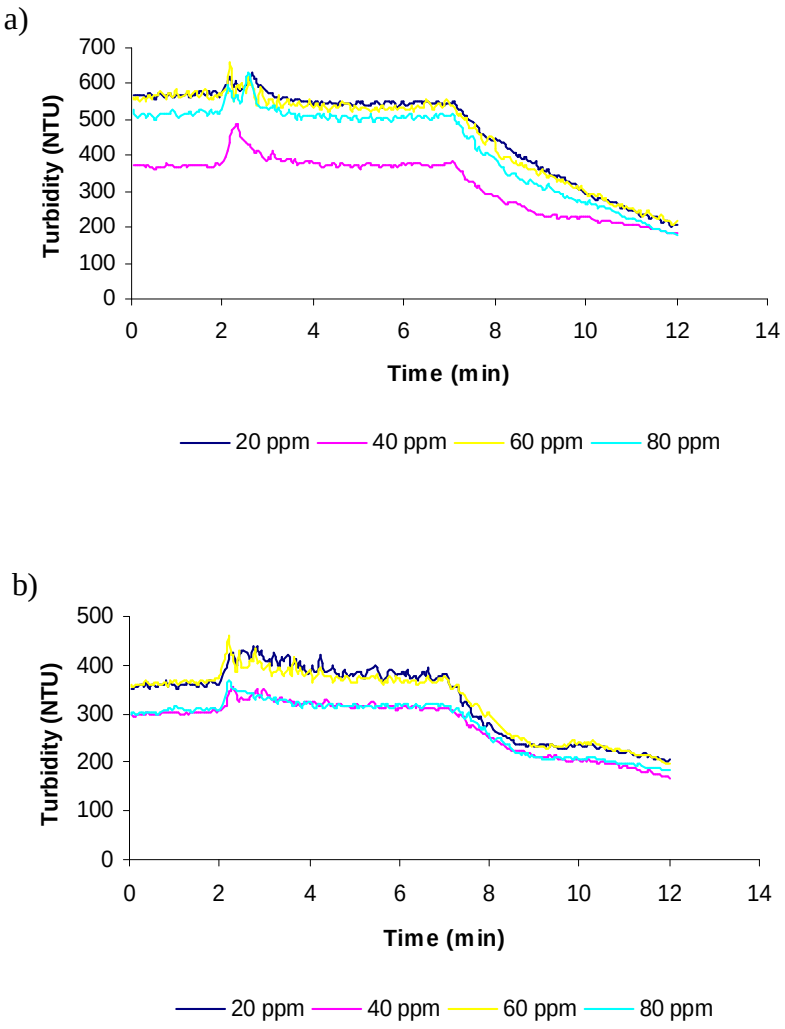


Figure 3: Flocculation activity of alum (a) and ferric (b) at 0.500 g/L of kaolin solution.

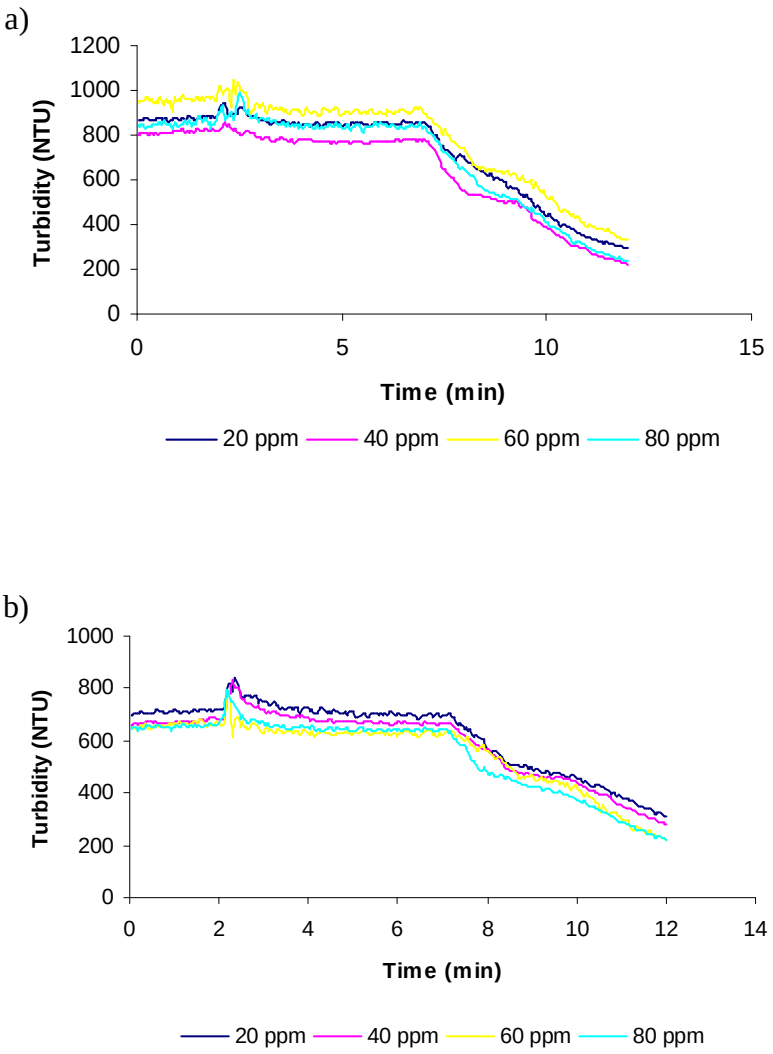


Figure 4: Flocculation activity of alum (a) and ferric (b) at 1 g/L of kaolin solution.

a)

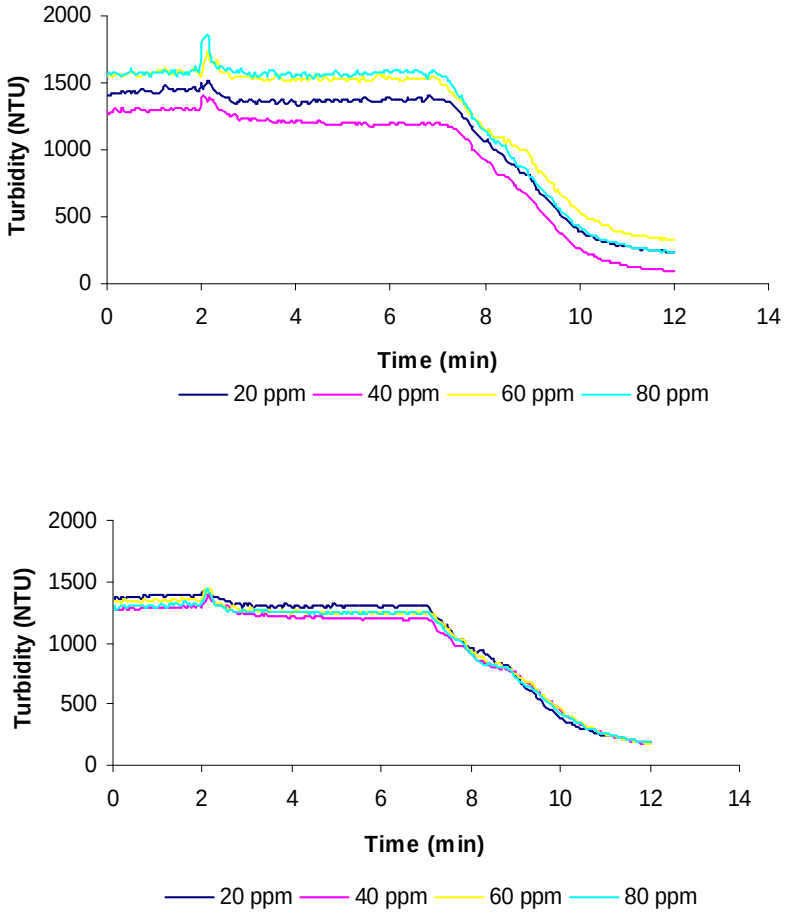
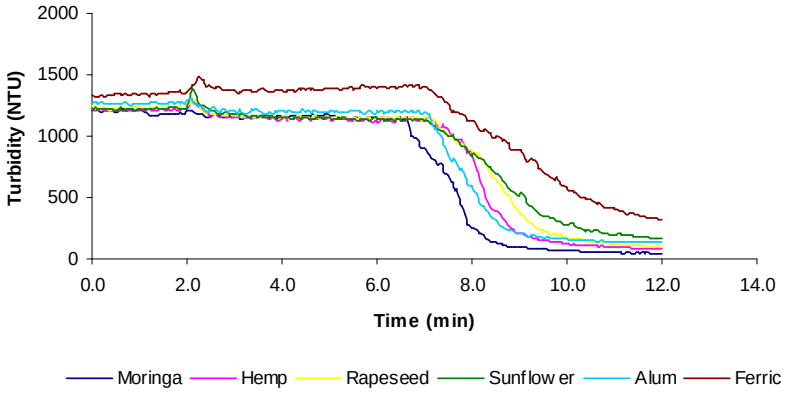


Figure 5: Flocculation activity of alum (a) and ferric (b) at 2 g/L of kaolin solution.

Appendix E

Flocculation activity of PC extracts (10 and 40 ppm) at pH 5 and pH 9 under high ionic strength (**Chapter 5**, **Section 5.3.2.1**).

a)



b)

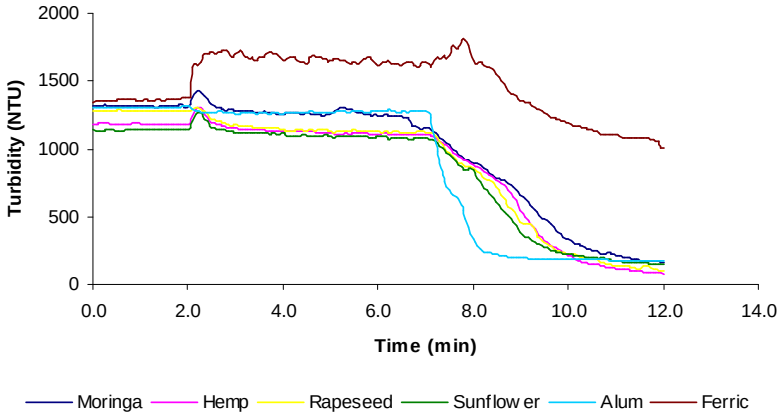
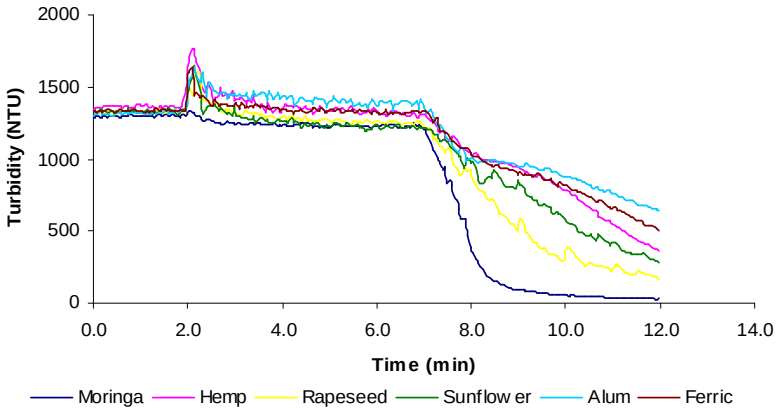


Figure 1: Flocculation activity of PC extracts at pH 5 at 10 ppm (a) and 40 ppm (b).

a)



b)

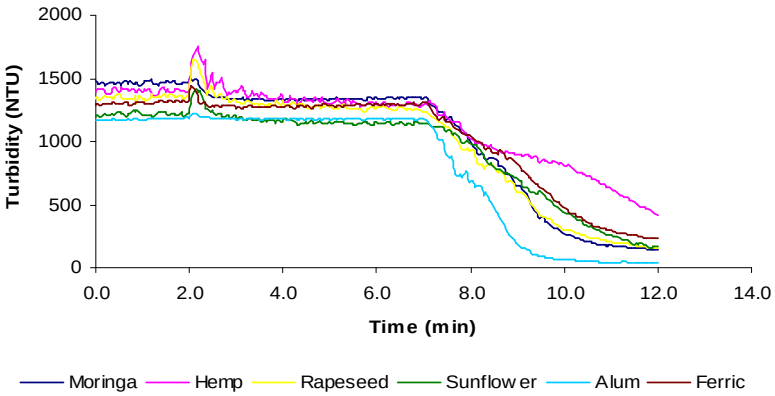
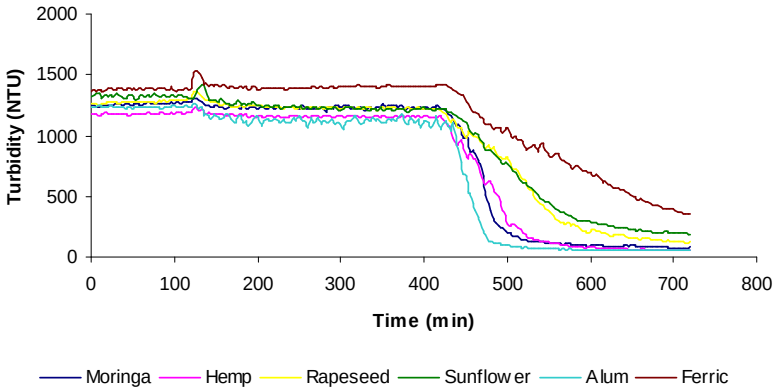


Figure 2: Flocculation activity of PC extracts at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix F

Flocculation activity of PC extracts (10 and 40 ppm) at pH 5 and pH 9 under low ionic strength (**Chapter 5**, **Section 5.3.2.2**).

a)



b)

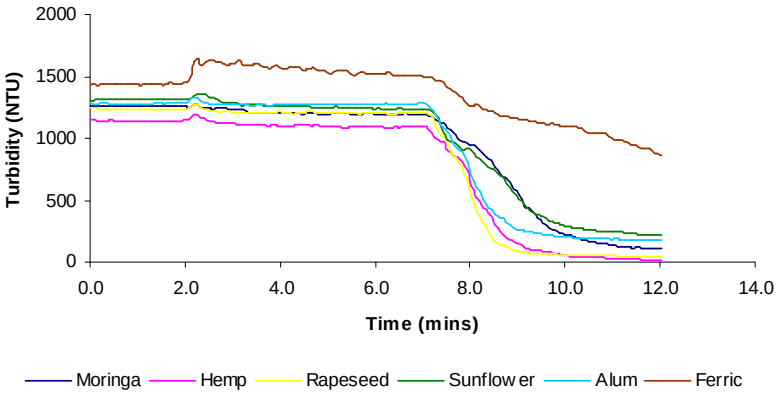
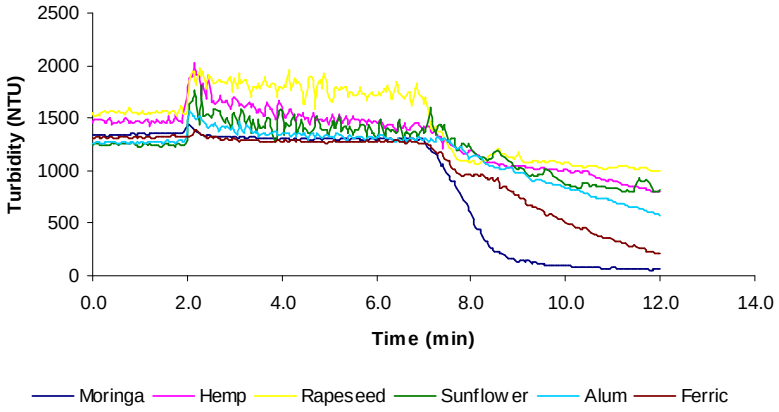


Figure 1: Flocculation activity of PC extracts at pH 5 at 10 ppm (a) and 40 ppm (b).

a)



b)

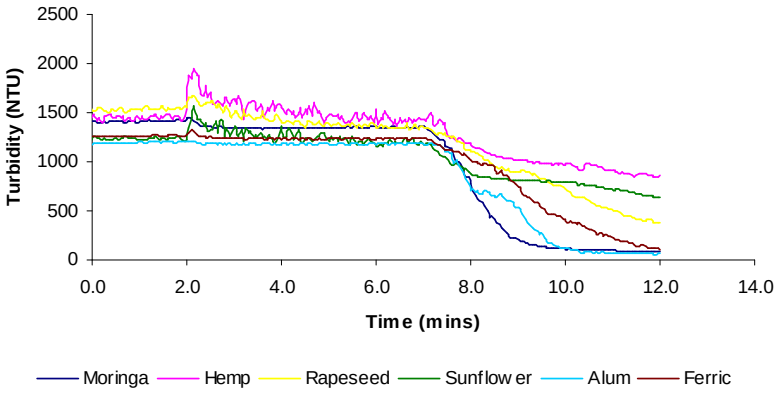
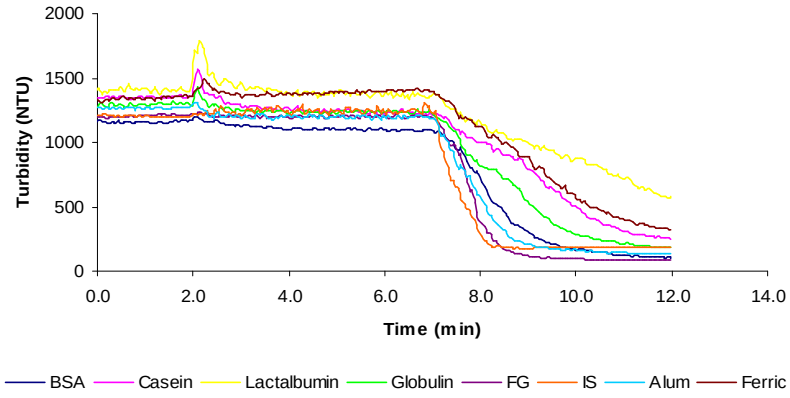


Figure 2: Flocculation activity of PC extracts at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix G

Flocculation activity of purified commercial proteins (10 and 40 ppm) at pH 5 and pH 9 under high ionic strength (**Chapter 5**, **Section 5.3.3.1**)

a)



b)

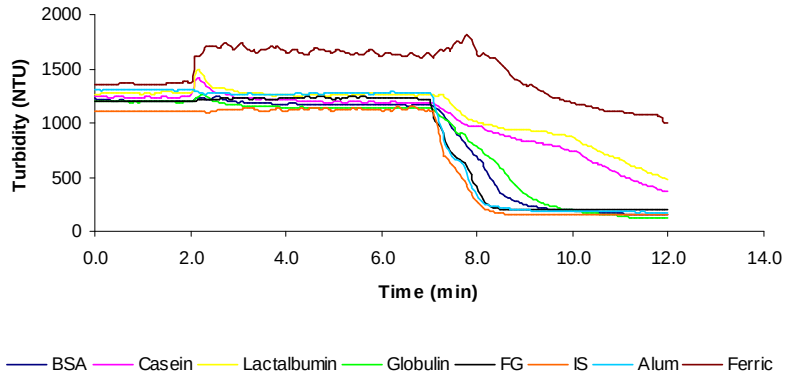
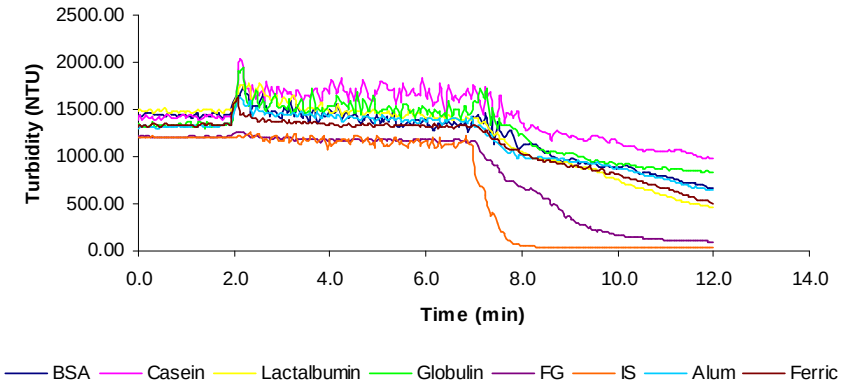


Figure 1: Flocculation activity of purified proteins at pH 5 at 10 ppm (a) and 40 ppm (b).

a)



b)

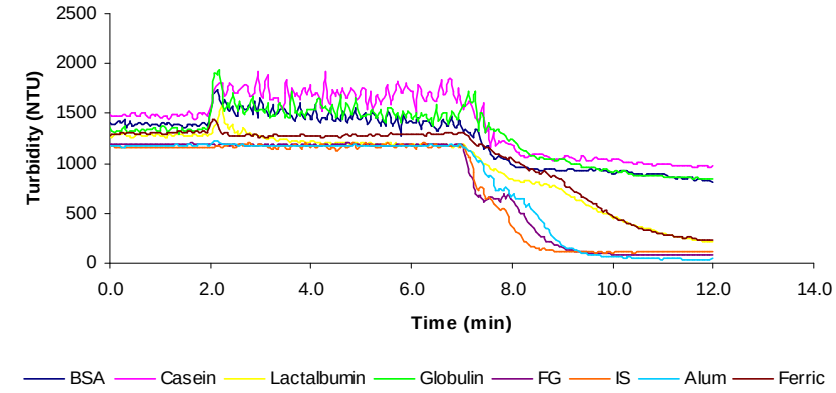
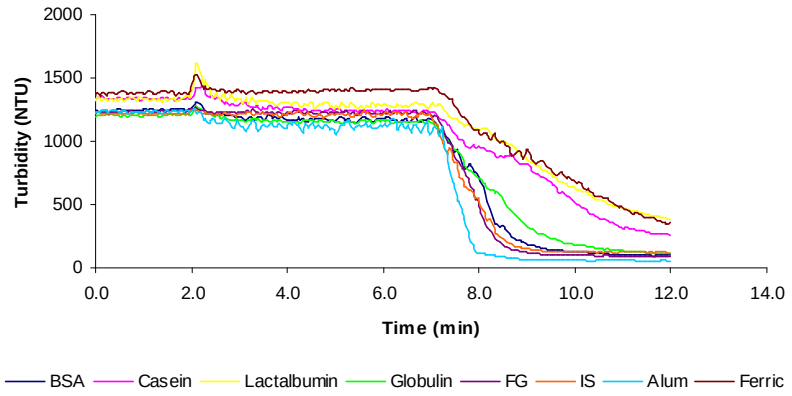


Figure 2: Flocculation activity of purified proteins at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix H

Flocculation activity of purified commercial proteins (10 and 40 ppm) at pH 5 and pH 9 under low ionic strength (**Chapter 5**, **Section 5.3.3.2**)

a)



b)

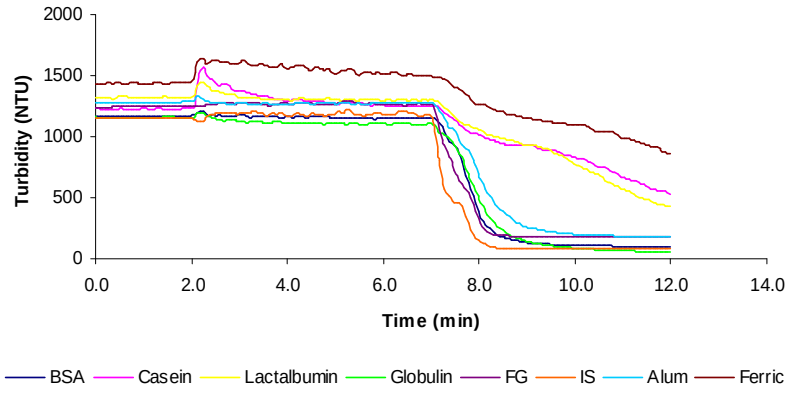
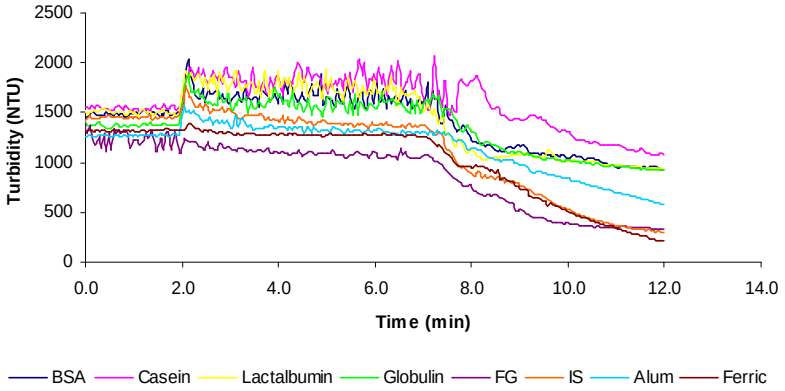


Figure 1: Flocculation activity of purified proteins at pH 5 at 10 ppm (a) and 40 ppm (b).

a)



b)

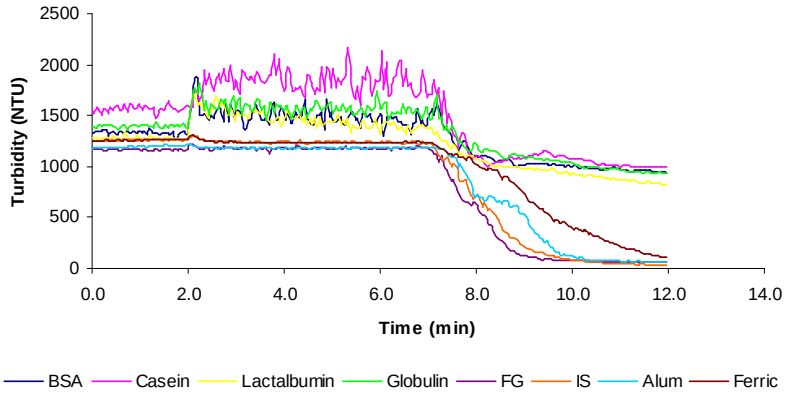
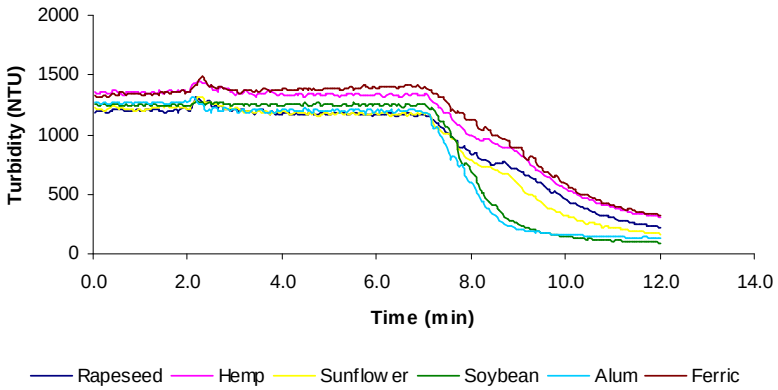


Figure 2: Flocculation activity of purified proteins at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix I

Flocculation activity of NSC extracted from hemp, sunflower, soybean and rapeseed under high ionic strength conditions at pH 5 and 9, for 10 and 40 ppm (**Chapter 6**, **Section 6.3.1**).

a)



b)

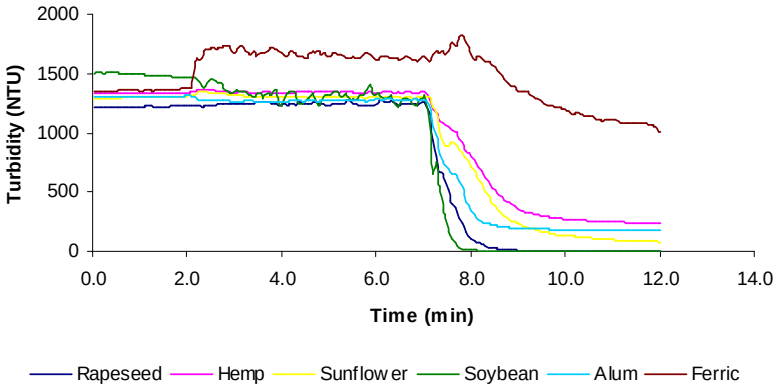
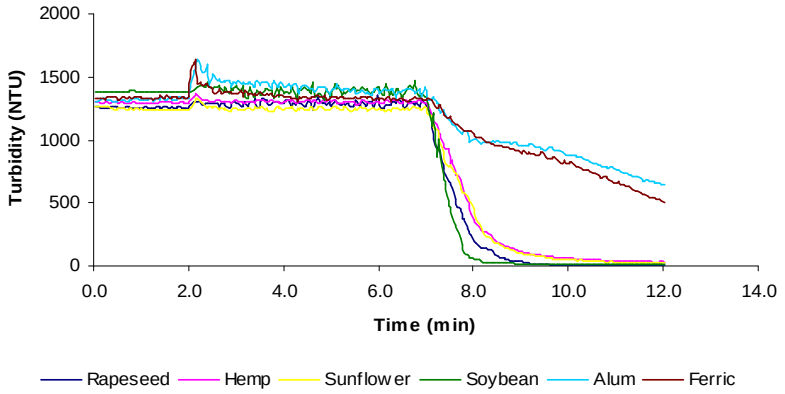


Figure 1: **Flocculation activity of NSC from different oleaginous seeds at pH 5 at 10 ppm (a) and 40 ppm (b).**

a)



b)

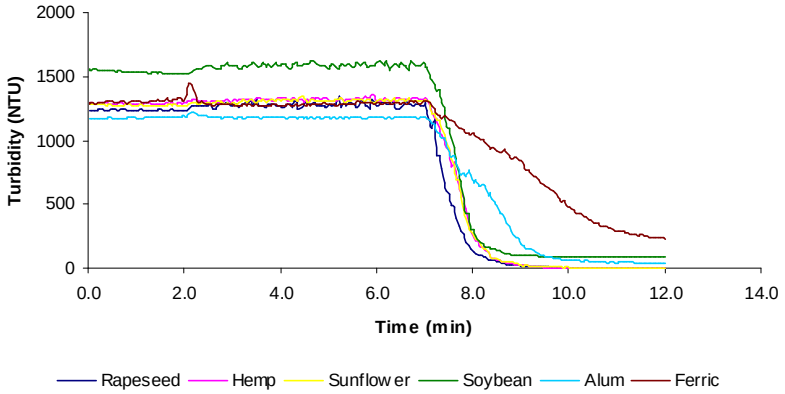
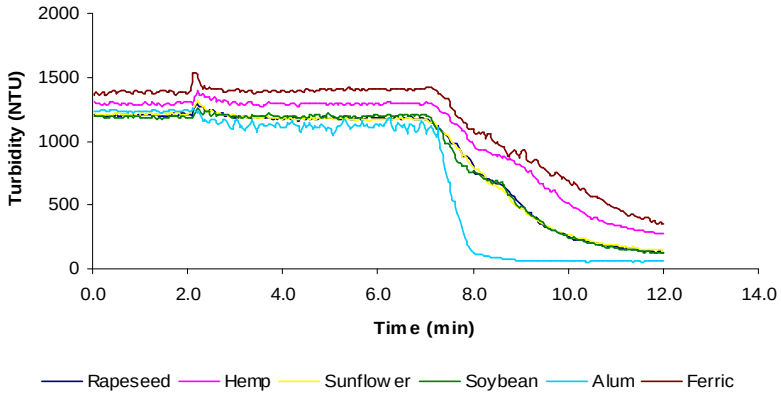


Figure 2: Flocculation activity of NSC from different oleaginous seeds at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix J

Flocculation activity of NSC extracted from hemp, sunflower, soybean and rapeseed under low ionic strength conditions at pH 5 and 9, for 10 and 40 ppm (**Chapter 6**, **Section 6.3.2**).

a)



b)

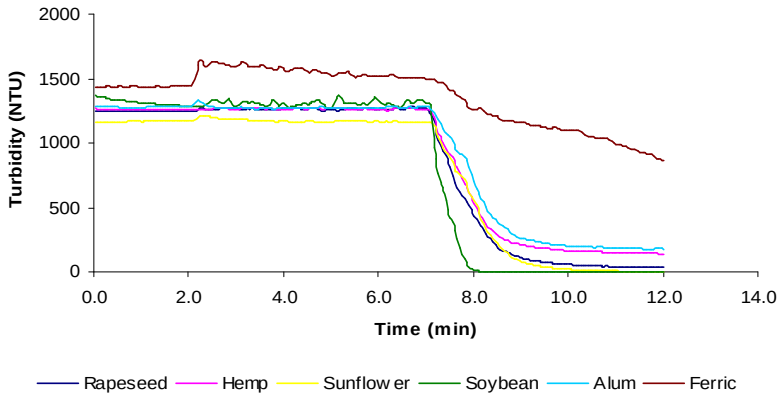
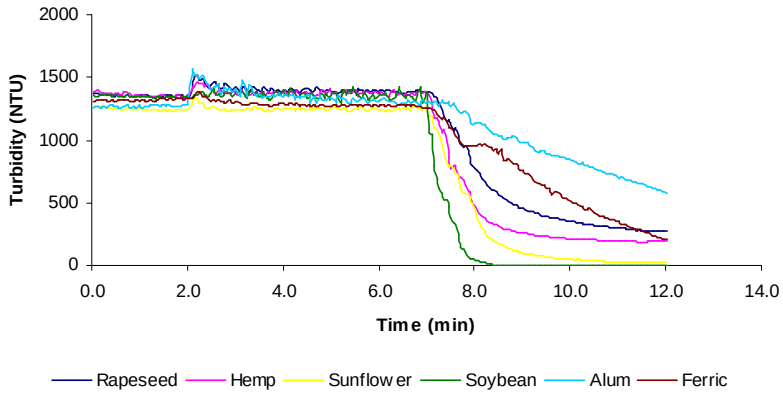


Figure 1: Flocculation activity of NSC from different oleaginous seeds at pH 5 at 10 ppm (a) and 40 ppm (b).

a)



b)

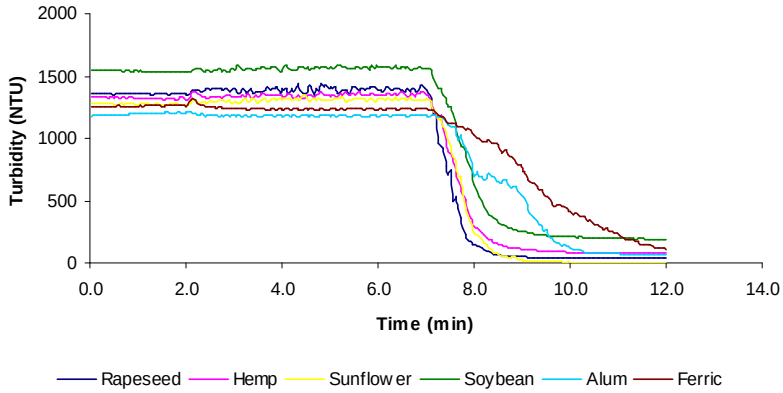


Figure 2: Flocculation activity of NSC from different oleaginous seeds at pH 9 at 10 ppm (a) and 40 ppm (b).

Appendix K

Comparison of flocculation activity of 10 ppm of protein soluble extracts and NSC extracted from rapeseed, hemp and sunflower (**Chapter 6**, **Section 6.3.3**).

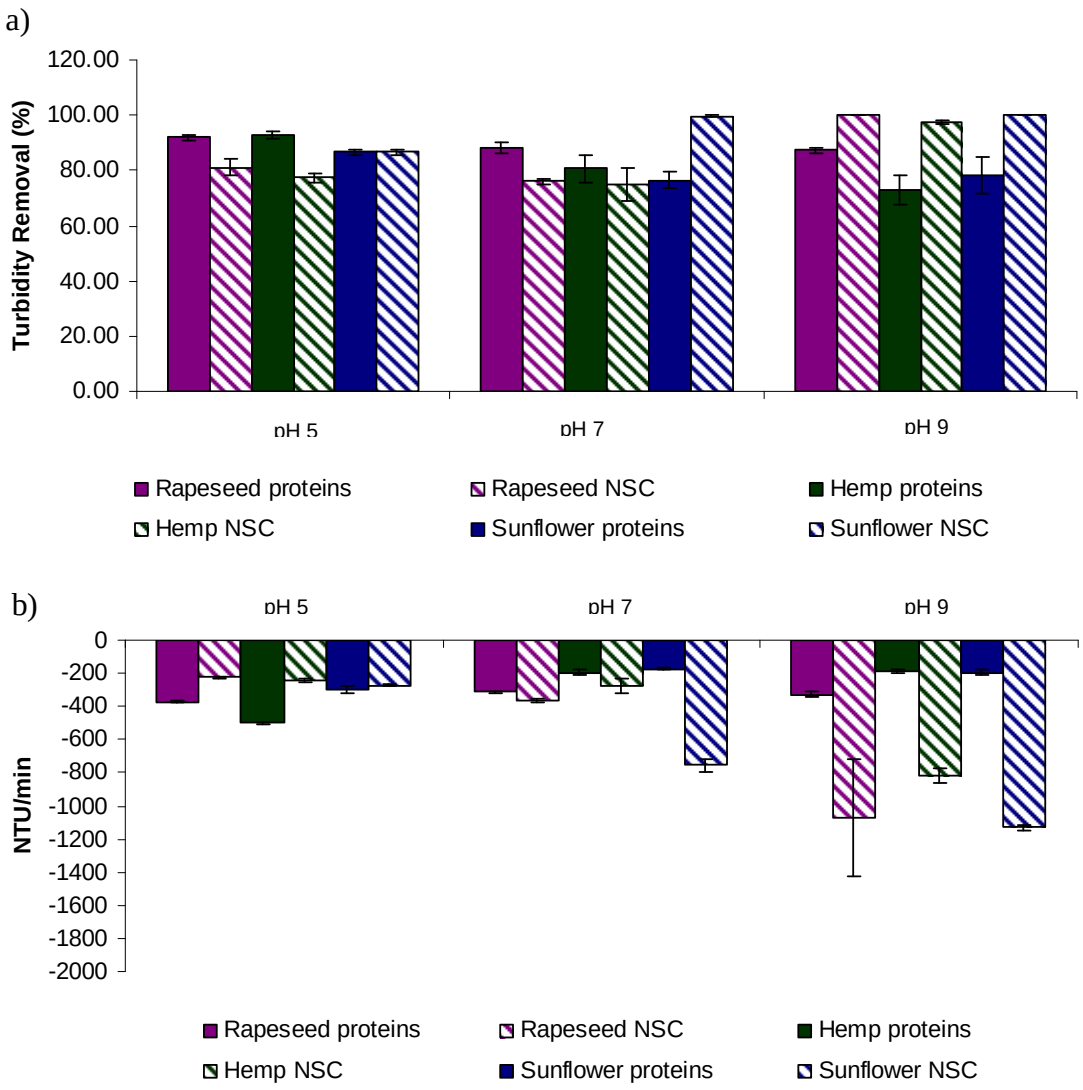


Figure 1: Flocculation activity of 10 ppm of protein soluble extracts and NSC under high ionic strength. a) Turbidity removal and b) sedimentation rates.

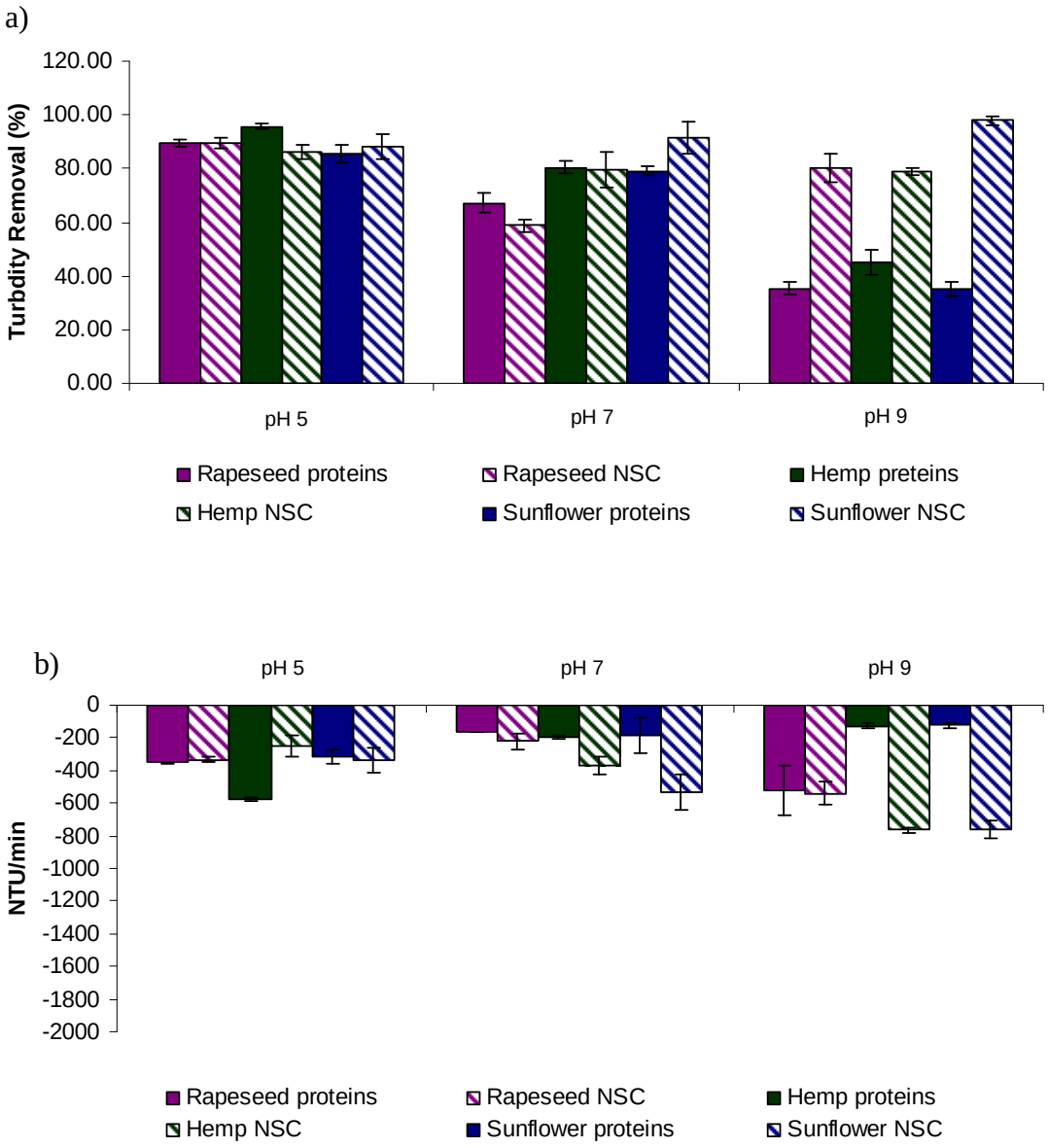


Figure 2: Flocculation activity of 10 ppm of protein soluble extracts and NSC under low ionic strength. a) Turbidity removal and b) sedimentation rates.

Appendix L

Determination of pharmaceuticals in water using RP-HPLC (**Chapter 7, Section 7.2.2**)

Compounds: Sulfamethoxazole, Metoprolol, Furosemide, Warfarin, Clofibric acid, Carbamazepine and Diclofenac.

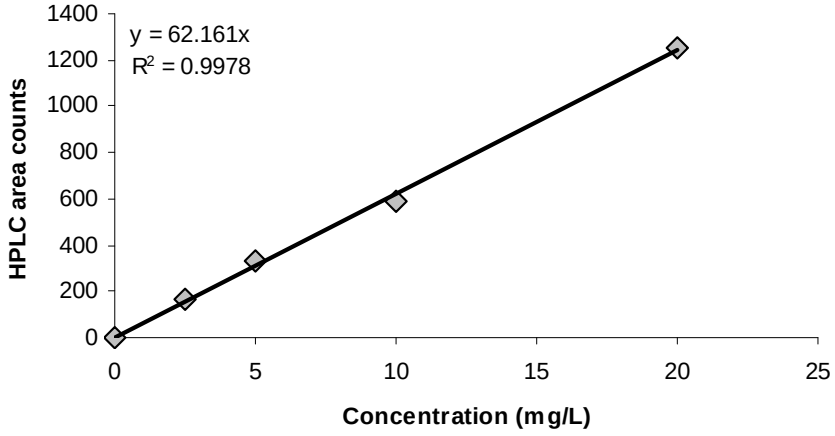


Figure 1: Standard curve for the quantification of sulfamethoxazole.

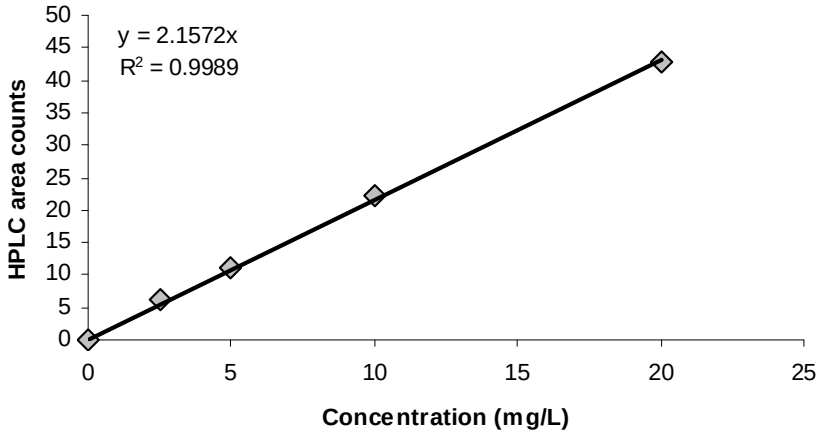


Figure 2: Standard curve for the quantification of metoprolol.

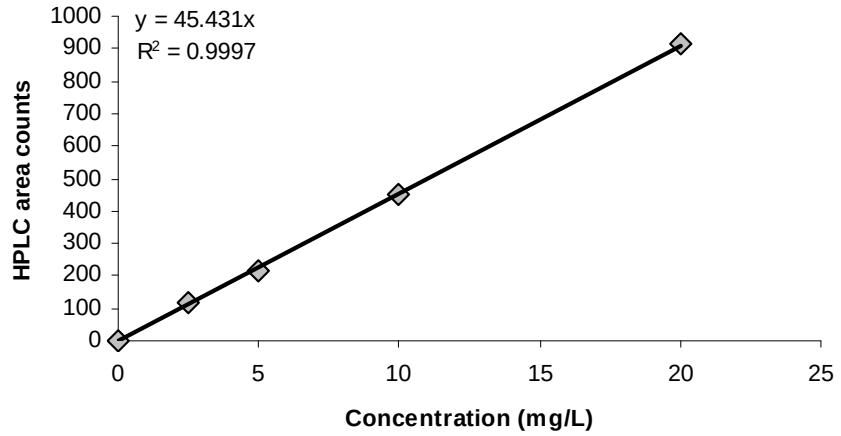


Figure 3: Standard curve for the quantification of furosemide.

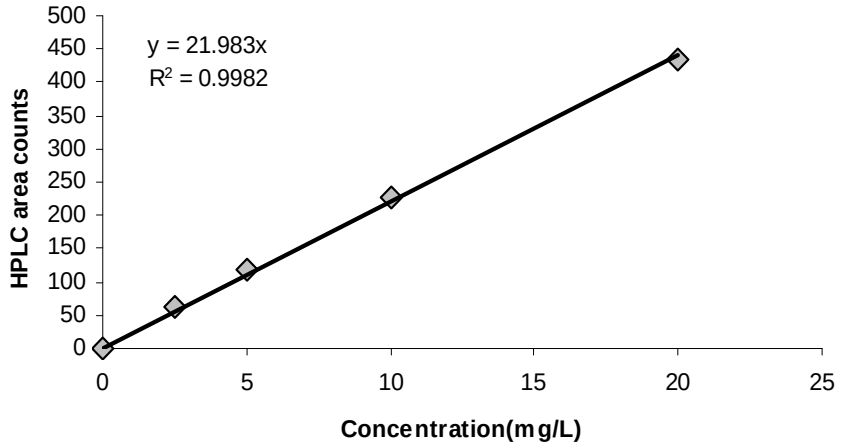


Figure 4: Standard curve for the quantification of carbamazepine.

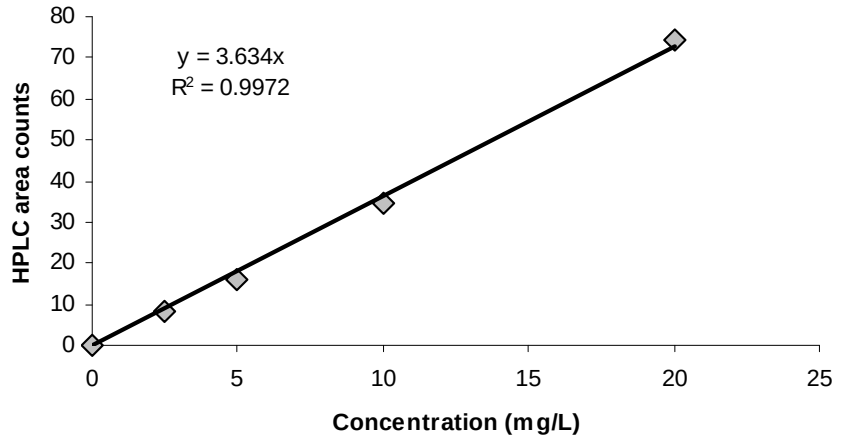


Figure 5: Standard curve for the quantification of clofibric acid.

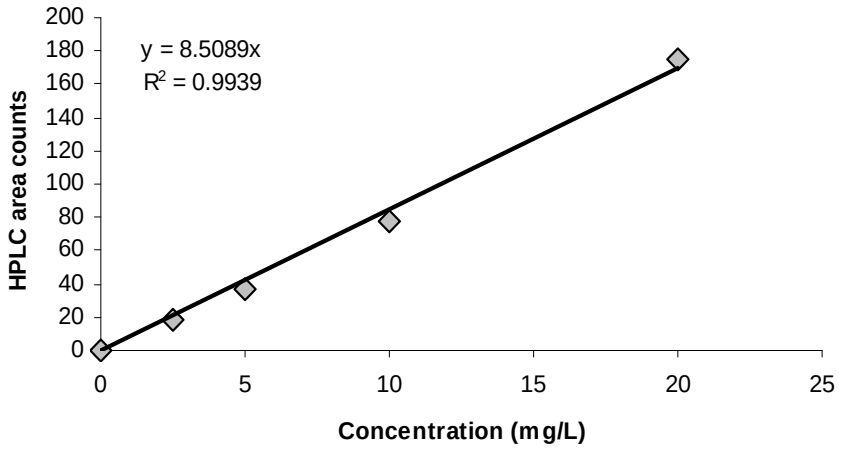


Figure 6: Standard curve for the quantification of warfarin.

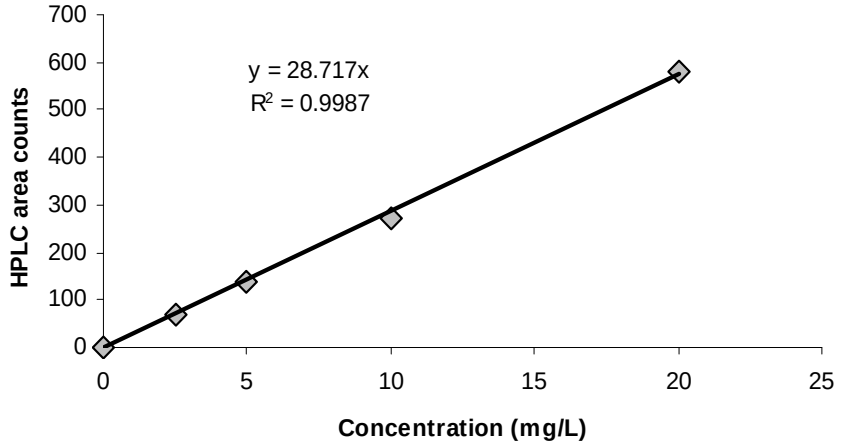


Figure 7: Standard curve for the quantification of diclofenac.

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