**Exploring the functionality of coconut proteins**

**Abstracts**

At present, coconut proteins are discarded as a waste product by the coconut oil industry. If the range of applications of coconut proteins is to be expanded, their potential functionalities should be investigated. Emulsions and gels are of the greatest interest in food industry.

Today the dry processing of copra at elevated temperatures is used to optimize the oil recovery. The functionalities of proteins in food are mainly determined by their structure and physicochemical properties, such as amino acid composition and sequence, protein size and conformation, physical and thermal stability, solubility and surface hydrophobicity. The harsh treatment leads to denaturation and loss of protein solubility and functionality.

Wet processing is an alternative method that may yield functional proteins in addition to oil. To explore the functionalities of coconut proteins, protein fractions were obtained using the wet processing method and analysed for molecular size and isoelectric points in electrophoresis, amino acid sequencing from mass spectrometry and also for their capacity to emulsify and stabilise oil-in-water emulsions.

The results showed minimum solubility at pH 3-4, increasing in both sides of the minimum, exhibiting a V-shaped profile. Mass spectrometry and protein fingerprint did not produce conclusive results, as coconut proteomes have not been sequenced and entered in the databases. Protein mapping only matched partially to glutelin OS, tr|Q9SNZ2|Q9SNZ2\_ELAGV and 7S globulin, tr|Q9AU64|Q9AU64\_ELAGV from oil palm, a cultivar close to *Cocos nucifera*.

SDS-PAGE showed results close to those already reported, especially for the skim coconut milk proteins. The insoluble protein resolved at 32 and 21 kDa, which correlates closely to the 11S globulin or cocosin, the major coconut globulin protein.

Native coconut proteins were not able to efficiently emulsify and stabilise oil in water emulsion. The droplets were large (13 µm) and high-pressure homogenisation only reduced the droplets size down to 3-5 µm, still not sufficiently small to prevent destabilisation. Flocculation and creaming were the predominant mechanisms of destabilisation. However, under heating to protein denaturation, at 95°C and homogenisation, concentrated coconut milk (40% fat) demonstrated improved physical stability due to formation of a colloidal glass structure.

Coconut protein also demonstrated the ability to form gels with appreciable elastic modulus (G’ > 1000 Pa), but only in neutral to alkaline environments.

The study shows that colloidal glass and gelation are functional properties of coconut proteins that may lead to novel products and potentially of the greater use.

Popular summary

The potential of coconut proteins as food ingredients was studied with the aim of evaluating how plant proteins, in particular those from coconut, could find industrial applications and compete with proteins of animal origin based on meat, fish, milk or eggs. Three research directions were chosen for investigation, to evaluate how coconut proteins would perform in each of the following situations:

Would coconut proteins be able to keep stable oil in water dispersions (emulsions), as in cows’ milk?

Do coconut proteins stiffen when heated, like when an egg is boiled?

Does full-fat coconut milk, heat-treated and homogenised, behave like mayonnaise, showing some initial resistance to flow under stress?

These questions address important issues, as they can provide a general picture regarding the potential of coconut proteins for further investigation. There is a growing segment of vegetarians demanding nutritious protein products, comparable to those of animal origin. Products comparable to cows’ milk, yoghurt, butter, cheese, but based on plant ingredients, are desirable. However, the results were only satisfactory for the final two issues. In particular we were able to manufacture an additive free stable coconut milk despite the challenging properties of the coconut protein. This achievement was possible by using heat treatments and by concentrating the emulsion to obtain a loose gel like system.

To understand the role of proteins in food systems, it is first necessary to determine the protein structure and intrinsic properties, such as amino acid composition, physical and thermal stability, and solubility. This then allows evaluation of the behaviour of protein in a particular food product. These properties are very important in food processing, as they determine characteristics such as texture, hardness, viscosity, and water and fat absorption.

Plant oilseeds, including coconut, store reserve proteins for embryo growth during germination, and these proteins also have great potential for human consumption. They provide well-balanced nutrients, are cheap, widely available, and renewable.

World oilseed production was 380 million tons in 2005 and protein meal 207 million, 69% derived from soya bean. Coconut protein has been overlooked for decades, unlike coconut oil, which is still the most important product in the sector. Despite the moderate protein content in the fresh coconut kernel (3-4%), the annual global output of 60 million tons of coconut provides a potential source of approximately 5000 tons of protein, which today is not utilized at all.

The technology used in oil extraction is detrimental to the quality of coconut protein, because of negative effects arising from high processing temperatures, traces of solvents, and insect contamination during copra desiccation. This makes the protein unfit for human consumption. Our experiments were carried out using mild wet processing conditions, on fresh materials to meet food quality standards.

**Keywords**

Coconut, coconut milk, extraction, proteins, emulsions, functional properties, denaturation, gels, yield stress, glass structure.

**List of papers**

This thesis is based on the following papers, which will be referred in the text by their Roman numerals and appended at the end of the thesis.

I. Edible proteins from coconut milk press cake; one step alkaline extraction and characterization by electrophoresis and mass spectrometry

*Chambal, B., Bergenståhl, B. and Dejmek, P.*

Food Research International, Vol.47, pages 146 – 151, 2012

II. Coconut press cake alkaline extract – Protein solubility and emulsification properties *Chambal, B., Bergenståhl, B. and Dejmek, P.*

Food and Nutrition Sciences, Vol. 4, 29 – 37, 2013

III. Heat induced gels from coconut press cake proteins

*Chambal, B., Bergenståhl, B. and Dejmek, P.*

Food and Nutrition Sciences, Vol. 5, 562 – 570, 2014

IV. Effect of thermal denaturation and homogenization of coconut proteins on the stability of coconut milk emulsion.

*Chambal, B., Gómez Galindo, F. and Bergenståhl, B.*

Manuscript

**The author’s contribution to the papers**

1. The author developed the wet processing method for coconut milk, coconut oil and coconut protein, performed all experimental work on physicochemical characterization of coconut protein and with contribution of the co-authors, wrote the paper.
2. The author designed, with suggestions from the co-authors, the assays for emulsification and solubility of the press cake proteins. The author took an active part in the discussion and wrote the paper with minor contribution of the co-authors.
3. The author carried out the experiments on gelation of press cake protein fraction and with the cooperation from the co-authors, evaluated the results and wrote the paper.
4. The author performed the experiments in cooperation with the co-authors on the emulsion glass structure, evaluated together with the co-authors the results. The author wrote the major part of the paper.

**Abbreviations and symbols**

1DE One dimension electrophoresis

2DE Two dimensions electrophoresis

APE Alkaline press-cake extract

FAO Food and Agriculture Organization of United Nations

G’, [Pa] Elastic module, [Pascal]

H-B model Herschel-Bulkley model

k, [Pa.sn] Consistency coefficient [Pascal.secondn]

kDa kilo-Dalton

MW Molecular weight

n [dimensionless] flow behavior index [dimensionless]

SDS-PAGE Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

USDA United States Department of Agriculture

VSERUM Serum phase volume

VTOTAL Total volume

WHO World Health Organization

δ, [º] Phase angle [degree]

τ, [Pa] Shear stress [Pascal]

τo, [Pa] Yield stress [Pascal]

γ, [1/s] Shear rate [1/second]

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1. **Introduction and objectives**

Plant oilseeds store proteins as a reserve material for embryo growth during germination, among carbohydrates, fat, minerals and vitamins. They represent an important protein source for human consumption, providing valuable nutrients with the advantage of being cheap, widespread and renewable. Global oilseed production was 380 million tons in 2005, and protein meal, 207 million tons in the same year (Moure, Sineiro, Domınguez, & Parajo, 2006). Soya beans and rapeseed are the most abundant protein meals, representing around 69 and 12% of the global protein meal production, respectively (Ash & Dohlman, 2005).

For decades, coconut protein has been subordinate to coconut oil, which is still the most important product in the sector. However, current trends show increasing demand for non-traditional coconut products like coconut milk, coconut water and food-grade coconut protein. An average annual output of 60 million tons of coconut (FAOSTAT, 2014) would provide approximately 5000 tons of protein, most of this currently lost during oil extraction. In addition, the technology used for oil extraction affects the quality of the final protein, which is currently unfit for human consumption due to high processing temperatures, traces of solvents, or by contaminations occurring during open-sky copra desiccation (Mepba & Achinewhu, 2003; Naik, Raghavendra, & Raghavarao, 2012; Naik, Venu, Prakash, & Raghavarao, 2014).

In order to find industrial applications and expand the range of coconut protein-based products, one approach is to develop oil extraction technology to meet the necessary quality standards for proteins. Their potential functionalities also need to be explored. However, as yet, studies on the emulsification of oil-in-water dispersions by coconut proteins have not reached satisfactory results. Information on gelation capabilities is also lacking.

Coconut milk, the second most important product of the sector, with appealing characteristics, faces the problem of physical instability because of large emulsion droplets (Onsaard, Vittayanont, Srigam, & McClements, 2005; Onsaard, Vittayanont, Srigam, & McClements, 2006; Tangsuphoom & Coupland, 2005; Tangsuphoom & Coupland, 2008a; Tangsuphoom & Coupland, 2008b). Heating and mechanical treatments such as homogenisation have not shown significant improvements, as the emulsion tends to flocculate in large aggregates, ending up with liquid phase separation by creaming. Attempts to reduce the instability of coconut milk in the industry have included the use of commercial emulsifiers and thickeners, some of them chemically synthesised or with restricted use in some countries. Information available on the functionality of coconut proteins refers only to the native state, and very little has been reported regarding the potential of denatured proteins as functional food ingredients. Therefore, in general, there is a lack of comprehensive information on the functionality of the coconut proteins.

This thesis tries to explore the potential of partial denaturation of coconut proteins in stabilising coconut milk emulsions and describes the wet processing method as an alternative to dry processing technology, with the aim of processing materials on a fresh basis and under controlled hygiene standards. The protein fractions needed to be obtained in a systematic manner, followed by evaluation of the related physicochemical and functional proprieties, such as the solubility profile, molecular sizes, amino acid composition and sequence, emulsification and gelation capabilities.

* 1. **Objectives**

The general objective of this work was to explore the functionality of native and denatured coconut proteins, with the aim of expanding the range of coconut-based products, mainly coconut milk, and thereby replacing the use of commercial additives.

To address tasks relevant to the general objective, specific objectives were formulated and detailed in the correspondent papers, as follows:

* To develop a protein extraction method based on wet processing, with minimum manipulation, and to characterise the protein with regard to amino acid profile, size and pattern (PAPER I).
* To produce, at bench-scale, the protein powder and assess its solubility behaviour, emulsifying capacity of oil in water (o/w) emulsions, and to evaluate the effect of pH (PAPER II).
* To evaluate the capacity of the protein powder to form heat-induced gels over a wide range of pH values (4-10) at low ionic conditions (PAPER III).
* To evaluate the combined effect of denatured proteins and high-pressure homogenisation on the stability of the coconut milk (PAPER IV).
  1. **Relevance of the objectives**

These specific objectives were undertaken to direct the work towards the final objective. The first step was to establish the wet processing method for protein, oil and coconut skim milk, and evaluate the preliminary physicochemical properties of the protein fractions.

The extraction method was designed to overcome the problems arising from the dry-processing technology of copra, and to yield more products than the simple coconut oil. For example, the extraction temperature (50°C) was selected to ensure that the protein would not denature and the pH was set in the regions favouring higher extraction yields. Paper I addresses this, discussing the wet processing method, the alkaline extraction, the amino acid profile and sequencing, and the molecular sizes of different coconut protein fractions obtained.

The solubility profile and emulsifying behaviour were studied on the native press-cake protein fraction, at pH ranging from 2-10, under low ionic strengths, to evaluate how the solubility was affected by the pH and how, in turn, this controlled the extension of the emulsification properties. Consequently, the study (Paper II) was important, since no sufficient data from previous studies were available regarding emulsification based on the alkaline protein fraction. The same importance was given to gelation in Paper III, describing the related proprieties on the press-cake protein fraction.

Of particular importance was the effect of partial denaturation of coconut milk protein fraction, induced by heat, in enhancing the stability of coconut milk emulsion (Paper IV), as this is close to the general objective. The study was to discuss whether or not the denaturation process influenced the stability of coconut emulsion and also to increase knowledge about the principles behind the property.

1. **Coconut as a source of protein for human consumption**

Seed storage proteins are cheaper than those deriving from animals. They may serve as an alternative source for human consumption, although some of the essential amino acids are limited, mostly the sulphur-containing amino acids (Alu’datt et al., 2013; Tandang-Silvas, Tecson-Mendoza, Mikami, Shigeru Utsumi, & Maruyama, 2011). Soya beans, rapeseed, cottonseed, sunflower seeds and peanuts are the most abundant protein meals, representing approximately 69, 12.4, 6.9, 5.3 and 2.8% of the world protein meal production, respectively (Ash & Dohlman, 2005).

Coconut is a basic commodity largely grown in many tropical countries, yielding an average annual output of about 60 million tons (FAOSTAT, 2014), of which more than 75% is devoted to oil extraction (Seow & Gwee, 1997). The remainder is used in many ways, including use as a condiment in cuisines in the form of coconut milk, or eaten fresh. Most Asian economies are greatly influenced by the coconut sector (FAOSTAT, 2014; Onsaard et al., 2005). This output corresponds to approximately 0.5 million tons of protein that would be available annually for various food applications, if the processing methods could be made to comply with the necessary quality and hygiene requirements.

The proximate composition of fresh coconut endosperm is shown in Table 1. The dry copra meal after oil extraction may reach around 30% protein, depending upon a number of factors, such as cultivar, age, growing and processing conditions.

Mozambique, located in the tropical belt, is one of the coconut producers with large potential to expand the crop from the highest yields of 500,000 tons achieved in the 1970s (Cepagri, 2014; FAOSTAT, 2014), if an appropriate farming system is developed, sustainable government policies promoted, and more gains achieved from the entire production chain. Currently, the coconut sector in Mozambique is mainly supported by family farmers lacking in the adequate techniques and skills required to ensure sustainable growth of the sector. Actual yields have fallen to 260,000 tons (FAOSTAT, 2014) in the past 20 years, partly due to problems arising from infestation caused by an epidemic coconut yellowing disease in the growing areas.

Table 1. Composition of fresh coconut endosperm and full-fat coconut milk 1 (USDA, 2014), 2 (Food agency of Sweden, 2015)

|  |  |  |  |
| --- | --- | --- | --- |
| Nutrient | Unit | 1 Value per 100 g | 2 Value per 100 g |
| Water  Energy  Protein  Total lipid (fat)  Ash  Carbohydrate, by difference  Fibres, total dietary  **Minerals**  Calcium, Ca  Iron, Fe  Magnesium, Mg  Phosphorus, P  Potassium, K  Sodium, Na  **Vitamins**  Vitamin C, total ascorbic acid  Niacin  Pantothenic acid | g  kcal  g  g  g  g  g  mg  mg  mg  mg  mg  mg  mg  mg  mg | 54  330  3.6  35  1.2  6.7  2.2  11  2.3  28  122  325  4  2.8  0.9  0.3 | -  352  3.4  33.5  -  6.1  9  14  1.8  43  113  355  0.05  3  0.5  - |
| Nutrient values and weights are per edible portion | | |  |

Proteins are important ingredients in the food industry, not solely because of their nutritional value, but also due to functional properties such as emulsification, gelation, water retention and foaming (Cabra, Roberto Arreguín, & Farres, 2008; Kosseva, 2001; Lad & Murthy, 2012; Tandang-Silvas et al., 2011). The nutritional value of food proteins is determined by factors such as their content, amino acid profile and score, bioavailability, and processing conditions.

Table 2 shows protein, energy and amino acid requirements for human nutrition as the daily intake recommended to provide adequate and sustainable nitrogen for healthy body function (Becker et al., 2004; WHO, 2007). Functional properties result from physicochemical interactions among proteins, affecting their behaviour in a food system during processing, storage and consumption. The properties can be categorised into three broad groups: the hydration properties – those correlated with the solubility behaviour or water retention; surface properties – associated with emulsification and foaming capabilities; and protein-protein interactions – related to ability in forming gels (Smith, 2003).

Functional properties are important in food processing, as they determine the food texture, hardness, viscosity, water and fat absorption. They are not easily predictable, as food is a complex system influenced by multiple factors such as composition, temperature, pH, and ionic strength.

However, almost all the coconut protein is lost with the spent copra meal (the desiccated coconut endosperm) during oil extraction, when the dry processing technology is used. In the dry processing technology, the copra may be treated with organic solvents or pressed at high processing temperatures, ultimately causing protein quality loss (Naik et al., 2012; Onsaard et al., 2005; Onsaard et al., 2006), due to denaturation or contamination by traces of solvents left in the end products. Furthermore, the desiccation of copra is often carried out in open-sky environments, posing serious concerns related to sanitary issues.

An alternative approach to overcome these problems is the wet processing method. The fresh endosperm is first processed into a coconut milk as an intermediate product, and then into oil and food-grade protein (Mepba & Achinewhu, 2003; Naik et al., 2012; Naik et al., 2014; Onsaard et al., 2006; Seow & Gwee, 1997). One advantage of this procedure is that all materials undergo processing while fresh and in a controlled environment, protecting against contaminants or oxidation with no need for organic solvents or high processing temperatures. This ensures the quality of the end or by-products, such as proteins.

However, further developments are needed before the wet processing method can become cost-effective with sound industrial application. For example, the yields for oil, the main product of interest in the coconut industry, remain lower compared to those produced through dry processing. Attempts have been made to improve yields, including the use of commercial enzymes to help in degrading the endosperm (Freitas & Coelho, 2003; Man, Suhardiyono, & Asbi, 1996; Raghavendra & Raghavarao, 2010; Raghavendra & Raghavarao, 2011; Tano-Debrah & Ohta, 1997). However, there are many other technical aspects to overcome, such as emulsion breakage, before oil can be recovered.

Table 2. Summary of adult protein, energy and indispensable amino acids requirements (WHO, 1985; WHO, 2007)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Moderate activities | | 2007  Present estimates | | 1985 FAO/WHO/UNU | |
| Males | Females | mg/kg a  per day | mg/g b protein | mg/kg a  per day | mg/g b  protein |
| Energy (kJ/kg per day )  Protein (g/kg per day)  Histidine  Isoleucine  Leucine  Lysine  Methionine + cysteine  Methionine  Cysteine  Phenylalanine + tyrosine  Threonine  Tryptophan  Valine  Total indispensable amino acids | 175  0.66 | 148  0.66 | 10  20  39  30  15  10  4  25  15  4  26  184 | 15  30  59  45  22  16  6  38  23  6  39  277 | 8-12  10  14  12  13  -  -  14  7  3.5  10  93.5 | 15  15  21  18  20  -  -  21  11  5  15  141 |
| a mg/kg body weight; b mean nitrogen requirement of 107 mg nitrogen per day (0.66 g protein/kg per day) | | | | | | |

1. **Physicochemical characterisation of coconut proteins**

Physicochemical characterisation of proteins is an important step in understanding their functionalities. Proteins can undergo many changes during manufacturing, some of them desirable when they contribute to the textural and sensory attribute of the final products. Electrophoresis and mass spectrometry are useful methods for characterising proteins or analysing modifications that may occur in terms of their physical characteristics, such as the molecular size, isoelectric point, peptides subunit composition and amino acids sequence.

The amino acid composition and sequence determine the protein backbone, called the primary structure (Buxbaum, 2007; Hettiarachchy, Sato, Marshall, & Kannan, 2012; Moure et al., 2006). The protein backbone may assume certain local conformations due to internal interactions among amino acid groups, by sulphur or hydrogen bridges. This is the secondary structure that results in α-helix or β-sheets. Long-term distance interactions of elements from the secondary structure give rise to the tertiary structure, characterised by global conformations of the protein backbone. The protein may also evolve to the quaternary structure, which describes how different polypeptides can interact together to form a single functional protein.

Coconut proteins, like the proteins from many plant oilseeds, are classified as storage proteins, as their function is to provide carbon, nitrogen and sulphur resources to the embryo during germination (Balasundaresan, Sugadev, & Ponnuswamy, 2002; Tandang-Silvas et al., 2011). They represent a cheap and valuable alternative source of protein for human consumption (Cabra et al., 2008; González‐Pérez & Arellano, 2009; Joye & McClements, 2014; Sharma, SU, Joshi, Roux, & Sathe, 2010; Wanasundara, 2011).

There are two major types of storage proteins in plant oilseeds, the vicilins and legumins, which are classified according to their sedimentation coefficients as 7S/11S; oligomeric organisation, as trimeric/hexameric, or by their polypeptide chain structure, as single chain/disulphide linked pair of chains (Balasundaresan et al., 2002; González‐Pérez & Arellano, 2009; Gonzalez-Perez & Vereijken, 2007; Tandang-Silvas et al., 2011). The major coconut endosperm protein is the 11S globulin or cocosin, compared to the legume class reserve protein (Balasundaresan et al., 2002) accounting for 86% and the 7S, 14% (Garcia, Arocena, Laurena, & Tecson-Mendoza, 2005), as shown in Table 3. Cocosin is a hexamer for which the molecular weight is, as yet, not precisely defined. Sjögren (Sjögren & Spychalki, 1930) reported a molecular weight of 208 kDa, but Garcia suggested a higher value of 326 kDa for cocosin and 156 kDa for the trimeric 7S globulin (Angelia et al., 2010; Garcia et al., 2005)*.*

Table 3. Coconut protein – as a legume seed storage protein

|  |  |  |
| --- | --- | --- |
| Coconut storage globulins | Cocosin | 7S globulin |
| Sedimentation coefficient  Oligomeric organisation  Polypeptide chain structure  Occurrence  Molecular weight | 11S a  Hexameric a  Disulphide-linked chains a  86% total protein b  208 c, 326 b | 7S a  Trimeric a  Single chain  14% b  156 b |

a (Balasundaresan et al., 2002; Tandang-Silvas et al., 2011); b (Garcia et al., 2005); c (Sjögren & Spychalki, 1930)

Coconut proteins can provide well-balanced amino acids, although with limited score for some of the amino acids, such as methionine and cysteine (Hagenmaier, Mattil, & Carter, 1974; Kwon, Park, & Rhee, 1996; Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996).

1. **Functional properties of proteins – emulsions, gels and colloidal glass emulsions**

Functional properties are generally defined as those physical and chemical properties that influence protein behaviour in a food system during processing, storage, and consumption, bringing to food new characteristics in terms of rheology, texture and sensory perceptions (Damodaran, 1997; Kinsella, 1976; Moure et al., 2006).

* 1. **Emulsions**

Emulsions form the basis of wide range of food products, and those stabilised by proteins are of particular interest. Two basic factors govern the emulsification process of oil in water dispersions: the reduction in the interfacial tensions due to the adsorption of proteins at the oil-water interface, and the electrostatic interactions, a structural and mechanical barrier opposing the destabilisation process (Cabra et al., 2008; Dalgleish, 2003).

Knowledge about the physicochemical properties of protein, such as the size, structure, flexibility and surface hydrophobicity, is very important, as it can help in understanding the behaviour of proteins as emulsifiers. However, since food is a complex system, the functionality of a given protein in a food product is difficult to predict, so experimental determinations are necessary.

Proteins are macromolecules that may bear charged groups in the same structure, as well as the non-polar amino acids, which provide specific characteristics as emulsifiers (Buxbaum, 2007). The amphiphilic nature of protein is an essential property for food emulsification, and originates from the well-balanced distribution of the hydrophilic and hydrophobic groups in the protein molecule that, in a way, contributes to emulsion stabilisation (Hettiarachchy et al., 2012)

One of the most important functional properties in food technology is that related to the capacity of the proteins to form and stabilise emulsions. Emulsions are thermodynamically unstable systems due to unfavourable contact between, at least, two immiscible phases, one dispersed in the other (Cabra et al., 2008; Lad & Murthy, 2012). Typically the dispersed phase is in the form of oil droplets and the continuous phase is water, a dispersion referred to as oil-in-water (o/w) emulsion.

To prevent the emulsion system destabilising and reverting to the original state, by collapsing the droplets (coalescence) and causing separation of the oil phase and water, a third component, partially soluble in both phases (emulsifier), is necessary. Proteins are one of many emulsifiers, the most widely used in food systems (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014; Joye & McClements, 2014). To form stable emulsions, a mechanical work should be applied to the system to disintegrate the oil phase into very small droplets, with a greater interfacial area on which the proteins adsorb and conform, creating the necessary barrier that prevents the droplet from aggregating/coalescing and thus stabilising the emulsion (Benjamin et al., 2014; Joye & McClements, 2014; Raikos, 2010; Zayas & Lin, 1989).

The emulsifying capacity of proteins can be determined by a number of different methods, such as by measuring their capacity to efficiently cover the interfacial oil droplet surface and create the network structure. Typically, the surface coverage can reach values as high as 2-3 mg protein/m2, as in for example cow’s milk casein (Beverung, Radke, & Blanch, 1999), which reflects how well the proteins are adsorbed and conformed at the interface to stabilise the emulsion by steric hindrance and electrostatic repulsion. A number of different factors, such as the surface hydrophobicity of a protein, the charge heterogeneity, and structure flexibility, are seen as important factors controlling the efficiency of adsorption (Beverung et al., 1999; Cabra et al., 2008; Wilde, Mackie, Husband, Gunning, & Morris, 2004), thereby determining the structure and thickness of the protective barrier layer (Zayas & Lin, 1989).

* 1. **Gels**

Gelation is another functional property of great interest in food processing under which a protein solution is turned up, irreversibly, into a soft-solid, space filling network structure, normally setup by heat (Chodankar, Aswal, Kohlbrecher, Vavrin, & Wagh, 2009; Gosal & Ross, 2000; Ikeda & Nishinari, 2001; Nicolai & Durand, 2013), bringing to food new sensory and rheological properties. Gel formation of globular proteins is affected by environmental conditions such as temperature, protein concentration, pH and ionic strength of the medium. Pressure has also recently been reported as a gel-inducing factor (Devi, Buckow, Hemar, & Kasapis, 2014; J. He et al., 2013; X. H. He et al., 2014).

Heat-induced gelation from globular proteins follows, primarily, protein denaturation, where the proteins first unfold, flipping the hydrophobic groups normally hidden in the inner core of the protein domain outwards against the aqueous solution, which is energetically unfavourable (Green, Hopkinson, & Jones, 1999; X. Li, Li, & Hua, 2007; Nicolai & Durand, 2007; Nicolai & Durand, 2013). This is followed the protein rearrangement at the interface and, finally, the aggregation by intermolecular association, building up the network gel according to the mechanism shown in (1). The aggregation also follows the primary stage and, if the protein concentration is sufficient, it may follow the second stage, known as secondary aggregation to form the gel.

N I D (1)

N - native structure; I - intermediate; D - denatured state

The denaturation process and gel formation do not affect the primary amino acid sequence or the protein backbone. It is mostly the tertiary and quaternary structures that are involved in various protein conformations, resulting in different associations and gel formation, but very little from the secondary structure, as summarised in Table 4, modified after Foegeding and Davis (Foegeding & Davis, 2011).

Two important factors for gelation are electrostatic conditions (pH and salt concentration) and the protein concentration.

Table 4. Molecular properties associated with native (N), intermediate (I) and denatured (D) protein structures, modified after Foegeding and Davis (Foegeding & Davis, 2011).

|  |  |  |  |
| --- | --- | --- | --- |
| Property of the native state | Description | Change in N I | Change in I D |
| Molecular weight  Isoelectric point  Primary structure  Secondary structure  Tertiary structure  Quaternary structure | Molecular size  Net charge of the molecule  Amino acid sequence  Amount α-helix, β-sheets, others  Grouping of chains in space  Grouping of sub-structures assembling in space | No  Possible, pKa changes  No  Very little  Yes  Yes | No  Possible, pKa changes  No  Yes  Yes  Yes |

When the pH is far from the isoelectric point, where electrostatic interactions are favoured and under low ionic strengths, fine-stranded gels are formed. Under high ionic strength and pH close to the isoelectric point, spherical and particulate gels are formed (Foegeding & Davis, 2011; Gosal & Ross, 2000; Nicolai & Durand, 2013).

* 1. **Colloidal glass emulsions**

Emulsions and gels are complex heterogeneous multicomponent and multiphase systems consisting of dispersed phases and macromolecules in different stage of organization (Dickinson, 1992). Oil-in-water emulsions at low concentrations are liquids. With progressive increase in the dispersed phase beyond a critical concentration, emulsions turn into soft-solid-like materials due to an increase in viscosity, finally obtaining a semi solid character. Normally, their consistency is such that the material practically does not flow until sufficient stress is applied (Joshi, 2014). In addition, because of their disordered structure, they may evolve to a more relatively stable state over time. These soft-solid-like dispersions with disordered structure are broadly termed ‘colloidal glass’ and include a wide variety of products such as concentrated emulsions, gels, mayonnaise, and paints (Dawson, 2002; McClements, 2010). Solidification of this soft matter occurs without formation of crystalline order of the dispersed phase, resembling glass solidification (Fielding, Sollich, & Cates, 2000). Fig. 1 illustrates the transition liquid-glass states, one to another.

High-fat concentrated emulsions in the range of 60% fat may change from liquid to arrested state. The transition may occur through attractive or repulsive interactions, to form a structure of loosely connected droplets, displaying high viscosity and stability towards creaming. In the glass structure the particles pack and interact together, which prevent the free settling of the particles or the counter diffusion through the continuous phase. However, although the colloidal glass displays some stability to creaming, this does not reflect an equilibrium situation, because of the disordered structure. Therefore it may evolve to more stable conditions.

Various approaches have been used to expand the application of emulsion principles to reach a wide variety of food products. Recently, there has been increasing interest in developing novel structured emulsions, aimed at improving or extending the functional performance of food by exploring such concepts as multilayer emulsions, solid lipid particles, and multiple emulsions (McClements, 2010). The structured emulsions are more sophisticated systems than the simple o/w or w/o, and are being developed toward new applications in food manufacturing (McClements, 2010).

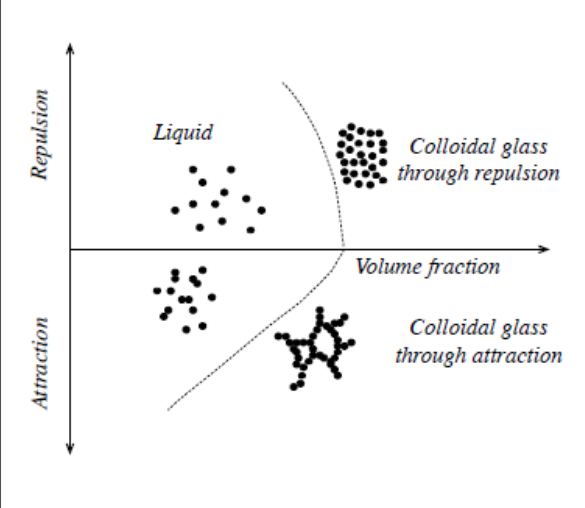


Fig. 1. Effect of oil volume fraction on the equilibrium of liquid and colloidal glass emulsions.

1. **Wet processing method used to produce coconut protein fractions**

Protein by-products obtained from the traditional dry processing of copra have been underutilised due to their low quality, caused by denaturation or sanitary issues that make them unfit for human consumption. They are therefore thrown away to the environment with the spent copra meal or used in feeding animals (Mepba & Achinewhu, 2003; Naik et al., 2012; Raghavendra & Raghavarao, 2011). The quality of these coconut proteins has been affected by the extraction methods.

Most of the protein extraction methods described in the literature were performed on laboratory scales for scientific studies, and none were tested on industrial scale for food purpose applications. The extraction procedures normally involve protein fractionation based on the solubility in different solvents, as shown in Table 5 (Kwon et al., 1996; Mepba & Achinewhu, 2003; Sant’Anna, Freitas, & Coelho, 2003) or apply enzymes for simultaneous recovery of oil and protein (Mepba & Achinewhu, 2003; Samson, Khaund, Cater, & Mattil, 1971; Sant’Anna et al., 2003).

Table 5. Protein fractionation based on the modified method of Hu and Essen (Kwon et al., 1996).

|  |  |  |
| --- | --- | --- |
| Protein fraction | Extraction solvent | Total protein extracted (%) |
| Albumins  Globulins  Prolamines  Glutelins-1  Glutelins-2  Unextractable protein | Deionised water  0.5M NaCl  70% isopropyl alcohol  50% Acetic acid  0.1M NaOH | 21.0 (0.4)a  40.1 (0.3)  3.3 (0.2)  14.4 (0.4)  4.8 (0.7)  8.1 (0.9) |

a Values in parenthesis are standard deviations obtained from triplicates (Kwon et al., 1996)

The wet processing method for protein extraction developed here was designed to be simple and to expand the diversity of coconut products, recovering not only the protein but also the oil and skim coconut milk on a fresh basis and under controlled processing conditions.

White shredded coconut endosperm

Mass: 100 g Protein: 3.5 %

Full-fat coconut milk

Mass: 40 g Prot.: 4%

Press-cake

Mass: 60 g Prot.: 3%

Pressing at 40°C, 10 bar, 15 min

Water

Warming and centrifugation

Protein extraction, pH=10

Press-cake residue

Mass: 58 g Prot.: 1.5%

Alkaline protein extract (APE) Mass: 300 g Prot.: 0.3%

Milk sediment (insoluble fraction)

Mass: 1.5 g Prot.: 60% Yield: 26%

Coconut skim milk

Mass: 24.1 g Prot.: 2.9% Yield: 20%

Ultrafiltration and freeze drying

Oil

Mass: 14.4 g Prot.: 0%

Alkaline protein powder (APE)

Mass: 2.1 g Prot.: 43% Yield: 26%

Fig. 2. Flow-sheet used to prepare different protein fractions from coconut endosperm, in the wet processing method. The yields for protein were determined in relation to the total protein in the starting material (adapted from Fig. 1, Paper I).

Fig. 2 describes the process used to obtain different protein fractions. The white coconut endosperm was the starting material, treated with no water addition, as illustrated. Coconut skim milk protein fraction, the insoluble protein sediment and the alkaline protein powder were the three protein fractions obtained and used in different characterisation of the coconut proteins. In order to improve protein yields, the comminuted white endosperm was pressed with no water addition, while in common practice warm water is added during coconut milk production. Water causes the precipitation of the globulins, the major protein fraction in coconut, decreasing the extraction yields.

For the alkaline protein extraction, the press-cake was dispersed in water in a water/press-cake ratio of 1:5, the pH adjusted to 10, and the protein extracted at a constant temperature of 50°C, as detailed in Paper I. The pH was selected in order to displace the extraction from the isoelectric conditions, thereby favouring the extraction yields.

A simple pressing of the endosperm released about 45% of the total protein with the coconut skim milk and the protein sediment fractions, while 26% were additionally recovered by the alkaline extraction, giving an overall yield of 72% of total protein from the starting material. This yield compares favourably to those obtained in more complex extraction procedures, shown in Table 5, assuming that the albumins, globulins and glutelins-2 were extracted. It was found that the temperature also increased the extraction yields, but special care was taken in setting the extraction temperature to avoid protein denaturation, as detailed in Paper I.

1. **Characterization of the coconut protein fractions from wet processing method**
   1. **Amino acid composition.**

The experiments performed on the three protein fractions showed comparable amino acid profiles to those shown in previous studies, as illustrated in Table 6 (Chambal, Bergenståhl, & Dejmek, 2012; Hagenmaier et al., 1974; Kwon et al., 1996; Santoso et al., 1996). The results showed that glutamic acid and arginine were the most abundant amino acids in the coconut proteins, and also that the alkaline method has extracted threonine to nearly twice the quantity of what has previously been reported (Paper I).

In addition, some of the amino acids may have been underestimated because of the conditions of the method used. For example, threonine, serine, cysteine and methionine may have undergone oxidation or destruction because of the acid hydrolysis used to digest the proteins (Coward-Kelly, Agbogbo, & Holtzapple, 2006; Kabaha, Taralp, Cakmak, & Ozturk, 2011; Rasyid, Manullang, & Hansen, 1992). On the other hand, the alkaline conditions may also have degraded or formed new amino acids during the alkaline extraction, since the experiment was performed with no precautions taken to protect those sensitive amino acids from degradation (Coward-Kelly et al., 2006; Macuha & Chudziak, 1977; Rasyid et al., 1992).

The results also showed that the amino acid composition re-calculated on the basis of the essential amino acids pool provided a balanced profile to meet or suppress the nutritional requirements (WHO, 1985; WHO, 2007), as in the case of histidine, (phenylalanine + tyrosine) and threonine (Fig. 3). However, the sulphur-containing amino acids such as methionine and cysteine were lower, as seen from Fig. 3, which is likely to apply to oilseed proteins.

Coconut protein can play an important role as valuable adjuvant in human nutrition, when used in combination with other protein sources and provided that suitable processing technologies for protein extraction are established.

Table 6 – Amino acid profile of the wet processing protein fractions.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Amino acid composition  (%, w/w) | Experimental results 1 | | | Literature 2  Fresh Endosperm | Literature 3  Fresh Endosperm |
| Skim milk | Insoluble protein fraction | Alkaline protein fraction |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Asp  Thr  Ser  Glu  Pro  Gly  Ala  Val  Cys  Met  Ile  Leu  Tyr  Phe  Lys  His  Arg | 8.86  3.00  4.15  25.58  1.48  5.12  4.04  4.06  1.00  0.61  2.46  6.11  1.88  5.19  7.38  3.86  15.21 | 9.91  3.99  5.38  21.79  1.56  5.38  3.88  5.15  0  0  3.57  7.99  1.98  5.76  4.78  2.60  16.86 | 9.60  8.02  5.38  18.32  1.73  5.32  4.48  4.45  0.47  0  2.95  7.26  3.02  5.47  5.61  2.82  15.03 | 8.51  3.17  4.79  18.58  3.36  3.86  4.80  4.57  0.90  1.71  3.10  6.20  1.82  3.87  11.76  2.77  16.42 | 9.21  3.43  4.86  21.57  3.90  4.47  4.81  5.72  1.87  1.77  3.69  6.99  1.09  4.79  4.16  2.16  15.48 |

1 Corresponding to table 4, Paper I, 2 (Santoso et al., 1996) 3 (USDA, 2014)

Fig. 3. Essential amino acids profile of the three protein fractions obtained on the wet processing of coconut (recalculated from Table 2 and 6).

* 1. **Solubility profile of the press-cake protein**

Protein solubility is probably the first physicochemical property of interest when characterising the functionality of a protein, since it controls the extent to which functional properties such as emulsification, gelation and water retention are performed (Moure et al., 2006). It means that any change in the protein solubility (concentration) will directly influence the related property of interest.

The major coconut protein fraction, cocosin, a storage globulin, is water insoluble, but soluble in aqueous salt solutions at a given ionic strength, as shown in Table 5. The solubility of globular proteins is pH-dependent, exhibiting minimum solubility at the isoelectric point.

The isoelectric point of the coconut proteins is still not well defined. For example, early studies on solubility in relation to pH by Kwon et al. (Kwon et al., 1996) reported minimum solubility of cocosin under low ionic strength, in the range of pH 4-5. More recently, Angelia et al. (Angelia et al., 2010) found a minimum in the range of pH 5-6 and Onsaard, at 4-5, working with coconut skim milk protein powders (Onsaard et al., 2005). The authors also observed that at higher ionic strengths the isoelectric points were shifted to the acidic regions, close to pH 3.

The three-protein fraction studied here displayed different degrees of solubility: the skim milk protein fraction was mainly composed of the soluble coconut proteins, which remained in the aqueous phases after the separation (warming and centrifugation) of the insoluble protein sediment and oil. The protein sediment fraction was practically insoluble in deionised water, while the APE was partially soluble in water, but affected by the pH and salt concentration.

The solubility behaviour discussed here refers to the alkaline protein fraction (APE), which was analysed by dispersing the APE in deionised water, followed by pH adjustment, as detailed in Paper II. The results (Fig. 4) showed that the solubility profile of APE generally accords with the data reported in the literature, but differs slightly in terms of minimum solubility.

Fig. 4. Solubility of the press-cake protein powder over the pH values, at three concentration series: (  ) 0,01 % APE ; ( ) 0,05 % ; ( ) 0,08 %. The alkaline protein powder contained 43% of protein. (Corresponding to Fig. 1, Paper II)

The minimum solubility of APE was found at pH 3 to 4 in all samples, at low ionic strength. The solubility also increased on both sides of the minimum, exhibiting V-shaped profiles. This type of profile is typical for many plant proteins, with the minimum solubility at the corresponding isoelectric points (Angelia et al., 2010). For example the 11S globulins from a wild variety of soya bean and rapeseed also exhibit minimum solubility in the range of acidic pH (Maruyama et al., 2004; Mori et al., 2004; Rickert, Johnson, & Murphy, 2004; Salleh et al., 2002). It can be argued that the profile is correlated with the higher amount of acidic amino acids in the protein structure, like cocosin (Angelia et al., 2010).

The common practice of adding warm water for coconut milk extraction has a detrimental impact on protein yields. If, on the one hand, this helps to disaggregate the endosperm, on the other, it causes severe precipitation of proteins, which are then lost with the endosperm press-cake, discharged as a waste. The effect of precipitation is due to the insolubility of cocosin in water, as this is a globulin class protein, and also due to the pH effect, since it is driven close to the isoelectric point conditions (pH < 6).

* 1. **One dimension SDS-PAGE of the coconut protein fractions**

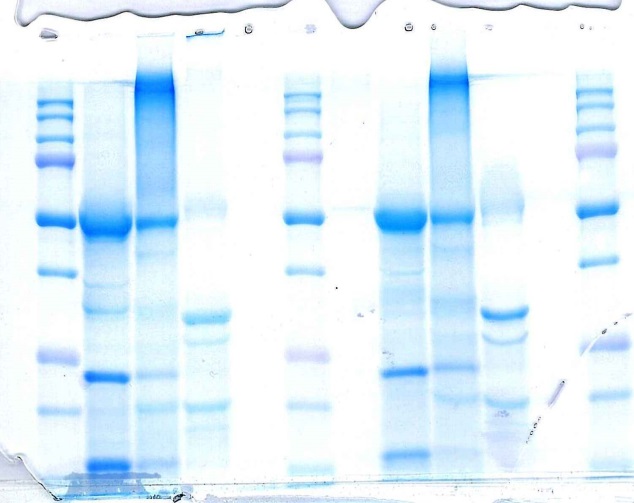
In order to characterise the molecular sizes and isoelectric points, one and two dimensional SDS-PAGE electrophoresis were carried out on the three protein fractions. The one dimension electrophoresis (1DE) was run according to the same principles developed by Laemmli (Laemmli, 1970), after modifications by Hames (Hames, 1998), loaded in a pre-cast criterion mini gels (12.5% Tris-HCl, pH 6.8, Bio-Red, Sweden). The results of 1DE are summarised in Table 7, based on Fig. 5.

Table 7. SDS-PAGE summary for the 3 protein fractions I. **+ Strong band;** + light band, (Adapted from Fig. 6, Paper I)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| MW (kDa) approx. | A  50 | B  42 | C  34 | D  32 | E  24 | F  21 | G  16 |
| Skim milk fraction  Milk sediment  APE | **+**  **+** | + | +  + | **+** | **+**  + | +  + | **+**  + |

The coconut skim milk proteins were made up of the soluble proteins, which remained in the liquid phase after separation of the insoluble fraction. These soluble proteins resolved in clear bands at approximately 50, 24 and 16 kDa, showing one light band at 34. The insoluble fraction resolved in two sets at 32 and 21 kDa, which correlated very closely to the cocosin globulin, as the polypeptide sub-units (D) and (F) compare well with those of the purified cocosin used by Angelia (Angelia et al., 2010). Previous studies by Garcia had reported that each polypeptide unity of cocosin was composed of acidic (C) and basic (E) polypeptides, and that the subunit (A) was a result from the combination of both (Garcia et al., 2005)*.*

Table 7 also shows that the APE fraction contained additional protein sub-unit (B), at 42 kDa, which was not present in the original fat milk. It means that this protein was bound to the endosperm press-cake and only extracted by the alkaline method. The isoform was characterised by MS-MS, matching partially to the glutelin fraction (Paper I)*.*



1 2 3 4 5 6 7 8 9 10 11 12

kDa

50

37

25

20

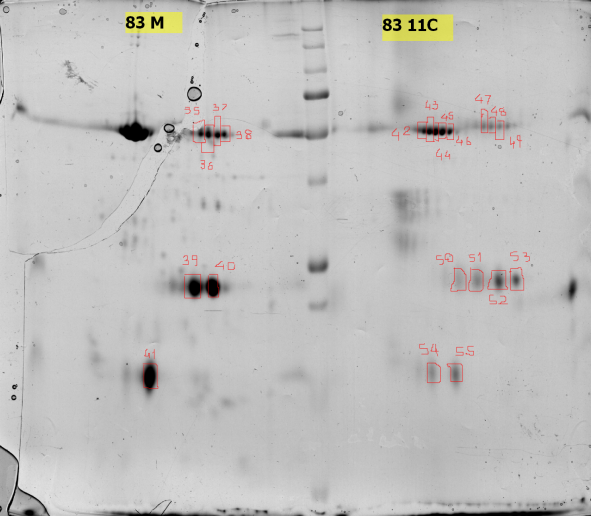
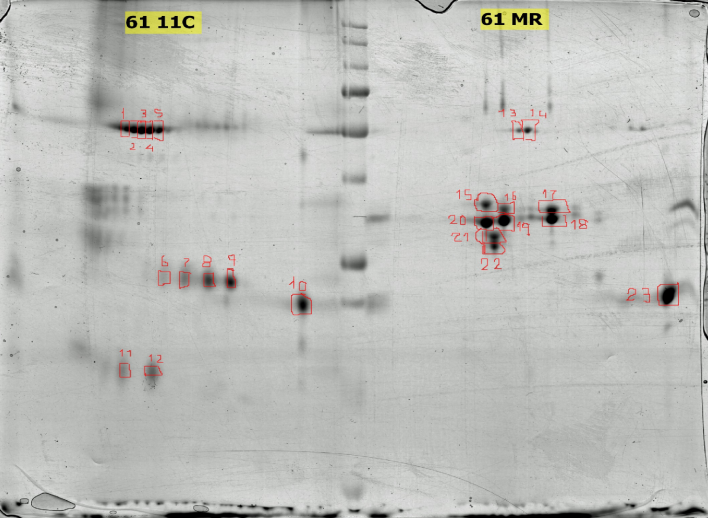
15

Fig. 5. 1DE SDS-PAGE on the three protein fractions. 1, 6, 12 – Molecular weight standard (Bio-Rad Laboratories, Vintergatan, Sweden); 2, 8 – Coconut skim milk; 3, 9 – APE; 4, 10 – Coconut milk sediment. (Corresponding to Fig. 6, Paper I).

The enzymatic digestibility of a protein is also an important indication of its nutritive quality. Digestion of cocosin with α-chymotrypsin was found to be more effective on the acidic polypeptides; the digestion was greater than for the basic polypeptides (Angelia et al., 2010)*.* The basic polypeptides at 24 and 21 were more resistant to enzymatic degradation than the acidic at 34 and 32 kDa. In particular, the polypeptide at 34 kDa was the one very rapidly digested, suggesting differences in the susceptibility to enzymatic proteolysis, which also reflects differences in amino acid sequence and the abundance of cleavage sites.

* 1. **Two dimensions SDS-PAGE and mass spectrometry**

Two dimension electrophoresis (2DE) were run in the three protein fractions, using 7 cm, non-linear pH-gradient (3–10) gel strip isoelectric focusing, as the first dimension electrophoresis. The proteins migrated along the strip length, ceasing the mobility at the corresponding isoelectric points. The gel strip with the proteins span over the isoelectric points were loaded in the second dimension, the SDS-PAGE, to allow migration according their sizes, as shown in Fig. 6 and detailed in Paper I.

****

3 pH 10

3 pH 10 3 pH 10

kDa

75

50

37

25

20

Fig. 6 – 2D Electrophoresis on 7 cm, NL pH gradient (3-10) gel strips (Bio-Rad, Sweden) on the (61 11C – press cake fraction), (61 MR – milk sediment fraction) and (83 M – skim milk fraction).

The results showed some similarities between the press-cake proteins and the coconut milk fractions in terms of molecular weight span or even in terms of isoelectric points. However, the milk sediment protein fraction was completely different. This fraction was dominated by polypeptide subunits in the range of 20 – 30 kDa, confirming previous results from 1DE SDS-PAGE (Fig. 5). The isoelectric points were found concentrated in the acidic regions. However, this fraction showed one polypeptide subunit at about 20 kDa in the alkaline region, similarly to the press-cake protein fraction, which was not detected in the coconut milk.

Mass spectrometry (MS-MS) was run in each spot, taken from the 2D gels. The spots were numbered to make them easy to track along the mass spectrometry assy. Each spot was separately placed in the mould target, mixed with ionizing matrix and enzymatically digested to be degraded in small peptides. Trypsin was used for the digestion. Peptides were ionized and accelerated over the mass spectrometry and finally detected as a mass/charge ratio and therefore, experimentally sequenced.

Peaks searching engine (Bioinformatics) was used to compare to the theoretical peptides sequences, from the reference databases Mascot (Matrix Science, ), with those experimentally obtained and evaluated for the significance of the protein matches (Paper I).

Protein fingerprint matched partially to 7S Globulin, tr|Q9AU64|Q9AU64\_ELAGV with 73% (significant) significance, from the coconut milk protein fraction and the Glutelin OS, tr|Q9SNZ2|Q9SNZ2\_ELAGV with 37% (not significant) from the press-cake fraction. However, the genome used in the data base was that from oil palm. Therefore no conclusive results were achieved from the mass spectrometry, probably because the coconut proteomes have not been systematically sequenced and entered in the databases. Oil palm is a cultivar close but different from *Cocos Nucifera L*.

1. **Functionality of the coconut protein fractions**

The functionality of proteins is determined by their physicochemical properties, such as molecular size and structure, amino acid composition and sequence, net charge and distribution, surface hydrophobicity, flexibility and their response to the environmental conditions (temperature, pH, and ionic strength). The ability to form or stabilise dispersions, such as emulsions or gels, is viewed as the main classical functionality of proteins in food systems, which is associated with changes in structural and rheological attributes of food (Cabra et al., 2008; Joye & McClements, 2014; Wilde et al., 2004).

The functionality of the coconut protein fraction was determined by their emulsifying and gelation capacities on the press-cake protein fraction, under selected ranges of pH, temperature and at low ionic strength.

* 1. **Emulsifying capacity of the native press-cake proteins (APE)**

Paper II describes the emulsification process of oil in water dispersions stabilised by the native press-cake protein fraction. The systems consisted of diluted dispersions, at three concentration levels and low ionic strength: 0.01; 0.05 and 0.08% APE (the APE powder contained 43% protein) in water, at different pH values (2-10). To these APE solutions, 3% v/v of n-hexadecane was introduced and homogenised by a shear mixer.

The results showed that the coconut protein fraction did not efficiently emulsify the systems below a droplet size of 10 µm during homogenisation. The volume distributions were dominated by a population of droplets with mode values in the range of 10-15 µm and by a second insignificant population of small droplets, with mode values of about 1 µm, as described in Paper II and illustrated in Fig. 7.

The emulsification properties of the native press-cake protein fraction were strongly correlated to the solubility profile over the pH. As can be seen from Fig. 6, the smallest droplets, and therefore the ones leading to relatively more stable emulsions, were only achieved at pH > 7, while in the acidic regions, close to the isoelectric points, no emulsion was formed at all. The isoelectric conditions corresponded to the minimum protein solubility. However, the formed emulsions quickly underwent destabilisation by flocculation and creaming, although they were quite stable facing coalescence. This indicates that the emulsifying capacity of the press-cake proteins was not good enough.

Fig. 7. Mode values of the volume droplet size distribution over the pH, of the representative population, at three concentration series: (  ) 0,01 % APE ; ( ) 0,05 % ; ( ) 0,08 %. APE contained 43% protein. The dispersion contained 3% v/v n-hexadecane (diagram corresponds to Fig. 2, Paper II).

Similar results from previous studies and different coconut protein fractions have been reported, showing limited emulsifying capacity. For example, fresh coconut milk exhibits high instability and quickly separates into a cream layer and serum aqueous phase after less than 4 h (Lad & Murthy, 2012; Onsaard et al., 2005; Tangsuphoom & Coupland, 2008a; Tangsuphoom & Coupland, 2008b).

The SDS-PAGE run on the initial press-cake protein solutions before the emulsification process showed that the cocosin polypeptides at 35 and 24 kDa were very sensitive to the isoelectric conditions under which they precipitated (Fig. 10a and Fig. 10b Paper II). ), but were found in the gels/precipitate (Fig. 10b). These polypeptides might seem to be the most important for emulsification, as the systems emulsified poorly under the conditions where only the 7S polypeptides (24, 22 and 16) kDa were present.

The experiments also showed that the initial protein concentration (the protein availability) was not the limiting factor for an effective emulsification, since non-adsorbed proteins left after the emulsification were found, especially as the initial concentration increased. The ratios of adsorbed proteins during emulsification decreased with the initial protein concentration, as seen from Fig. 8.

Fig. 8. Protein solutions before emulsification at pH 6 and 10 and protein adsorbed after emulsification with 3% v/v n-Hexadecane. The press-cake powder contained 43% protein. (Data taken from Table 1, Paper II).

* 1. **Heat set gel from the press-cake protein (APE)**

To assess the gel formation, 15 % of the press-cake powder (approx. 6,5 % native protein) was dispersed in water, at pH values varying from 4 to 9. The protein concentration was chosen after preliminary experiments, and run with a lower concentration (10% press-cake, corresponding to 4.3% protein in the mixture), which also formed gel at 75°C. The dispersions were directly heated in a rheometer in order to monitor the heat set temperature and the gel strength at a given pH. The low strain oscillatory method was applied, where the storage modulus G’ and phase angle δ were monitored from 30°C to 75°C during each run, as shown in Fig. 9 (for more details see Paper III).

Fig. 9. Gel development during heating and cooling, on 15% APE dispersions in water, at different pH values. ( **T**). Adapted from Fig. 1, paper III.

The results (Fig. 9), presented in detail in Paper III, showed that the press-cake protein fraction only had good capacity to form heat-induced gels (G’> 1000 Pa; δ < 20°) at pH 8-9. Below pH 8, weak gels were observed, according to Fig. 9. The onset temperatures for gelation for the native coconut press-cake proteins were found in the range of 65°C to 70°C for the stronger gels, meaning that more than one protein participated in the gelation. This is shown by a range gel set temperatures instead of a single and fixed temperature. This range of temperatures compares well with the values reported in the literature for other globular proteins; for example, for β-lactoglobulin (Foegeding, Gwartney, & Errington, 1998; Nicolai & Durand, 2013)*.*

The gel strengths were also assessed by a texture analyser in back extrusion mode, where portions of the same sample had been heated to 75°C before loading into the texture analyser. The results also showed gel strengths in the same order as those from rheometry. Tough gels were those at pH 8-9 and weak gels were below pH 8 (details in Paper III).

The SDS-PAGE profile on the initial solutions and final gels, Fig. 10a and 10b, showed that the proteins responsible for gelation were those at 53, 32-34 and 24 kDa, named A, C-D and E, respectively, according to Table 7. These polypeptide sub-units were sensitive to acidic conditions as they precipitated under isoelectric environments, as illustrated in the solubility curve in Fig. 4. Therefore, as the pH was being lowered for the necessary adjustments before gelation, the sensitive polypeptides were precipitating, so the final result from the process was a mixture of gel and precipitate. From the SDS-PAGE it was not easy to distinguish the gels from the precipitates, as they were the same proteins participating in both processes.



Fig. 10a. SDS-PAGE on the initial solutions (15% APE) before gelation, over the pH (corresponding to Fig. 3, Paper III).



Fig. 10b. SDS-PAGE on the gels/precipitate, at different pH values (corresponding to Fig. 4, PaperIII.

The morphology and gel strengths varied widely over the tested pH. The stronger gels, at higher pH (8 and 9), were more homogeneous, less prone to syneresis and exhibited higher elastic modules, while the weaker (below pH 8) showed particulate structure, with liquid phase separation. Microscope images of the gel structure and the Fourier transforms showed clearly the differences in gel morphology (see Paper III, for details). The necessary adjustments to pH in the initial solutions before gelation caused the protein to precipitate, so the heating of the system resulted in a mixture of gel and the already formed precipitate, which probably affected the structure, as seen from Fig. 11 and 12.

Generally, the press-cake protein fraction showed appreciable capacity to form gels under heating, in the range 65-75°C, although homogeneous and strong gels were obtained at the neutral to alkaline environments. This could limit to some extent their application in food formulation, as food is generally mildly acidic.



Fig. 11. Gel image on particulate, with liquid phase separation structure, at pH 4, taken at 50x magnification (corresponding to Fig. 5, Paper III)



Fig. 12– Gel image on smooth and homogenous gel structure, at pH9, taken at 50x magnification (corresponding to Fig. 6, Paper III).

1. **Heat denaturation of coconut milk proteins, and homogenisation to enhance stability**

Coconut milk is a traditional oil-in-water emulsion extracted from the coconut endosperm, stabilised by globulins, albumins and some phospholipids (Seow & Gwee, 1997). It is a quite unstable emulsion, which readily separates into a cream layer on top and light serum phase, due to low stabilising quality of the proteins, and exhibiting large oil droplet sizes (10-20 µm) (Tangsuphoom & Coupland, 2005; Tangsuphoom & Coupland, 2008a; Tangsuphoom & Coupland, 2008b).

Attempts to increase the stability of coconut milk emulsion have led to different treatments, including the use of commercial additives, such as sodium caseinate, polyoxyethylene sorbitan monolaurates, esters of fatty acids, carboxymethyl cellulose, and acacia gum (Aoki et al., 2007; Lad & Murthy, 2012; Tipvarakarnkoon, Einhorn-Stoll, & Senge, 2010). Some of these are chemically synthesised and others have restricted use in food applications. The use of high pressure homogenisation only slightly improves the stability, as the original droplet size is only reduced down to 2-3 µm (Onsaard et al., 2006). This droplet size is still too large to prevent coconut milk destabilisation. Furthermore, because of poor emulsifying property of coconut protein, the homogenised emulsion flocculates in large aggregates, ending up by creaming. Application of acoustic cavitation for emulsification is another approach reported (Lad & Murthy, 2012; M. K. Li & Fogler, 1978), but has not produced more stable coconut milk emulsion.

Very little attention has been devoted to the study of the effect of partial denaturation of coconut proteins on the stability of coconut milk emulsions. Previous studies (Tangsuphoom & Coupland, 2005; Tangsuphoom & Coupland, 2008b) were only conducted on diluted systems, under mild heat treatments below the denaturation conditions. Under these conditions, the studies reported an increasing instability by flocculation and creaming, because the coconut proteins were unable to efficiently cover an increased surface area generated by homogenisation.

Paper IV attempts to describe the stabilising effect from denaturation of full-fat coconut milk proteins, conjugated with high-pressure homogenisation on coconut milk against creaming, flocculation and coalescence. The approach relies on the principle of colloidal glass structure (Dawson, 2002) shown in Fig. 1, where the droplets are immobilised in stable although amorphous structure, rendering the emulsion stable over a period of time.

Although the protein denaturation is mostly assessed to be a loss in protein quality, because of aggregation combined with concentrated oil droplets, the system of concentrated emulsions can be regarded as a colloidal glass, characterised by an arrested state, with potential to stabilise emulsions and gels (Dawson, 2002). If proteins are partially denatured, their interactions may change from repulsive to attractive. More adhesive interactions may result in the formation of a network of loosely connected emulsion exhibiting soft-solid-like consistency. For reasons not yet clearly understood, the system may solidify without the formation of a crystalline structure of the lipid phase, resembling the glass state. These soft-solid-like materials do not flow over a time scale unless a sufficient stress is applied (McClements, 2010). Typically, these systems would show a yield stress.

Full-fat coconut milk batches were used to prepare coconut milk samples at fat contents – 13, 19, 29 and 38%, by dilution with water. The samples were heated at 70, 80, 90 and 95°C to reach the protein denaturation. The heated samples were homogenised at 300/30 bars in a two-stage valve homogeniser to reduce droplet size and promote contact between the newly formed interfacial area and the different system phases, as described in Paper IV. Creaming index, droplet size distribution and yield stress on the treated emulsions were used to assess the emulsion stability and rheological properties.

* 1. **Size distribution**

The size distribution of the heat-treated and homogenised coconut milk was measured only on the full-fat samples by laser light diffraction, using the Malvern Mastersizer (Mastersizer 2000, Malvern Instruments, UK). An adequate amount of homogenised emulsion was dispersed either in water for effective droplet and agglomerate sizes, or in 1% SDS solution for primary droplet size (Tangsuphoom & Coupland, 2008b). The dispersions were added until 15% of the laser beam obscuration was obtained.

Mode values of the volume distribution from the representative population were used to characterise the emulsion size pattern, as the volume weighted mean diameter, described by Equation (2) (Benjamin et al., 2014; Bortnowska, Balejko, Tokarczyk, Romanowska-Osuch, & Krzeminska, 2014; Lad & Murthy, 2012; Tangsuphoom & Coupland, 2008a).

( 2)

The results showed that it is difficult to homogenise coconut milk emulsion below 2 µm to provide stability (typically this should be below 1 µm). This can be achieved using emulsifiers, but this does not preserve product identity.

Fig. 13. Primary droplet size distribution of a batch of heated and homogenised full-fat coconut milk (apprx. 40% fat). ( ) not heated – but homogenised ; ( ) 70°C – homogenized ; ( ) 70°C – homogenised, but no SDS (effective droplet size) ; ( ) 90°C – homogenized ; ( ) 95°C – homogenized. (Paper IV)

From Fig. 13 it can be seen that the samples heated at 95°C and homogenised showed smaller primary droplet sizes, in the range of 3 μm, in comparison with the other coconut milk samples investigated. At this temperature it was also observed that the coconut milk proteins reached denaturation conditions, as they coagulated after heating. In general the heating below denaturation conditions (below 90°C) and homogenisation resulted in more flocculation and creaming.

* 1. **Creaming index**

Creaming is one of the destabilising mechanisms of emulsions, consisting of gravitational separation of the oil droplets and the serum phase. Creaming rates are correlated with the oil volume fraction and decrease with the droplet concentration (McClements, 2010; Moschakis, 2013). Concentrated emulsions in highly viscoelastic states exhibit low creaming rates. Protein denaturation brings new functionalities to the system by exposing new hydrophobic domains to the water phase. This results in new protein-protein interactions, leading to increased viscosity and significant improvement in stability (Gosal & Ross, 2000; Liang, Patel, & Matia-Merino, 2013; Nicolai & Durand, 2013). Below the denaturation temperature, the heat treatments do not improve stability, and may even deteriorate the situation by promoting more aggregation and, therefore, more creaming. For dense systems at high fat contents, precise creaming behaviour should be determined experimentally.

Creaming indexes were determined for all coconut milk samples covering the range of the fat concentration according to Equation (3) (Lad & Murthy, 2012; Tangsuphoom & Coupland, 2008b) to evaluate the stability of the emulsion under combined effects of temperature, high pressure homogenisation and fat content, as shown in Fig. 14. VSERUM and VTOTAL are the volumes of separated serum phase after a given length of time related to the total initial volume of emulsion, respectively. The data were collected on the third day, with the samples stood upright at a room temperature.

(3)

The results (Fig. 14) showed that the higher stability was obtained for the high-fat concentrated emulsions, treated at higher temperature, while those with lower fat content and heated at the lower temperatures (below 90°C) exhibited greater instability towards creaming. As recognised in previous studies, the relative creaming rates depend on the concentration of the dispersed phase (McClements, 2010), and have 0.6 fixed as the limit value of oil volume fraction for stability, below which an emulsion will undergo separation (Bortnowska et al., 2014). The stability is mainly attributed to the effect of arrested particles emulsion, which hinders the free settling or by the counter diffusion of the droplets over the continuous phase.

High-pressure homogenisation on the heat set emulsion had two opposing effects. It disrupted the soft-solid-like structure, turning it into a fluid-like (destabilising effect), but also reduced the original droplets to the smallest size (stabilising effect), dispersing them over a texturised medium which prevented the emulsion destabilisation to some extent.

Fig. 14. Creaming of the heat treated emulsion at different temperatures and fat concentration, for 30 minutes, prior to homogenization at 40C, for all samples. (Corresponding to Fig. 3, Manuscript IV)

It was also observed that the samples that were not heat-treated but homogenised showed some relative stability, but less than that at 95°C. This relative stability was solely gained because of the reduction in the original droplet size, from approximately 13±2 µm (fresh milk) (Onsaard et al., 2005; Onsaard et al., 2006; Simuang, Chiewchan, & Tansakul, 2004; Tangsuphoom & Coupland, 2008b; Tipvarakarnkoon et al., 2010) to nearly 4-6 µm, on the primary droplet size .

The higher stability of the concentrated emulsion, heated at 95°C, seems to correlate closely to the glass structure, which describes the effect of transition from liquid state to glass state. The reduction in droplet size was not the cause of the stability, as this was not below 1 µm. This sample appeared to be a solid-like material after heating to denaturation of coconut milk proteins. However, the samples with same composition, treated at lower temperatures (< 90°C) did not exhibit the same consistency, because they did not reach the denaturation conditions. A colloidal system such as an emulsion is described as a liquid, if the droplets are allowed to diffuse or cream freely through the continuous phase. Such system has liquid properties and displays more or less Newtonian rheology. With the droplets in arrested state the polydisperse material becomes disordered solid-like that can be classified as colloidal glass.

The arrested state is normally observed at high fat concentration (55 – 75%) and can be originated from repulsive or attractive droplet-droplet interactions, as illustrated in Fig. 1. Although full-fat coconut milk was only 32 - 45 % fat, the arrested state was formed, possibly promoted by gel particles formed by coconut milk protein denaturation.

* 1. **Yield stress**

Yield stress is encountered in a wide range of products such as toothpaste, cement, mayonnaise, and mortar, and is defined as the initial stress required to make the material flow (Coussot, 2014; Joshi, 2014; Ovarlez, Cohen-Addad, Krishan, Goyon, & Coussot, 2013). Materials exhibiting yield stress have a fundamental characteristic: they do not flow unless a stress is applied above a critical value. Above this value they will flow like fluids and, below, they will behave as solid materials. The material in arrested glass state exhibits yield stress.

Yield stress and other rheological properties were determined only for the full-fat coconut milk (32-45%) treated at 70, 80, 90 and 95°C, as those with low fat content could not reach the glass state.

The rheological determination was performed on the Kinexus rheometer (Kinexus, Malvern Instruments, UK), using 4 blades vane geometry (4V21SC000SS), inserted in a serrated cup (PC25C0052AL). Data were acquired under log-progression in a controlled shear stress ramp, run at a constant temperature of 25°C. In preliminary experiments, the stress ramp was set to 0.4-45 Pa for the samples treated at 90 and 95°C, while for those at 70 and 80°C it was set at 0.1-5 Pa.

The Herschel-Bulkley (H-B) model (Equation 3) (Herschel & Bulkley, 1926) was used to characterise the flow properties of the heat-treated and homogenised coconut milks, where *Ƭ* – is the shear stress [Pa], *Ƭo* – the yield stress [Pa], *ϒ* – the shear rate [1/s], k – the consistency coefficient [Pa.s n] and *n* – flow behaviour index [dimensionless].

(3)

The yield stress of the samples was obtained directly from the plot of apparent viscosity versus shear stress, as the stress corresponded to the inflexion point, similar to the procedure adopted by Gamonpilas et al. (Gamonpilas et al., 2011). The yield stress values were then entered into the model to calculate k and n, by fitting the experimental data to the plot (Ƭ – Ƭo) versus ϒ, in the range of 1-300 [1/s] shear rate. A power regression from the plot provided the values for k and n.

The yield stress was determined only on the high-fat concentrated emulsions after heat treatments and homogenization, since coconut milk has fat content below that critical concentration (approx.. 60% fat) necessary for the transition to glass emulsion.

The results, Fig. 15, showed that all samples exhibited shear thinning behaviour, as the apparent viscosity decreased over the range of shear rate (implicitly represented by shear stress) tested.

Fig. 15. Effect of shear stress on apparent viscosity of heat treated and homogenised full-fat coconut milk batch (approx. 38 % fat). The heating was performed at 70, 80, 90 and 95°C. (Corresponding to Fig. 4 Manuscript IV).

The yield stresses obtained from different full-fat coconut milk batches, using the flow curves as in Fig. 15 and the inflexion points, are reported in Table 8. The inflexion points corresponded to the stage when the material was changing the deformation pattern from solid-like (Hook’s Law) to fluid-like. It was also found that the yield stress determined using this procedure always occurred at shear rates close to, but below, 1 [1/s].

The samples treated at 95° showed the highest yield stress. The (H-B) model parameter (k) also showed an increase with temperature while (n), the non-Newtonian shear thinning behaviour, was (n ˂ 1), as seen from Table 8. Therefore it can be concluded that these samples treated at 95°C reached the arrested glass structure, as they exhibited the yield stress.

The variability of the (H-B) model parameters observed from Table 8 was a result of different compositions of the full-fat coconut milk batches. Full-fat coconut milk can exhibit a wide range of fat content: 32-45% (Paper I). Fig. 16 shows the application H-B parameters obtained at 95ºC, from two different full-fat coconut milks batches (two replicates per batch).

Table 8 – Average values for Herschel-Bulkley parameters, equation [3], determined from different coconut milk batches, (different fat contents), treated at 95, 90, 80 and 70°C. In parenthesis: [number of samples], (standard error of the mean). (Corresponding to Table 2, Manuscript IV)

|  |  |  |  |
| --- | --- | --- | --- |
| Temperature  [°C] | **Ƭo [Pa]** | **k [Pa.sn]** | **n** |
| 95°C ; [5]  90°C ; [5]  80°C ; [6]  70°C ; [4] | 5,7 ( 1.0)  2,9 ( 0,6)  0,90 (0,2)  0,67 (0,2) | 1,10 (0,18)  0,92 ( 0,26)  0,35 (0,06)  0,39 (0,14) | 0,55 (0,02)  0,61 (0,02)  0,69 (0,03)  0,62 (0,05) |

Fig. 16 – Experimental data (dots) at 95°C from two different full-fat coconut milks (in duplicates) and the H-B model (line), applying averaged (H-B) model parameters from Table 8. (Corresponding to Fig. 5 Manuscript IV).

We can assume that the heat treatment of the coconut milk to denaturation modified the inter-particle interactions between droplets. The heat treatment increased droplet-droplet adhesion, which promoted the stability.

The theory of colloidal glasses is used in paper IV to obtain a structured discussion about the role of concentration, particle size and interaction for the obtained stability and rheology

1. **Conclusions**

The main objective of this study was to explore some potential functionalities of coconut proteins, with the aim of expanding the range of applications of coconut-based products. The objective was partially achieved, as coconut milk could be stabilised against creaming, as shown by the potential of protein denaturation under arrested state. Gel formation was another potential functionality where the coconut proteins performed well. These findings enable possible replacement of some commercial additives currently used in the coconut milk industry. The limitation involved emulsification, as coconut proteins did not show good emulsifying capacity. The coconut protein-based emulsions flocculated and creamed.

Fresh coconut milk is a very unstable emulsion because of large droplet sizes, in the range of 10-20 µm. High-pressure homogenisation only reduces the droplets down to 3-5 µm (primary droplet size), which is still far from the size of those of homogenised cow milk (< 1 µm), because the coconut proteins have limited emulsifying quality. High-pressure homogenisation applied to emulsions as a single treatment is ineffective, even combined with heat treatment, if the arrested colloidal glass state is not reached.

The wet processing method developed here was able to recover an overall yield of 70% of total coconut endosperm protein, in the form of coconut milk proteins, milk sediment proteins and press-cake protein fraction. Wet processing of coconut is a promising technology, which adds the advantages of simultaneously recovering coconut milk, coconut oil and food grade coconut protein, on a fresh basis. However, further studies are required to develop this technology to meet the oil yields currently provided by the dry processing technology.

Protein solubility, an important physicochemical property, was different from the three protein fractions obtained under the wet processing method. The alkaline press-cake fraction was water soluble over a wide range of pH, but showed minimum solubility at pH 3-4, which is close to isoelectric conditions. The coconut milk sediment was practically insoluble in deionised water, a property of globulins. This protein fraction was closely related to cocosin, the major coconut globulin protein, even in terms of SDS-PAGE pattern.

Heating full-fat coconut milk to protein denaturation at 95°C was found to exhibit yield stress in the range of 1-10 [Pa], supporting the idea of coconut milk emulsion forming a glass structure with stabilising capacity. This structure showed great improvement in the stability of coconut milk against creaming. High-pressure homogenisation, although breaking the glass structure, converting it into a fluid-like state, this helped in preventing coalescence by dispersing the droplets over a texturised medium. The structure was immediately reconstituted as soon as the shear stress was suspended.

1. **Future outlook**

The physical instability of coconut milk is certainly the most important issue of concern in the coconut industry. Industries wish to bring to the market more natural and stable products with appealing characteristics. The use of commercial additives for stabilisation of coconut milk appears to be controversial, and thorough studies and eventually a replacement by more natural treatments that preserve the identity of coconut products are recommended.

This study shows the possibilities of expanding the utilisation of coconut-based products, by exploring appropriate functionalities of coconut proteins such as the denaturation and glass structure, and the gelation. However, further work is required to provide precise information on the properties of such systems. A full characterisation of coconut proteins is an important issue for expanding the coconut sector. Complete coconut proteome sequencing is a long-term goal to reach a comprehensive characterisation of coconut proteins and their potential functionalities as ingredients in the food industry.

1. **Acknowledgements**

The author would like to thank his family for the constant support throughout the project period, despite the long distance. Warm love and hugs to his wife and sons, Sonia Chambal, Nilsa Chambal, Yuri Chambal and Winnil Chambal…

He also would like to thank the supervisors, Professors Petr Dejmek, Björn Bergenståhl and Federico Gómez Galindo, for their clear guidance through all the steps of the work. Thanks are also extended to SIDA (The Swedish International Development Agency) for funding the present work and also the support provided by the Eduardo Mondlane University, the author’s home institution.

The author acknowledges the close and stimulating interactions with the colleagues at the Department of Food Technology of Lund University. Special thanks to Katarzyna Dejmek who he shared for years, the same office. The innebandy team, Eric, Emma, Petr, Marilyn, Juan, Stina and Sander is unforgettable, for the great times they enjoyed the games.

He also thanks Tec-Pro, the program coordinated by Dr. Carlos Lucas and Jose da Cruz Francisco for the entire support for the project. The Finance Direction at Eduardo Mondlane University was grateful in providing the necessary funds, right in time, to cover all the expenditures during project development. Director Orton Malipa, Chissano and Benedito Zavala are especially thankful for their grateful job.

So, in general the author would like acknowledge everyone who has directly or indirectly been involved in this project. Many thanks to Mr Samuel, in behalf of “Associacao Josina Machel”, in Inhambane, who provided coconuts in the early stages of the project. Special thanks to Leslie Walk who helped in polishing the English writing, to make the thesis more readable. Thank you all!

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