Exploring novel food proteins and processing technologies

A case study on quinoa protein and high pressure – high temperature processing

Geraldine Avila Ruiz

Thesis committee

Promotor

Prof. Dr M.A.J.S. van Boekel

Professor of Product Design and Quality Management

Wageningen University

Co-Promotors

Dr G. Sala

Researcher, Wageningen UR Food & Biobased Research

Wageningen University

Dr M.A. Stieger

Associate Professor, Division of Human Nutrition

Wageningen University

Other members

Prof. Dr H. Gruppen, Wageningen University

Dr H. de Vries, INRA, Montpellier, France

Dr L. Pouvreau, NIZO, Ede

Dr A. Sein, DSM, Delft

This research was conducted under the auspices of the Graduate School Vlag (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Exploring novel food proteins and processing technologies

A case study on quinoa protein and high pressure – high temperature processing

Geraldine Avila Ruiz

Thesis

submitted in fulfillment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus Prof.

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 4 November 2016

at 1.30 p.m. in the Aula.

Geraldine Avila Ruiz

Exploring novel food proteins and processing technologies,

152 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)

With references, with summary in English

ISBN: 978-94-6257-909-5

DOI: 10.18174/388766

Contents

Chapter 1

General Introduction

1. The demand for novel food proteins and processing technologies

A growing world population and urbanisation have resulted in the need for sustainable alternatives for protein production to ensure world food security. Therefore, efforts are required to develop safe protein foods with high quality. In the framework of a research programme initiated by Wageningen University and Research Centre, the IPOP/Customised Nutrition programme, such efforts were made for human and animal nutrition. As part of this programme, this thesis focussed on protein foods, and their processing, for human nutrition.

Alternative protein sources are being explored for their potential as more sustainable and healthy alternatives compared to traditional protein sources. This process has been termed as the protein transition [1]. Novel proteins can be sourced from plants, fungi, algae, microorganisms or more sustainable animals (e.g. insect) [2]. Before a novel protein from a more sustainable source can be used in production of foods, the main questions that need to be answered are: what are the properties that the alternative protein sources provide and can those help in the development of high-quality protein foods?

For protein foods, a high quality refers to a high nutritional value, but it also includes aspects such as being appealing, tasty and safe to consume. Food quality is an important factor that is influenced by processing. Novel processing technologies have been investigated to improve food quality compared to traditional thermal techniques [3]. Examples are high pressure processing, pulsed electric fields, radiofrequency and cold plasma. Before a novel processing technology can be applied in food production, the main question that needs to be answered is: can the novel processing technologies improve the quality of protein food?

The general aim of this thesis was to explore the properties of a novel food protein and the potential of a novel processing technology for the development of high-quality protein foods. For this, quinoa was chosen as an alternative protein source and HPHT processing was chosen as a novel processing technology.

2. Quinoa protein

Quinoa (Chenopodium quinoa Willd.) is a grain native to the Andean highlands in South America and has recently gained global popularity due to its high nutritional value [4]. In this respect, quinoa has been claimed to be able to contribute to world food security by the FAO, which named 2013 the "International Year of Quinoa" [5]. Quinoa protein is often described as being high in quantity and quality compared to other protein sources. Several studies have emphasised the high protein content of quinoa, typically around 15 w/dw%, compared to common cereals, like rice, maize, barley, rye and sorghum [6-9]. However, the protein content of quinoa is lower or comparable to several other plant-based protein sources such as soybean, pea, lupine and algae [1]. Quinoa protein is high in lysine, a limiting amino acid in cereal grains, as well as in methionine and cysteine, two limiting amino acids in legumes [6-9]. Therefore, quinoa has been considered a complete protein source, having a similar essential amino acid composition and protein efficiency ratio to casein.

The protein in quinoa seed is mainly found in the embryo (57% of total protein) and endosperm (Figure 1), where it is stored in the form of protein bodies [8]. The structure, exact location or association of quinoa protein bodies with other seed components is not known. Quinoa protein consists of two major protein fractions (44-77% of total protein): the salt-soluble 11S globulin (about 37% of total protein), called chenopodin, and the water-soluble 2S albumin (about 35% of total protein). Chenonpodin is similar in structure to glycinin, the 11S globulin of soy. As a hexamer, it consists of six pairs of acid and basic polypeptides, which have molecular weights of 20-25 kDa and 30-40 kDa, respectively. The polypeptides are linked to each other by disulphide bonds. The 2S albumin fraction is composed of polypeptides with molecular weight of 8-9 kDa. Minor protein fractions have been reported to be glutelins (13-29% of total protein) and prolamins (0.5-7% of total protein) [10]. Due to the low prolamin content, quinoa is recognised as being gluten-free and, therefore, non-allergenic [10-12].

The physicochemical and functional properties of quinoa protein had been studied to a limited extent when this thesis started in 2012 [8] (Figure 2). The properties were protein

purity, protein yield, amino acid composition, thermal properties, solubility, water holding capacity, and foaming and emulsifying properties. These will be described in the following. All studies used solvent-based extraction (conventional wet fractionation) to isolate quinoa protein from the seed and examine it.

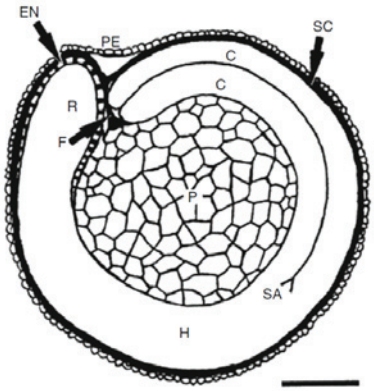


Figure 1. Chenopodium quinoa: median longitudinal section of the grain. Pericarp (PE) covers the seed. The embryo consists of a hypocotylradicle axis (H) and two cotyledons (C). Endosperm (EN) is present in the micropylar region. F, Funicle; P, perisperm; PE, pericarp; R, radicle ; SA, shoot apex. Bar = 500 µm. (Copyright Oxford University Press. Reproduced with permission.) [13]

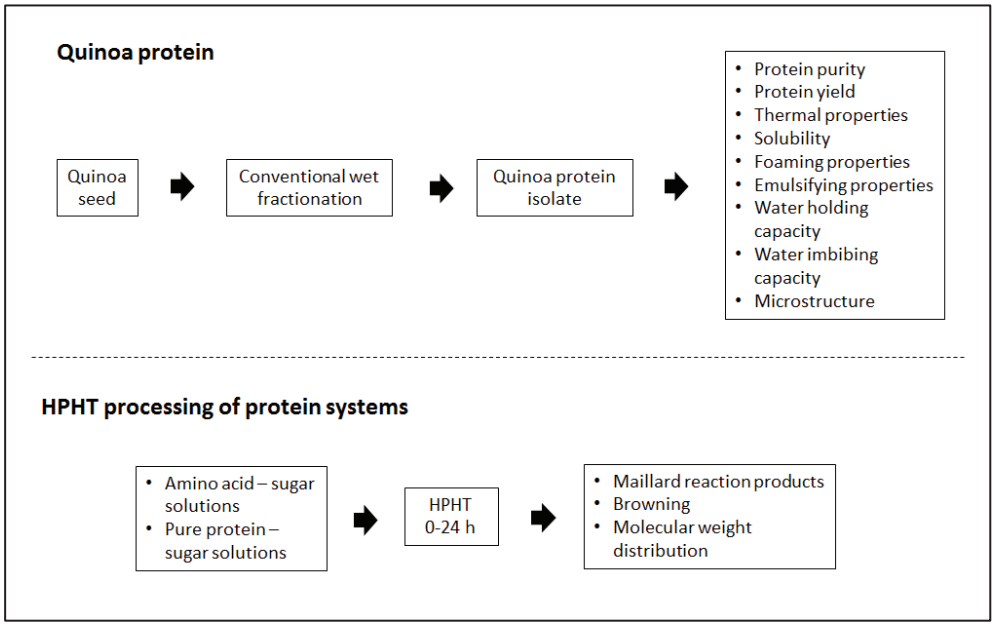


Figure 2. Overview of aspects described in literature on quinoa protein and HPHT processing of protein systems up to 2012.

Protein purities (protein content on a dry matter basis) from 46 to 89 w/dw% in quinoa protein isolates (QPI) were obtained by conventional extraction [14-16]. Protein purities in the same order of magnitude have been reported for the extraction of pea and lupine protein [17,18]. Protein yields (% protein obtained from seed) for QPIs varied from 24 to 56%. These protein yields are overall higher than for pea protein isolates (28%) [19]. For QPI, it was shown that protein purity and yield increased with extraction pH [16,20], similar to lupine protein extraction [18].

QPIs obtained at extraction pH 9 and 11 contained essential amino acid levels that were sufficient, according to FAO and WHO, when consuming the recommended amount of protein for adults and children of 10 to 12 years [8]. When comparing the essential amino acid compositions of the two QPIs to the quinoa seed, the compositions were found to be similar [20,9]. This means that the extraction process does not significantly affect essential amino acid content. An extraction pH of 11 resulted in similar contents of essential amino acids as found in soy protein isolate and similar or higher contents of histidine, methionine, cysteine and tryptophan than in casein. At extraction pH 9, QPI has a more

balanced composition in essential amino acids than commercially available pea protein isolate [21] and a slightly more balanced composition than QPI obtained at pH 11.

When analysing the thermal properties of quinoa protein by DSC, QPI obtained at pH 9 showed an endotherm between 85.6 and 103.1°C, whereas QPI obtained at pH 11 showed no endotherm. The endotherm was attributed to chenopodin, as it was found to have a denaturation temperature of 98.1 ± 1°C and a denaturation enthalpy of 12.4 ± 1.6 J/g [20]. This means that the thermal stability of quinoa protein is higher compared to whey protein (76.4°C for whey proteins overall) and in the range of other globulins of vegetable origin, such as those in soy (92°C), sunflower (95°C) and broadbean (94°C) [22,23]. The fact that QPI obtained at pH 11 showed no endotherm peak with DSC indicates complete denaturation compared to extraction pH 9. A positive correlation between extraction pH and protein denaturation has also been shown by Martinez & Anon (1996) for protein isolates from amaranth, a grain from the same family as quinoa. An explanation for this might be that at higher pH, more negative charges on the protein repulse each other, thus the protein structure unfolds.

Solubility profiles of QPI in a pH range between 3 and 12 mostly showed an inversed bell shape curve with the highest solubility at pH 7-12 [24,25,16]. However, Abugoch et al. (2008) reported that solubility continuously increased from pH 3 to 11. The solubility of QPIs at pH 7 varied considerably from 20 to 95%, depending on the presence of saponins, the extraction technique and the quinoa variety. The solubility of QPI is comparable to the solubility of soy protein isolate (80%) and of commercially available pea protein isolate (60% at pH 7) [21,26].

The water holding capacity of QPIs obtained at extraction pH 9 and 11 was similar (3-4 ml water/g protein) in a pH range between 3 and 9, and comparable to that of soy protein isolates [20]. The water imbibing capacity was higher for protein extracted pH 11 (1.7 ml of water/g of isolate) compared to protein extracted at pH 9 (2.6 ml of water/g of isolate) [20]. The values were in the range reported for soy protein isolates.

Foaming capacity (defined as the percentage of initial solution volume) of QPI obtained at extraction pH 9 was found to be 204-246%, which is higher than the foaming capacity of egg white (92%) under similar conditions [15]. Foam stability (defined as percentage of initial foam volume after 30 min standing at room temperature) was shown to be 35%,

which is higher compared to foam stability of soy protein (28%) and lower than that of egg white protein (60%) under similar conditions. Another study reported much lower values of foaming capacity (25%) and foam stability (35%) for QPI's obtained at presumably higher extraction pH [25]. In the study in question, NaOH was used for protein extraction yielding an estimated extraction pH of around 12. However, the buffering effect of proteins will probably lower the pH but it is not certain to which extent.
Emulsifying properties of QPI obtained at extraction pH 9 varied among QPIs with and without saponins and were higher or lower compared to the emulsifying properties of soy and egg white protein [15].

As a result, QPI has been claimed to be a promising functional ingredient to be used in several foods and beverage products, depending on extraction conditions [20,15,14]. However, to this date, to the best of our knowledge, quinoa protein in a concentrated form does not yet exist on the market [27]. On the other hand, the studies mentioned above focussed only on some physicochemical and functional properties of QPIs and under a limited set of conditions. Functional properties of food proteins, which are in turn determined by their physicochemical properties, can also be influenced by processing conditions post-extraction, as during the processing of final products. Therefore, it is important to further study the impact of extraction as well as processing conditions on a variety of physicochemical and functional properties. The effect of several extraction pH values and heat treatment on protein properties has not yet been investigated. Therefore, in this thesis the effects of extraction pH and heat treatment on several of physicochemical and functional properties were studied to validate previously studied protein properties (protein purity, protein yield, solubility and thermal properties) and to explore new functional protein properties (digestibility, protein aggregation and gelation behaviour) of QPIs.

The conventional wet fractionation method to obtain quinoa protein might not be the best for applications at large scale because the use of organic chemicals, such as hexane, NaOH and HCl, often denatures the protein, possibly leading to a lower protein digestibility. Also, the use of organic chemicals is not in line with consumer demand for

"clean-label" and "natural" food, which have become top trends since 2009, according to trend reports from Innova Market Insights [27]. The use of organic chemicals, which are used in the extraction process of ingredients and are subsequently removed from the end product, does not need to be declared on the label. Nevertheless, the increasing demand for transparency might not work in favour of using such chemicals. Furthermore, the conventional wet fractionation method uses high amounts of energy and water, which is costly and not environmental-friendly [28]. A milder and more resource-efficient method might be a hybrid dry and aqueous fractionation method, which was shown to be effective for obtaining protein-rich fractions from pea [19]. This thesis investigated whether the hybrid dry and aqueous fractionation method can be used to obtain protein-rich fractions from quinoa.

3. High pressure – high temperature processing of protein systems

Mild processing of foods and beverages to better preserve food quality has been successfully achieved with various non-thermal techniques. However, high pressure processing has been reported to be the most developed emerging technology [29]. It has been implemented in industry to process food and beverage products, which in number have been growing since 2002 (Figure 3). A total of 645 products supposedly treated with HPP are nowadays on the market. The top five market categories in which the products are segmented are soft drinks (61.6%), followed by meat, fish and eggs (9.5%), dairy (4.0%), sauce and seasonings (3.6%), and hot drinks (3.3%) [27]. To the best of our knowledge, there are no commercial products available that have been treated with HPP at high temperatures (>100°C) for sterilisation purposes. Yet, the use of high temperature – high pressure (HPHT) has been found to be promising for milder processing of a variety of foods compared to traditional thermal techniques, as it is claimed to lead to improved nutritional and sensorial food properties [30-32].

Due to the increasing demand for protein foods, it is worth exploring the potential of HPHT processing to improve their quality compared to traditional thermal techniques. In protein foods containing also reducing sugars, major quality aspects, such as flavour,

appearance, nutritional value and toxicity, are associated with Maillard reactions (MR) [33]. In sterilized foods, e.g. in dairy-based beverages, high-protein beverages, puddings, creams etc. MR are usually undesired due to browning. In this thesis it was hypothesized that HPHT processing is able to reduce browning in protein-containing model foods compared to traditional retorting. Literature on the effect of high pressure on MR is limited [34]. Generally, it was found that the rates of some MR pathways can be increased or decreased by high pressures depending on the predominant mechanism and specific processing conditions. Some studies showed that pressure accelerated the condensation reaction between amino groups and reducing sugars leading to the formation of Amadori products (the first important intermediates in MR), while other studies found that pressure decelerated amino acid-sugar conjugation, the Amadori rearrangement and the degradation of Amadori rearrangement products. Regarding the formation of advanced MR products (i.e., beyond Amadori products) and browning, it was reported that pressure retards or promotes these processes, depending on the pH.

However, experimental conditions chosen in all these studies were far away from the conditions occurring in industrial applications. By studying for example solutions of single amino acids and sugars, the influence of structure and conformation of proteins on MR is not taken into account. Furthermore, in these studies processing times ranged from 0 to 24 h. For industrial applications, processing times of 3-5 min have been shown to be sufficient [35]. Therefore, this thesis investigated the effects of pressure on MR and physicochemical properties of protein-sugar solutions under conditions closer to industrial applications (i.e. processing time of 3-15 min).

To the best of our knowledge, only one study investigated the influence of pressure on MR in solutions of proteins and sugars. Buckow et al. (2011) found that protein (BSA) – sugar (glucose) conjugation decreased with increasing pressure during HPHT treatments (0.1-600 MPa, 110°C, 0-50 min, pH 9). They also studied protein unfolding and aggregation. However, they did not really link their findings to the chemical properties of the protein. Pressures of 600 MPa for up to 45 min at temperatures higher than 70°C accelerated protein unfolding, possibly exposing more lysine groups, and an increase in high molecular weight compounds were found after HPHT treatment compared to heat treatment. This

seems contradicting, as a decreased protein – sugar conjugation shown for HPHT treatment would actually suggest less exposed amino groups and less protein crosslinking. Proteins had already been found to denature and aggregate by a different mechanism under high pressure treatment compared to heat treatment. It would thus be interesting to investigate more on the relationship between physical protein properties and MR under HPHT. Furthermore, physical protein properties can also determine rheological properties of protein-containing foods. Rheological properties are related to food texture and mouthfeel, which are other major food quality aspects.

In conclusion, literature on HPHT processing of protein systems has up to now almost exclusively focussed on the effect of pressure on MR under conditions far from processing conditions typical of industrial applications (Figure 2). To further explore HPHT processing as an alternative technology to traditional thermal techniques, it was deemed necessary to investigate the impact of HPHT processing on protein properties under more realistic processing and product conditions.

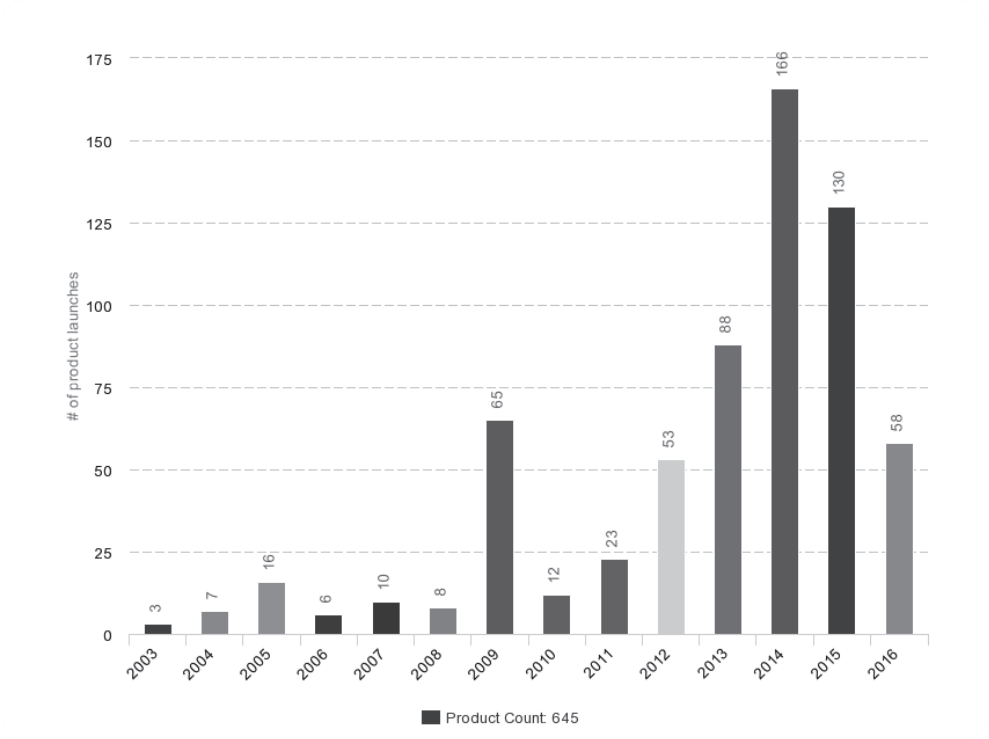


Figure 3. Product launches containing the word "HPP" and synonyms according to the Innova Database on 21 June 2016 [27]. Free text search was used with the formulation: HPP OR "high pressure" OR "high pressure treated" OR "cold pressurised" OR "cold pressurized" OR "fresher under pressure" OR "high pressure pasteurized" OR pascalisation OR pascalization OR "high-pressure". The option "Find exact words" was checked and the market category "Supplements" was unchecked.

4. Aims and outline of this thesis

As mentioned above, the overall aim of this thesis was to explore the properties of a novel food protein and the potential of a novel processing technology for the development of high-quality protein foods. The following specific aims of the thesis were formulated:

1) To study the effect of extraction pH of conventional solvent extraction on physicochemical (protein purity, protein yield, solubility and thermal properties) and functional (digestibility, protein aggregation and gelation behaviour) properties of QPI and to explore a hybrid dry and aqueous fractionation method for obtaining protein-rich fractions from quinoa

2) To examine the effect of pressure during HPHT processing on Maillard reactions, browning and physical protein properties under processing conditions close to industrial applications

This leads to the following outline of this thesis.

In **Chapter 2** the effect of extraction pH on protein purity, protein yield, solubility, thermal properties of untreated QPIs, and on aggregation, gelation and microstructure of heat-treated QPI suspensions is described.

In **Chapter 3** the *in vitro* gastric protein digestibility, thermal properties and protein aggregation of untreated and heat-treated suspensions of QPIs obtained at various extraction pH is assessed. The protein purity and yield of the untreated QPIs were determined. The *in vitro* gastric protein digestibility of wholemeal quinoa flour was assessed and compared to that of the quinoa protein isolates.

In **Chapter 4** a hybrid dry and aqueous fractionation method for obtaining protein-rich fractions from quinoa is examined. Protein purity and yield were evaluated at each step of the process. The hybrid dry and aqueous fractionation method is compared to the conventional wet fractionation method for protein purity, protein yield and water use.

In **Chapter 5** the effect of pressure during HPHT processing on browning, Maillard reaction products, pH, viscosity and aggregation in whey protein isolate – glucose/trehalose solutions was analysed. To elucidate the impact of pressure, HPHT treatments were compared to HT treatments.

In **Chapter 6** the main findings of this thesis are summarised, which are then used to discuss the findings within a wider context. The discussion covers the optimisation of protein yield and purity of QPIs, the potential to replace current food proteins with QPIs, the market potential of QPIs and the potential of HPP to design protein foods. The chapter is closed with opportunities and challenges for the future.

5. References

1. van de Velde F (2016) Protein Transition: How Fesible are the Alternatives? The World of Food Ingredients.
2. Boland MJ, Rae AN, Vereijken JM, Meuwissen MPM, Fischer ARH, van Boekel MAJS, Rutherfurd SM, Gruppen H, Moughan PJ, Hendriks WH (2013) The future supply of animal-derived protein for human consumption. Trends in Food Science & Technology 29 (1):62-73.
3. Sun D-W (2014) Preface to the 2nd Edition. In: Emerging Technologies for Food Processing (Second Edition). Academic Press, San Diego, p xi.
4. González JA, Eisa SSS, Hussin SAES, Prado FE (2015) Quinoa: An Incan Crop to Face Global Changes in Agriculture. In: Quinoa: Improvement and Sustainable Production. John Wiley & Sons, Inc, pp 1-18.
5. Ruiz KB, Biondi S, Oses R, Acuña-Rodríguez IS, Antognoni F, Martinez-Mosqueira EA, Coulibaly A, Canahua-Murillo A, Pinto M, Zurita-Silva A, Bazile D, Jacobsen S-E, Molina-Montenegro MA (2014) Quinoa biodiversity and sustainability for food security under climate change. A review. Agronomy for Sustainable Development 34 (2):349-359.
6. Graf BL, Rojas-Silva P, Rojo LE, Delatorre-Herrera J, Baldeón ME, Raskin I (2015) Innovations in Health Value and Functional Food Development of Quinoa (Chenopodium quinoa Willd.). Comprehensive Reviews in Food Science and Food Safety.
7. Vega-Galvez A, Miranda M, Vergara J, Uribe E, Puente L, Martinez EA (2010) Nutrition facts and functional potential of quinoa (Chenopodium quinoa willd.), an ancient Andean grain: a review. Journal of the science of food and agriculture 90 (15):2541-2547.
8. Abugoch L, Castro E, Tapia C, Añón MC, Gajardo P, Villarroel A (2009) Stability of quinoa flour proteins (*Chenopodium quinoa* Willd.) during storage. International Journal of Food Science and Technology 44 (10):2013-2020.
9. Bhargava A, Srivastava S (2013) Chemistry. In: Bhargava A, Srivastava S (eds) Quinoa - Botany, Production and Uses. CABI, Oxfordshire, pp 185-239.
10. Kozioł MJ (1992) Chemical composition and nutritional evaluation of quinoa (Chenopodium quinoa Willd.). Journal of Food Composition and Analysis 5 (1):35-68.
11. Galwey NW (1992) Proceedings of the European Symposium on Industrial Crops and Products The potential of quinoa as a multi-purpose crop for agricultural diversification: a review. Industrial Crops and Products 1 (2):101-106.
12. Berti C, Ballabio C, Restani P, Porrini M, Bonomi F, Iametti S (2004) Immunochemical and Molecular Properties of Proteins in Chenopodium quinoa. Cereal Chemistry Journal 81 (2):275-277.
13. Prego I, Maldonado S, Otegui M (1998) Seed Structure and Localization of Reserves inChenopodium quinoa. Annals of Botany 82 (4):481-488.
14. Scanlin L, Stone M (2009) Quinoa protein concentrate, production and functionality. Google Patents.

15. Lindeboom N (2005) Studies on the characterization, biosynthesis and isolation of starch and protein from quinoa (Chenopodium quinoa Willd.). Ph.D. Thesis, University of Saskatchewan, Canada
16. Aora JCC, Alvarado K (2009) Aislados proteínicos de granos altoandinos *chenopodiaceas*; quinua "*chenopodium quinoa*" – cañahua "*chenopodium pallidicaule*" por precipitación isoeléctrica. Revista Boliviana de Química 26 (1):12-20.
17. Boye J, Zare F, Pletch A (2010) Pulse proteins: Processing, characterization, functional properties and applications in food and feed. Food Research International 43 (2):414-431.
18. Berghout JAM, Pelgrom PJM, Schutyser MAI, Boom RM, van der Goot AJ (2015) Sustainability assessment of oilseed fractionation processes: A case study on lupin seeds. Journal of Food Engineering 150:117-124.
19. Pelgrom PJM, Boom RM, Schutyser MAI (2015) Functional analysis of mildly refined fractions from yellow pea. Food Hydrocolloids 44:12-22.
20. Abugoch LE, Romero N, Tapia CA, Silva J, Rivera M (2008) Study of some physicochemical and functional properties of quinoa (Chenopodium quinoa willd) protein isolates. Journal of Agricultural and Food Chemistry 56 (12):4745-4750.
21. Roquette Nutralys Pea Protein. http://www.pea-protein.com/. Accessed 17 June 2016
22. Wijayanti HB, Bansal N, Deeth HC (2014) Stability of Whey Proteins during Thermal Processing: A Review. Comprehensive Reviews in Food Science and Food Safety 13 (6):1235-1251.
23. Martínez EN, Añón MC (1996) Composition and Structural Characterization of Amaranth Protein Isolates. An Electrophoretic and Calorimetric Study. Journal of Agricultural and Food Chemistry 44 (9):2523-2530.
24. Chauhan GS, Eskin NAM, Mills PA (1999) Effect of saponin extraction on the nutritional quality of quinoa (*Chenopodium quinoa* Willd.) proteins. J Food Sci Tech 36 (2):123-126.
25. Aluko RE, Monu E (2003) Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science 68 (4):1254-1258.
26. Jiang J, Xiong YL, Chen J (2010) pH Shifting Alters Solubility Characteristics and Thermal Stability of Soy Protein Isolate and Its Globulin Fractions in Different pH, Salt Concentration, and Temperature Conditions. Journal of Agricultural and Food Chemistry 58 (13):8035-8042.
27. Innova Database (2016) Innova Market Insights. http://www.innovadatabase.com/. Accessed 21 June 2016
28. Schutyser MAI, van der Goot AJ (2011) The potential of dry fractionation processes for sustainable plant protein production. Trends in Food Science & Technology 22 (4):154-164.
29. Knorr D, Froehling A, Jaeger H, Reineke K, Schlueter O, Schoessler K (2011) Emerging Technologies in Food Processing. Annual Review of Food Science and Technology 2 (1):203-235.
30. Vervoort L, Van der Plancken I, Grauwet T, Verlinde P, Matser A, Hendrickx M, Van Loey A (2012) Thermal versus high pressure processing of carrots: A comparative

pilot-scale study on equivalent basis. Innovative Food Science & Emerging Technologies 15:1-13.
31. Sevenich R, Bark F, Crews C, Anderson W, Pye C, Riddellova K, Hradecky J, Moravcova E, Reineke K, Knorr D (2013) Effect of high pressure thermal sterilization on the formation of food processing contaminants. Innovative Food Science & Emerging Technologies 20:42-50.
32. Kebede BT, Grauwet T, Mutsokoti L, Palmers S, Vervoort L, Hendrickx M, Van Loey A (2014) Comparing the impact of high pressure high temperature and thermal sterilization on the volatile fingerprint of onion, potato, pumpkin and red beet. Food Research International 56:218-225.
33. Hellwig M, Henle T (2014) Baking, Ageing, Diabetes: A Short History of the Maillard Reaction. Angewandte Chemie - International Edition 53 (39):10316-10329.
34. Martinez-Monteagudo SI, Saldaña MDA (2014) Chemical Reactions in Food Systems at High Hydrostatic Pressure. Food Engineering Reviews 6 (4):105-127.
35. De Vleeschouwer K, Van der Plancken I, Van Loey A, Hendrickx ME (2010) The Effect of High Pressure−High Temperature Processing Conditions on Acrylamide Formation and Other Maillard Reaction Compounds. Journal of Agricultural and Food Chemistry 58 (22):11740-11748.

Chapter 2

Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein isolate from quinoa

This chapter has been published as: Avila Ruiz G, Xiao W, van Boekel M, Minor M, Stieger M (2016) Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein from sweet quinoa (Chenopodium quinoa Willd) Food Chemistry 209:2013-10.

1. Abstract

The aim of this study was to determine the influence of extraction pH on heat-induced aggregation, gelation and microstructure of suspensions of protein isolates extracted from quinoa (Chenopodium quinoa Willd). Quinoa seed protein was extracted by alkaline treatment at various pH values (pH 8 (E8), 9 (E9), 10 (E10) and 11 (E11)), followed by acid precipitation. The obtained protein isolates were freeze dried. The protein isolates E8 and E9 resulted in a lower protein yield as well as less protein denaturation. These isolates also had a higher protein purity, more protein bands at higher molecular weights, and a higher protein solubility in the pH range of 3 to 4.5, compared to the isolates E10 and E11. Heating the 10% w/w protein isolate suspensions E8 and E9 led to increased aggregation, and semi-solid gels with a dense microstructure were formed. The isolate suspensions E10 and E11, on the other hand, aggregated less, did not form self-supporting gels and had loose particle arrangements. We conclude that extraction pH plays an important role in determining the functionality of quinoa protein isolates.

2. Introduction

Quinoa is an Andean grain that has recently been gaining in popularity around the world. Quinoa is considered to have a high nutritional value, mainly because of the large amount of good quality proteins [1]. The total protein content of quinoa (12-23%) is, on average, higher than that of rice, corn and barley. The amino acid profile of quinoa has been reported to be better than most cereal and leguminous protein sources. Moreover, quinoa is gluten-free. Therefore, proteins isolated from quinoa have the potential to be used to enrich foods and beverages with protein, improving their nutritional value.

Quinoa protein isolates (QPI) consist mainly of 11S globulins (37% of total protein) and 2S albumins (35% of total protein) [2,3]. Quinoa's 11S globulin, also referred to as chenopodin, has a similar structure to glycinin, the 11S globulin of soy. It is a hexamer consisting of six pairs of acid and basic polypeptides. The acid and basic polypeptides have molecular weights of 20 to 25 kDa and 30 to 40 kDa, respectively, and are linked to each other by disulphide bonds. Quinoa's 2S albumin fraction consists of a heterogeneous population of polypeptides with molecular weights of 8 to 9 kDa [1].

QPIs are obtained from quinoa grains by extraction under alkaline conditions, concentration by acid precipitation and subsequent drying. The potential applications of QPIs in foods and beverages depend on the functional properties of the QPIs, which are in turn affected by the protein's physical, chemical and structural properties [4,5]. These properties are influenced by the extraction conditions, such as the pH of the aqueous extraction liquid [6,4,5,7]. Obtaining QPIs at an extraction pH of 11 leads to protein denaturation, a higher protein content, a lower solubility of the QPIs (in a pH range of 4 to 11), and a higher water imbibing capacity, compared to QPIs extracted at a pH of 9 [4]. Valenzuela et al. (2013) also found extensive protein denaturation but, in addition to this, they observed changes in aggregation, dissociation and structure of quinoa protein extracted at a pH higher than 10. Aora and Alvarado (2009) observed an increasing protein yield as they increased the extraction pH from 7.5 to 10.5. For amaranth protein isolates, an increase in the extraction pH resulted in a decreased thermal stability for pH values of 8 and higher, and a decreased enthalpy of denaturation at pH 11. Furthermore, extraction at pH 8 resulted in albumin-1 and part of the globulins, whereas at a pH higher than 8, albumin-2, glutelin and the remaining globulins were obtained [6].

To the best of our knowledge, the heat-induced aggregation, gelation and microstructure of QPIs have not yet been investigated. Only the cold-induced aggregation and gelation properties (at pH 8.5 and 10.5) have been described [8]. For potential commercial applications of QPIs in foods and beverages, it is important to explore the functional properties of QPIs that have not been further processed, both during and after thermal treatment, as this simulates the processing that food products containing QPIs would undergo.

These studies of functional properties were all carried out on bitter quinoa varieties. Sweet quinoa varieties are saponin-free (<0.11%), and thus need to be processed less after harvesting, which facilitates large-scale production [9,10]. Wageningen University and Research Centre in the Netherlands has been developing sweet quinoa varieties suitable to be grown on a commercial scale in northwest Europe [11,12]. The functional properties of sweet quinoa varieties have not yet been studied. The post-harvest removal of saponins from traditional bitter quinoa varieties has been demonstrated to increase the protein efficiency ratio, but to decrease the nitrogen solubility, emulsifying and foaming

properties [13-15]. Therefore, it is important to verify the influence of the inherent absence of saponins on the functional properties, as well as on the underlying physico-chemical properties and protein content, of protein isolates from sweet quinoa.

The aim of this study was to determine the effect of extraction pH on both the previously studied QPI properties (protein purity, protein yield, molecular weight distribution, thermal properties, solubility) and on the not-yet-studied heat-induced properties (aggregation, gelation, microstructure) of suspensions of QPIs obtained from sweet quinoa. We used the sweet quinoa variety *Atlas*, which is based on breeding lines designed and tested by Mastebroek et al. (2002), and which shows a good agronomic performance.

3. Material and methods

3.1. Materials

Quinoa seeds (*Chenopodium quinoa* Willd) of the sweet variety *Atlas* were supplied by the Agricultural Research Institute (INIA) in Santiago, Chile. Petroleum ether (boiling range 40-60°C) was used (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany).

3.2. Preparation of quinoa protein isolates

Quinoa protein isolates were prepared using a modified method previously described [4]. Quinoa seeds were ground with a Fritsch Mill Pulverisette 14 (Idar-Oberstein, Germany) using a speed of 7000 rpm, and sieved through a 200 µm sieve, to produce flour. The flour was defatted in a soxhlet extractor for 24 hours, using petroleum ether and 17% w/w flour [16]. After defatting, the petroleum ether was removed by evaporation. The defatted flour was suspended in deionized water (10% w/w), and the pH adjusted to 8, 9, 10 and 11 by the addition of 2 N NaOH. These suspensions were stirred for 4 hours at room temperature and stored at 4°C for 16 hours to maximize protein solubilization. Then the suspensions were centrifuged at 10°C for 30 min at 6000g. The pH of the supernatants was adjusted to pH 4.5 using 2N HCl, and the supernatants were centrifuged for 30 min at 13000g and 10°C. The precipitated pellets were re-suspended in deionized water (5%

w/w). To rinse remaining salts the suspensions were centrifuged for 30 min at 13000g and 10°C, re-suspended in deionized water (5% w/w) and neutralized by the addition of 2 N NaOH. The suspensions were frozen by dipping them into liquid nitrogen, and were subsequently freeze-dried for 72 h (Chris Epsilon 2-6D Freeze Dryer, Osterode am Harz, Germany). Finally, the dried protein isolates were ground with a kitchen blender for 1 min to turn them into powder.

3.3. Determination of protein yield and purity

Amounts of 8 to 15 mg QPI were weighed in tin cups and dried overnight at 60°C. The nitrogen content was determined using the Dumas methodology by sample combustion in a Dumas Flash EA 1112, Series NC analyzer (Wigan, UK), and converted to a crude protein percentage using a protein factor of 5.85 [17,18,4]. Measurements were performed in duplicate for isolates obtained in duplicate from two separate extractions. The protein yield was calculated as follows:

$$\text{Protein yield (\%)} = \frac{\text{isolate protein content (\%)} \times \text{isolate weight (g)}}{\text{flour protein content (\%)} \times \text{flour weight (g)}} \times 100$$

$$\text{Protein purity (\%)} = \frac{\text{isolate protein content (\%)} \times \text{isolate weight (g)}}{\text{isolate weight (g)}} \times 100$$

The protein loss was calculated as follows:

$$\text{Protein loss (\%)} = \frac{\text{protein weight at start of each step (g)} - \text{protein weight after each step (g)}}{\text{flour protein content (\%)} \times \text{flour weight (g)}} \times 100$$

3.4. Determination of molecular weight distribution

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine the molecular weight distribution of the quinoa protein isolate fractions, using a method previously described [19]. Polyacrylamide gel electrophoresis (PAGE) was performed using a NuPAGE Electrophoresis System (Invitrogen Corp., Carlsbad, CA). First, the protein suspensions (1% w/w) were prepared in deionized water (pH 6.5±0.1) and centrifuged for 1 min at 13000g. Then the supernatants were diluted with 1 x NuPAGE® LDS Sample Buffer and deionized water, before applying the samples to the gel. NuPAGE® Novex® Bis-Tris Gels (1–200 kDa), containing 12% acrylamide (4% acrylamide stacking gel), were used. The molecular weight markers were from NuPAGE® Novex® (Mark 12™

Unstained Standard, 2.5–200 kDa). The protein bands produced by the electrophoresis were stained with Simply Blue™ SafeStain.

3.5. Solubility measurements

The solubility of the QPIs was determined using a modified method previously described [4]. The QPIs were suspended in deionized water (1% w/w) and stirred for 1 h at room temperature (pH 6.5±0.1). The suspensions were mixed with an Ultra Turrax for 3 min at 4000 rpm, and homogenized (Labho Scope Homogenizer, Delta Instruments, Drachten, Netherlands) at 150 bar for 10 runs. The homogenized suspensions were adjusted to a pH range from 3 to 9, and centrifuged for 30 min at 8500g and 10°C. The protein purity of the supernatants was determined as described in section 2.3., using 200 µl of sample. Measurements were performed in duplicate for isolates obtained in duplicate. The solubility at each pH was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{supernatant protein content (\%)} \times \text{supernatant weight (g)}}{\text{isolate protein content (\%)} \times \text{isolate weight (g)}} \times 100$$

We define solubility as the percentage protein remaining in solution (protein solubility) after centrifuging the protein suspension for 30 min at 8500g and 10°C, using a Centrifuge 5430 R (Eppendorf AG, Hamburg, Germany), assuming that not all protein is molecularly dissolved but in suspension. To obtain the mass of the supernatant, the supernatant was weighed.

3.6. Particle size determination

The protein suspensions (1% w/w) were prepared in the same way as for the solubility analysis, for a pH range of 3 to 9. Instead of centrifuging, the suspensions were filtered with a 0.45 µm diameter filter. The particle size of the filtrates was quantified with a Malvern Zetasizer Nano (Malvern Instruments, Worcestershire, UK), using a modified method previously published [20]. The z-averaged hydrodynamic diameter (z-average) in nm was recorded. Data were collected at 20°C using a material refractive index of 1.45, a dispersant refractive index of 1.330 and a measurement angle of 173° (backscatter). For each sample, three measurements were performed. Measurements were performed in duplicate for isolates obtained in duplicate.

3.7. Determination of thermal properties

The thermal properties of the QPIs were assessed by Differential Scanning Calorimetry (DSC), using a modified method previously described [4]. Hermetically sealed aluminum pans were filled with 25-50 mg of 20% w/w suspensions of isolates, dispersed in deionized water. The DSC samples were heated from 20 to 140°C at a rate of 10°C/min, using a PerkinElmer Diamond series differential scanning calorimeter, equipped with an intracooler 2P. A double, empty pan was used as reference. The denaturation parameters were calculated using Pyris Software (Version 11, PerkinElmer), with the denaturation temperature (T_d) value corresponding to the maximum transition peak, and the transition enthalpy (ΔH) calculated from the area below the transition peaks. Measurements were performed in duplicate for isolates obtained in duplicate.

3.8. Effect of heating on particle size and gelation properties

For the particle size measurements, 1% w/w suspensions were prepared as described in Section 2.6. The suspensions were filtered through a 0.2 µm-diameter filter. The measurements were made at temperatures from 20 to 90°C, at intervals of 10°C, with an equilibration time of 5 min after each heating step. To avoid evaporation, the samples were covered with a thin layer of paraffin oil and sealed with a plastic stopper.

For the gelation measurements, a modified previously described method was used [19]. The protein suspensions (10% w/w) were prepared in deionized water and stirred for 1 h at room temperature (pH 6.5±0.1). Oscillatory strain tests were performed using a stress-controlled rheometer (Physica MCR 300, Anton Paar, Graz, Austria) equipped with stainless steel and titanium concentric cylinder geometry (CC-10, diameter inner cylinder: 10.00 mm; diameter cup: 10.845 mm). To prevent evaporation, samples were covered with a thin layer of paraffin oil. The samples were heated from 20 to 90°C at a heating rate of 1°C/min, kept at 90°C for 5 min, and cooled to 20°C at a rate of 3°C/min. During the temperature ramp, the storage modulus G’ and loss modulus G’’ were determined, by applying a strain amplitude of 1% at a frequency of 0.1 Hz. The temperature at which G’ started to increase considerably and became greater than the background noise was

designated as the gelation temperature [21]. Measurements were performed in duplicate for isolates obtained in duplicate.

3.9. Determination of microstructure of heat treated quinoa protein isolates

The microstructure of the heat-treated QPIs was analyzed using a modified method previously described [22]. Suspensions of the isolates were prepared in the same way as for the gelation measurements, except that rhodamine B was added to the suspensions before heat treatment. After performing the oscillatory strain tests, the micrographs of the heat-treated suspensions E8, E9, E10 and E11 were obtained using a Confocal Scanning Light Microscope (Zeiss LSM510, Jena, Germany), with an excitation wavelength of 488 nm, emission channel 1 of ≥635 nm (red), emission channel 3 of 545-635 nm (green) and an emission channel 2 of 505-545 nm (cyan). The resolution of the obtained micrographs was 250 x 250 µm.

3.10. Statistical data analysis

Statistical data analysis was performed using SPSS (V19, SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by least-squares difference posthoc testing (LSD) were performed to identify significant differences between mean values. A significance level of $p < 0.05$ was chosen.

4. Results and discussion

4.1. Protein yield and purity

The protein yield significantly increased as the extraction pH increased ($F(3,4)=205.5$; $p < 0.001$), from 36.3 % (g protein/100 g flour) for E8, to 52.0 % for E11 (Figure 1.). This suggests that the solubility of the proteins increased in more extreme alkaline conditions [23,7]. At a more alkaline pH, proteins are increasingly negatively charged due to ionization of the carboxyl groups and deprotonation of the amine groups. As a result, electrostatic repulsion between the negatively charged proteins is enhanced. This increases protein-water interactions and thereby protein solubility.

The protein yield range is in agreement with a previous study on bitter quinoa (*Chenopodium quinoa* Willd) from which a protein yield of 47 % at extraction pH 8 and 0.5 N NaCl was estimated [2]. The protein yields of the present study are slightly lower than the ones calculated based on the data of Aora & Alvarado (2009). A very recent study reported a maximum protein yield of 76.3% at extraction pH 11 and 0.1 N NaCl [24]. This is a very similar maximum yield to that obtained in the present study (at pH 11 yield is 74.3%). For other protein sources, such as paprika and soybean, Guerreo-Ochoa et al. (2015) reported maximum protein yields of 12.2% and 33.0% for extraction pH 9. The protein yield of QPI E9 calculated in the same way was 63.1%. An increase in protein yield with increasing extraction pH was also found by Aora and Alvarado (2009) for quinoa protein, and by Martínez and Añón (1996) for amaranth protein.

The vast majority of protein was lost during the alkalinization and precipitation steps (Figure 1.). In the alkalinization step, the protein loss decreased with increasing extraction pH. The protein yield increased with extraction pH by about the same ratio as the protein loss in the alkalinization step decreased. This indicates that more protein was solubilized from the grain matrix and ended up in the final protein concentrate.

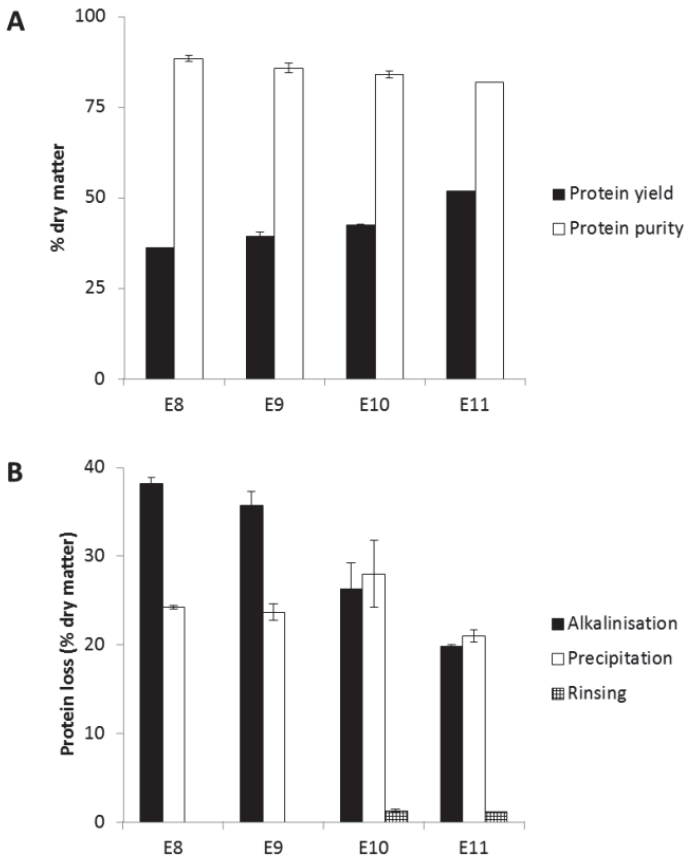


Figure 1. (A) Protein yield and protein purity on dry matter basis of the quinoa protein isolates E8, E9, E10 and E11. (B) Protein loss expressed as amount of protein lost relative to total protein in flour determined as protein content in the pellet of the alkaline suspension (alkalinization), in the supernatant of the precipitated protein (precipitation) and in the supernatant of the rinsed protein (rinsing) of the QPIs E8, E9, E10 and E11. Error bars represent the standard deviation.

Protein purity of the QPIs significantly decreased with increasing extraction pH ($F(3,4)=9.9$; $p < 0.05$) from 88% for E8, to 82% for E11. The decrease in purity may be caused by an increase in the amount of non-protein components co-precipitating with the protein isolates at pH values higher than 9, as theorized by Lestari et al. (2010).

The purity of the saponin-free QPIs obtained in our study was higher than the values previously reported in literature (52 to 85%), even with some studies that used protein factor of 6.25, as compared to the protein factor of 5.85 used in the present study [13,25,15,4,5]. The higher protein purity in the present study might be due to a longer

alkalinization time (16 h) than in most other studies (8-120 min), which allowed more protein to be solubilized from the grain.

The protein purity decreased slightly with increasing extraction pH, in contrast to results shown in the literature [6,4,5].

4.2. Molecular weight distribution

The SDS-PAGE analysis (Figure 2) showed numerous bands of varying intensity in the protein isolates E8, E9, E10 and E11. There are bands at 6kDa, 33kDa, 38 kDa, and 50kDa. For E8, E9 and E10, the most intense bands were found at 50 kDa. These bands could correspond to 11S globulin [2,1]. The bands for E11 were more diffuse and, at lower molecular weights, were more pronounced than the bands of the protein extracts obtained at lower pH. For E8, the high molecular weight fractions dominated the 6kDa fractions. As the extraction pH increased, the protein fractions of lower molecular weight became more prominent, and for E11 they dominated the 50kDa fractions. The SDS profiles indicated that globulin and other high molecular weight protein fractions could be obtained at extraction pH values ranging from 8 to 10. At extraction pH 11 these fractions might have been hydrolyzed into fractions with lower molecular weights, as well as associated through increased hydrophobic interactions and intermolecular disulfide bonds into insoluble aggregates that were removed by centrifugation before performing the electrophoresis [26,23,7]. This would explain the fainter bands at high and intermediate molecular weights for a higher extraction pH. At higher pH values, proteins of lower molecular weight might be more successfully extracted, similar to albumin-2 [6].

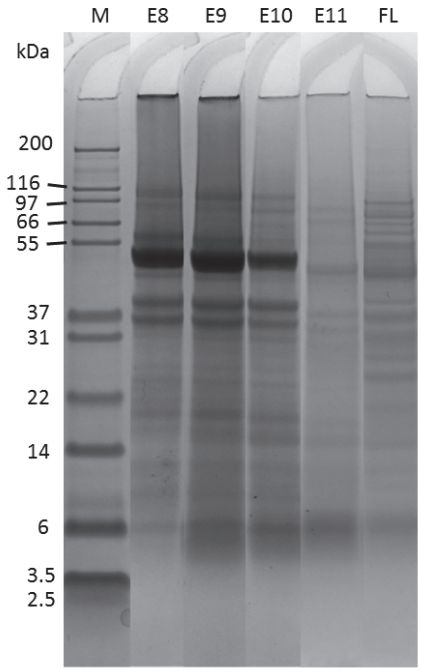


Figure 2. SDS-PAGE profile of the QPIs E8, E9, E10, E11 and defatted quinoa flour. Lane M: molecular weight marker; lane FL: defatted quinoa flour.

For all SDS gels, a considerable amount of protein remained at the top of all lanes that did not penetrate the gel. As 1-200kDa gel was used, this means that a considerable amount of proteins with molecular weights higher than 200 kDa was present in the isolates and in the defatted flour. The SDS profiles of the QPIs were similar to profiles published by Abugoch et al. (2008) and Valenzuela et al. (2013).

The profile of the defatted flour showed even more bands than the protein isolates, however, their intensities were more evenly distributed, probably as a result of the much lower protein concentration. Some of the flour's protein fractions (66-116 kDa, 26-30kDa) were not visible (or were hardly visible) in the isolates, while other fractions (50 kDa, 38 kDa and 33 kDa) were much more prominent in the isolates (E8, E9 and E10) than in the flour. The comparison of the isolates with the defatted flour shows that the alkaline extraction generated a different protein composition to the one originally present in the quinoa grain.

4.3. Thermal properties of quinoa protein isolates

A single endotherm peak at around 97°C (denaturation temperature T_d) was observed for E8, E9 and E10, but not for E11 (Figure 3). This is in agreement with Abugoch et al. (2008), who reported a single endotherm at 98°C for extraction pH 9, and no endotherm at extraction pH 11 for QPIs. Another study, analyzing protein isolates from amaranth, also observed endotherms from 94 to 100°C for extraction pH 9 to 11 [6]. A single peak generally suggests that the protein isolates consisted either of one protein species, or of several species with similar thermostability. The SDS-PAGE results showed that globulin appeared to be the most prominent protein species in isolates E8, E9 and E10. Furthermore, isolated globulin from amaranth has been found to have a major endotherm at 97°C [6]. Globulins from other plant sources have also been shown to have a T_d in this temperature range (soybean T_d = 92°C, broadbean T_d = 94°C, sunflower T_d = 95°C) [27]. Therefore, it is very likely that the endotherm peak from the present QPIs can be attributed to globulin. The high T_d of quinoa globulin shows that the protein is stable up to 97°C. This is the result of numerous remaining hydrophobic interactions and disulfide bonds that connect globulin's acidic and basic subunits to each other. [28,6,4].

There is no obvious relationship between the denaturation temperature and the extraction pH of the QPIs (Figure 3). For amaranth protein, Martínez & Añón (1996) observed only a slight overall decrease of T_d (by 2-3°C) from extraction pH 8 to 11. Other studies, of suspensions from amaranth and sunflower protein, reported a much sharper decrease in T_d (by 10-20°C) from pH 8 to 11 [18,29]. It seems that the extraction pH has much less effect on T_d than the pH of the protein suspension.

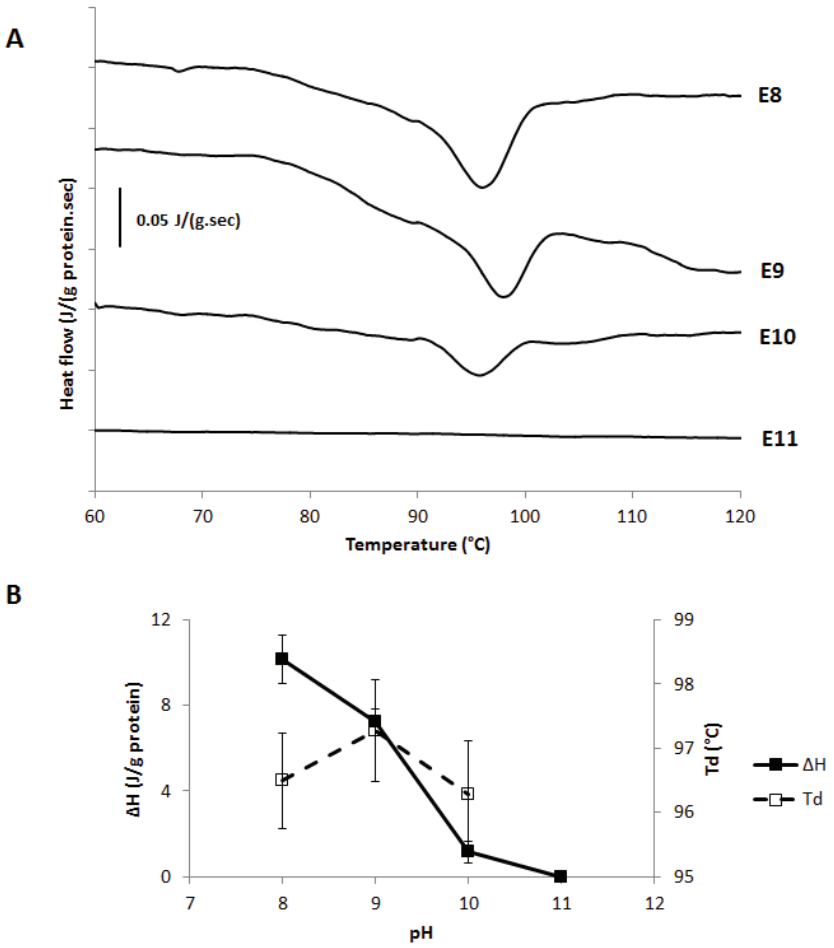


Figure 3. (A) DSC thermograms of QPIs E8, E9, E10 and E11. (B) Enthalpy (ΔH) and denaturation temperature (Td) of the QPIs. Error bars represent standard deviation.

The denaturation enthalpies (ΔH) for the isolates ranged from 0 to 10.2 J/g protein (Figure 3). For E9 the denaturation enthalpy was 7.2 J/g, which is lower than the value that Abugoch et al. (2008) reported for extraction pH 9 (12.4 J/g). For extraction pH 11, no endotherm could be observed in the present study, which is in agreement with Abugoch et al. (2008). The lower denaturation enthalpies compared to the literature might be due to the longer alkalinization step used in our study (16 h in the present study compared to 30 min in the study of Abugoch et al. (2008), which led to more protein denaturation. The denaturation enthalpy is known to be correlated with the content of ordered secondary

structure of a protein [30]. Alkaline treatment with subsequent acid treatment decreases the extent of ordered secondary structure of proteins through disruption of hydrogen bonds and hydrophobic interactions, to the point of irreversible changes in conformation, leading to a more denatured state of the proteins [6].

The denaturation enthalpy significantly decreased with increasing extraction pH ($F(3,4)=47.8$; $p < 0.001$). A higher extraction pH leads to more protein denaturation, which reduces the amount of heat necessary to denature the remaining native protein structure. The thermogram of E11 indicates that the proteins were already denatured, as no endotherm could be detected. The decrease in denaturation enthalpy with an increase in extraction pH is in agreement with studies on quinoa, amaranth and sunflower protein [6,18,29,4].

4.4. Solubility and particle size of QPIs

The solubility curves of the protein isolates E8, E9, E10 and E11 in aqueous solution, over a pH range of 3 to 9, have an inverse bell shape (Figure). The solubility values for all isolates ranged from 20 to 60% at pH 3, and from 35 to 73% at pH 7 to 9. The lowest protein solubility, of around 5%, was observed at pH 4 to 6. The low solubility plateau can be attributed to globulins, as they have been found to have the lowest solubility at pH 4 to 6 [2,6,31,32]. Isolate E8 had the highest solubility at pH 3 and 4, while E9 had the highest solubility at pH 7 and 8 compared to the other isolates ($F(2,3)=27.0$; $p < 0.05$) with the exception of E10. The low solubility plateau was at a higher pH value for E8 than for the other isolates. From soybean it is known that the association of the basic subunit with the acidic subunit of the 11S soy protein tends to increase solubility of the basic subunit [28]. SDS-PAGE showed the highest amount of protein fractions corresponding to intact 11S globulin for E8 and E9, which might explain the higher solubility of E8 and E9 at many pH values, compared with E10 and E11. It is known that solubility decreases with molecular weight and increases with surface polarity [28]. Therefore, the lower solubility of E10 and E11 may have resulted from their low molecular weight protein fractions, and the greater degree of denaturation of proteins in general, leading to the exposure of hydrophobic groups and thus decreased surface polarity. The consequence would be increased protein

aggregation, via hydrophobic interactions, to big, insoluble particles, which is in line with the fainter bands of E10 and E11 for higher molecular weights on the SDS-PAGE gel.

The solubility profiles are consistent with those of QPIs reported previously by Chauhan et al. (1999a), Aluko & Monu (2003), Mäkinen et al. (2015), Aora & Alvarado (2009), and in contrast to the solubility profiles reported by Abugoch et al. (2008), where solubility increased with pH continuously from pH 3 to 11. The trend of a higher solubility at lower extraction pH is in agreement with Abugoch et al. (2008), who observed a significantly higher solubility for extraction pH 9 than for pH 11.

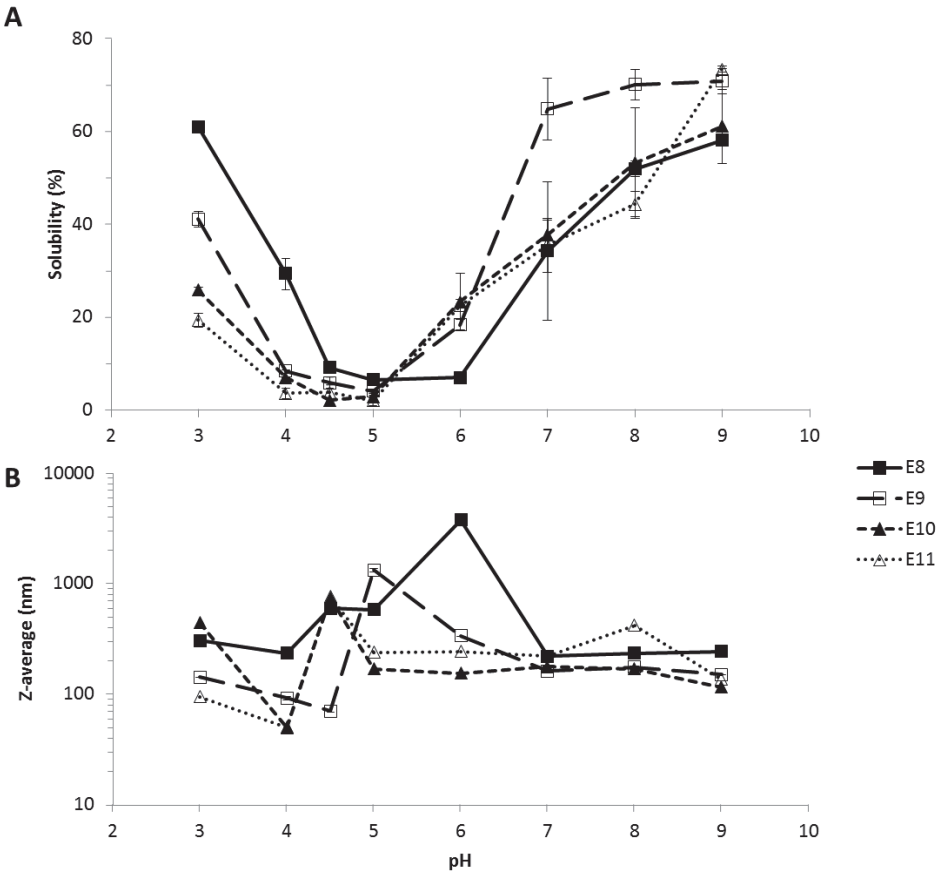


Figure 4. Solubility (A) and z-averaged particle size (B) of the QPIs E8, E9, E10 and E11 in suspension at pH values ranging from 3 to 9.

The z-averaged particle size for the QPIs varied from 50 to 3761 nm over a pH range of 3 to 9 (Figure). The z-averaged particle sizes above 450 nm in the pH range from 4.5 to 6 may be explained by the occasional passage of particles larger than 450 nm through the filter (pore size 450 nm), due to slightly more pressure applied to the syringe to filter the protein suspensions, as a result of a higher resistance. This particularly occurred at pH values where solubility was the lowest, and thus more big particles were present in the protein suspensions. This hypothesis about the correlation of the high z-averaged particle sizes with the low solubility plateau in the pH range of 4.5 to 6 is further confirmed by the observation that the largest particle size of E8 shifted to a higher pH in the same way as its corresponding low solubility plateau.

4.5. Effect of heating on particle size and gelation behavior of quinoa protein isolates

The z-averaged particle size of the QPI suspensions at pH 6 remained constant up to 50°C (Figure 5). From 60°C onwards, the z-averaged particle size was significantly higher for E8, and especially E9, compared with E10 and E11 ($F(3,8)=919.0$; $p < 0.001$).

This suggests that heating induced protein aggregation at temperatures of 50°C and higher, for particles (smaller than 450 nm) extracted at a low pH, while it induced less or no aggregation for QPI particles extracted at a higher pH. It seems that the more denatured proteins resulting from extraction at a higher pH (E10 and E11) could not undergo further association and aggregation at higher temperatures, while the less denatured proteins resulting from extraction at a lower pH (E8 and E9) still had the functional capacity to do so. The aggregation of E8 and E9 may be the consequence of increased disulfide bond formation. In line with this, Mäkinen et al. (2015) reported significant reductions of the free and total SH group content of QPI suspensions that had undergone heat-treatment at pH 8.5. We could also infer that an extraction pH of 9 caused the highest degree of aggregation from 70°C upwards.

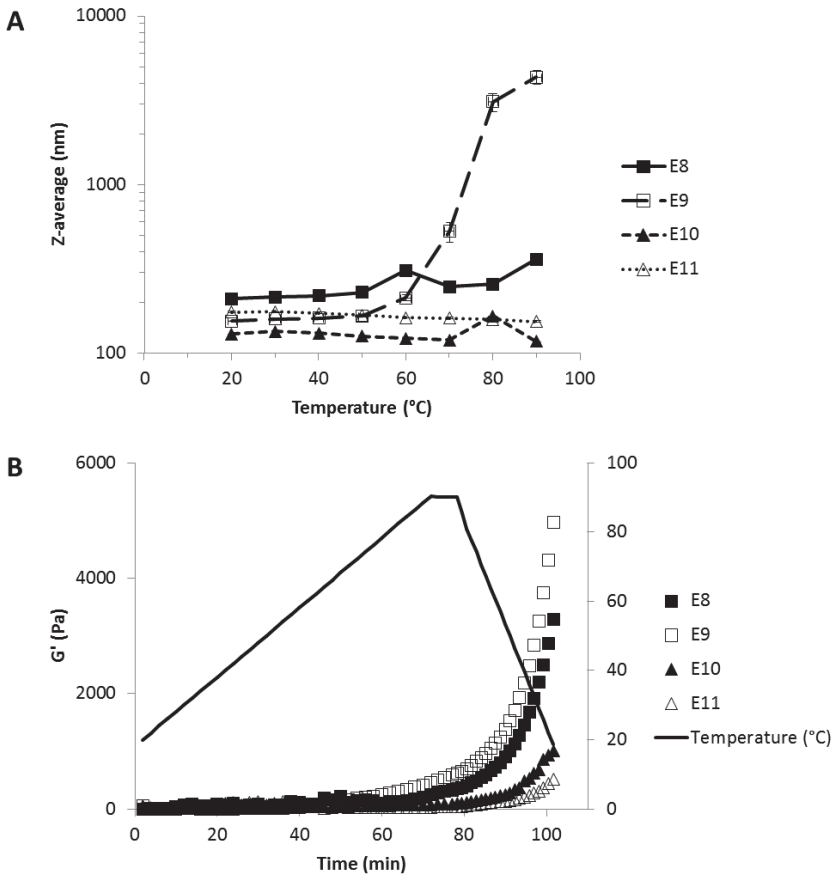


Figure 5. (A) Z-averaged particle size of the QPIs E8, E9, E10 and E11 in suspension at pH 6 as a function of temperature. (B) Storage modulus G' of the QPIs E8, E9, E10 and E11 in suspension (10% w/w) at pH 6.5 as a function of time. Heating and cooling temperature is plotted as a secondary axis.

The G' moduli of the isolate suspensions during heating and subsequent cooling are shown in
Figure 5. The G' values increased considerably for E8 and E9 at around 70°C, while for E10 and E11 the G' value increased only during the cooling phase. The gelation temperature of E8 and E9 (around 70°C) is similar to that of amaranth and pea protein isolates [33,34].

The G' values at the end of the cooling phase for E8 (5000 Pa) and E9 (3300 Pa) were similar to, or higher than, those for amaranth protein (up to 3800 Pa, 10% protein suspension, extraction pH 9), pea protein (2000 Pa, 7.5 and 9.9% protein suspension, extraction pH 8) and sunflower protein (500 Pa, 10% protein suspension, extraction pH 9) reported previously for similar heating profiles [33,31,34]. This suggests that stronger gels

can be formed from quinoa protein than from other plant proteins at comparable protein concentrations.

The G' values of E10 and E11 showed that quinoa protein extracted under strongly alkaline conditions did not gel during heating, and only formed a soft gel during cooling. A reason for this seems to be the higher extent of protein denaturation, which may have led to flocculation and sedimentation of larger particles, and to reduced aggregation of smaller particles, resulting in a weaker tendency to form a network. The higher G' values of E8 and E9 compared to E10 and E11 seemed to result from higher initial solubility and particle sizes, favoring the interaction and aggregation of proteins during heating. When comparing these results to the DSC results, we observed a difference between gelation temperature (around 70°C) and denaturation temperature (around 97°C) for E8 and E9. This difference may be explained by an initial hydration and swelling of the proteins from 60°C to 70°C (as indicated by an increasing particle size), leading to more protein-protein interactions. At about 75°C, DSC thermograms showed the beginning of a heat flow decline with isolates E8 and E9, indicating the start of a phase transition (protein unfolding). The sequence and overlap of the two events could be responsible for an exponential rise of the degree of network formation.

4.6. Microstructure

The microstructure of the heat-treated QPIs differed considerably for E8 and E9, compared with E10 and E11 (Figure 6). The heat treated suspensions of isolates E8 and E9 revealed irregular particles of 15-30 μm embedded in a dense protein matrix, with larger pores deprived in protein. By contrast, the suspensions of isolate E10 and E11 revealed many particles of a smaller size (5-15 μm) and rounder shape, which seemed more loosely arranged in a matrix, with a few small pores deprived in protein.

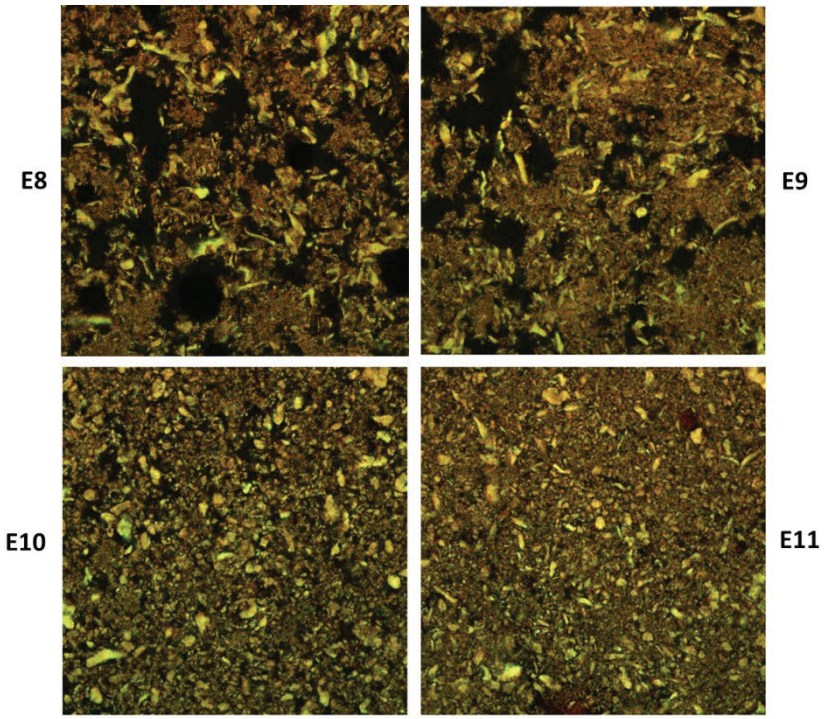


Figure 6. 10% w/w suspensions of the QPIs after heat treatment. Size of pictures is 250 x 250 µm. In green the protein phase is shown.

This suggests that the QPIs obtained at low extraction pH (E8 and E9) formed denser quinoa protein networks during the heat treatment, via particle association, yielding an agglomerated network structure. This structure seems to be responsible for the high G' values. At a high extraction pH, small particles do not seem to interact with each other, while big particles may have flocculated into the background plane, giving the whole a more continuous structure. As a result, this loose and inhomogeneous mass may explain the low G' final values.

Mäkinen et al. (2015) observed a more irregular, aggregated gel structure, with larger pores, for cold-induced QPI gels previously heat-treated at pH 8.5 compared to pH 10.5. This morphology is similar to what CSLM pictures show in the present study at similar pH values, but then of protein extraction instead of heat treatment post-extraction. The heat-treated QPI suspensions from both studies differ, however, in their gelation behaviour,

which reveals the impact of varying the pH at different steps of QPI production and processing on a functional level.

5. Conclusion

We conclude that the extraction pH affected the previously studied properties of QPIs (purity, yield, molecular weight distribution, denaturation and solubility) in a similar way to literature findings. The variation of heat-induced properties (aggregation, gelation and microstructure) with extraction pH, which had not previously been studied, revealed new insights into these properties. QPIs obtained from extraction at pH values below 9 could be used to prepare semi-solid gelled foods. QPIs obtained from extraction at pH values higher than 10 lost the capacity to form a strong gelled network upon heating. These QPIs could be used for beverages or other liquid food applications. Future research could focus on finding such applications for QPIs, but also on maximizing protein yield and purity, while minimizing protein loss.

6. Acknowledgments

The authors gratefully acknowledge the financial support of the “IPOP Customized Nutrition” program of Wageningen University and Research Centre.

7. References

1. Abugoch L, Castro E, Tapia C, Añón MC, Gajardo P, Villarroel A (2009) Stability of quinoa flour proteins (*Chenopodium quinoa* Willd.) during storage. International Journal of Food Science and Technology 44 (10):2013-2020.
2. Brinegar C, Goundan S (1993) Isolation and characterization of chenopodin, the 118 seed storage protein of Quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 41 (2):182-185.
3. Brinegar C, Sine B, Nwokocha L (1996) High-Cysteine 2S Seed Storage Proteins from Quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 44 (7):1621-1623.
4. Abugoch LE, Romero N, Tapia CA, Silva J, Rivera M (2008) Study of some physicochemical and functional properties of quinoa (Chenopodium quinoa willd) protein isolates. Journal of Agricultural and Food Chemistry 56 (12):4745-4750.

5. Aora JCC, Alvarado K (2009) Aislados proteínicos de granos altoandinos *chenopodiaceas*; quinua "*chenopodium quinoa*" – cañahua "*chenopodium pallidicaule*" por precipitación isoeléctrica. Revista Boliviana de Química 26 (1):12-20.
6. Martínez EN, Añón MC (1996) Composition and Structural Characterization of Amaranth Protein Isolates. An Electrophoretic and Calorimetric Study. Journal of Agricultural and Food Chemistry 44 (9):2523-2530.
7. Valenzuela C, Abugoch L, Tapia C, Gamboa A (2013) Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence on film formation. International Journal of Food Science & Technology 48 (4):843-849.
8. Mäkinen O, Zannini E, Arendt E (2015) Modifying the Cold Gelation Properties of Quinoa Protein Isolate: Influence of Heat-Denaturation pH in the Alkaline Range. Plant Foods for Human Nutrition:1-7.
9. Wright KH, Pike OA, Fairbanks DJ, Huber CS (2002) Composition of *Atriplex hortensis*, Sweet and Bitter Chenopodium quinoa Seeds. Journal of Food Science 67 (4):1383-1385.
10. Arendt EK, Zannini E (2013) Quinoa.409-438.
11. Limburg H, Masterbroek HD (1997) Breeding high yielding lines of *Chenopodium quinoa* Willd. with saponin free seed. In: Stølen O, Bruhn K, Pithan K, Hill J (eds) Small Grain Cereals and Pseudo-Cereals. Proc COST 814 workshop, 22–24 February 1996, Copenhagen, Denmark, pp 103–114.
12. Mastebroek HD, van Loo EN, Dolstra O (2002) Combining ability for seed yield traits of *Chenopodium quinoa* breeding lines. Euphytica 125 (3):427-432.
13. Chauhan GS, Cui W, Eskin NAM (1999) Effect of saponin on the surface properties of quinoa proteins. International Journal of Food Properties 2 (1):13-22.
14. Chauhan GS, Eskin NAM, Mills PA (1999) Effect of saponin extraction on the nutritional quality of quinoa (*Chenopodium quinoa* Willd.) proteins. J Food Sci Tech 36 (2):123-126.
15. Lindeboom N (2005) Studies on the characterization, biosynthesis and isolation of starch and protein from quinoa (*Chenopodium quinoa* Willd.). University of Saskatchewan, Canada
16. Pelgrom PJM, Wang J, Boom RM, Schutyser MAI (2015) Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes. Journal of Food Engineering 155 (0):53-61.
17. Becker R, Wheeler EL, Lorenz K, Stafford AE, Grosjean OK, Betschart AA, Saunders RM (1981) A Compositional Study of Amaranth Grain. Journal of Food Science 46 (4):1175-1180.
18. Castellani OF, Martínez EN, Añón MC (1998) Structural Modifications of an Amaranth Globulin Induced by pH and NaCl. Journal of Agricultural and Food Chemistry 46 (12):4846-4853.
19. Yi L, Lakemond CM, Sagis LM, Eisner-Schadler V, van Huis A, van Boekel MA (2013) Extraction and characterisation of protein fractions from five insect species. Food chemistry 141 (4):3341-3348.

20. Zhou C, Qi W, Neil Lewis E, Carpenter JF (2015) Concomitant Raman spectroscopy and dynamic light scattering for characterization of therapeutic proteins at high concentrations. Analytical Biochemistry 472 (0):7-20.
21. Renkema JMS, Knabben JHM, van Vliet T (2001) Gel formation by β-conglycinin and glycinin and their mixtures. Food Hydrocolloids 15 (4–6):407-414.
22. Auty MA, Twomey M, Guinee TP, Mulvihill DM (2001) Development and application of confocal scanning laser microscopy methods for studying the distribution of fat and protein in selected dairy products. The Journal of dairy research 68 (3):417-427.
23. Lestari D, Mulder W, Sanders J (2010) Improving *Jatropha curcas* seed protein recovery by using counter current multistage extraction. Biochemical Engineering Journal 50 (1-2):16-23.
24. Guerreo-Ochoa MR, Pedreschi R, Chirinos R (2015) Optimised methodology for the extraction of protein from quinoa (*Chenopodium quinoa* Willd.). International Journal of Food Science & Technology:n/a-n/a.
25. Aluko RE, Monu E (2003) Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science 68 (4):1254-1258.
26. Mauri AN, Anon MC (2008) Mechanical and Physical Properties of Soy Protein Films with pH-Modified Microstructures. Food Science and Technology International 14 (2):119-125.
27. Ma CY, Harwalkar VR, John EK (1991) Thermal Analysis of Food Proteins. In: Advances in Food and Nutrition Research, vol Volume 35. Academic Press, pp 317-366.
28. Kinsella JE, Damodaran S, German B, Wilcke AMAL (1985) V - Physicochemical and Functional Properties of Oilseed Proteins with Emphasis on Soy Proteins. In: New Protein Foods. Academic Press, pp 107-179.
29. Molina MI, Petruccelli S, Añón MC (2004) Effect of pH and Ionic Strength Modifications on Thermal Denaturation of the 11S Globulin of Sunflower (*Helianthus annuus*). Journal of Agricultural and Food Chemistry 52 (19):6023-6029.
30. Wang M, Hettiarachchy NS, Qi M, Burks W, Siebenmorgen T (1999) Preparation and Functional Properties of Rice Bran Protein Isolate. Journal of Agricultural and Food Chemistry 47 (2):411-416.
31. González-Pérez S, Vereijken JM (2007) Sunflower proteins: overview of their physicochemical, structural and functional properties. J Sci Food Agric 87 (12):2173-2191.
32. Nishinari K, Fang Y, Guo S, Phillips GO (2014) Soy proteins: A review on composition, aggregation and emulsification. Food Hydrocolloids 39:301-318.
33. O'Kane FE, Happe RP, Vereijken JM, Gruppen H, van Boekel MAJS (2004) Heat-Induced Gelation of Pea Legumin: Comparison with Soybean Glycinin. Journal of Agricultural and Food Chemistry 52 (16):5071-5078.
34. Shevkani K, Singh N, Rana JC, Kaur A (2014) Relationship between physicochemical and functional properties of amaranth (*Amaranthus hypochondriacus*) protein isolates. International Journal of Food Science & Technology 49 (2):541-550.

Chapter 3

Denaturation and in vitro gastric digestion of heat-treated quinoa protein isolates obtained at various extraction pH

This chapter is based upon: Avila Ruiz G, Opazo-Navarrete M, Meurs M, Minor M, Sala G, van Boekel M, Stieger M, Janssen A (2016) Denaturation and in vitro gastric digestion of heat-treated quinoa protein isolates obtained at various extraction pH. Food Biophysics 11:184-197.

1. Abstract

The aim of this study was to determine the influence of heat processing on denaturation and digestibility properties of protein isolates obtained from sweet quinoa (*Chenopodium quinoa* Willd) at various extraction pH values (8, 9, 10 and 11). Pretreatment of suspensions of protein isolates at 60, 90 and 120°C for 30 min led to protein denaturation and aggregation, which was enhanced at higher treatment temperatures. The *in vitro* gastric digestibility measured during 6 hours was lower for protein extracts pre-treated at 90 and 120°C compared to 60°C. The digestibility decreased with increasing extraction pH, which could be ascribed to protein aggregation. Protein digestibility of the quinoa protein isolates was higher compared to wholemeal quinoa flour. We conclude that an interactive effect of processing temperature and extraction pH on *in vitro* gastric digestibility of quinoa protein isolates obtained at various extraction pH is observed. This gives a first indication of how the nutritional value of quinoa protein could be influenced by heat processing, protein extraction conditions and other grain components.

2. Introduction

Quinoa has a balanced amino acid profile with high amounts of lysine and methionine. Sweet varieties of quinoa are more promising to provide high-quality protein in a more economic and sustainable way than the bitter quinoa varieties. More economic because saponins do not have to be removed, which saves in post-harvest processing. More sustainable because sweet varieties have been successfully adapted to North West European climates and soils, and could also be adapted to other regions in the world, making local quinoa production possible [1,2].

Protein functionality is an important aspect to evaluate the potential of a new protein and give guidance for usage in applications. To avoid influences from other grain components in assessing the protein potential as a food ingredient, the protein can best be isolated from the grain for subsequent analysis. Conventionally, solvent extraction is used to isolate protein from plant material. During this process, protein properties and thus functionality can be affected [3]. Only a few studies have examined the impact of extraction conditions on functional properties of quinoa protein so far, and only our

previous study has investigated properties of quinoa protein from sweet quinoa (saponin-free) [4-6]. The absence of saponins has been found to influence protein efficiency ratio, nitrogen solubility, emulsifying and foaming properties [3]. Next to extraction conditions, post-extraction processing can also influence protein properties. A few recent studies have investigated the effects of post-extraction heating on some properties of Quinoa Protein Isolates (QPI). We previously found that QPI suspensions started to gel at about 70°C when extracted at pH 8 and 9 but no gelation was observed when extracted at pH 10 or 11. Maekinen et al. (2015) reported that cold-set QPI gels were finer, more regularly structured and had a higher storage modulus when QPI suspensions were heat-treated (100°C, 15 min) at pH 10.5 than when heat-treated at pH 8.5 [7]. Silva et al. (2015) found that heat treatments (100°C, 30 min) of quinoa protein fractions containing anti-nutritional factors increased *in vitro* protein digestibility. To the best of our knowledge, no studies have investigated the effect of varying heat processing parameters on protein denaturation and digestibility of QPIs. Protein denaturation and digestibility are main determinants of protein quality and would be important for application of quinoa (protein) in food products [8]. Gastric protein digestibility is a first indicator of overall protein digestibility and nutritional value of the protein [9,10] [11-13]. Therefore, in the present study, we examined how heat processing at different temperatures influenced denaturation properties and *in vitro* gastric digestibility of sweet quinoa protein isolated at various extraction pH values. Based on literature, we hypothesize that heat processing in the temperature range of 60 to 120°C increases *in vitro* gastric digestibility of the quinoa protein at mildly alkaline extraction pH and decreases the digestibility at strongly alkaline extraction pH.

3. Material and methods

3.1. Materials

Quinoa seeds (*Chenopodium quinoa* Willd) of the sweet variety *Atlas* were supplied by the Agricultural Research Institute (INIA) in Santiago, Chile. Petroleum ether (boiling range 40-60°C) was used (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). Chemicals for preparation of the simulated gastric juice were purchased from Sigma-Aldrich, Inc. (St.

Louis, MO, U.S.A.).

3.2. Preparation of quinoa protein isolates

Quinoa seeds were ground with a Fritsch Mill Pulverisette 14 (Idar-Oberstein, Germany) using a speed of 7000 rpm and sieved through a 200 µm sieve. The flour was defatted in a Soxhlet using petroleum ether with a sample-to-solvent mass ratio of 1:5 for 24 h [14]. The petroleum ether was removed by evaporation. The defatted flour was suspended in deionized water (10% w/w) and the pH was adjusted to 8, 9, 10 and 11 by addition of 1 N NaOH. The suspensions were stirred for 1 h at room temperature and centrifuged for 20 min at 6000 g and 10 °C. The obtained supernatants were acidified to pH 5.5 by addition of 1 N HCl. The suspensions were centrifuged for 30 min at 13000 g and 10°C. The precipitated pellets were re-suspended in deionized water (5% w/w). To rinse remaining salts the suspensions were centrifuged for 20 min at 11000 g and 10°C, re-suspended in deionized water (5% w/w) and neutralized by addition of 1 N NaOH. The suspensions were frozen by dipping into liquid nitrogen and subsequently freeze-dried for 72 h (Chris Epsilon 2-6D Freeze Dryer, Osterode am Harz, Germany). The dried protein isolates were ground with a spoon for about 30 s to obtain powders. Isolates were obtained in duplicate from two separate extractions.

3.3. Determination of protein yield and purity

8 to 15 mg QPI was weighed in tin cups and dried overnight at 60°C. The nitrogen content was determined by sample combustion in a Dumas Flash EA 1112, Series NC analyzer (Wigan, UK) and converted to crude percentage of protein using a protein factor of 5.85 [4,15,16]. Measurements were performed in duplicate. Protein yield and protein purity were calculated as follows:

$$\text{Protein yield (\%)} = \frac{\text{protein content isolate (\%)} \times \text{dry isolate (g)}}{\text{protein content flour (\%)} \times \text{flour (g)}} \times 100$$

$$\text{Protein purity (\%)} = \frac{\text{protein content isolate (\%)} \times \text{dry isolate (g)}}{\text{dry isolate (g)}} \times 100$$

3.4. Heat processing of quinoa protein isolates

Suspensions of the QPIs obtained at the different extraction pH values were prepared at protein concentrations 1, 5 and 20% w/w in deionized water and stirred for 1 h at room temperature. For the heat processed samples, the suspensions were heat-treated in an Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany) for 30 min at 60, 90 and 120°C and then cooled down to room temperature. The temperatures were selected based on temperatures used in applications and to test within a wide range of temperatures. A temperature of 90°C represents pasteurization conditions, while a temperature of 120°C is representative for sterilization conditions. Treatment at 60°C was chosen as mild heating temperature without causing denaturation of the quinoa protein. The terms “processing temperature of 20°C” and “unprocessed” refer to the incubation of QPI suspensions at 20°C without further treatment.

3.5. Determination of molecular weight distribution

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine the molecular weight distribution of the quinoa protein isolate fractions. Heat-processed and unprocessed suspensions of 1% w/w protein concentration were prepared. The suspensions were then re-suspended in deionized water (pH 6.5±0.1) and centrifuged for 1 min at 13000 g to obtain the solubilized protein. The supernatants were diluted with 1 x NuPAGE® LDS Sample Buffer and deionized water before applying the samples to the gel. NuPAGE® Novex® Bis-Tris Gels (1–200 kDa) containing 12 % acrylamide (4% acrylamide stacking gel) were used. The molecular weight markers were from NuPAGE® Novex® (Mark 12™ Unstained Standard, 2.5–200 kDa). Protein bands were stained with Simply Blue™ SafeStain.

3.6. Determination of thermal properties

The thermal properties of the QPIs were assessed by Differential Scanning Calorimetry (DSC). Heat-processed and unprocessed suspensions of 20% w/w protein concentration were prepared. Hermetically sealed aluminum pans were filled with 25-50 mg of heat-processed or unprocessed QPI suspensions. DSC samples were heated at a rate of

10°C/min from 20 to 140°C using a PerkinElmer Diamond series differential scanning calorimeter equipped with an intracooler 2P. A double, empty pan was used as reference. The denaturation parameters were calculated using Pyris Software (Version 11, PerkinElmer) with the denaturation temperature (T_d) value corresponding to the maximum transition peak and the transition enthalpy (denaturation enthalpy ΔH) calculated from the area below the transition peaks. Measurements were performed in duplicate for isolates obtained in duplicate.

3.7. Determination of *in vitro* gastric protein digestibility

Simulated gastric juice was prepared according to [17,18]. Pepsin (1 g L-1), mucin (1.5 g L-1), and NaCl (8.775 g L-1) were dissolved in Milli-Q water and the pH was adjusted to 2.0 with 2 M HCl. Heat-processed and unprocessed QPI suspensions, as well as suspensions of whole meal quinoa flour (5% w/w protein, 2 mL), were prepared and added to 50 mL of simulated gastric juice in a jacketed glass vessel connected to a water bath at 37°C (Julabo GmbH, Seelbach, Germany). The vessel was sealed with Parafilm (Pechiney Plastic Packaging, Inc., IL, U.S.A.) to avoid evaporation and the gastric juice solutions were stirred at 100 rpm. Samples of 1 mL were taken after 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240 and 360 min and heated under stirring in a pre-heated Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany) at 90 °C and 1400 rpm for 5 min to inactivate pepsin [19]. All measurements were performed in triplicate.

3.8. Determination of concentration of free amino groups

To compare the relative digestibility of different quinoa protein samples treated at different temperatures with each other, the concentration of free amino groups was determined using the OPA method as described by Luo et al. (2015). The OPA reagent was prepared and stored in a bottle covered with aluminum foil to protect the reagent from light. A spectrophotometer DU 720 (Beckman Coulter Inc. Pasadena, CA, U.S.A) was set at 340 nm with 1.5 mL OPA reagent + 0.2 mL Milli-Q water. Serine standard solutions of 200 µL of 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L were added to 1.5 mL OPA reagent and mixed. The solutions were measured with the spectrophotometer after standing for 3 min.

The samples were pipetted into the Amicon Ultra-0.5 10K Centrifugal Filter Units (Millipore, USA) and centrifuged for 20 min at 14000 g. A filtration step was used to avoid interference from mucin, as has been was observed by Luo et al. (2015) in unpublished work. We corrected for the OPA response by ε-amino groups by subtracting the OPA response of a sample at digestion time zero (blank). All measurements were performed in triplicate.

To compare the digestibility of quinoa protein with other proteins, the "apparent degree of hydrolysis (DH)" was estimated. We use an apparent DH as aggregates larger than 10 kDa might have been removed by the filtration step and thus might not have been detected in the OPA analysis. The "apparent DH" is defined as the percentage of cleaved peptide bonds over the total number of peptide bonds (h_{tot}). The latter was calculated as follows:

$$h_{tot}\ (\text{eqv/kg protein}) = \frac{1000\ \text{g protein}}{\text{average molecular weight of anhydro amino acids (Da)}}$$

The average molecular weight of an amino acid in quinoa protein was calculated using the sum of products of molecular weight and proportion of the amino acids in quinoa protein (Lindeboom, 2005). The molecular weight of water was subtracted from the average molecular weight of amino acids to obtain the average molecular weight of anhydro amino acids. The h_{tot} of quinoa protein was found to be 8.6 eqv/kg protein.

3.9. Size exclusion chromatography (SEC)

The peptide profile after digestion was analyzed using SEC Ultimate 3000 UHPLC system (Thermo Scientific, MA, U.S.A.) equipped with a TSKgel G2000SWxl column (Tosoh Bioscience LLC, PA, U.S.A.). 0.1 mL sample was used for analysis. The running buffer consisted of acetonitrile and 70% Milli-Q water with 0.1% Trifluoro Acetic Acid (TFA). The flow rate of the running buffer was 1 mL/min and the UV detector was set at 214 nm. In order to standardize the molecular weight range of the chromatographic separation, the following purified proteins and amino acids were used for calibration: carbonic anhydrase (29 kDa), α-lactalbumin (14.1 kDa), aprotinin (6.51 kDa), insulin (5.7 kDa), bacitracin (1.42 kDa) and phenylalanine (165 Da) (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.). The area under the curves was determined and the relative area for each segment calculated. All

measurements were done in triplicate.

4. Results and discussion

4.1. Protein yield and purity

When extracting quinoa protein in a pH range of 8-11, a protein purity of 90-93% was obtained (Figure 1). These values are the highest reported in literature so far [20,4,6,21-23]. In our previous study, we used a similar extraction protocol, only the alkalinization time was longer and the precipitation pH lower, resulting in a lower protein purity (82-88%) [20]. Protein yield increased from 24-37% when increasing the extraction pH from 8 to 11. These values are lower than in our previous study (35-50% going from extraction pH 8 to 11) but they also increased with extraction pH. For industrial production of quinoa protein isolates, this means that the extraction pH would need to be controlled carefully.

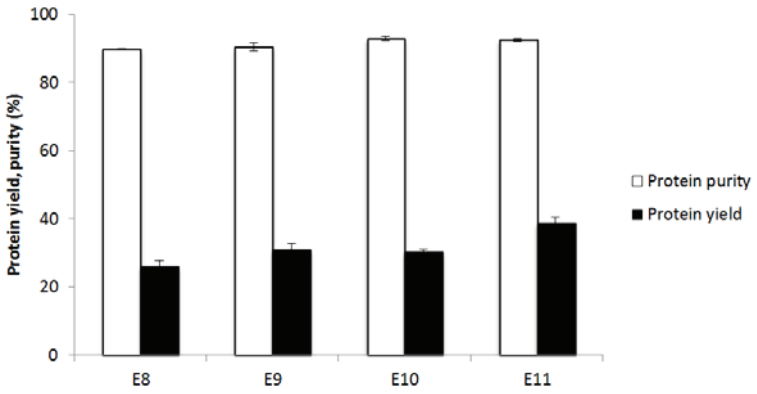


Figure 1. Protein yield and protein purity on dry matter basis of the quinoa protein isolates E8, E9, E10 and E11. Error bars represent the standard deviation based on duplicate extraction experiments

4.2. Thermal properties

Unprocessed and processed 20% QPI suspensions showed an endotherm from 96-102°C (denaturation temperature range) (Figure 8-12), which is in line with denaturation temperatures (T_d) previously found for quinoa, amaranth and sunflower protein. These denaturation temperatures have been attributed to 11S globulin [20,4,24,16,25]. Therefore, we assume that the endotherm found in our study also mainly corresponds to

11S globulin. There was no significant change in T_d with processing temperature, but T_d decreased with increasing extraction pH. This decrease was also observed by Martínez & Añón (1996) for amaranth protein and indicates that protein is less heat-stable when extracted at higher pH [24].

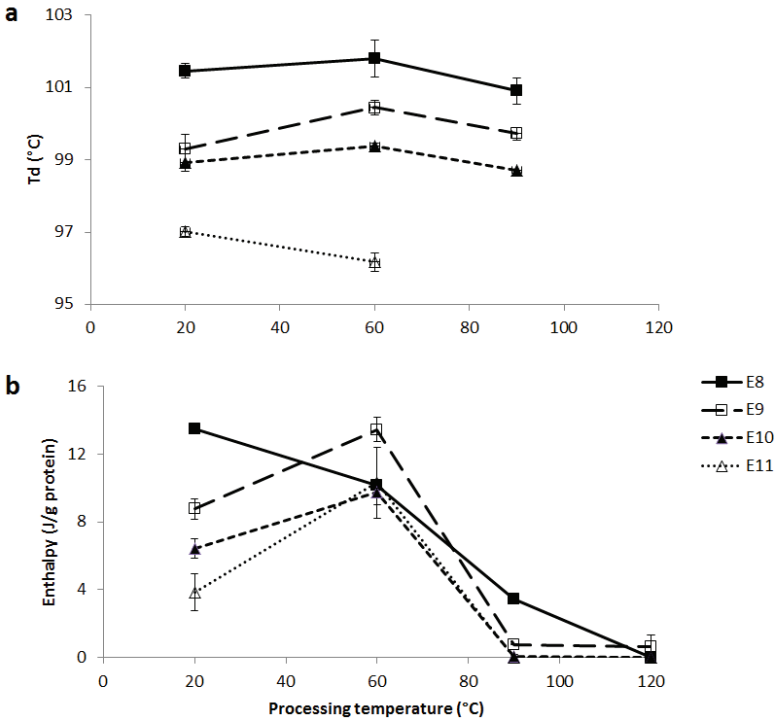


Figure 2. (A) Denaturation temperature (Td) and (B) denaturation enthalpy (ΔH) of 20% w/w suspensions of QPI E8, E9, E10 and E11 after processing at different temperatures. Data were obtained from DSC measurements

The denaturation enthalpy of the unprocessed QPI suspensions decreased considerably from 13.5 to 3.8 J/g protein with increasing extraction pH (Figure). This trend has also been observed in several other studies on quinoa, amaranth and sunflower protein, showing that the protein is more denatured at higher extraction pH [20,4,24,16,25]. When QPI suspensions were processed at 90 and 120°C, the denaturation enthalpy was reduced to 0-3.4 J/g protein. However, the enthalpy was significantly higher after processing at 60°C than at 20°C for E9, E10 and E11.

Martínez & Añón (1996) have summarized the notion of denaturation enthalpy to be the

result of endothermal processes, e.g. disruption of hydrogen bonds, and exothermal processes, e.g. protein aggregation and disruption of hydrophobic interactions. The higher denaturation enthalpy (or transition enthalpy) of E9, E10 and E11 at 60°C might thus indicate a conformation of the protein that was stabilized by a greater extent of hydrophobic interactions and/or hydrogen bonds and that cost more transition energy than at 20, 90 or 120°C. The exception was E8, which showed a continuous decrease in enthalpy from 20 to 120°C. Based on the notion of denaturation enthalpy of Martínez & Añón (1996) it might be that at an extraction pH of 8 the protein initially contained a higher degree of hydrophobic interactions and/or hydrogen bonds as compared to the protein obtained at other extraction pH values. These molecular interactions might have decreased in number from a processing temperature of 20 to 60°C in contrast to the other extraction pH values, where the protein initially had undergone more extensive conformational changes due stronger alkaline extraction conditions, resulting in a different degree of molecular interactions after processing at 60°C. In summary, the effect of processing temperature on the thermal properties of QPIs seemed to depend on the protein properties predetermined by the extraction pH.

4.3. Protein fractions

SDS profiles showed major bands at 50 kDa for all QPIs and at 37 kDa for E8, E9 and E10 (Figure 3). The bands of E8 were the most intense and decreased in intensity with increasing extraction pH. The SDS profiles were similar to the ones of previous quinoa protein studies, suggesting a correspondence of the bands at 50 kDa to 11S globulin [20,4,26]. Furthermore, bands at 37 kDa might correspond to the acidic subunit and bands at 23 kDa might be attributed to the basic subunit of 11S globulin. Alkali is known to cause disulfide bond cleavage, resulting in the dissociation of 11S globulin into acidic and basic subunits of 32-39 kDa and 22-23 kDa, respectively [27].

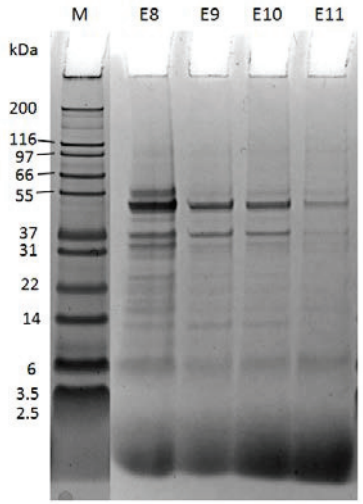


Figure 3. SDS-PAGE profile of the unprocessed QPIs E8, E9, E10 and E11. Lane M: molecular weight marker

After heat processing, the SDS profiles showed less bands with less intensity for all QPIs (Figure 4). In some lanes specific bands were even not visible anymore. The disappearance of bands with increasing processing temperature indicates enhanced protein aggregation to protein particles larger than 200 kDa or to insoluble protein particles that remained in the pellet after centrifuging the heat-processed protein suspensions. Protein aggregation might have resulted from increased protein dissociation and subunit interactions and re-association to larger (insoluble) aggregates as reported for heat-processed soy protein (0-30 minutes at 80 and 100°C) [28,29]. DSC results showed higher denaturation enthalpies of the unprocessed and 60°C-processed QPI suspensions compared to the suspensions processed at 90 and 120°C. As described before, the higher enthalpies might result from more hydrophobic interactions, hydrogen bonds but also from increased protein aggregation, according to Martínez & Añón (1996). Based on the results of SDS and DSC, it seems likely that protein aggregation leads to insoluble particles remaining in the pellet, especially at 120°C (less protein on the SDS gels), while the aggregates seem to be less capable to undergo a heat-induced phase transition up to a temperature of 140°C (maximum temperature reached during DSC measurements) compared to protein treated at 60°C.

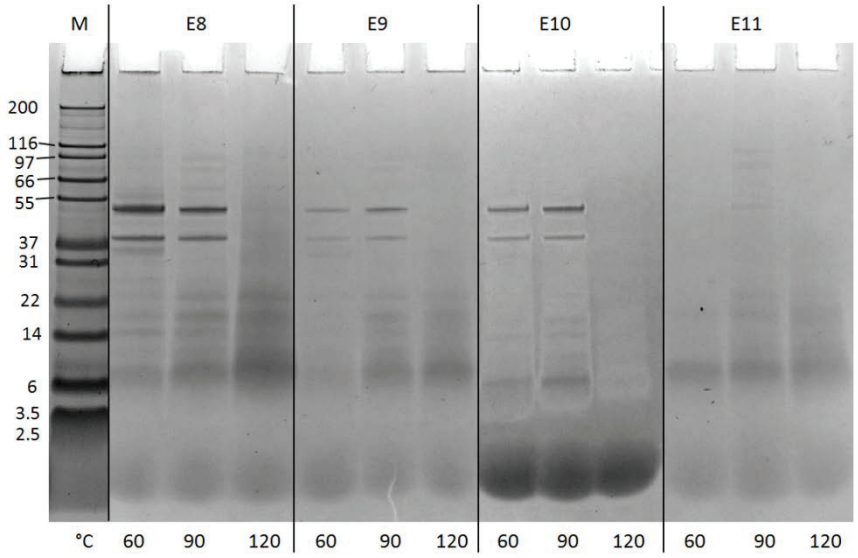


Figure 4. SDS-PAGE profile of the QPIs E8, E9, E10 and E11 heat-treated for 30 min at 60, 90 and 120°C. Lane M: molecular weight marker. The gel of E10 seems to be overloaded at the bottom. E10 was run on a different gel and is shown in Figure 13.

4.4. *In vitro* gastric protein digestibility of quinoa protein isolates

The focus of the present study was not to quantify the true degree of hydrolysis but to compare the relative digestibility of different quinoa protein samples (treated at different temperatures). Therefore, gastric digestibility of the QPIs *in vitro* simulating physiological conditions was indicated as concentration of free amino groups which was determined using the OPA method. However, to compare the digestibility of quinoa protein to other proteins such as whey and eg-white protein, "apparent DH" values were estimated. Only DH values were available for whey and egg-white proteins. The presence of protein aggregates larger than 10 kDa in the quinoa samples might influence the true DH values for quinoa protein, consequently only apparent DH is reported. The concentration of free amino groups of unprocessed and processed 5% QPI suspensions sharply increased within the first 20 min and further increased at a slower rate in the following hours (Figure 5).

HPLC chromatograms showed that when digesting unprocessed and processed QPI suspensions for 5-360 min higher amounts of peptides ranging from 0.5 to 5 kDa were obtained (Figure 6, 14-16). The peaks in the molecular size range of 0.5-5 kDa became

larger and moved to a smaller size range with increasing *in vitro* digestion time. As digestion progressed, pepsin cleaved increasingly more peptide bonds, resulting in smaller molecules. When comparing processing temperatures, the chromatograms did not significantly change from 20 to 60°C. However, at 90 and 120°C, the response areas were significantly smaller compared to 20 and 60°C. This is most clearly visible after 5 and 20 minutes of digestion. This finding could be confirmed by measurements of free amino group concentration (Figure 5): the concentration of free amino groups was reduced overall at 90 and 120°C compared to 20 and 60°C. Similar observations were made for lupine protein [30]. A heat treatment at 60°C for 30 min did not change the digestibility of lupine protein compared to the untreated sample, while a heat treatment at 90°C for 30 min did reduce the digestibility. The reduction in the concentration of free amino groups at higher processing temperature was enhanced at higher extraction pH.

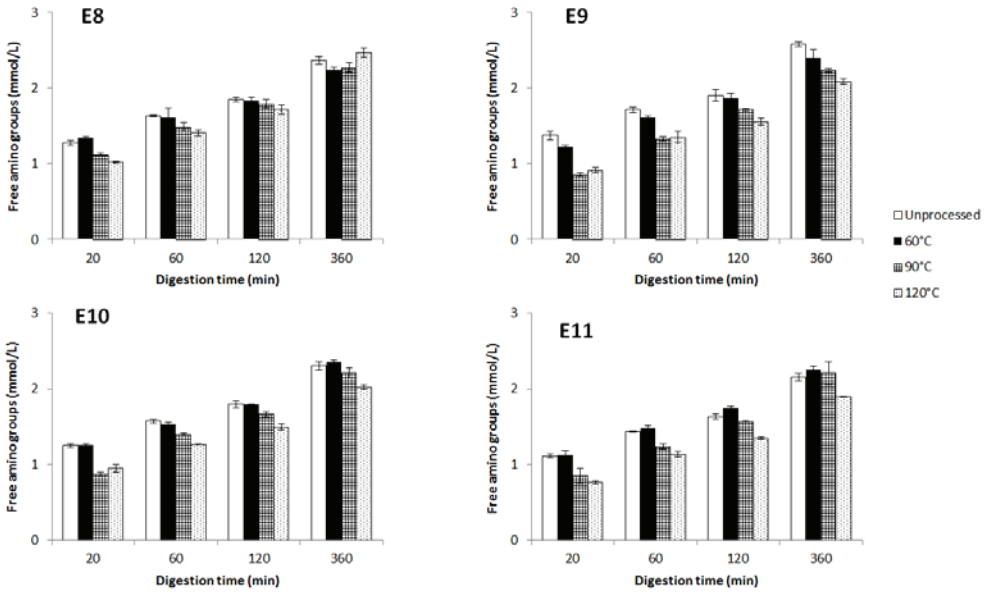


Figure 5. Concentration of free amino groups of 5% w/w suspensions of QPI E8, E9, E10 and E11 processed at different temperatures and subsequently digested for different time periods

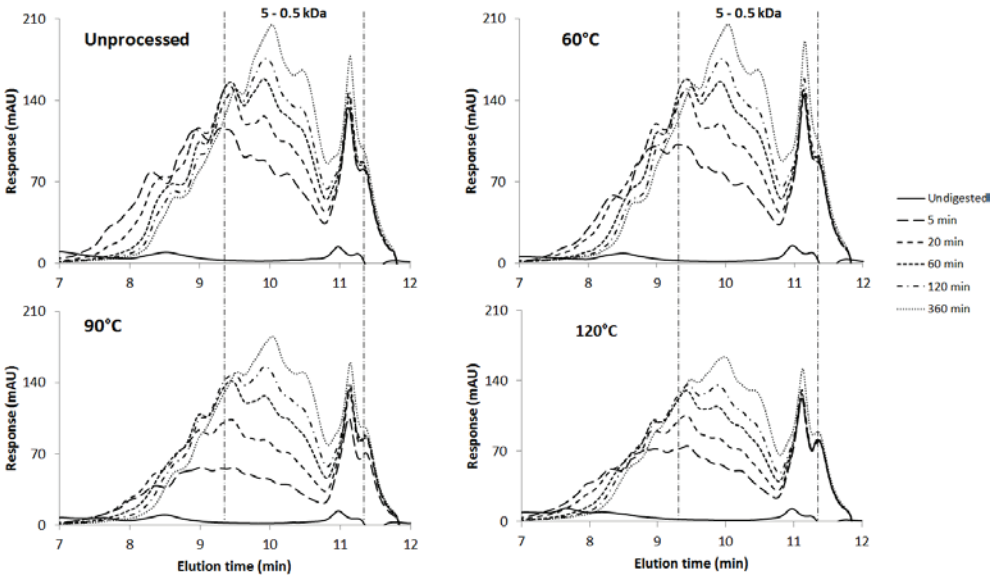


Figure 6. HPLC chromatograms of 5% w/w suspensions of QPI E9 processed at different temperatures and subsequently digested for different time periods. Size exclusion chromatography is used for separation. This means that larger peptides have a low elution time. See Figure 14-16 for the HPLC chromatograms of E8, E10 and E11.

These results suggest that pepsin was less effective after heat-treatment of the QPI suspensions. This might be explained by the heat-induced change in protein conformation, molecular interactions and protein aggregation as indicated by DSC and SDS results. Increased protein aggregation after the heat treatments might have reduced the accessibility of pepsin. Impairment of protein digestibility for pepsin has already been previously correlated with stronger protein crosslinking when cooking sorghum [31]. The *in vitro* digestibility of sorghum protein using pepsin has therefore been validated as an indicator for the degree of protein crosslinking. This relation might also be valid for quinoa protein.

If this is the case, the fact that the reduction in the concentration of free amino groups at higher processing temperature was enhanced at higher extraction pH can be explained with increased protein crosslinking. This might also be deduced from SDS results: with an increasing extraction pH and processing temperature, the degree of protein aggregation, possibly as a result of protein crosslinking, seemed to be higher. However, DSC results implied that the protein suspensions from a high extraction pH (10 and 11) and processing temperature (90 and 120°C) were only slightly capable or not capable at all to undergo a

heat-induced phase transition. Therefore, not a greater extent of protein aggregation or crosslinking seemed to be impairing enzyme action more under these harsher conditions, but a more heat-resistant type of protein aggregation or crosslinking.

The extraction pH had almost no influence on the concentration of free amino groups when comparing pH values of the unprocessed suspensions and of the processed suspensions at 60 and 90°C (Figure 5). This means that the effects of extraction pH observed on the physical properties of unprocessed QPIs and processed QPIs at 60 and 90°C were not clearly transferred to *in vitro* gastric digestibility. At 120°C, the rate of free amino group concentration was only slightly reduced at extraction pH 11 compared to the other extraction pH values. These results show a bigger impact of processing temperature on the concentration of free amino groups of quinoa protein compared to extraction pH.

We conclude that heat treatment for 30 min at 90 and 120°C impairs *in vitro* gastric digestibility of protein in QPIs. The hydrolysis profiles of quinoa protein compare to those of whey and egg white protein obtained by Luo et al. (2015) at the same protein concentration, and under the same digestion and measurement conditions. When interpolating the "apparent DH" values of the QPI suspensions treated at 90°C to a digestion time of 3 h, the values for quinoa protein (11.8 – 14.1%) were in the range between DH values of egg white protein (11%) and whey protein (15%), both pre-treated for 30 min at 90°C and digested for 3 h.

4.5. Gastric *in vitro* protein digestibility of whole quinoa flour

To examine how protein digestibility in QPIs compares to that in whole quinoa flour, we performed the digestibility study with wholemeal quinoa flour at the same protein concentration. The concentration of free amino groups also increased in time and looked similar to that of the QPIs. However, the concentration of free amino groups was overall lower, especially at 120°C (Figure 7). This reduction in free amino groups concentration might be due to the other components present (in higher amounts) in the quinoa flour (mainly starch, fiber and fat). The mere presence of much higher amounts of starch and fiber in the quinoa flour compared to the QPIs might be the responsible factor, but also the behavior of these components at the different processing temperatures might have

had an impact on digestibility [32]. The gelatinization of quinoa starch starts from 45-54°C, peaks from 51-62°C and concludes from 64-71°C [33]. At processing temperatures of 60 and 90°C, there was no large difference in the concentration of free amino groups compared to the protein isolates, indicating that gelatinization did not affect protein digestibility significantly. There was a larger drop in amount of free amino groups from 90 to 120°C for the quinoa flour compared to the protein isolates. As starch gelatinization did not seem to have an impact on digestibility at lower temperatures, it is possible that at higher temperatures the gelatinized starch interacted with denatured protein (T_d=96-102°C), thereby hindering enzyme action. Another explanation might be that in contrast to the protein in the flour, the protein in the protein isolates underwent conformational changes during the extraction, which limited the effect of processing temperature on protein digestibility.

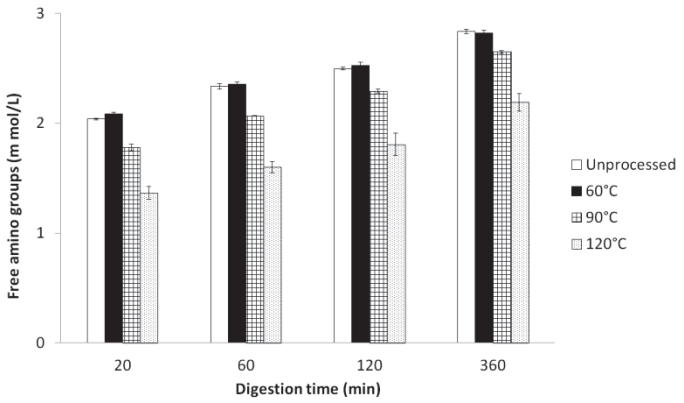


Figure 7. Concentration of free amino groups of wholemeal quinoa flour (5% w/w protein) processed at different temperatures and subsequently digested for different time periods.

5. Conclusions

Using the extraction protocol from the present study, we could achieve a very high protein purity, but at the expense of a low protein yield. The degree of denaturation and molecular weight profiles of the QPIs were strongly affected by processing temperature and extraction pH, individually and combined. For QPI's, extraction pH and processing temperature showed an interactive effect on *in vitro* gastric digestibility of the protein.

Extracting protein from quinoa flour results in a higher protein digestibility when compared to keeping the protein in the flour. For applications, the present findings mean that extraction and processing conditions need to be controlled to optimize protein digestibility. Future research could investigate other functional properties of quinoa protein but also examine ileal and *in vivo* protein digestibility under various conditions to verify the present findings in more real-life digestion conditions.

6. Acknowledgments

Mauricio Opazo Navarrete was financially supported by a PhD scholarship of CONICYT (Formation of Advanced Human Capital Program), Chile. The authors gratefully acknowledge the financial support of the "IPOP Customized Nutrition" program of Wageningen University and Research Centre.

7. Appendix

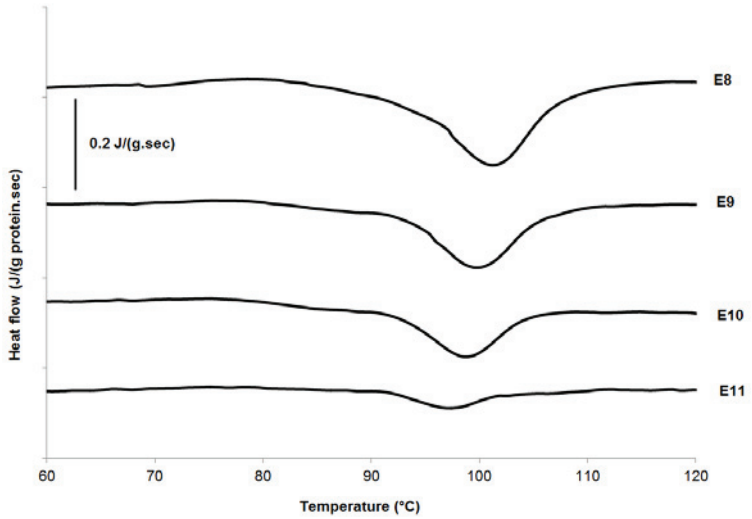


Figure 8. DSC thermograms of untreated 20% w/w suspensions of QPI E8, E9, E10 and E11.

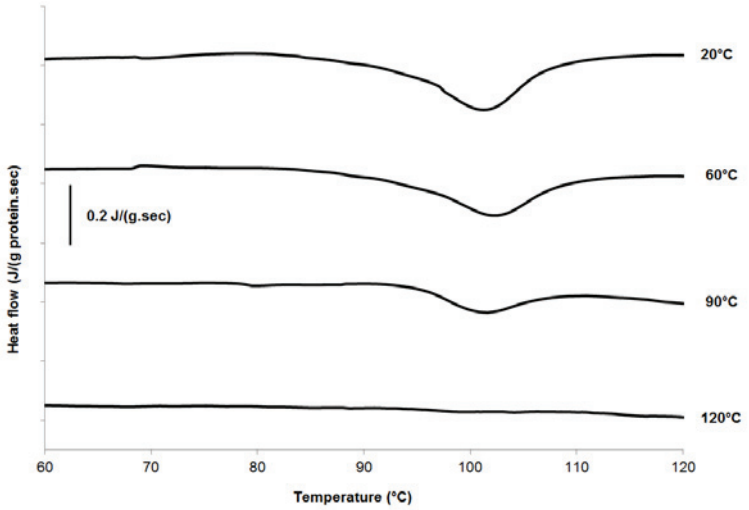


Figure 9. DSC thermograms of 20% w/w suspensions of QPI E8 after processing at different temperatures.

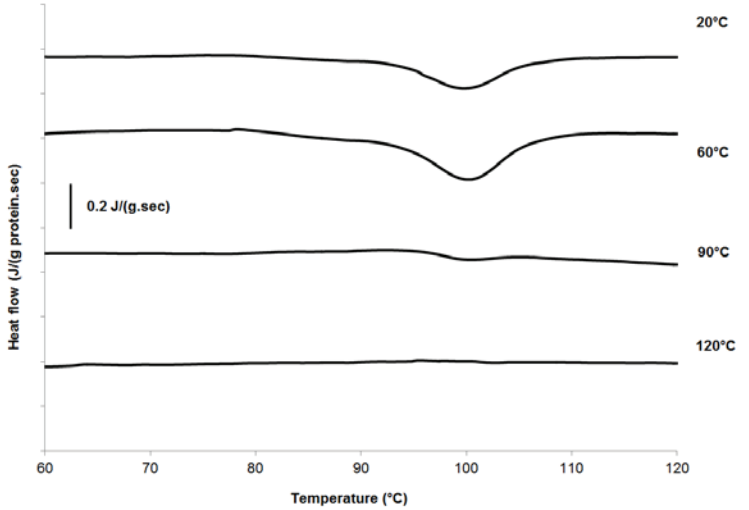


Figure 10. DSC thermograms of 20% w/w suspensions of QPI E9 after processing at different temperatures.

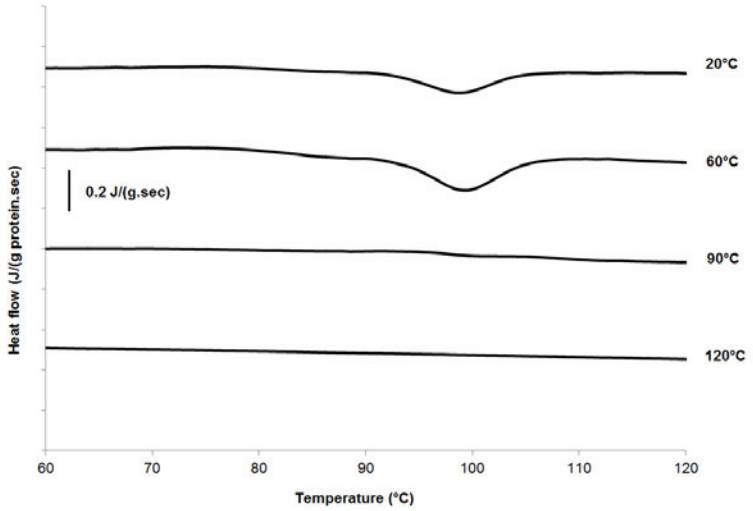


Figure 11. DSC thermograms of 20% w/w suspensions of QPI E10 after processing at different temperatures.

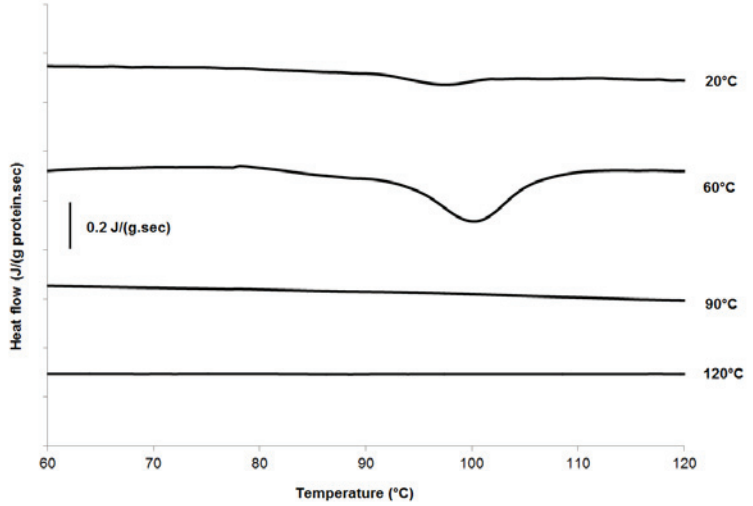
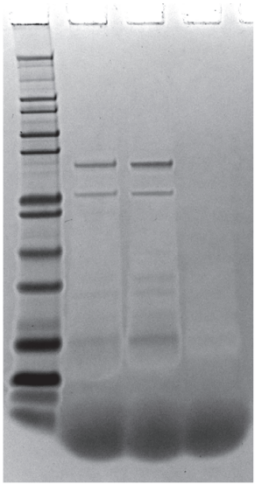


Figure 12. DSC thermograms of 20% w/w suspensions of QPI E11 after processing at different temperatures



F

Figure 13. SDS-PAGE profile of the QPIs E10 heat-treated for 30 min at 60, 90 and 120°C. Lane M: molecular weight marker

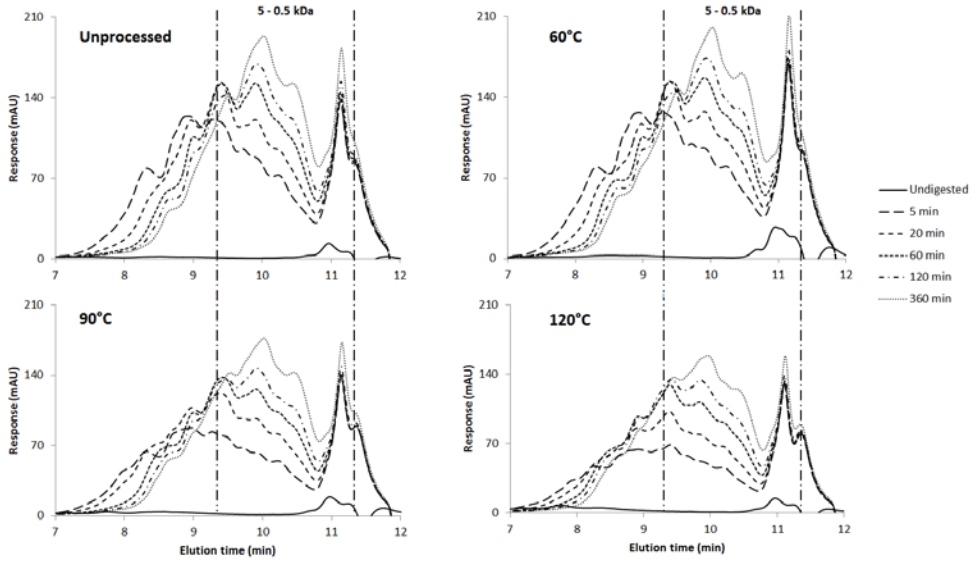


Figure 14. HPLC chromatograms of 5% w/w suspensions of QPI E8 processed at different temperatures and subsequently digested for different time periods. Size exclusion chromatography is used for separation. This means that larger peptides have a low elution time

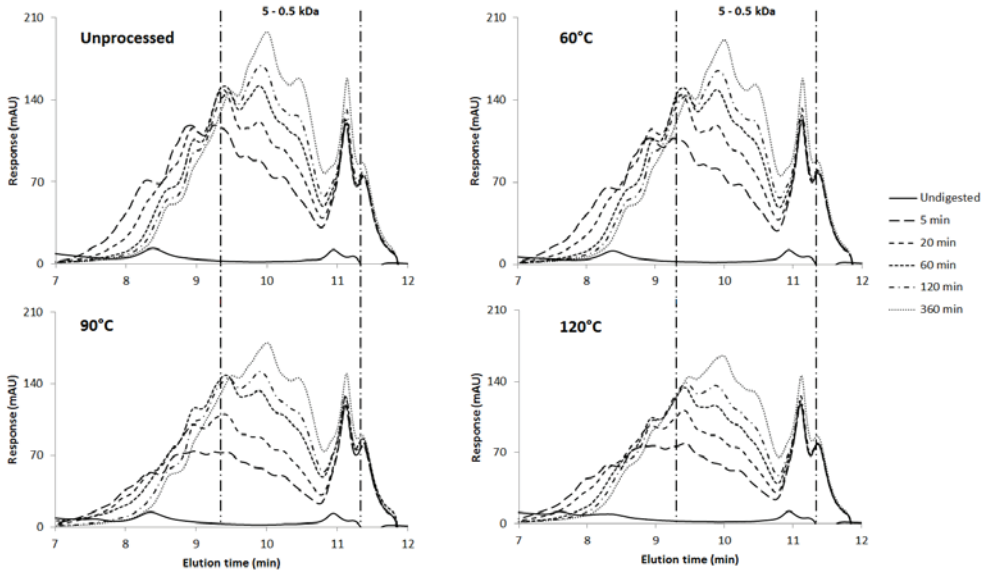


Figure 15. HPLC chromatograms of 5% w/w suspensions of QPI E10 processed at different temperatures and subsequently digested for different time periods. Size exclusion chromatography is used for separation. This means that larger peptides have a low elution time

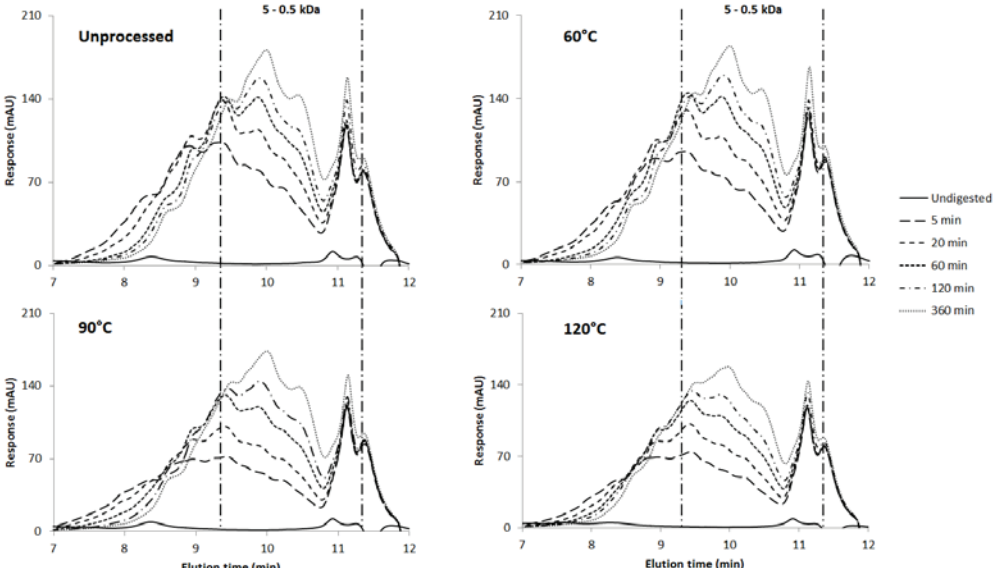


Figure 16. HPLC chromatograms of 5% w/w suspensions of QPI E11 processed at different temperatures and subsequently digested for different time periods. Size exclusion chromatography is used for separation. This means that larger peptides have a low elution time

8. References

1. Limburg H, Masterbroek HD Breeding high yielding lines of *Chenopodium quinoa* Willd. with saponin free seed. In: Stølen O, Bruhn K, Pithan K, Hill J (eds) Proc COST 814 workshop on Small Grain Cereals and Pseudo-Cereals, Copenhagen, 24 February 1997. pp 103–114.
2. Mastebroek HD, van Loo EN, Dolstra O (2002) Combining ability for seed yield traits of *Chenopodium quinoa* breeding lines. Euphytica 125 (3):427-432.
3. Avila Ruiz G, Xiao W, van Boekel M, Stieger M, Minor M (2016) Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein from sweet quinoa (*Chenopodium quinoa* Willd) (submitted).
4. Abugoch LE, Romero N, Tapia CA, Silva J, Rivera M (2008) Study of some physicochemical and functional properties of quinoa (Chenopodium quinoa willd) protein isolates. Journal of Agricultural and Food Chemistry 56 (12):4745-4750.
5. Valenzuela C, Abugoch L, Tapia C, Gamboa A (2013) Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence on film formation. International Journal of Food Science & Technology 48 (4):843-849.
6. Aora JCC, Alvarado K (2009) Aislados proteínicos de granos altoandinos *chenopodiaceas*; quinua "*chenopodium quinoa*" – cañahua "*chenopodium pallidicaule*" por precipitación isoeléctrica. Revista Boliviana de Química 26 (1):12-20.
7. Mäkinen O, Zannini E, Arendt E (2015) Modifying the Cold Gelation Properties of Quinoa Protein Isolate: Influence of Heat-Denaturation pH in the Alkaline Range. Plant Foods for Human Nutrition:1-7.
8. Guo X, Yao H, Chen Z (2007) Effect of heat, rutin and disulfide bond reduction on in vitro pepsin digestibility of Chinese tartary buckwheat protein fractions. Food Chem 102 (1):118-122.
9. Mokrane H, Amoura H, Belhaneche-Bensemra N, Courtin CM, Delcour JA, Nadjemi B (2010) Assessment of Algerian sorghum protein quality [*Sorghum bicolor* (L.) Moench] using amino acid analysis and *in vitro* pepsin digestibility. Food Chem 121 (3):719-723.
10. Sarker AK, Saha D, Begum H, Zaman A, Rahman MM (2015) Comparison of cake compositions, pepsin digestibility and amino acids concentration of proteins isolated from black mustard and yellow mustard cakes. AMB Express 5:22.
11. Budryn G, Nebesny E, Rachwał D (2013) Pepsin Digestibility and Antioxidant Activity of Egg White Protein in Model Systems with Green Coffee Extract. Int J Food Prop 17 (7):1529-1546.
12. Hoppe A, Jung S, Patnaik A, Zeece MG (2013) Effect of high pressure treatment on egg white protein digestibility and peptide products. Innovative Food Science & Emerging Technologies 17:54-62.
13. Mertz ET, Hassen MM, Cairns-Whittern C, Kirleis AW, Tu L, Axtell JD (1984) Pepsin digestibility of proteins in sorghum and other major cereals. Proceedings of the National Academy of Sciences of the United States of America 81 (1):1-2.

14. Pelgrom PJM, Wang J, Boom RM, Schutyser MAI (2015) Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes. J Food Eng 155 (0):53-61.
15. Becker R, Wheeler EL, Lorenz K, Stafford AE, Grosjean OK, Betschart AA, Saunders RM (1981) A Compositional Study of Amaranth Grain. J Food Sci 46 (4):1175-1180.
16. Castellani OF, Martínez EN, Añón MC (1998) Structural Modifications of an Amaranth Globulin Induced by pH and NaCl. J Agric Food Chem 46 (12):4846-4853.
17. Kong F, Singh RP (2009) Modes of Disintegration of Solid Foods in Simulated Gastric Environment. Food Biophysics 4 (3):180-190.
18. Luo Q, Boom RM, Janssen AEM (2015) Digestion of protein and protein gels in simulated gastric environment. LWT - Food Science and Technology 63 (1):161-168.
19. Casey EJ, Laidler KJ (1951) The Kinetics and Mechanism of the Heat Inactivation of Pepsin1. J Am Chem Soc 73 (4):1455-1457.
20. Avila Ruiz G, Xiao W, van Boekel M, Stieger M, Minor M (2015) Effect of extraction pH on yield, denaturation properties and functionality of quinoa protein (Chenopodium quinoa Willd) Food Chem.
21. Chauhan GS, Cui W, Eskin NAM (1999) Effect of saponin on the surface properties of quinoa proteins. Int J Food Prop 2 (1):13-22.
22. Lindeboom N (2005) Studies on the characterization, biosynthesis and isolation of starch and protein from quinoa (*Chenopodium quinoa* Willd.). University of Saskatchewan, Canada
23. Aluko RE, Monu E (2003) Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science 68 (4):1254-1258.
24. Martínez EN, Añón MC (1996) Composition and Structural Characterization of Amaranth Protein Isolates. An Electrophoretic and Calorimetric Study. Journal of Agricultural and Food Chemistry 44 (9):2523-2530.
25. Molina MI, Petruccelli S, Añón MC (2004) Effect of pH and Ionic Strength Modifications on Thermal Denaturation of the 11S Globulin of Sunflower (*Helianthus annuus*). J Agric Food Chem 52 (19):6023-6029.
26. Brinegar C, Goundan S (1993) Isolation and characterization of chenopodin, the 118 seed storage protein of Quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 41 (2):182-185.
27. Kinsella JE, Damodaran S, German B, Wilcke AMAL (1985) V - Physicochemical and Functional Properties of Oilseed Proteins with Emphasis on Soy Proteins. In: New Protein Foods. Academic Press, pp 107-179.
28. Utsumi S, Damodaran S, Kinsella JE (1984) Heat-induced interactions between soybean proteins: preferential association of 11S basic subunits and .beta. subunits of 7S. J Agric Food Chem 32 (6):1406-1412.
29. Wolf WJ, Tamura T (1969) Heat Denaturation of Soybean 11S Protein. Cereal Chemistry 46:331-343.
30. Pelgrom PJM, Berghout JAM, van der Goot AJ, Boom RM, Schutyser MAI (2014) Preparation of functional lupine protein fractions by dry separation. LWT - Food Science and Technology 59 (2, Part 1):680-688.

31. Zhao R, Bean SR, Ioerger BP, Wang D, Boyle DL (2008) Impact of Mashing on Sorghum Proteins and Its Relationship to Ethanol Fermentation. J Agric Food Chem 56 (3):946-953.
32. Lev Elkonin L, Italianskaya J, Fadeeva I (2012) Gel Electrophoresis as a Tool to Study Polymorphism and Nutritive Value of the Seed Storage Proteins in the Grain Sorghum. In: Mageldin S (ed) Gel Electrophoresis - Advanced Techniques. InTech, Russia, pp 463-478.
33. Bhargava A, Srivastava S (2013) Chemistry. In: Bhargava A, Srivastava S (eds) Quinoa - Botany, Production and Uses. CABI, Oxfordshire, pp 185-239.

Chapter 4

A hybrid dry and aqueous fractionation method to obtain protein-rich fractions from quinoa

This chapter has been published as: Avila Ruiz G, Arts A, Minor M, Schutyser M (2016) A hybrid dry and aqueous fractionation method to obtain protein-rich fractions from quinoa (Chenopodium quinoa Willd) Food Bioprocess Technology:1-9.

1. Abstract

Combination of dry and aqueous fractionation is investigated to obtain protein-rich fractions from quinoa in a milder and more sustainable way compared to conventional wet fractionation. Dry fractionation of quinoa involved milling and subsequent air classification, generating a protein-enriched embryo fraction. Subsequently, this fraction was milled, suspended and further fractionated by aqueous phase separation. The efficiency of aqueous phase separation could be improved by addition of NaCl (0.5 M). Finally, the top aqueous phase was decanted and ultrafiltered, resulting in a protein purity of 59.4 w/dw% for the 0.5 M NaCl-protein solution and a protein yield (g protein obtained/g protein in seed) of 62.0%. Having used 98% less water compared to conventional wet extraction, the hybrid dry and aqueous fractionation is a promising method for industry to create value from quinoa in a more economic and sustainable friendly way while minimising the impact on quinoa's native protein functionality.

2. Introduction

The nutritional properties of quinoa are unique since it contains all essential amino acids, trace elements and vitamins (B6, folate, riboflavin and niacin) [1]. As a result its popularity and cultivation area are expanding rapidly. A promising quinoa variety to use on a large scale is sweet quinoa (virtually saponin-free). This variety could be a more sustainable and economic raw material to use in industry due to savings in post-harvest processing (not necessary to remove saponins), in seed transport and availability (it can be cultivated in different regions and also in temperate climates) [2].

To stimulate more extensive use and create added value of (sweet) quinoa in the production of foods, ingredients derived from quinoa by fractionation have been explored by several studies, in particular, the production of protein isolates [3-11,2,12]. In all these studies the conventional wet fractionation method was applied. It involves the use of a solvent for fat removal (hexane, petroleum ether, etc.), an alkali to solubilise the protein from the defatted flour (mostly NaOH) and an acid to purify the protein via precipitation (mostly HCl). However, this method consumes large amounts of water and energy and moreover often leads to denaturation of the protein [13].

Dry fractionation is milder and more sustainable for production of protein concentrates from cereals (wheat, barley etc.) and legumes (pea, lupine, chickpea etc.), although generally the purities obtained are less high [14-16]. A major advantage of this technique is that native functional properties of the proteins are retained [17]. Dry fractionation involves fine milling of the seeds to disclose protein-rich particles and subsequent dry separation of the flour in fractions of different particle size using air classification. The dissociation of seed components is critical to enable separation and is dependent on seed structure and the milling conditions.

For pea seeds (23.7 w/dw% protein), dissociation of protein bodies from starch granules can be achieved by very fine impact milling, which is followed by air classification, generating a protein-rich fine fraction (55.6 w/dw% protein) with smaller particle size and a starch-rich course fraction with a larger particle size [18]. For quinoa seeds (~15 w/dw% protein) it is extremely difficult to separate protein bodies from starch granules as these are similar in size [19]. However, quinoa protein bodies are concentrated in the embryo of the seed (~23.5 w/dw% protein), while starch granules are concentrated in the perisperm [20]. Therefore, we propose rotor milling followed by sieving or air classification to dissociate and separate the embryo from the perisperm. Using rotor milling we aim at clear dissociation of embryo and perisperm and in this way can produce protein-enriched fractions with either sieving or air classification.

Attempts to further dry fractionate the embryo fraction into higher protein enriched-fractions were hitherto unsuccessful, because protein bodies and starch granules in the quinoa seed are similar in size [7]. To achieve higher protein purities, wet fractionation may be applied. However, instead a hybrid method of dry fractionation and aqueous phase separation followed by ultrafiltration is investigated here. This approach is inspired by successful aqueous phase separation of dry-enriched pea fractions and is reported milder and more sustainable [17,21]. The dissolution and subsequent centrifugation of the pea fine fraction obtained by air classification provided a phase separated system with four layers, where the protein was concentrated in the top two layers. Via this method pea protein purity could be increased from 49.7 w/dw% in the fine fraction to 68.6 w/dw% in the combined two top layers. After ultrafiltration a final protein purity of 77.4 w/dw% could be achieved.

The aim of this study was thus to develop a hybrid separation process for quinoa to obtain high protein-rich fractions. The novelty of this method consists especially of the combination of dry fractionation and aqueous fractionation for obtaining protein–rich quinoa fractions, which to the best of our knowledge has not been done before. Purity and yield were evaluated at every step of the new proposed hybrid separation process. Finally, the hybrid fractionation route is compared to conventional wet fractionation of quinoa for its efficiency.

3. Material and methods

3.1. Materials

Quinoa seeds (*Chenopodium quinoa* Willd) of the sweet variety *Atlas* were supplied by the Agricultural Research Institute (INIA), Santiago, Chile. Sodium chloride was obtained from Sigma Aldrich Chemie GmbH, Schnelldorf, Germany. De-ionised water was used throughout the fractionation process.

3.2. Milling of quinoa seeds and air classification of quinoa flour

Quinoa seeds were milled using a 100UPZ Rotor Mill (Hosokawa-Alpine, Augsburg, Germany) with an air flow of 40 m^3/h and a built-in sieve with a screen aperture of 2.0 mm. These optimal settings were derived from previous unpublished work. The obtained flour was air-classified using an ATP50 Classifier (Hosokawa-Alpine, Augsburg, Germany) with a classifier wheel speed of 1000 rpm and an air flow of 80 m^3/h. The fine fraction from this air classification step is in this study referred to as the non-milled fraction. This because the majority of the generated embryo-rich fine fraction from the air classification step was further milled using a ZPS50 Impact Mill (Hosokawa-Alpine, Augsburg, Germany) with an air flow of 52 m^3/h and a classifier wheel speed of 2500 rpm to facilitate dissolution of the protein. The extra impact milling was applied to facilitate disclosure of the protein-rich components from the surrounding matrix and thus subsequent dissolution during suspension.

3.3. Aqueous phase separation of the fine and coarse quinoa fractions

Milled and non-milled fine fractions obtained by air classification were further fractionated by aqueous phase separation. Suspensions of fine fractions (20 w/w%) were prepared in de-ionised water with and without the addition of NaCl (0.15, 0.35 and 0.5 M). They were stirred for 3 h at room temperature and subsequently centrifuged for 30 min at 4500 rpm [17].

3.4. Ultrafiltration of the liquid layer of the phase-separated fractions

The liquid layers of the phase-separated impact-milled fine fractions with 0, 0.15 and 0.5 M NaCl were carefully decanted and utrafiltered at room temperature using an Amicon Ultrafiltration Cell with a regenerated cellulose membrane (PLBC, Ultracel PL Membrane, NMWL Cutoff of 3 kDa) (Millipore Corporation, Billerica, MA, USA). A pressure of 350 kPa was applied for approximately 165 minutes. This ultrafiltration time was slightly varied to obtain enough permeate volume. The average permeability during the experiments was 0.11 $L.m^{-2}.h^{-1}.bar^{-1}$, which is not very high due to the continuous increasing component concentrations in the batch process.

3.5. Determination of the particle size distribution

To determine the particle size distributions of the milled and non-milled quinoa seeds, a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK) was used. All measurements were performed in duplicate.

3.6. Image analysis

Scanning electron micrographs (SEM) were obtained using a Phenom Pure G2 desktop Scanning Electron Microscope (Eindhoven, The Netherlands).

3.7. Determination of protein purity and protein yield

Protein purity was defined as mass protein / mass dry matter (w/dw%) and corresponds to the term “protein content” used in the literature mentioned in the present study. To

determine the protein content (mass protein) of a sample, the Dumas method was used. Nitrogen content was measured using a Nitrogen Analyser (FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands). The conversion factor used to convert nitrogen to protein was 5.7 [22]. All measurements were performed in duplicate.

The protein yield after each step in the fractionation process was calculated as follows:

$$\text{Protein yield (\%)} = \frac{\text{\% protein purity of fraction} \times \text{g fraction}}{\text{\% protein purity of starting material} \times \text{g starting material}} \times 100\%$$

(Equation 1)

3.8. Determination of starch purity

Starch purity was defined as the ratio of mass starch and mass dry matter (w/dw%) and determined using a Total StarchAssay Kit (Megazyme International Ireland Ltd, Bray, Ireland). All measurements were performed in duplicate.

3.9. Statistical analysis

Error bars for all data points were calculated by taking the standard deviation of the average value of duplicates. If the error bars of two data points did not overlap, we concluded they were significantly different.

4. Results and discussion

4.1. Milling and air classification

Quinoa seeds were milled using a rotor mill with an air flow of 40 m^3/h and a sieve screen aperture of 2.0 mm. The objective of the milling was to separate the protein-rich embryo from the protein-poor perisperm. SEM was performed to assess the efficiency of the milling. In the SEM pictures it can be observed that the rotor milling has the potential to achieve neat dissociation of the embryo from the perisperm (Figure 1). Particle size analysis showed a decrease in the volume fraction of particles of around 1000 µm and an increase in the volume fraction of particles of 100-600 µm (Figure 2a). This change in particle size distribution also reflects the dissociation of quinoa seed into smaller perisperm and embryo particles. However, the broadening of the particle size distribution

after milling indicated that dissociation of the embryo from the perisperm was not complete. Instead of rotor milling, also roller milling might be applied. In a previous unpublished study in our laboratory this was investigated and it was concluded that rotor milling of quinoa seeds provides better results in terms of complete disclosure than roller milling. It is assumed that predominant shear and low compression forces applied by the rotor mill dissociates the embryo while most of the perisperm remains intact. In comparison, the roller milling applies high compression forces, which provide also dissociation of the embryo but at the same time lead to more breakage of the perisperm particles.

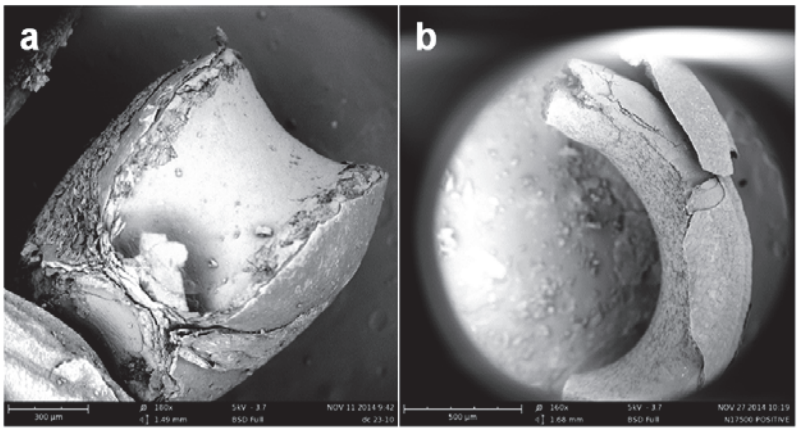


Figure 1. After impact milling of the quinoa seeds: (A) Perisperm hull (magnification: 180x) and (B) Embryo particle (magnification: 160x)

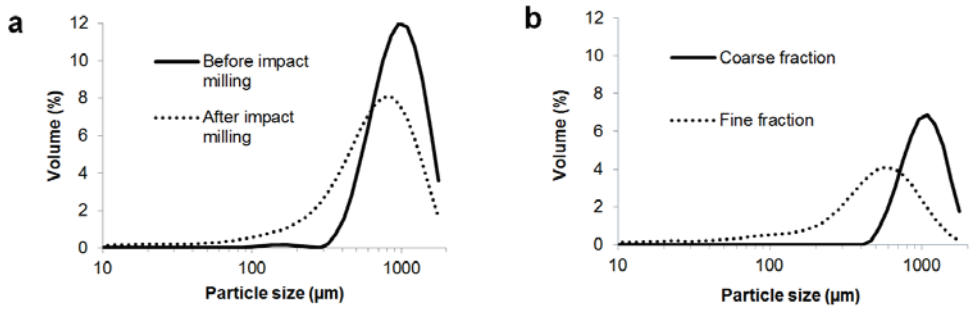


Figure 2. Particle size distribution (A) before and after impact milling of quinoa seeds and (B) of the quinoa coarse and fine fractions obtained after air classification

Air classification of the milled quinoa flour produced a coarse perisperm-rich fraction and a fine embryo-rich fraction (Table 1 and Figure 2b). As the protein content of the quinoa embryo (23.5 w/dw%) is higher than that of the perisperm (7.2 w/dw%), the fractionation resulted in almost a doubling of the protein purity in the fine fraction, with a factor five times higher protein yield than in the coarse fraction. The cut size characterizes the air classification process by defining the size where particles have equal chance of ending up in either the coarse or fine fraction. Because the yield of both fractions is equal, the cut size is comparable to the mass median diameter of the quinoa seed after milling (704.5 µm).

Table 1. Experimental characterisation of the whole quinoa flour, the fine fraction and the coarse fraction after air classification, with ± is equal to the standard deviation.

Material	Yield (%)	Protein yield (%)	Protein purity (w/dw%)	Starch purity (w/dw%)	$D_{0.5}$ (µm)
Whole flour	100.0	100.0	14.5 ± 0.6	53.7	996.1 ± 18.5
Coarse fraction	48.3 ± 1.8	17.1	6.6 ± 3.6	70.6	1035.3 ± 10.5
Fine fraction	50.4 ± 2.5	82.9	23.9 ± 1.3	24.9	558.5 ± 6.2

4.2. Aqueous phase separation

As observed for pea fractionation, it was hypothesized that aqueous suspension of quinoa flour would lead to phase separation of protein, starch and fibre into soluble and insoluble fractions. This phase separation can be explained by differences in density between non-dissolved particles and possible enthalpic and entropic effects between different dissolved biopolymers [17]. However, for quinoa it was found that an additional fine milling step was critical to facilitate protein dissolution and would thus increase enrichment of dissolved protein by subsequent aqueous phase separation. In this fine milling step the average particle diameter decreased from 559 µm down to 30 µm.

When suspending the non-milled and milled fine fractions, phase separation into three distinct layers, a liquid layer (layer 1), a white solid layer (layer 2), and a beige solid layer (layer 3), was observed for both fractions (Figure 3). Layer 1 had the highest protein purity in both fractions, showing protein enrichment in the top layer at either particle size (Figure 4). However, protein purity and protein yield were higher in layer 1 of the finely milled fine fraction (41.2 w/dw% and 40.3%, respectively) compared to the non-milled

fraction, indicating enhanced protein dissolution. This can be explained by the disruption of cells upon milling and thus the easier dissociation of starch granules and protein bodies during suspension.

Quinoa protein consists of 35% water-soluble albumins and 37% globulins soluble in salt solutions [1], while from the experiments it appeared that the dissolved (only water) protein in the top layer presents 40.3% of all proteins (Figure 4). This might at least be partially explained by the quinoa variety being higher in water-soluble protein.

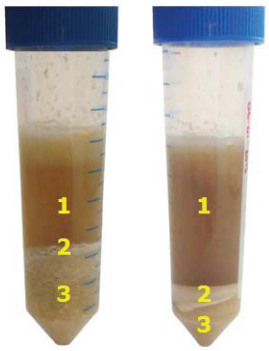


Figure 3. Aqueous phase separation of the suspended fine fractions with and without milling before suspension. Left: non-milled fine fraction ($D_{0.5}$: 559 µm), right: milled fine fraction ($D_{0.5}$: 30 µm). Numbers indicate the layers formed

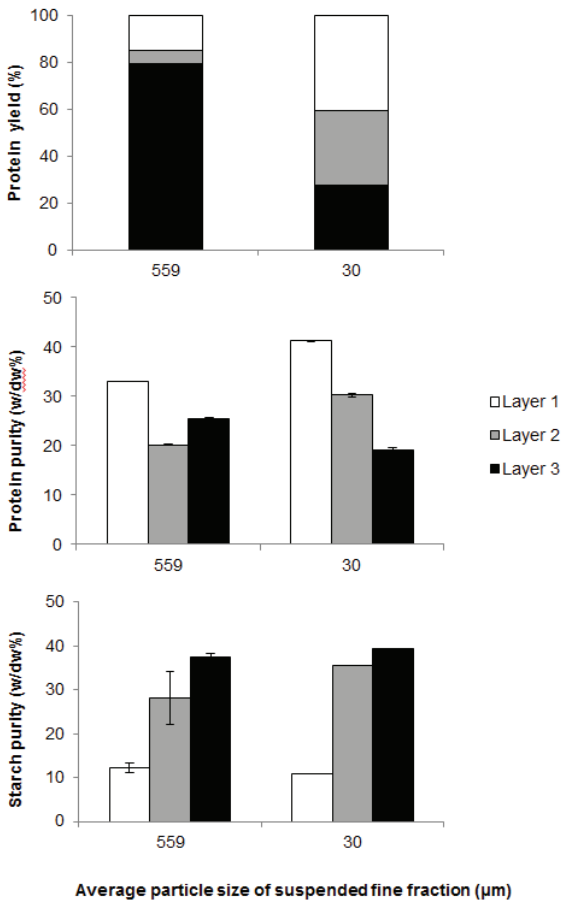


Figure 4. Protein yield (%), protein purity (w/dw%) and starch purity (w/dw%) of the non-milled and milled fine fractions

Previous research on quinoa protein showed that protein solubility could be increased by the addition of salt [3]. It was observed that when adding up to 0.5 M NaCl to quinoa flour suspensions, protein yield increased steadily. Higher NaCl concentrations did not increase the yield significantly. Therefore, we added NaCl to suspensions of the milled fine fraction to reach different concentrations in the range 0-0.5 M. Similar as observed for the suspensions without salt addition, the suspensions phase-separated into three layers; however the dry matter content of the top layer increased with increasing salt concentration (Figure 5). Protein purity and protein yield of the layers were calculated by correcting for the added salt. For layer 1, protein yield increased considerably from 40.3 to

80.3% going from 0 to 0.5 M NaCl, respectively (Figure 6). Protein purity in the same layer did not increase as strongly with increased salt content but it was higher for 0.35 and 0.5 M NaCl than for 0 and 0.15 M NaCl. These results indicate higher protein solubility at higher salt concentrations and are line with the findings from literature. The increased protein solubility can be explained by the salting in-effect [23-25]. The added salt ions interact with the charged groups of the protein molecule, leading to less interactions of the protein molecule with the surrounding water molecule, which results in an increased solubility of the protein.

As 37% of quinoa protein is salt-soluble, the addition of salt facilitates the solubilisation of globulins, which can be added to the amount of solubilised albumins, as albumin dissolution behavior was found not to be affected by the salt content [3]. The smaller increase in protein purity compared to protein yield might be due to the additional solubilisation of non-protein components. Starch purity did not clearly increase with higher salt concentrations (Figure 6), which suggests that possibly the dissolution of soluble fibres might have been influenced by the NaCl concentration.

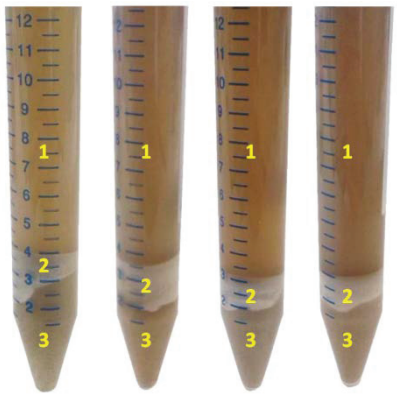


Figure 5. Aqueous phase separation of the suspended milled fine fraction with varying NaCl concentrations. Left to right: 0M, 0.15 M, 0.35 M and 0.5 M NaCl. Numbers indicate the layers formed

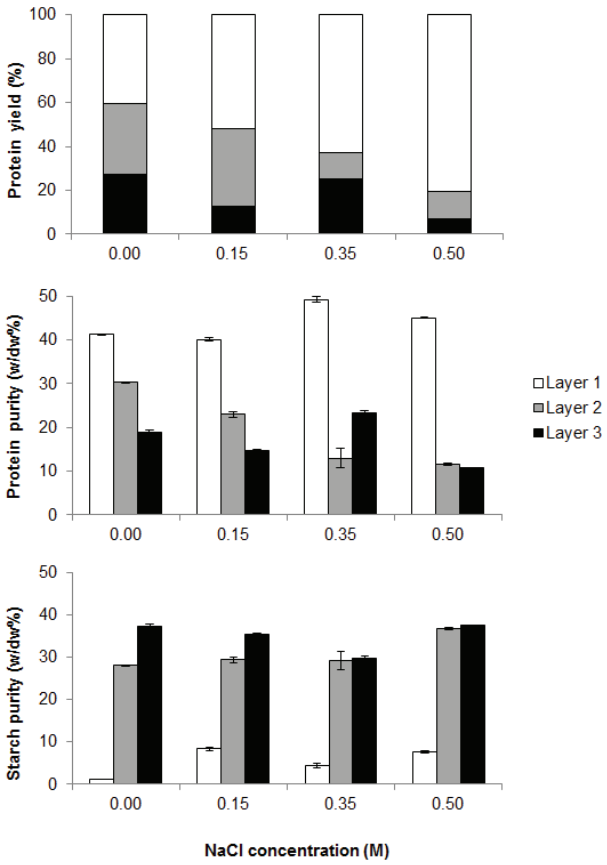


Figure 6. Protein yield, protein purity (w/dw%) and starch purity (w/dw%) of the suspended milled fine fraction with varying NaCl concentrations

4.3. Ultrafiltration

To further increase protein purity, the liquid top layer of the phase-separated suspensions with and without added salt were carefully decanted and subjected to ultrafiltration. The idea behind this step was that small solutes would be removed and proteins would be retained by the membrane, thereby increasing the protein concentration in the retentate. The ultrafiltration was carried out in a batch system for approximately 165 minutes after which a retentate volume of 55% compared to the initial feed volume was obtained. Because the filtration time was not always exactly 165 min for each sample small corrections were made to obtain protein purity and protein yield values for an exact final

retentate volume of 55%. On the basis of 55% retentate yield, the protein concentration in the retentate could be increased from 41 to 46 w/dw% without addition of salt, and from 35 to 59 w/dw% for 0.5 M NaCl (Figure 7). It should be emphasized that the latter values are the protein contents without correction for the presence of NaCl.

It can be concluded that the protein purity after ultrafiltration increases significantly, which is caused obviously by the loss of salt via the permeate flow. However, in addition also the total protein yield increased, which may be explained by the different size of the globulins and albumins. Globulins range from 8-100 kDa in size, while albumins are 8-9 kDa in size [3]. The cut-off of the ultrafiltration membrane was 3 kDa, so some smaller albumins were probably lost during the ultrafiltration. Because at high salt concentrations there are relative more globulins compared to albumins, the relative loss of protein will substantially decrease at higher salt concentrations. Concluding, the use of salt during aqueous phase separation and subsequent ultrafiltration is considered very promising as it provides higher protein purity and yield.

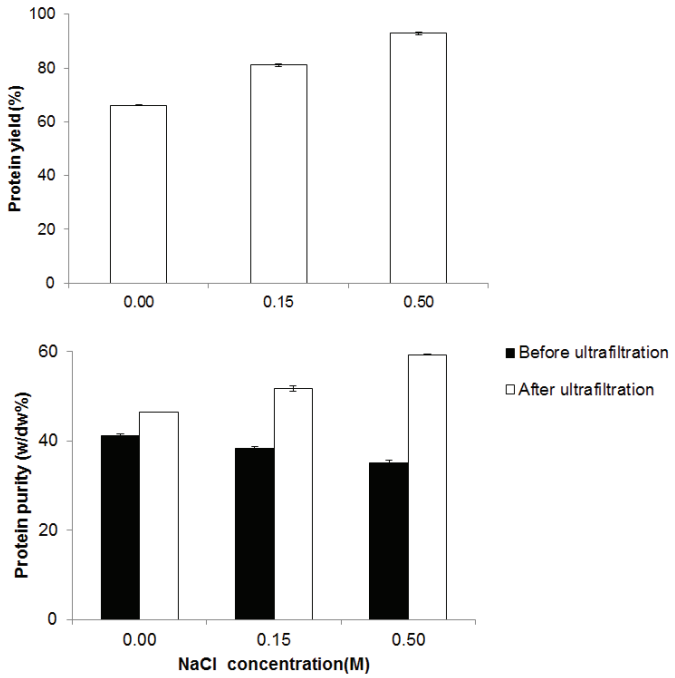


Figure 7. Protein yield and protein purity (w/dw%) of layer 1 of the phase-separated suspensions containing different salt concentrations

4.4. Process review

A mass flow analysis was carried out and visualized in a Sankey diagram to review the entire hybrid dry and aqueous fractionation process of quinoa. This was specifically done for the aqueous phase separation with 0.5 M NaCl for extracting protein from the milled fine fraction (Figure 8). The protein yield and protein purity starting from the seed to the final ultrafiltration are shown in Figure 9. It can be observed that a large amount of material (48.1%) was lost during impact milling of the fine fraction (Figure 8). This material loss can be explained by the relatively small particle size of the fraction, which increases the attractive van der Waals forces between particles and particles and wall of the mill interior, thus resulting in fouling [26]. However, when feeding larger amounts of material (compared to the 287 g that was fed during our experiment) the loss due to fouling is expected to be much less. This can be explained by the development of a steady state situation during which no further accumulation of material will occur. If we exclude losses during impact milling, then, 24.4% protein from the total quinoa protein could be recovered without salt use in the process and 62.0% with use of 0.5 M NaCl during aqueous phase separation (Figure 9).

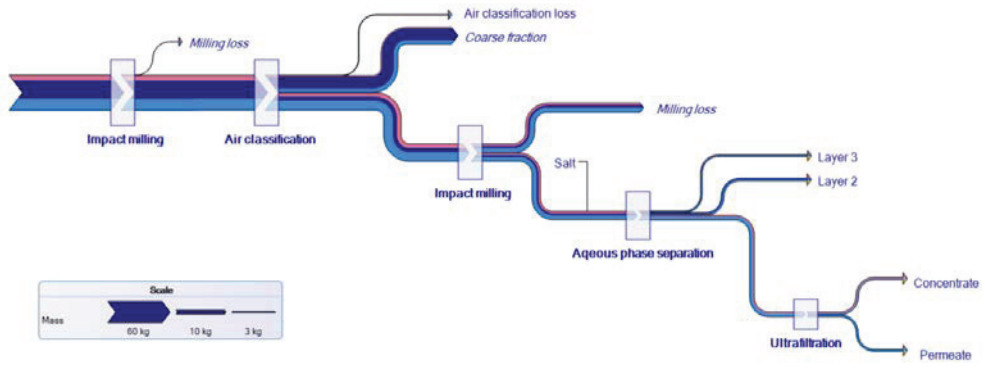


Figure 8. Sankey diagram of the hybrid dry and aqueous fractionation process for the production of protein-rich fractions of quinoa. The arrow thickness corresponds to the mass of the flow. Red: protein, dark blue: starch, light blue: rest

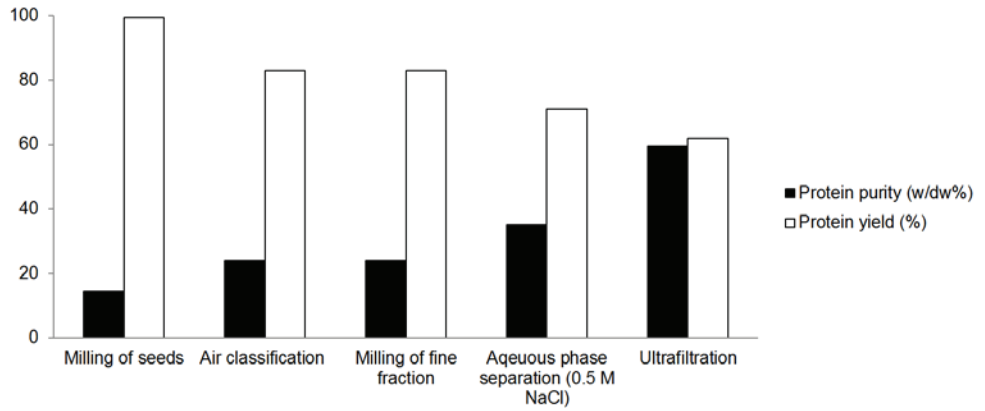


Figure 9. Protein purity (w/dw%) and protein yield (g protein obtained / g protein in the seed) after each step of the hybrid dry and aqueous fractionation process, assuming that fouling at higher throughputs is negligible

The proposed hybrid fractionation is a milder and more sustainable way compared to wet fractionation, although the protein purity obtained is still lower compared to conventional wet fractionation. Further process optimization can be carried out to increase the protein purity even more. Optimizations might be performed from the very beginning, before even milling the seed. A recent study applied a moist conditioning treatment to quinoa before milling [11]. By raising the moisture content from 12.3 to 15 w/w% the protein purity of the bran fraction obtained after milling increased from 24 to 28 w/dw%. The higher moisture content was related to increased elasticity of the outer cell tissues, providing better dissociation of the embryo from the perisperm during milling. In another study on pea the moisture content prior to milling was increased to shift the protein to the rubbery state. This treatment facilitated disentanglement from the glassy starch granules during milling, providing higher separation efficiency [27].

Another step, where the protein purity may be further increased, is during ultrafiltration. One may increase the concentration factor or apply diafiltration to completely wash out the salt. Increasing the concentration factor leads to a smaller retentate volume. For example for a final retentate volume of 20% (in combination with 0.5 M NaCl) protein purity may further increase from 59.4 to 78.2 w/dw%. The drawback of an increased concentration factor is that the permeate flux will decline severely due to the accumulating solute concentration [28]. For 55% retentate volume, diafiltration and

thereby removal of all salt would increase protein purity from 59.4 to 65.5 w/dw% in combination with 0.5 M NaCl aqueous phase separation. However, removal of the salt will lead to precipitation of the salt-soluble globulins, which may not always be desirable. Still, the calculations show there is room for further optimization of the process towards protein purities that are approaching protein concentrations from conventional wet fractionation.

4.5. Comparison to conventional wet fractionation

To compare the efficiency of the proposed hybrid dry and aqueous fractionation method to conventional fully wet fractionation for protein isolation, protein yield, protein purity and water consumption were compared with literature data (Table 2). Recent studies have analysed protein yield and protein purity from quinoa during wet fractionation with varying conditions [11,9,2]. With wet fractionation very high protein purities (68-93 w/dw%) can be achieved, but at the expense of a lower protein yield (g protein obtained / g protein in the seed) (24-61%). Furthermore, during wet fractionation 9-9.5 ml of water was used per gram of quinoa flour (depending on the fat content of the quinoa seeds used) to achieve a protein yield of 61%. The hybrid fractionation process proposed in this study resulted in a lower protein purity compared to literature values for wet fractionation but similar or higher protein yield compared to wet fractionation. But important to note is that only 0.2 ml of water per gram of quinoa flour was used to achieve the protein yield of 62%, which means 97.8% savings in water compared to wet fractionation. Even if using double the amount of water for ultrafiltration to remove salts remaining in the final quinoa fraction, savings of over 88.9% in water are possible. This reduction in water consumption is connected to an enormous potential reduction in energy consumption, as less water needs to be removed for drying the final protein ingredient suspension.

Table 2. Summary of the different methods for isolation of quinoa protein. Protein yields were recalculated according to equation 1 and using a nitrogen-to-protein conversion factor of 5.7 for fair comparison. Water use was recalculated to ml water per g non-defatted quinoa flour, assuming an average fat content of 5-7.2% in the quinoa seed used [29]

Method for protein isolation	Study	Protein solubilisation conditions	Protein yield %	Protein purity (w/dw%)	Water use (ml/g quinoa flour)
Wet fractionation	Avila Ruiz et al., 2015	pH 8-11	23-36	88-91	21-22
	Aora & Alvarado, 2009	pH 7.5-10.5	37-56	67-79	10-11
	Scanlin, 2009 (patent)	pH 8-12	No data	46-82	10-11
Dry and wet fractionation	Föste et al., 2015	pH 10	63	70	10-11
Dry and aqeuous fractionation	Present study	0-0.5 M NaCl	24-62	47-59	0.2

Another main difference between our process and the conventional extraction is that mild conditions are used in contrast to wet fractionation (avoiding addition of chemicals for fat extraction and to induce pH shifts). This not only is more cost effective for the producer but also in line with clean-label and sustainability trends among consumers. Moreover, by avoiding harsh conditions also native properties of the quinoa protein are retained as much as possible. Finally, we recommend exploring the application of the side-streams of our hybrid fractionation process to maximise sustainability. Such side-streams are for example the perisperm starch-rich fraction obtained after air classification and the aqueous phases that are enriched in starch.

5. Conclusions

We succeeded in developing a hybrid separation process for quinoa to obtain high protein-rich fractions. The method proposed in the present study can provide a protein concentrate with a purity of 59 w/dw% and a protein yield of 61%. This yield is similar or higher compared to conventional wet fractionation. Although the purity is lower compared to conventional extraction with further process optimisation, the product obtained is still relevant for the food industry. This is because higher protein purities will not always be required or even desired, as food producers may also wish to keep some of the quinoa fibre, starch, oil and micronutrients in the protein concentrate for functional or

nutritional benefits depending on the application. In this case, the advantages of mild fractionation are obvious in providing reduction in water, energy and chemicals consumption and retention of native functional properties. Finally, we estimated that the protein purity may be further increased up to 78 w/dw% by process optimization.

6. Acknowledgements

The authors gratefully acknowledge the financial support of the "IPOP Customized Nutrition" program of Wageningen University and Research Centre.

7. References

1. Abugoch L (2009) Chapter 1 Quinoa (Chenopodium quinoa Willd.): Composition, Chemistry, Nutritional, and Functional Properties. Advances in Food and Nutrition Research 58:1-31.
2. Avila Ruiz G, Xiao W, van Boekel M, Stieger M, Minor M (2016) Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein from sweet quinoa (*Chenopodium quinoa* Willd) Food Chemistry.
3. Brinegar C, Goundan S (1993) Isolation and characterization of chenopodin, the 118 seed storage protein of Quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 41 (2):182-185.
4. Brinegar C, Sine B, Nwokocha L (1996) High-Cysteine 2S Seed Storage Proteins from Quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 44 (7):1621-1623.
5. Chauhan GS, Cui W, Eskin NAM (1999) Effect of saponin on the surface properties of quinoa proteins. International Journal of Food Properties 2 (1):13-22.
6. Aluko RE, Monu E (2003) Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science 68 (4):1254-1258.
7. Lindeboom N (2005) Studies on the characterization, biosynthesis and isolation of starch and protein from quinoa (*Chenopodium quinoa* Willd.). University of Saskatchewan, Canada
8. Abugoch LE, Romero N, Tapia CA, Silva J, Rivera M (2008) Study of some physicochemical and functional properties of quinoa (Chenopodium quinoa willd) protein isolates. Journal of Agricultural and Food Chemistry 56 (12):4745-4750.
9. Aora JCC, Alvarado K (2009) Aislados proteínicos de granos altoandinos *chenopodiaceas*; quinua "*chenopodium quinoa*" – cañahua "*chenopodium pallidicaule*" por precipitación isoeléctrica. Revista Boliviana de Química 26 (1):12-20.
10. Valenzuela C, Abugoch L, Tapia C, Gamboa A (2013) Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence on film formation. International Journal of Food Science & Technology 48 (4):843-849.

11. Föste M, Elgeti D, Brunner A-K, Jekle M, Becker T (2015) Isolation of quinoa protein by milling fractionation and solvent extraction. Food and Bioproducts Processing 96:20-26.
12. Avila Ruiz G, Opazo-Navarrete M, Meurs M, Minor M, Sala G, van Boekel M, Stieger M, Janssen A (2016) Denaturation and *in vitro* gastric digestion of heat-treated quinoa protein isolates obtained at various extraction pH. Plant Foods for Human Nutrition.
13. Schutyser MAI, van der Goot AJ (2011) The potential of dry fractionation processes for sustainable plant protein production. Trends in Food Science & Technology 22 (4):154-164.
14. Tyler R, Youngs CG, Sosulski FW (1981) Air Classification of Legumes. I. Separation Efficiency, Yield, and Composition of the Starch and Protein Fractions. Cereal Chemistry 58:144-148.
15. Wu YV, Stringfellow AC (1992) Air Classification of Flours from Wheats with Varying Hardness: Protein Shifts. Cereal Chemistry 69 (2):188-191.
16. Pelgrom PJM, Vissers AM, Boom RM, Schutyser MAI (2013) Dry fractionation for production of functional pea protein concentrates. Food Research International 53 (1):232-239.
17. Pelgrom PJM, Boom RM, Schutyser MAI (2015) Functional analysis of mildly refined fractions from yellow pea. Food Hydrocolloids 44:12-22.
18. Pelgrom PM, Boom R, Schutyser MI (2015) Method Development to Increase Protein Enrichment During Dry Fractionation of Starch-Rich Legumes. Food and Bioprocess Technology 8 (7):1495-1502.
19. Prego I, Maldonado S, Otegui M (1998) Seed Structure and Localization of Reserves in *Chenopodium quinoa*. Annals of Botany 82 (4):481-488.
20. Ando H, Chen Y-C, Tang H, Shimizu M, Watanabe K, Mitsunaga T (2002) Food Components in Fractions of Quinoa Seed. Food Science and Technology Research 8 (1):80-84.
21. Schutyser MAI, Pelgrom PJM, van der Goot AJ, Boom RM (2015) Dry fractionation for sustainable production of functional legume protein concentrates. Trends in Food Science & Technology 45 (2):327-335.
22. Chauhan GS, Eskin NAM, Tkachuk R (1992) Nutrients and Antinutrients in Quinoa Seed. Cereal Chemistry 69:85-88.
23. Arakawa T, Timasheff SN (1982) Preferential interactions of proteins with salts in concentrated solutions. Biochemistry 21 (25):6545-6552.
24. Collins KD (1997) Charge density-dependent strength of hydration and biological structure. Biophysical Journal 72 (1):65-76.
25. Li W, Mu Y (2011) Hydration patterns and salting effects in sodium chloride solution. The Journal of Chemical Physics 135 (13):134502.
26. Pelgrom PJM, Berghout JAM, van der Goot AJ, Boom RM, Schutyser MAI (2014) Preparation of functional lupine protein fractions by dry separation. LWT - Food Science and Technology 59 (2, Part 1):680-688.
27. Pelgrom PM, Schutyser MI, Boom R (2013) Thermomechanical Morphology of Peas and Its Relation to Fracture Behaviour. Food and Bioprocess Technology 6 (12):3317-3325.

28. Suki A, Fane AG, Fell CJD (1984) Flux decline in protein ultrafiltration. Journal of Membrane Science 21 (3):269-283.
29. Bhargava A, Srivastava S (2013) Chemistry. In: Bhargava A, Srivastava S (eds) Quinoa - Botany, Production and Uses. CABI, Oxfordshire, pp 185-239.

Chapter 5

High pressure – high temperature processing of whey protein – sugar solutions: influence of pressure on Maillard reaction and protein aggregation

This chapter is based upon: Avila Ruiz G, Xi B, Minor M, Sala G, van Boekel M, Fogliano V, Stieger M (2016) High pressure – high temperature processing of whey protein – sugar solutions: Influence of pressure on Maillard reactions and protein aggregation. Journal of Agricultural and Food Chemistry 64(38):7208-7215.

1. Abstract

The aim of the study was to determine the influence of pressure in high pressure-high temperature (HPHT) processing on Maillard reactions and protein aggregation of whey protein-sugar solutions. Solutions of whey protein isolate containing either glucose or trehalose at pH 6, 7 and 9 were treated by HPHT processing or conventional high temperature (HT) treatments. Browning was reduced, and early and advanced Maillard reactions were retarded under HPHT processing at all pH values compared to HT treatment. HPHT induced a larger pH drop than HT treatments, especially at pH 9, which was not associated with Maillard reactions. After HPHT processing at pH 7, protein aggregation and viscosity of whey protein isolate-glucose/trehalose solutions remained unchanged. It was concluded that HPHT processing can potentially improve the quality of protein-sugar containing foods, for which browning and high viscosities are undesired, such as high-protein beverages.

2. Introduction

The use of high pressure-high temperature (HPHT) processing to sterilize foods is a promising alternative to conventional retort heating [1]. HPHT processing combines high temperatures (90-121°C) with pressures ≥ 600 MPa to inactivate pathogens and spores. Compression heating allows reducing heating-up times leading to shorter processing times and lower heat loads compared to conventional retort sterilization. It was reported that lower heat loads are the main advantage of HPHT processing [2], which can consequently improve sensorial and nutritional food properties [3,1,2]. However, it remains unclear whether pressure itself or the lower heat load contributes to the improved sensory and nutritional properties of HPHT processed foods.

Maillard reactions (MR) are an important factor contributing to sensory quality of foods and beverages [4]. In sterilized foods MR are usually undesired, e.g. in dairy-based beverages, high-protein beverages, puddings, creams etc. Studies on the effect of high pressure on MR are not extensive and were reviewed recently [5]. The rates of some MR pathways can be increased or decreased by high pressures depending on the predominant mechanism and specific processing conditions. Some studies showed that pressure

accelerated the condensation reaction and the formation of Amadori products, while other studies found that pressure decelerated amino acid-sugar conjugation, the Amadori rearrangement and the degradation of Amadori rearrangement products [6-10]. Several studies reported that for amino acid-sugar solutions, pressure retards or promotes the formation of advanced MR products and browning, depending on the pH [11-14]. To the best of our knowledge, the influence of pressure on MR products in protein-sugar solutions has been investigated only by two studies, whereas several studies have examined MR products in amino acid-sugar solutions. Proteins were found to denature and aggregate by a different mechanism under high pressure treatment compared to heat treatment [15]. Changes in protein structure can be associated with the extent of MR under HPHT [9,10]. The focus of these studies was on the chemical properties of protein-sugar solutions and the Maillard reaction kinetics. Buckow et al. (2011) also studied physical properties of the solutions using SDS-PAGE. An increase in high molecular weight compounds after HPHT treatment (30 min, 200 and 600 MPa at 110°C) of BSA-glucose solutions compared to heat treatment (10 and 30 min, 0.1 MPa at 110°C) was found. Aggregation, and a potential change in rheological properties, in sterilized food might be desirable or not, depending on the type of food. For liquid, sterilized foods containing protein, usually, viscosity increases are only desired to a certain extent, e.g. in high-protein beverages.

The aim of the study was to determine the influence of pressure in HPHT processing on Maillard reactions and protein aggregation of whey protein-sugar solutions. Browning, pH, Maillard reaction products (furosine, Nε-(Carboxymethyl)-ι-lysine (CML) and Nε-(Carboxyethyl)-ι-lysine (CEL)), viscosity and particle size of whey protein isolate solutions containing glucose (reducing sugar) or trehalose (non-reducing sugar) were quantified. Different HPHT treatment conditions (700 MPa, 0-15 min, 123°C) were compared with different high temperature (HT) treatments (0-15 min, 123°C). Processing times similar to those used in industry (3-5 min) were chosen [14].

3. Materials and methods

3.1. Materials

Whey protein isolate (WPI) (BiPRO) was purchased from Davisco, Foods International, Inc. (Minnesota, USA). Glucose and trehalose were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). MilliQ water was used.

3.2. Preparation of WPI-glucose/trehalose solutions

Aqueous solutions of 6% (w/w) WPI and 5% (w/w) glucose or 5% (w/w) trehalose were adjusted to pH 6, 7 and 9 by addition of 1 N HCl or 0.1 N NaOH, respectively, and stirred for 3 h. WPI – glucose (WPI/G) and WPI – trehalose (WPI/T) solutions were stored overnight at 4°C before processing to ensure dissolution of WPI.

3.3. HPHT treatment of WPI/G and WPI/T solutions

WPI/G and WPI/T solutions (10 ml) were sealed in small polyethylene bags after removal of air. Solutions were HPHT-treated using a Resato high-pressure apparatus (Resato FPU-100-50, Resato International B.V., Roden, The Netherlands). Pressure build-up rate was 4.5 MPa/s. Water was used as pressure medium. Solutions were first preheated at 90°C for 3 min in a water bath and subsequently high-pressure treated at 700 MPa for 0, 1.5, 3, 9 and 15 min. The time point at which the solutions reached 123°C was taken as processing time zero.

It was not possible to measure the temperature or pH of the solution during the HPHT treatment experimentally. To estimate temperature-time profiles for all processing times, two assumptions were made: 1) the adiabatic heat increase was uniformly transmitted to the solution without time delay; 2) the heat-transmitting properties of the WPI/G and WPI/T solutions were similar to those of water. To estimate the maximum temperature reached in the HPHT treatment, the temperature of the pressure medium during pressurization was measured using a lab-scale high-pressure unit (volume 180 ml, maximum pressure 1000 MPa, Resato International B.V., Roden, The Netherlands) (Figure S1). In previous studies the temperature of water after applying different pressures at

various initial temperatures was measured [16,17]. When extrapolating the data of Esthiagi et al. (2001), a maximum temperature of 122.5°C during HPHT treatment at 700 MPa was obtained. The maximum temperature measured by Knoerzer et al. (2010) at 700 MPa was 125.0°C. Through combination of our experimental data and the data from literature, the maximum temperature in our study was estimated to be 123°C ± 2°C.

The temperature loss was determined by measuring the temperature of the pressure medium before pressure-build up and after pressure release. The difference in temperature was assumed to be equal to the temperature loss experienced during the processing times. The calculated temperature difference was linearly correlated to the initial temperature (Figure S2).

3.4. HT treatment of WPI/G and WPI/T solutions

WPI/G and WPI/T solutions were heated in a heating block (Liebisch Labortechnik, type: 53186301, Germany) to 123°C. Solutions were treated for 0, 1.5, 3, 9 and 15 min. To mimic the temperature-time profile of the HPHT treatment during the processing times, the heating block was set to lower temperatures during these times. Subsequently, solutions were cooled down to room temperature using a water bath at 15°C. The temperature of the solutions was monitored during the entire treatment. Temperature measurements were performed in triplicate.

3.5. Determination of browning

Browning intensity of HPHT and HT treated WPI/G and WPI/T solutions was determined by quantifying the absorbance at 420 nm with a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) [18]. To compare browning rates between the treatments, linear regression of absorbance as a function of processing time was performed.

3.6. Determination of Maillard reaction products

Furosine, Nε-(Carboxymethyl)-ι-lysine (CML) and Nε-(Carboxyethyl)-ι-lysine (CEL) were quantified using a previously described method with small modifications [19].

3.6.1. Sample preparation

A solution volume of 100 µl was mixed with HCl (6 N) in a screw capped flask with PTFE septa. The mixture was saturated with nitrogen (15 min at 2 bar) and hydrolyzed in a heating block (Liebisch Labortechnik, type: 53186301, Germany) for 20 h at 110°C. The mixture was centrifuged for 10 min at 4000 rpm at 4°C and the supernatant was subsequently filtrated using polytetrafluoroethylene filters (0.45µm, Phenomenex, USA). A volume of 200 µl filtered sample was dried under nitrogen flow in order to prevent the oxidation of the constituents. The sample was reconstituted in 190 µl of water and 10 µl of a mixed internal standard (d4-lys, d2-CML, d2-CEL and d2-furosine) was added. The sample was loaded onto equilibrated Oasis HLB 1 cc cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described in detail [19]. Eluted solutions were dried under nitrogen overnight. Samples were dissolved in 150 µl of an acetonitrile – water (90:10) solution. Then, 5 µl were injected into the LC-MS/MS system.

3.6.2. Liquid chromatography tandem mass spectrometry (LC–MS/MS)

Separation of furosine, CML, CEL, lysine and their respective internal standards was achieved on a Hydrophilic Interaction Liquid Chromatography column using the following mobile phases: A) 0.1 % acetic acid in water, B) 50 mM ammonium acetate in water, and C) 0.1% acetic acid in acetonitrile.

The compounds were eluted and the chromatographic profile was recorded according to the method of Troise et al. (2015).

3.6.3. Analytical performances

CML, CEL and furosine were quantified using a linear calibration curve obtained with solutions of purified CML, CEL and furosine at different concentrations. The limit of detection (LOD) and the limit of quantitation (LOQ) were monitored according to Troise et al. (2015).

3.7. Determination of pH

pH of WPI/G and WPI/T solutions was determined at 20°C using a pH meter (Conductivity

Proline Plus, QiS, The Netherlands). Measurements were performed in duplicate.

3.8. Determination of viscosity

Viscosity of the solutions was determined using an Ubbelohde viscometer (SI Analytics GmbH, Germany) at 25°C. The constant of the viscometer capillary was 0.004639 mm^2s^{-2}. Measurements were performed in triplicate. Viscosity was calculated using the following formula:

υ_{kin} (m^2s^{-1}) = t (s) × capillary constant (mm^2s^{-2}) ×10^{-6} [1]

where υ_{kin} is the kinematic viscosity and t is the flow-through time,

η (Pa.s) = υ_{kin} (m^2s^{-1}) × ρ ($kg.m^{-3}$) [2]

where η is the dynamic viscosity and ρ the density of the solutions.

Density was determined using a density meter (DMA 5000, AntonPaar, Graz, Austria) at 25°C. When setting equation 1 equal to equation 2, the viscosity was obtained and converted to mPa.s.

3.9. Determination of particle size

WPI/G and WPI/T solutions were diluted to a protein concentration of 0.5% (w/w) with MilliQ water and subsequently filtered using 0.2 µm RC filters. Particle size of the heated solutions was determined by High Pressure – Size Exclusion Chromatography (HP-SEC) fitted with an Ultimate 3000 pump and a UV detector (Thermo Scientific, USA). The HP-SEC columns (TSKGel G3000SWXL, 5µm, 300 x 7.8 mm, and TSKGel G2000SWXL, 5 µm, 300 x 7.8 mm) were equilibrated with 30% acetonitrile in MilliQ water and 0.1% trifluoroacetic acid as eluents. Samples were loaded and eluted at 1.5 ml/min at 30°C, and the eluates were monitored at 214 nm.

4. Results and discussion

4.1. Determination of processing conditions

The temperature-time profiles of HT treatments were experimentally determined, while those of HPHT treatments were estimated (Figure 1). The main difference between the

temperature-time profiles of HT and HPHT treatments was in the heating-up phase. The HT treatment took about 6 min to reach the target temperature (123°C), and it took about 3 min for the temperature to increase from 90 to 123°C. In contrast, the HPHT treatment took about 3 min to reach the target temperature (123°C), and it took only about 30 s for the temperature to increase from 90 to 123°C. This fast temperature rise in the HPHT treatments is due to adiabatic heating accompanied by pressure build-up [16,17]. The temperature decrease during the pressure-holding time was successfully matched in the HT treatments. During the cooling phase the temperature decreased from about 120 to 90°C faster for HPHT treatments compared to HT treatments.

Due to the differences in the heating-up phase between the two treatment techniques, the time point at which the solutions reached 123°C (t = 6 min) was taken to compare HT and HPHT treatments in terms of heat load. The matching of the temperature-time profiles of the HT-treated solutions in the pressure-holding phase time also ensured a fair comparison between HT and HPHT treatments.

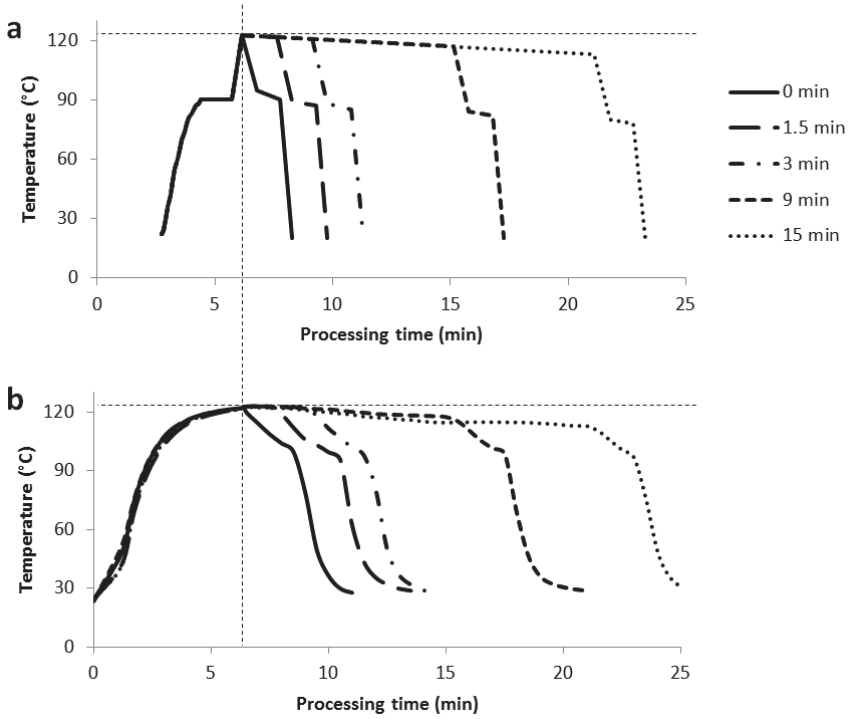


Figure 1. Temperature-time profiles of (a) HPHT treatments estimated using experimental and literature data, and (b) HT treatments experimentally determined. Profiles of HPHT treatments start at 3 min to show the starting point for processing when 123°C is reached.

4.2. Browning

The absorbance of WPI/G solutions treated with HT was higher compared to that of WPI/G solutions treated with HPHT for all processing times at pH 7 and 9 (Figure 2). At pH 7 and 9, the browning rates were 15 times and 3.5 times higher for HT than for HPHT treatment, respectively. The difference in browning rate was also evident by eye (Figure S3). The absorbance of the WPI/G solutions at pH 6 could not be measured due to turbidity of the solutions. However, a reduced browning rate was observed by eye for the HPHT treatment compared to the HT solutions (Figure S3).

The browning kinetics of the WPI/G solutions treated with HT were comparable to those of casein (3% w/w) – glucose (150 mM) solutions (pH 6.8) heated to 120°C for up to 40 min [18]. It is noteworthy that in our study the heating-up time was excluded from the reported processing times. The higher absorbance of HT solutions compared to HPHT solutions shows that pressure at high temperature had a retarding effect on browning. The higher browning rates of HPHT solutions indicate that the retarding effect of pressure was stronger than the promoting effect of heat on browning, especially at pH 7.

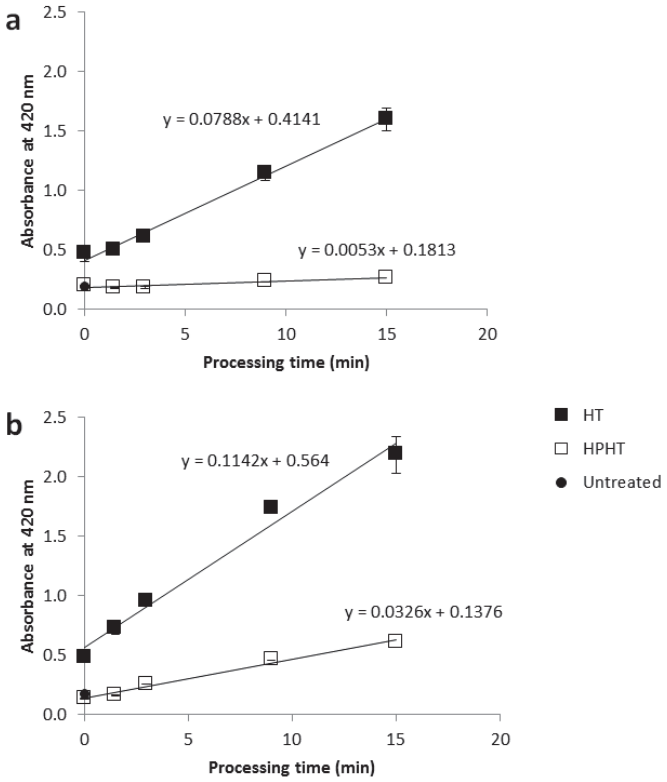


Figure 2. Absorbance of WPI/G solutions at (a) pH 7 and (b) pH 9 as a function of processing time after HT and HPHT treatments. Means of two measurements are shown with standard deviations.

4.3. Maillard reaction products

Concentrations of furosine, CML and CEL were higher in WPI/G solutions treated with HT than in solutions treated with HPHT at pH 6, 7 and 9, paralleling the browning development (Figure 3). The concentrations of furosine, CML and CEL increased about linearly with processing time for HPHT treatment, whereas for HT treatment, they first increased steeply and then approached a plateau value. At pH 9, the concentrations of MR products in HT solutions dropped at 15 min processing.

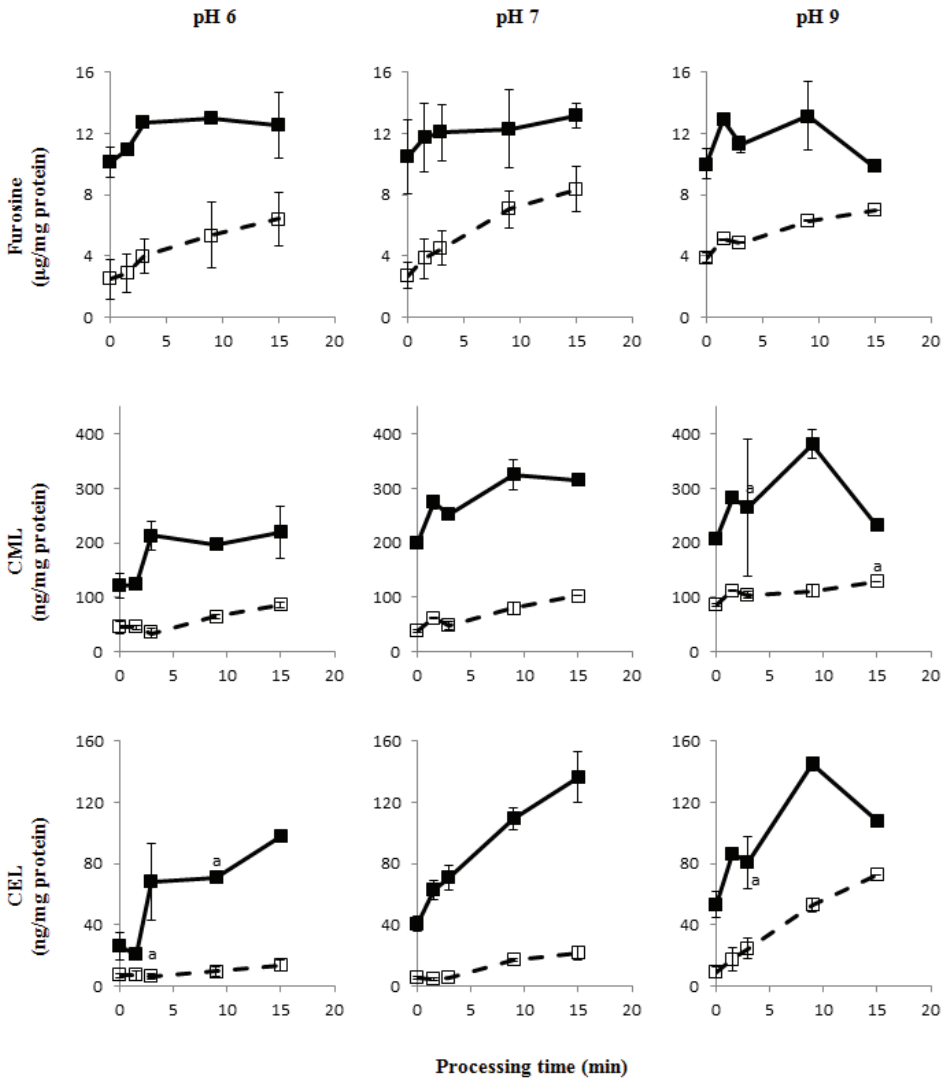


Figure 3. Concentrations of furosine, CML and CEL as a function of processing time in WPI/G solutions prepared at pH 6, 7 and 9 and treated with either HT (■) or HPHT (□). Means of two measurements are shown with standard deviations. a = single measurements.

Furosine, CML and CEL concentrations were comparable to those measured in UHT milk [19]. Furosine concentrations were in the same order of magnitude compared to concentrations measured by Brands and van Boekel (2001) in casein (3%) – glucose (150 mM) solutions (pH 6.8) heated for 0-40 min at 120°C. CML and CEL concentrations were also comparable to results obtained in a previous study with heated casein (3%) – glucose

(2.7%) solutions (pH 6.8) for 0-30 min at 120°C [20]. The plateau observed for HT treatment can be explained with the rate of MR product formation being equal to the rate of degradation, after which the rate of degradation becomes dominant. Such a behavior has been previously reported for the Amadori products [21].

The lower concentrations of MR products for HPHT treatment show that pressure had a retarding effect on the generation of furosine, CEL and CML. The difference in concentration profiles between the two treatment techniques was similar to that for furosine in heat-treated milk at different temperatures. At 130°C, furosine concentration increased linearly from 0 to 18 min processing, whereas at 140°C, the concentration increased sharply from 0 to 8 min, after which it reached a plateau [22]. Compared to previous studies, which investigated HPHT treatment using amino acids or purified proteins and long treatment times (0-24 h), in our study a retarding effect of HPHT treatment for a mixture of proteins was observed using treatment times closer to industrial applications. Previous studies ascribed this effect to pressure favoring the side of the reactants in Maillard reactions due to the smaller volume occupied compared to the volume occupied by the products (positive activation volume) [5]. In our systems, the volume of native and denatured proteins might also play a role. It has been reported that pressure has a synergistic effect with heat on whey protein denaturation and unfolding [23,24,9]. Buckow et al. (2011) reported that pressures of 600 MPa for up to 45 min at 70°C did not lead to significant unfolding of BSA. However, at higher temperatures, protein unfolding was accelerated, possibly exposing more lysine groups. In the same study, it was found that protein-sugar conjugation was decelerated under HPHT treatment compared to HT treatment. This could mean that although more reactive groups become available under high pressure at high temperature, they will not all react with the sugars, as a larger resulting volume is not favorable.

The differences in the concentration profiles between the HPHT and HT treatments, especially for furosine and CEL at pH 6 and 7, were in agreement with the observed differences in browning rates and indicate that pressure at high temperature had a stronger retarding effect on overall MR compared to the promoting effect of heat.

4.4. pH change after HT and HPHT treatment

The pH of WPI/G and WPI/T solutions decreased after HT and HPHT treatments (Figure 4). The pH decrease was larger at pH 9 compared to pH 7. For WPI/G solutions, the pH decrease was larger after HT treatment than after HPHT treatment, while the opposite was found for WPI/T solutions.

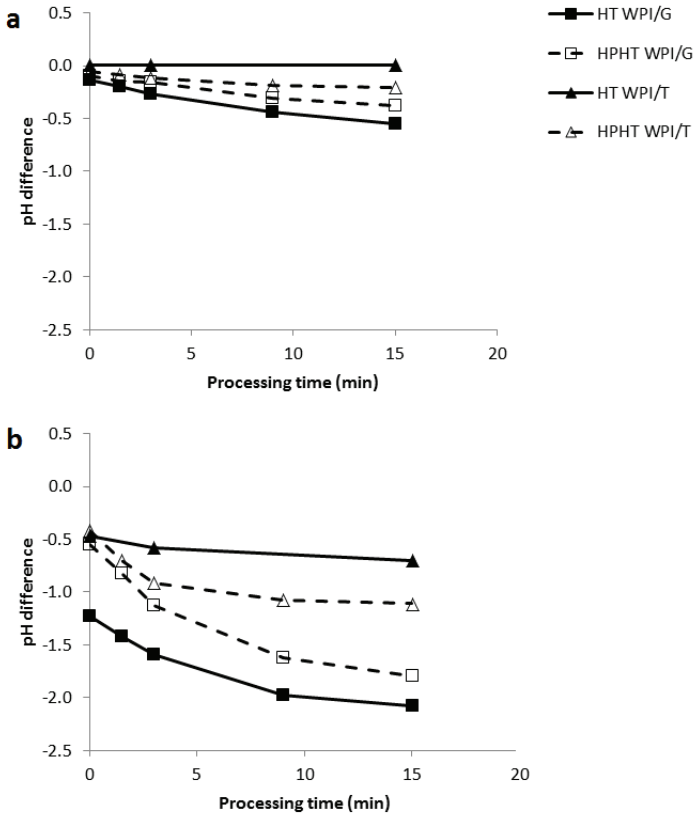


Figure 4. pH difference as a function of processing time for WPI/G and WPI/T solutions treated with either HT or HPHT prepared at (a) pH 7 and (b) pH 9 . As the standard deviations were smaller than the data point markers, they are not shown.

A pH decrease after HT and HPHT treatment has been associated with enhanced MR at longer processing times and increasing temperatures, resulting in a higher production of organic acids [14]. For WPI/T solutions, the larger pH drop after treatment compared to HT treatment can be directly ascribed to the effect of pressure on pH rather than to the

effect of MR. This pressure-induced pH drop might be due to pressure promoting the dissociation of ionizable compounds such as salts, acids, bases and polyelectrolytes [5]. According to a previous study, pressure shifts the dissociation equilibrium to the dissociated species, resulting in a pH decrease. However, Hill et al. (1996) and Moreno et al. (2003) could only explain the reduced MR at pH ≤ 8 on the basis of this mechanism. At higher pH values, pressure was found to accelerate MR. In later studies, the mechanism of pressure influencing particularly acid-base reactions, leading to changes in pH and protein reactions, has repeatedly been supported [25,26]. However, the pH drop may also be due to a change in ionic strength, which would have an effect on ion activities.

Another mechanism associated with the pressure-induced pH drop might be irreversible changes in the protein structure caused by pressure. Pressures beyond 150 MPa, 400 MPa and 800 MPa cause irreversible loss of native structure for β-LG, α-LA and BSA, respectively [27]. Such irreversible changes in protein structure and conformation might affect ion charges and ion-solvent interactions leading to permanent pH changes [25,26].

4.5. Protein aggregation

WPI/G solutions at pH 7 treated with HT contained larger particles and displayed higher viscosities compared to WPI/G solutions treated with HPHT (Figure 5 and 6). At pH 9, particle size and viscosity of samples treated with HT and HPHT did not differ considerably. With respect to particle size and viscosity, WPI/T solutions displayed similar behavior as WPI/G solutions (data not shown). The viscosity at pH 6 could not be measured due to the presence of large, coagulated particles.

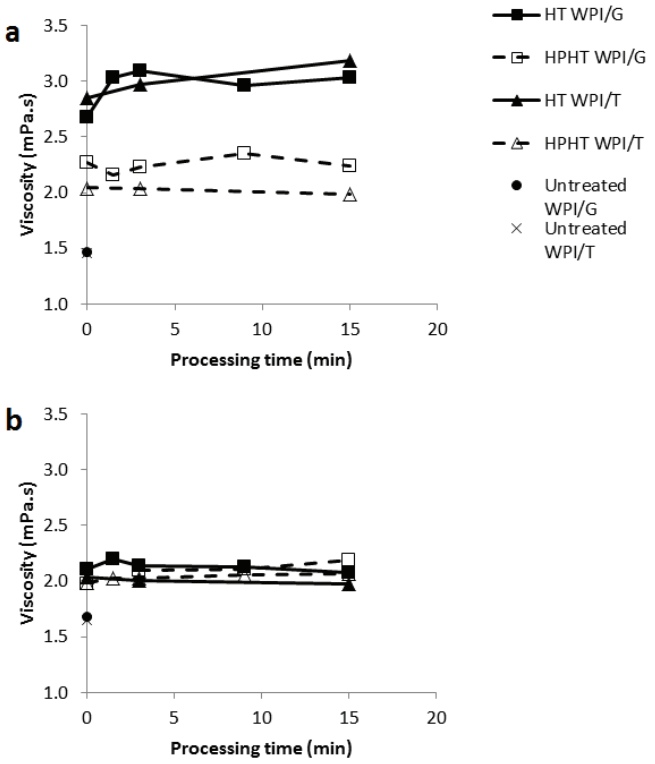


Figure 5. Viscosity as a function of processing time for WPI/G and WPI/T solutions treated with HT or HPHT prepared at (a) pH 7 and (b) pH . As the standard deviations were smaller than the data point markers, they are not shown.

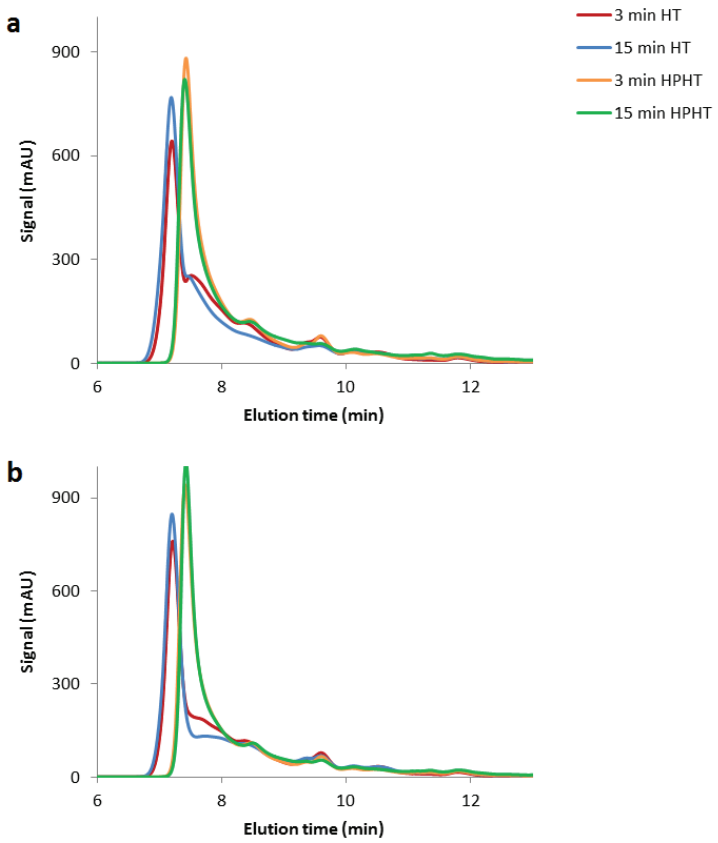


Figure 6. Size-exclusion chromatograms of (a) WPI/G solutions and (b) WPI/T solutions prepared at pH 7 treated for various times by HT and HPHT.

At pH 9, pressure did not have an effect on particle size and viscosity of WPI/G and WPI/T solutions. At pH 7, the smaller particle size and lower viscosity of WPI/T and WPI/G solutions treated with HPHT compared to WPI/T and WPI/G solutions treated with HT show that pressure at high temperature inhibited protein aggregation, hence viscosity development. A linear dependence of viscosity on particle size has been described previously for protein-enriched liquids [28]. The inhibitory effect of HPHT could have been, at first glance, associated with the retardation of MR. Reduced crosslinking of proteins and sugars might have been responsible for less aggregate formation. However, the similar trends of WPI/G and WPI/T solutions with regards to the effect of processing time on particle size and viscosity suggest that MR did not play a major role in aggregate formation and viscosity development. However, a positive correlation between protein glycation and

aggregate formation was found by Buckow et al. (2011) at pH 9. HT and HPHT treatments resulted in increased protein-sugar conjugation and formation of high molecular weight compounds in BSA-glucose solutions. In contrast to our results at pH 9, increased protein aggregation was reported after HPHT treatment (30 min, 200 and 600 MPa, 110°C) compared to HT treatment (10 and 30 min, 0.1 MPa, 110°C). The increased protein aggregation was associated with changes in the protein conformation under HPHT. Another study showing a positive correlation between protein-sugar conjugation and molecular weight stands in contrast to our results at pH 7 [29]. While Hofmann (1998) found higher molecular weights in casein-glucose solutions after HT treatment (4 h, 95°C), no differences in particle size were found in the WPI/G solutions treated with HT of our study. This difference can be due both to the different type of treatment and to the different proteins. Casein cannot denature and unfold in contrast to whey protein.

The larger particle size of WPI/T and WPI/G solutions treated with HT at pH 7 compared to pH 9 is in line with the finding from a previous study [30]. When heating β-LG solutions at pH 6.5, high molecular weight aggregates were formed compared to pH 7.5. This seemed to be associated with different degrees of hydrophobic interactions and disulfide bond formation. The smaller particle size and lower viscosity of the solutions treated with HPHT might be thus associated with a reduced degree of such phenomena. As mentioned in section 3.3, pressure has been found to act synergistically with heat on whey protein denaturation and unfolding. To the best of our knowledge, no study has investigated protein aggregation during HPHT treatment at and above 100°C and whether the synergistic effect of pressure and heat on protein denaturation and unfolding also leads to protein aggregation. However, it can be anticipated that the particle size and viscosity of solutions treated with HPHT and HT are associated with pH-dependent differences in protein conformation, protein-protein interactions as well as with differences in the pressure and heat sensitivity of whey proteins [15,31]. Data showed that protein-sugar conjugation played a minor role in this respect.

5. Conclusion

To summarize, the influence of pressure at high temperature on Maillard reaction products, browning and physicochemical properties of whey protein isolate glucose/trehalose solutions was evaluated comparing HPHT and HT treatments. A pressure of 700 MPa at about 123°C had a significant influence on browning, MR, pH, particle size and viscosity by acting on its own or in combination with heat. The novelty with regards to previous studies is that pressure at high temperature retarded browning and MR under conditions closer to application, namely the use of a protein-sugar mixtures and shorter processing times. The retarding effect of pressure on MR development was stronger than the promoting effect of heat. Interestingly, pressure initially induced a pH decrease in WPI/G solutions via a mechanism not related to MR. Pressure at high temperature inhibited protein aggregation and, thereby, viscosity development. These findings suggest that HPHT treatment can improve food quality when browning and high viscosities are undesired. We showed that the uniqueness and added value of HPHT treatment lies in the impact of pressure on the MR itself rather than the smaller heat load resulting from the mere presence of pressure. HPHT processing of liquid products containing protein and sugar, where browning and viscosity increases are undesired, could be introduced in the future.

6. Acknowledgments

The authors gratefully acknowledge the technical assistance of Geert Meijer in the LC-MS/MS experiments and the financial support of the “IPOP Customized Nutrition” program of Wageningen University and Research Centre.

7. Appendix

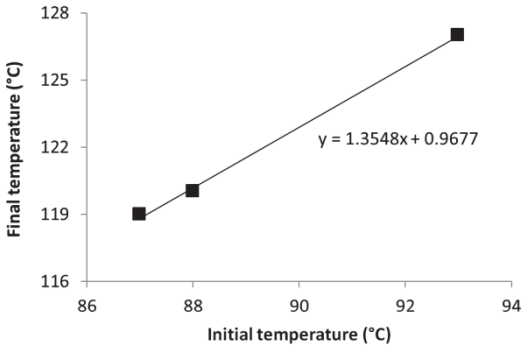


Figure S1. Temperature of pressure medium at 700 MPa for different initial temperatures.

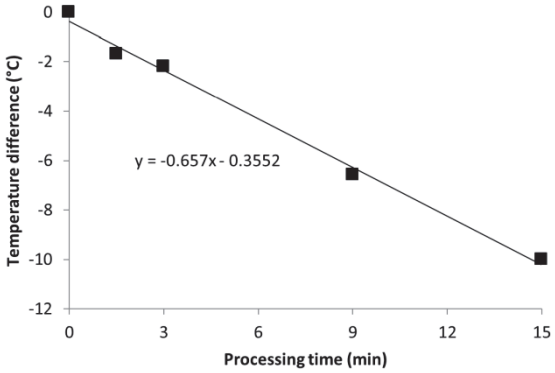


Figure S2. Temperature difference before pressure build-up and after pressure release for different processing times using a pre-treatment at 90°C.

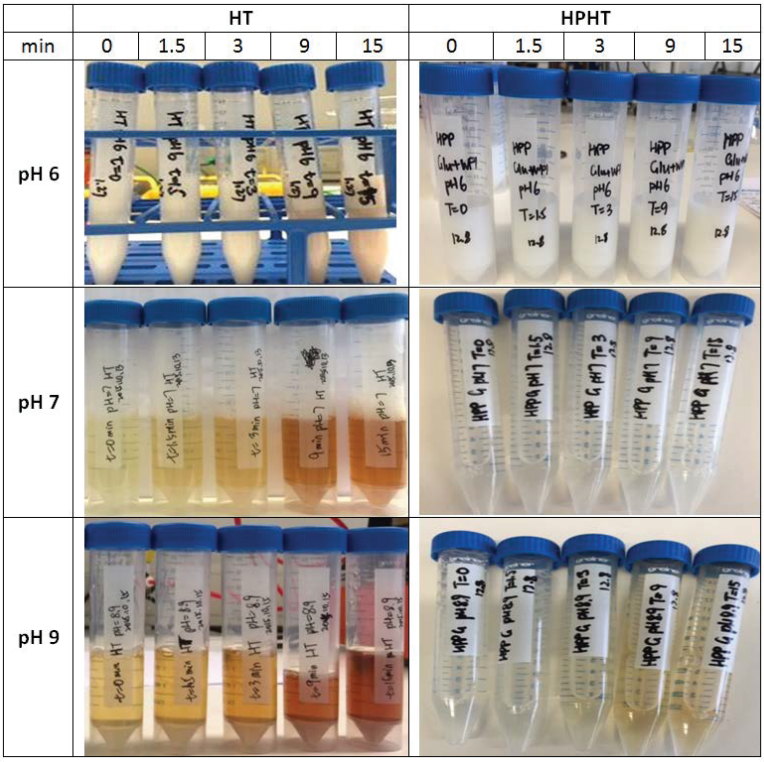


Figure S3. WPI/G solutions prepared at different pH values and treated for various times using HT and HPHT treatment.

8. References

1. Sevenich R, Bark F, Crews C, Anderson W, Pye C, Riddellova K, Hradecky J, Moravcova E, Reineke K, Knorr D (2013) Effect of high pressure thermal sterilization on the formation of food processing contaminants. Innovative Food Science & Emerging Technologies 20:42-50.
2. Kebede BT, Grauwet T, Mutsokoti L, Palmers S, Vervoort L, Hendrickx M, Van Loey A (2014) Comparing the impact of high pressure high temperature and thermal sterilization on the volatile fingerprint of onion, potato, pumpkin and red beet. Food Res Int 56:218-225.
3. Vervoort L, Van der Plancken I, Grauwet T, Verlinde P, Matser A, Hendrickx M, Van Loey A (2012) Thermal versus high pressure processing of carrots: A comparative pilot-scale study on equivalent basis. Innovative Food Science & Emerging Technologies 15:1-13.
4. Hellwig M, Henle T (2014) Baking, Ageing, Diabetes: A Short History of the Maillard Reaction. Angewandte Chemie - International Edition 53 (39):10316-10329.
5. Martinez-Monteagudo SI, Saldaña MDA (2014) Chemical Reactions in Food Systems at High Hydrostatic Pressure. Food Engineering Reviews 6 (4):105-127.

6. Bristow M, S. Isaacs N (1999) The effect of high pressure on the formation of volatile products in a model Maillard reaction. Journal of the Chemical Society, Perkin Transactions 2 (10):2213-2218.
7. Isaacs NS, Coulson M (1996) Effect of pressure on processes modelling the Maillard reaction. Journal of Physical Organic Chemistry 9 (9):639-644.
8. Schwarzenbolz U, Klostermeyer H, Henle T (2000) Maillard-type reactions under high hydrostatic pressure: Formation of pentosidine. Eur Food Res Technol 211 (3):208-210.
9. Buckow R, Wendorff J, Hemar Y (2011) Conjugation of Bovine Serum Albumin and Glucose under Combined High Pressure and Heat. J Agric Food Chem 59 (8):3915-3923.
10. Devi AF, Buckow R, Singh T, Hemar Y, Kasapis S (2015) Colour change and proteolysis of skim milk during high pressure thermal–processing. J Food Eng 147:102-110.
11. Tamaoka T, Itoh N, Hayashi R (1991) High Pressure Effect on Maillard Reaction. Agric Biol Chem 55 (8):2071-2074.
12. Hill VM, Ledward DA, Ames JM (1996) Influence of High Hydrostatic Pressure and pH on the Rate of Maillard Browning in a Glucose−Lysine System. J Agric Food Chem 44 (2):594-598.
13. Moreno FJ, Molina E, Olano A, López-Fandiño R (2003) High-Pressure Effects on Maillard Reaction between Glucose and Lysine. J Agric Food Chem 51 (2):394-400.
14. De Vleeschouwer K, Van der Plancken I, Van Loey A, Hendrickx ME (2010) The Effect of High Pressure−High Temperature Processing Conditions on Acrylamide Formation and Other Maillard Reaction Compounds. J Agric Food Chem 58 (22):11740-11748.
15. Considine T, Patel HA, Anema SG, Singh H, Creamer LK (2007) Interactions of milk proteins during heat and high hydrostatic pressure treatments — A Review. Innovative Food Science & Emerging Technologies 8 (1):1-23.
16. Eshtiagi MN (2001). High Pressure.
17. Knoerzer K, Buckow R, Versteeg C (2010) Adiabatic compression heating coefficients for high-pressure processing – A study of some insulating polymer materials. J Food Eng 98 (1):110-119.
18. Brands CMJ, van Boekel MAJS (2001) Reactions of Monosaccharides during Heating of Sugar−Casein Systems: Building of a Reaction Network Model. J Agric Food Chem 49 (10):4667-4675.
19. Troise AD, Fiore A, Wiltafsky M, Fogliano V (2015) Quantification of Nε-(2-Furoylmethyl)-l-lysine (furosine), Nε-(Carboxymethyl)-l-lysine (CML), Nε-(Carboxyethyl)-l-lysine (CEL) and total lysine through stable isotope dilution assay and tandem mass spectrometry. Food Chem 188:357-364.
20. Nguyen HT, van der Fels-Klerx HJ, van Boekel MAJS (2016) Kinetics of Nε-(carboxymethyl)lysine formation in aqueous model systems of sugars and casein. Food Chem 192:125-133.
21. van Boekel MAJS (2001) Kinetic aspects of the Maillard reaction: a critical review. Food / Nahrung 45 (3):150-159.
22. Van Boekel MAJS (1998) Effect of heating on Maillard reactions in milk. Food Chem 62 (4):403-414.

23. Huppertz T, Fox PF, Kelly AL (2004) High pressure treatment of bovine milk: effects on casein micelles and whey proteins. J Dairy Res 71 (1):97-106.
24. Hinrichs J, Rademacher B (2005) Kinetics of combined thermal and pressure-induced whey protein denaturation in bovine skim milk. Int Dairy J 15 (4):315-323.
25. Stippl VM, Delgado A, Becker TM (2005) Ionization equilibria at high pressure. Eur Food Res Technol 221 (1):151-156.
26. Oey I (2016) Effects of High Pressure on Enzymes. In: Balasubramaniam VM, Barbosa-Cánovas VG, Lelieveld LMH (eds) High Pressure Processing of Food: Principles, Technology and Applications. Springer New York, New York, NY, pp 391-431.
27. Patel HA, Creamer LK (2008) High-Pressure-Induced Interactions Involving whey Proteins. In: Milk Proteins. pp 205-238.
28. Amin S, Barnett GV, Pathak JA, Roberts CJ, Sarangapani PS (2014) Protein aggregation, particle formation, characterization & rheology. Current Opinion in Colloid & Interface Science 19 (5):438-449.
29. Hofmann T (1998) Studies on the Relationship between Molecular Weight and the Color Potency of Fractions Obtained by Thermal Treatment of Glucose/Amino Acid and Glucose/Protein Solutions by Using Ultracentrifugation and Color Dilution Techniques. J Agric Food Chem 46 (10):3891-3895.
30. Laligant A, Dumay E, Casas Valencia C, Cuq JL, Cheftel JC (1991) Surface hydrophobicity and aggregation of .beta.-lactoglobulin heated near neutral pH. J Agric Food Chem 39 (12):2147-2155.
31. Yang J, Powers JR (2016) Effects of High Pressure on Food Proteins. In: Balasubramaniam VM, Barbosa-Cánovas VG, Lelieveld LMH (eds) High Pressure Processing of Food: Principles, Technology and Applications. Springer New York, New York, NY, pp 353-389.

Chapter 6

General Discussion

1. State-of-the-art after PhD project

The general aim of this thesis was to explore the properties of a novel protein and the potential of a novel processing technology for the development of high-quality protein foods. Knowledge on physicochemical and functional properties of quinoa protein and HPHT processing has been expanded by this thesis (Figure 1). With regards to quinoa protein, new functional properties, processing methods and fractionation techniques were studied. With regards to HPHT processing, the behaviour of a new protein-sugar system under processing conditions closer to industrial applications was determined.

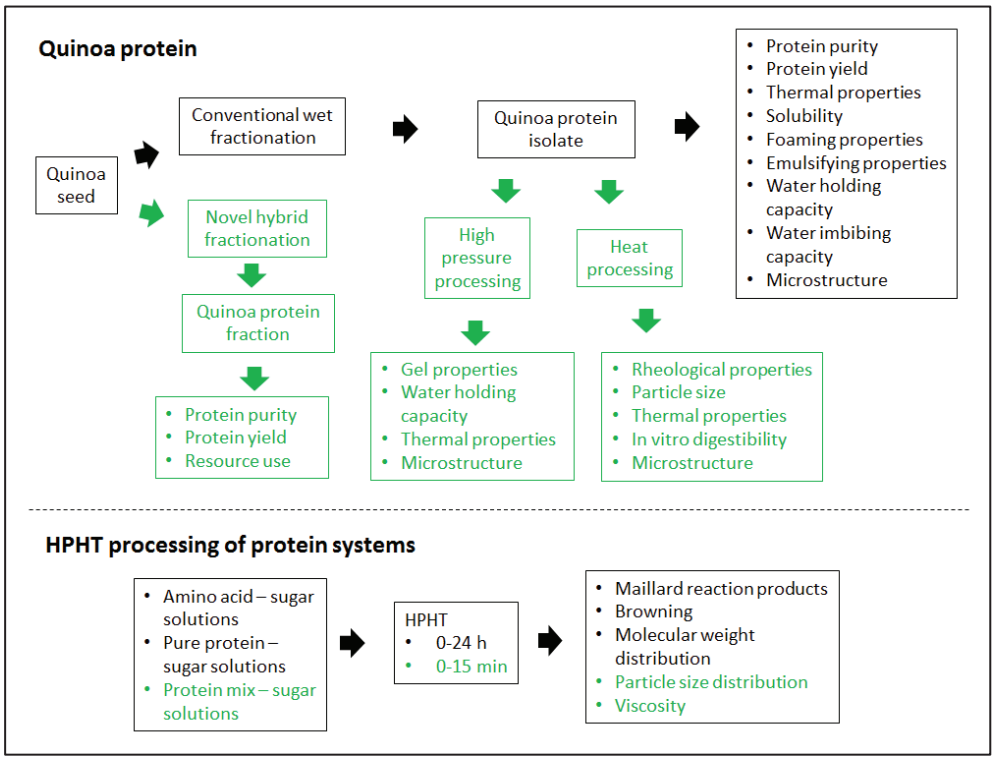


Figure 1. Overview of aspects described in literature on quinoa protein and HPHT processing of protein systems up to 2012 with knowledge added through this thesis (in green).

The aims of this thesis were:

1) To study the effect of extraction pH of conventional solvent extraction on physicochemical (protein purity, protein yield, solubility and thermal properties)

and functional (digestibility, protein aggregation and gelation behaviour) properties of QPI and to explore a hybrid dry and aqueous fractionation method for obtaining protein-rich fractions from quinoa

2) To examine the effect of pressure during HPHT processing on Maillard reactions, browning and physical protein properties under processing conditions close to industrial applications

The following main findings were obtained addressing the specific aims:

1) As extraction pH increased, quinoa protein yield increased, protein purity decreased or did not change and protein denaturation increased. Solubility was the highest at pH 7 for QPI extracted at pH 9. However, the optimal extraction pH depends on the application for which the QPI is used. When heating suspensions of QPI that were extracted at pH 8 and 9, increased protein aggregation was observed and heat-induced, semi-solid gels with a dense microstructure were obtained. When heating suspensions of QPI extracted at pH 10 and 11, limited aggregation was observed and the obtained gels were not self-supporting gels and had loose particle arrangements. After heat treatments denaturation and aggregation of quinoa protein was increased and *in vitro* gastric protein digestibility was decreased. It was concluded that extraction and processing conditions need to be controlled to optimise protein digestibility.

   Hybrid dry and aqueous fractionation of quinoa allowed to obtain protein-rich fractions with a protein purity lower and a protein yield similar or higher compared to conventional wet fractionation. Large water savings were made. Therefore, hybrid dry and aqueous fractionation is more resource-efficient, but the functionality of the obtained fractions might be different compared to QPIs obtained using conventional fractionation.

2) Pressure retarded early and advanced Maillard reactions and browning at pH 6, 7 and 9 in whey-protein-sucrose solutions, while it inhibited protein aggregation and, thereby, viscosity development at pH 7. It was concluded that HPHT processing under conditions close to industrial applications can potentially improve the quality

of protein-sugar containing foods, for which browning and high viscosities are undesired, such as high-protein beverages.

The mechanisms behind these findings will be discussed in the following.

1.1. Quinoa protein

To extract a protein concentrate from plant material, there are several methods available. Extraction methods can mainly be categorised in wet and dry fractionation methods [1]. For wet fractionation, solvents are used to dissociate the protein from the plant matrix and to purify the isolated protein. This process is based on properties of the protein, such as charge and protein conformation. These properties can be modified by factors, such as pH, type of solvent, solvent concentration and temperature, to increase protein solubility and thus protein recovery. Alkaline extraction followed by acid precipitation (conventional wet fractionation) has been commonly used to solubilise the protein from the plant material and subsequently purify it [2]. For alkaline extraction, often NaOH is used to increase the pH above 7. Proteins become negatively charged due to ionisation of the carboxyl groups of the amino acids leading to electrostatic repulsion between negatively charged groups, which increases protein-water interactions and thereby protein solubility [3] [4]. Therefore, as extraction pH increases, protein solubility also increases, as observed in Chapter 2 for quinoa protein. For subsequent acid precipitation, often HCl is used to decrease the pH to below 7. Proteins are then at or close to their isoelectric point, resulting in a net zero charge and thus leading to protein aggregation. In this way, proteins can be separated from non-protein components and purified. However, this process can also lead to increased protein denaturation due to conformational changes in the protein structure, so the precipitation pH might be chosen to be further away from the isoelectric point. This procedure was followed in Chapter 3 to reduce protein denaturation compared to the fractionation procedure followed in Chapter 2. Protein denaturation was indeed reduced when changing the precipitation pH from 4.5 to 5.5, as shown by a higher denaturation enthalpy of the extract (Chapter 2, Figure 2b). In addition, the shorter alkalinisation time (1 h instead of 16 h) might have also contributed to the reduction of protein denaturation.

An alternative method to wet protein fractionation is a dry fractionation method. According to this method, the protein is separated from the other plant components by air classification of the milled plant material [1]. The separation process is based on particle size. For pea, dry fractionation has been shown to be effective in obtaining protein-rich fractions (55.6 w/dw% protein), as the protein bodies and starch granules differ in size [5]. For quinoa, however, the protein bodies and starch granules are similar in size and thus more difficult to separate (Chapter 4). However, most of the protein is contained in the embryo and it was shown that the embryo can be neatly disentangled from the perisperm. Therefore, dry fractionation can be used to pre-concentrate quinoa protein. However, by using for quinoa this technique alone, protein contents as high as for pea might not be achievable due to the limited capacity to separate protein and starch based on size. Therefore, to optimise the separation process for quinoa it is necessary to add wet fractionation steps to further concentrate the protein. In the aqueous phase separation step using salt, advantage of quinoa's water- and salt-soluble proteins was taken to maximise protein yield and purity. In the ultrafiltration step, the size of quinoa proteins compared to remaining soluble compounds (e.g. soluble fibres, sugars and micronutrients) proved beneficial to further concentrate the protein.

1.2. HPHT processing

In HPHT processing, there are two characteristic processing parameters involved: pressure and heat. Separately, they are known to have a similar effect on whey proteins: they denature and unfold whey proteins, leading to protein aggregation [6]. Together, pressure and heat were shown to increase, reduce or not affect protein aggregation compared to heat alone, as reported by Buckow et al. (2011) and in Chapter 5. Similarly, the combination of heat and pressure affected MR differently in several studies. This implies that pressure and heat interact in different ways depending on the food system and the processing conditions. The processing conditions may influence protein structure as well as protein interactions with the solvent, sugar and/or other proteins in such a way that it

affects pressure- and heat-related physical phenomena, such as entropy and activation volume. The net sum of these events might determine the extent of chemical and physical interactions, such as MR, browning and protein aggregation.

In the next sections of this Chapter it is discussed how these findings can be used for the development of high-quality protein foods. With regards to quinoa protein, the mapping of more protein properties at more extraction pH and processing conditions provides further information for application possibilities. Therefore, in the following, it is examined which functional properties of the obtained QPIs can be useful for the design of high-quality protein foods and which current protein isolates they can replace. As for quinoa protein-rich fractions (QPFs) obtained using the hybrid fractionation method, protein yield and purity are relevant for the industrial applications. Furthermore, as protein yield of QPIs might not be optimal for large-scale production, it is examined how it can be optimised. Finally, a different, non-technical aspect of quinoa protein as a food ingredient is discussed: the market perspective.
With regards to HPHT processing, the effect of pressure on protein properties is evaluated in a broader context for the development of high-quality protein foods.

2. Comparison of quinoa protein properties to other food protein properties

In Chapter 2 and 3, it was shown that the properties of QPIs can vary according to their extraction and processing conditions and that these conditions can determine the application. Therefore, it is necessary to first define which properties would be desired for a certain application and at which level. Then, the properties of the QPIs can be compared to the properties of other food protein isolates to determine the potential of quinoa proteins to replace other proteins while maintaining functional properties.

Nutritional value of protein foods depends on many protein properties, such as composition, digestibility, bioavailability and utilisation. Generally, a high nutritional value is desired for protein foods. Protein foods should not only be nutritious but also taste

good. This usually includes flavour, appearance, texture and other sensorial properties. These can be related to physicochemical and functional properties of the proteins the food contains, such as aroma composition, gelation behaviour, emulsifying and foaming properties. Different food proteins can display different degrees and nuances of these protein properties. The degrees and nuances desired depend on the application the protein is used for. In the following the focus is on protein solubility, gelation behaviour, protein composition and digestibility.

It was chosen to mainly compare quinoa protein to a current standard plant protein (soy) and a more novel, but already commercialised plant protein (pea). The reason was that as these proteins have been successful on the market, comparing quinoa protein with them can better indicate the potential of quinoa protein. When the properties of QPI are similar to those of soy or pea protein isolates, the reason for replacement with quinoa would lay in the following. As mentioned in Chapter 1, QPI has a more balanced essential amino acid profile compared to pea protein isolate, which makes QPI potentially more nutritious. Regarding soy protein isolate, the main disadvantage compared to QPI is considered to be the allergenicity. Soy may cause allergic reactions and has been associated with positive as well as negative health effects [7]. Quinoa, in contrast, is free of all the major allergens in plants (gluten, soy and nut) and has, to the best of our knowledge, only positive health effects if the grain has been washed to remove the saponins.

2.1. Quinoa protein isolates

When for an application of a protein isolate in foods a high solubility and gel-formation ability is desired at neutral pH, QPI obtained at extraction pH 9 (E9) might be suitable to use as it has the highest solubility and gel-formation ability when dispersed at pH 7 compared to the other extraction pH (Chapter 2). The storage and elastic moduli of QPI gels at similar protein concentrations are comparable to those of soy and pea protein isolate gels [8,9]. This means that QPI E9 has the potential to replace soy and pea protein isolates while maintaining or exceeding gelation properties.

The *in vitro* gastric protein digestibility of untreated and heat-treated QPI obtained at any extraction pH seemed to be similar or higher compared to untreated soy and pea protein isolates after the same digestion time, even after severe heat treatment (30 min at 120°C) (Chapter 3) [10]. However, the gastric conditions applied by He et al. (2013) were not the same as those chosen for the study describe din in Chapter 3, so it is not possible to draw a definite conclusion. Even if the *in vitro* conditions between the different studies were the same, it should be noted that overall digestibility, including other phases of the gastrointestinal tract, were not studied. He et al. (2013) also measured the digestibility of soy and pea protein isolate under simulated duodenal conditions, subsequently to the simulated gastric conditions. While soy protein had a higher digestibility than pea in the gastric phase, it was lower in the duodenal phase. This means that the digestion kinetics of each protein can vary from one digestion phase to the other. Therefore, it is difficult to conclude from the present results on overall digestibility.

However, the results obtained in Chapter 3 indicate an initial high digestibility and thus nutritional value of QPI compared to pea and soy protein. Furthermore, heat treatment of QPI does not reduce digestibility to a large extent compared to the digestibility of untreated pea and soy protein under the *in vitro* gastric conditions. This means that even after harsh heat treatment, the digestibility of quinoa protein may be higher than that of pea and soy protein that has not been treated at all. However, the digestion kinetics of QPI should be studied in combination with other phases of the GI tract, too. Also, movements in the gut might influence digestion kinetics *in vivo* and could also be simulated in *in vitro* models. This would provide a more complete picture of quinoa protein digestibility.

Another aspect to pay attention to is the influence of Maillard reactions on digestibility. Heat treatment of whey protein – sugar solutions has been often found to lead to decreased protein digestibility [11,12]. One study reported increased digestibility as a result of Maillard reactions in the initial stages. As it is expected that the QPIs contain reducing sugars and quinoa protein digestibility was shown to be reduced by heat treatment, it might also apply here that Maillard reactions decrease protein digestibility. The extent of the impact of MR on digestibility might depend on the protein type, so this

aspect is difficult to compare between quinoa protein and other proteins at this stage but should be taken up in further research.

Regarding foaming properties, according to Lindeboom (2005), foaming capacity of quinoa protein is lower than that of soy protein, while foam stability of quinoa protein is higher than that of soy protein (Chapter 1). Regarding emulsifying properties, QPIs without saponins (corresponding to our QPIs) had a similar specific surface area (m^2/ml) but a lower emulsion stability (defined as percentage of initial specific surface area after 30 min standing at room temperature) of initial specific surface area after 30 min standing) compared to soy protein. This means that when a high foam stability is desired for a product, like whipped cream, QPI might be more suitable to use than soy protein. However, when a high emulsion stability is desired, like for mayonnaise, QPI might not be less suitable.

In conclusion, QPI obtained at extraction pH 9 can be an allergen-free or more nutritious option compared to pea or soy protein isolate, respectively, for applications requiring a high solubility and gel-formation ability in water. It may be a better option than soy protein if a high protein digestibility and a high foam stability is desired. However, as very little literature is available on these aspects, as well as on sensory properties of QPIs, these should be investigated further before replacement can really put be put into practice.

2.2. Quinoa protein – rich fractions obtained by the hybrid fractionation process

After having obtained a protein-rich fraction from quinoa using the hybrid fractionation method, the physicochemical and functional properties of this fraction were studied. Based on Chapter 2-4, unpublished work and previous literature, it is possible to speculate on some of the potential physicochemical and functional properties of the quinoa protein – rich fractions (QPF).

In Chapter 4, it is expected that by using the hybrid fractionation method the native protein functionality would be better retained compared to the conventional wet fraction method due to the absence of organic solvents. However, the question is if using 0.5 M NaCl might not affect protein properties. Salt is known to influence proteins via the salting-in and salting-out effect [13]. At high salt concentrations, proteins may precipitate (salting-out). In Chapter 4, we made use of the salting-in effect to increase protein solubility and no precipitation was observed at 0.5 M NaCl. Therefore, it is assumed that this salt concentration did not significantly affect protein structure, thus functionality. Also not when removing the salt through rinsing, which would be a necessary additional step before the QPF can be used as an ingredient. Protein structure and functionality was furthermore expected to be little affected when using a mild drying technique, such as hot-air drying. The protein would be denatured to smaller extent compared to less mild drying techniques such as spray-drying.

The QPF obtained in Chapter 4 had a protein purity of 59.4 w/dw% and starch content of 9.5% w/dw% (unpublished data). This means that the composition of QPF will probably lay between that of quinoa flour and that of QPI obtained at extraction pH 8 (E8). pH 8 is closest to the pH of water, which was used for hybrid fractionation. Therefore, the values for the properties of QPF will probably also lay between those of QPI E8 and quinoa flour. For example, the middle value for solubility of QPF and QPI E8 at pH 7 is calculated to be 30%, as quinoa flour has been found to have a protein solubility of 18% at pH 7 while QPI E8 showed a solubility of 35% [14] (Chapter 2). This means that QPF will have a very low protein solubility in water compared to QPI E9, soy and pea protein isolates. However, protein contents in soy and pea protein isolates similar to QPF might also have a lower protein solubility, so QPF should be rather compared to protein concentrates, which can have protein purities of 60 w/dw%. Using the same calculation for the middle value, QPF is estimated to have a degree of hydrolysis of 16% (360 min digestion) when untreated, and of around 15% when heat-treated for 30 min at 120°C. This means that protein digestibility of QPF will not be much different from QPI E9 before and after severe heat treatment. This indicates that initial protein digestibility will still be higher than that of untreated soy and pea protein isolates, while having a lower protein purity than them.

QPF probably has a higher starch content than QPIs. What is remarkable about quinoa starch is its high freeze-thaw and retrogradation stability [15]. This means that quinoa starch can be useful in frozen food products, sauces, cream soups, pie fillings and in emulsion-type food products (e.g. salad dressings). Also, quinoa starch has been found to exhibit a higher viscosity than wheat starch at the same starch concentration and at similar temperatures [15]. Therefore, QPF might be a better option than QPI for protein-enriching the mentioned food products and better than using wheat starch if high viscosities are desired.

Furthermore, QPF might contain more health-promoting compounds, such as fibres and micronutrients, as protein concentrates have been associated with health benefits compared to further refined ingredients [15,16]. Also, the oil was not extracted for QPF, in contrast to QPI. Quinoa oil has been claimed to be of high quality due to high contents in polyunsaturated fatty acids (similar composition to maize and soy oil) [15].

In conclusion, QPF might have to some extent different functional properties to QPI but be comparable to soy and pea protein concentrates. For certain applications, it may be better to use QPF than QPI, if a very high protein purity is not necessary, due to a potentially higher technical and nutritional functionality but this, as well as a comparison to other protein concentrates should be investigated further.

3. Optimisation of quinoa protein yield

As QPI E9 and QPF obtained in Chapter 2 and 3, respectively, show promise to be used in foods replacing other plant protein concentrates (section 2), the effectiveness and efficiency of the fractionation processes needs to be examined. Effectiveness and efficiency are important factors for economic and environmental reasons and can be a large barrier for the upscaling of any production process.

QPI E9 (Chapter 2) and QPF (Chapter 4) have a protein purity of 85% and 60%, respectively, which is less than soy (90%) but similar to pea (80-85%) [2,5]. The protein yield of QPI and QPF is 39% and 62%, respectively, which is less than soy (71-85%) but similar to pea (55-65%). This shows that although QPF has a lower protein purity than pea protein isolate, it compares to pea protein isolate in terms of protein yield. To increase protein purity and yield and thus make the hybrid fractionation process more effective and efficient, recommendations were made in Chapter 4.

QPI E9 (Chapter 2) has a similar protein purity compared to soy and pea protein isolates, however, the protein yield might be too low for large-scale production. This would result in the waste of a lot of protein. Therefore, it is advisable to increase protein yield of QPI. This, however, might decrease protein purity, as shown in Chapter 2 and in previous literature for lupine [17]. On the other hand, a negative correlation between protein yield and protein purity was not found in Chapter 3, 4 and also not in several previous studies on quinoa and amaranth (from the same family as quinoa) [18-20]. Some of those studies reported only a positive correlation of protein yield with extraction pH or even also with protein purity. It might be that the correlations depend on the individual extraction/fractionation conditions and plant material. However, more research should be performed on this for quinoa. For now, it is assumed that protein purity will not decrease significantly with increasing protein yield and the options for raising the protein yield will be analysed in the following.

During protein extraction from plant material, protein yield can be influenced by factors such as type of solvent, pH, time, temperature and biomass/solvent ratio [2]. For quinoa, the influencing factors in order of importance were found to be: solvent/meal ratio > pH > NaCl concentration > temperature > extraction time [21]. Because NaCl concentration and temperature showed a negative correlation with protein yield, these factors were discarded for determining the optimal extraction conditions. Optimal conditions for a maximum protein yield were: solvent/meal ratio of 19.6/1 (v/w), pH 11 and 149.1 min of extraction time. These yielded 76.9% of quinoa protein. As mentioned in Chapter 2, this protein yield is very similar to our obtained value (74.3%) at pH 11 in the alkalinisation stage. However, in our study the solvent/meal ratio (10/1 (v/w)) and the extraction time

(960 min) were lower compared to the optimal extraction conditions found by Guerreo-Ochoa et al. (2015). This suggests that there are other factors positively affecting protein yield than those reported significant or positively correlated to protein yield by Guerreo-Ochoa et al. (2015). All factors will be discussed in the following.

The temperature for optimal protein extraction determined by Guerreo-Ochoa et al. (2015) was 36.2°C. We extracted at ambient temperature (about 20°C). An increase in temperature from 20 to 25°C was shown to increase quinoa protein solubility at the alkalinisation stage by 6% [22]. Further temperature increase did not significantly raise solubility any further. This means that the protein yield as reported in Chapter 2 could be increased by 6% when the temperature was raised by 5°C. Also, a temperature raise from 20 to 25°C is unlikely to denature the protein and thus lead to functionality loss and it might not be that costly to realise in large-scale production.

One factor suggested by Guerreo-Ochoa et al. (2015) to be insignificant for protein yield but which might actually be significant is particle size of the meal. Guerreo-Ochoa et al. (2015) chose a particle size of 500 µm. Föste et al. (2015) found an increase in solubility from 62 to 68% with decreasing particle size from 750 to 250 µm. We used a sieve (200 µm) for the milling of the quinoa seed, which, according to the manual of the mill, means that approximately 2/3 of the particles had a smaller size than 100 µm. If it was assumed that the average particle size was 80 µm, decreasing the particle size by finer milling to a size of 30 µm, as used in Chapter 4, the protein yield would increase by 0.86%, according to Föste et al. (2015). This might be considered a small increase in protein yield, however, in industry this might be considered a large difference in profit. As the granule size of quinoa starch is about 1-1.5 µm [15]., the starch granules would not be affected and possible interactions with protein extractability or purity would be unlikely.

Solvent/meal ratio and extraction time were factors found to affect protein yield by Guerreo-Ochoa et al. (2015) and can thus also be adjusted to increase protein yield in Chapter 2 and 3. To know how they should be adjusted, the response surface plots of Guerreo-Ochoa et al. (2015) for the correlations between solvent/meal ratio and protein

yield, as well as for extraction time and protein yield were studied. Protein yield as a function of solvent/meal ratio was reported to have an optimum at about 20/1 (v/w) for pH 6.5-11.5, which suggests that increasing the solvent/meal ratio from 10/1 to 20/1 in Chapter 2 and 3 would increase protein yield at any of the extraction pH used. If the response surface plots applied to the conditions in Chapter 2, an increase in solvent/meal ratio from 10/1 to 20/1 would result in a protein yield increase by an average of 10% for extraction pH 8-11, which is a large gain. However, higher volumes of solvents (NaOH and HCl) are unlikely to be adopted by industry, even if they can be reused, because of the higher costs for chemicals, equipment, and energy [2]. Besides, overall costs will also increase due to an increase in costs for downstream processing for protein recovery.

With regards to extraction time, Guerreo-Ochoa et al. (2015) reported that protein yield increased continuously from 20-160 min for pH 8-11.5. It is not known what protein yields would be obtained after extracting for longer than 160 min. In contrast to Guerreo-Ochoa et al. (2015), Föste et al. (2015) found that protein solubility increased over the first hour but did not significantly increase further over the next three hours. This means that extending the extraction times used in Chapter 2 and 3, might not necessarily lead to higher protein yields. Besides, long extraction times might denature the protein more and increase costs in industrial production.

In Chapter 4 it was shown that when increasing NaCl concentration from 0 to 0.5 M, the yield of quinoa protein increased from 40.3 to 80.3%. In contrast, Guerreo-Ochoa et al. (2015) found a negative correlation between NaCl concentration and quinoa protein yield in the concentration range 0-2 M. It is not clear whether NaOH (2 M) or Tris-HCl buffer (0.2 M) was present in the study of Guerreo-Ochoa et al. (2015). The presence of any of these two solvents might have an influence on the effect of NaCl on protein yield. Therefore, the interactive effect between several solvents, such as NaCl, NaOH and Tris-HCl, on protein yield should be studied further. In case the combination of NaCl and NaOH does not influence protein yield negatively, an increase in protein yield by 30% might be achieved in Chapter 2 when using 0.5 M NaCl. As explained in section 2.2, it is assumed

that such NaCl concentrations do not affect protein structure and that they are reversible once the salt is washed out. However, this should be investigated.

In conclusion, to increase protein yield from quinoa using conventional wet fractionation, taking into account the feasibility in industry, it is recommended to test systematically the effect of temperature, particle size and NaCl concentration on protein yield. If the effect of these factors on yield can be confirmed in the magnitudes reported, protein yield of QPI E9 (Chapter 2) could be doubled from 39.4 to 81.1%, assuming there are no interactive effects between the factors. Interactive effects seem to be mostly positive, according to Guerreo-Ochoa et al. (2015). Therefore, even higher protein yields might be achievable. However, the protein quality should be checked again and extra costs for increase in temperature and NaCl concentration (and water for washing out NaCl) should be weighed against the gain. Also, it is not certain that the protein purity stays the same, protein purity should also be taken into account when optimising protein yield. It is, furthermore, not certain that the gain in protein yield at the alkalinisation stage will translate 1:1 to the final product.

4. Market perspective of quinoa protein isolates and fractions

Once all the technical aspects of quinoa protein as an alternative food protein have been dealt with, another aspect to take into account for industrial production is the market. There needs to be a market for quinoa protein and satisfying the market should be profitable. Therefore, to test the market, a quick analysis of the popularity of quinoa as a seed was performed. From this, the possible competitive advantage of quinoa protein was deduced. To compare the profitability between conventional and hybrid fractionation, the processing costs for the production of QPI and QPF were calculated.

A total of 8120 launches of products containing the word “quinoa” were found worldwide on 21 June 2016 [23]. The number of product launches grew exponentially from 2002 to 2015 (Figure 2). This growth might be explained by quinoa tapping into several of the 10 top trends in food that have emerged in the past 10 years, according to trend reports from

Innova Market Insights [23]. As a result of the marketing of quinoa's properties, quinoa has been in line with the top trends reported in whole grain, superfoods, high protein, "free-from", natural/organic and ancient/authentic/traditional foods. This is also reflected in the positioning of the current products labelled with quinoa (Figure 2).

It might be assumed that when concentrating the protein of quinoa, the reputation of the seed will be preserved for the protein product. A protein concentrate from quinoa then needs to compete with protein concentrates from other sources. The same inherent properties of quinoa might distinguish quinoa protein from other proteins: balanced in essential amino acids, hypoallergenic and originating from an "ancient grain". Although basically any grain can be called "ancient grain" as it started being consumed in ancient times, quinoa was until recently being consumed by a small amount of people (relative to the world population), who are still living according to indigenous (Incan) traditions. This stands in contrast to grains like wheat, maize or soy, which are not (only) being consumed by indigenous people anymore. So quinoa has still an advance in being exotic and authentic. It might thus be better to call quinoa an "indigenous grain", for the moment, and then it is justified and even advised to create a halo around quinoa with all the Incan history and tradition for marketing purposes. In this way people can learn where it comes from and what the Incan culture is like.

As a result, to the best of our knowledge, none of the current (novel) proteins has the combination of features mentioned above. Therefore, a quinoa protein concentrate would have a clear competitive edge over other current (novel) protein concentrates.

The costs for the production of QPI E9 and QPF on an industrial scale were calculated and compared (Table 1). Despite the different properties of the two products, it is useful to compare their costs, as those can then be weighed against the benefits and limitations of the two products. To compare the costs of the two production processes, only the processing costs (energy, water and chemicals) were taken into account. Capital (equipment) and labour costs were not taken into account here, as these were assumed to be similar for the two processing techniques. The defatting step in the conventional method was also not taken into account, as the cost would be high (USD 264/tonne quinoa) compared to the other processing costs [24]. It would not be fair to compare to

the hybrid method where defatting is not included.
The calculation results show that the hybrid fractionation method produces a product that is less than half the price of that of the wet method. This large cost difference is especially due to the drying cost (drying requires a lot of energy), and to the low protein yield for the wet method. Therefore, even if the hybrid fractionation method produces low protein purities, the gain in energy savings and reduced protein loss during processing makes the hybrid method more attractive for large-scale production.

However, this cost analysis does not take into account agronomic factors, which might play an important role. One is the protein yield per hectare (crop yield per hectare x crop's protein content). For quinoa grown in the Netherlands, it was calculated to be 0.6 t/ha, assuming a crop yield of 4 t/ha and a protein content of 15 w/dw% [25](Chapter 1). This protein yield of quinoa is higher than that of pea (0.4 t/ha), chickpea (0.2 t/ha) and rapeseed (0.4 t/ha), and equal to that of maize [26]. However, it is lower compared to soy (0.9 t/ha), lupin (1 t/ha), wheat (1.1 t/ha) and potato (1.2 t/ha). This means that the quinoa protein yield per hectare can compete with several novel plant proteins, while it is at this moment outperformed by mainstream or other novel plant proteins. However, if the protein yield per hectare is linked to the fractionation process of the crop, the actual protein yield per hectare, taking into account the protein loss during fractionation, might be different among protein sources. Also, other agronomic factors may vary, such as the use of water, fertilisers, pesticides etc., influencing the final cost of the protein product.

In conclusion, it is likely that quinoa protein can not only benefit from the promising protein functionality and technical scalability (section 2 and 3), but also from the current popularity of quinoa seed and therewith profile itself against other food proteins. In terms of financial scalability, it can be more advantageous to produce a quinoa protein product using the hybrid dry and aqueous fractionation method compared to the conventional method. The result would be higher profits. Even when considering the protein yield per hectare, quinoa protein performs better than pea but worse than soy. However, other agronomic factors should be taken into account for a better comparison to other proteins. Also, it is not certain if significant profits from quinoa protein production using any

fractionation method can be made at this moment. The price for quinoa seed is still very high (on average USD 3000/tonne) [27], which might now be the biggest barrier for industrial production of quinoa protein.

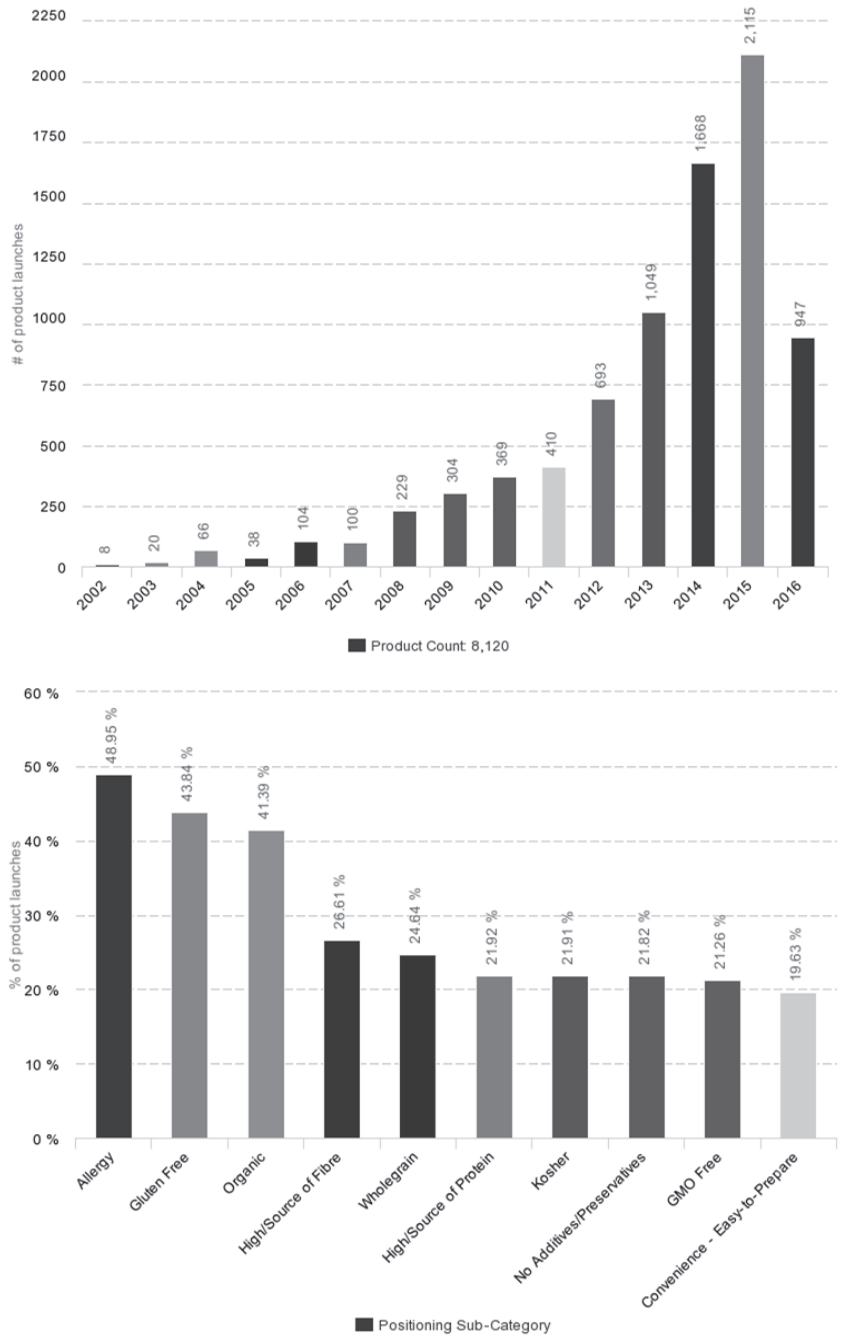


Figure 2. Product launches containing the word "quinoa" (free text search using the term "quinoa") according to the Innova Database on 21 June 2016. Above: number of product launches by year. Below: percentage of product launches (from a total of 8120) by the top 10 positioning sub-categories.

Table 1. Cost calculations for concentrated quinoa protein produced (in tonne = t) using the conventional wet or hybrid fractionation method (Chapter 2 and 4).

			kg/t quinoa seed		Cost/t quinoa seed (USD)	
Processing step or material	**MJ/t quinoa seed**	**Price (USD/t)**	**Conventional fractionation**	**Hybrid fractionation**	**Conventional fractionation**	**Hybrid fractionation**
Milling[a]	100.00				2.97	10.35
Centrifugation[a]	15.00				0.45	0.22
Ultrafiltration[a]	14.00				0.00	0.21
Spray-drying[a]	4800.00				141.30	70.82
Water[b]		0.95	10000.00	5012.00	9.50	4.76
NaOH[b]		453.00	6.40		2.90	0.00
HCl[b]		85.00	33.20		2.82	0.00
NaCl[b]		150.00		146.35	0.00	21.95
Total cost/t quinoa seed					**159.93**	**108.32**
Total cost/t product					**405.92**	**180.53**

[a] Energy requirement based on Schutyser et al. (2015) and electricity price (USD 0.106 per kWh) based on Ulrich and Vasudevan (2006) [28,29].
[b] Price based on ICIS [30]

5. Potential of high pressure processing of proteins for the development of protein foods

When developing high-quality protein foods, attention should be also paid to the processing of the final product. In Chapter 5, pressure was shown to have a unique and direct effect on WPI – sugar solutions during HPHT processing, which can be used to improve protein food quality compared to traditional processing. The question arises to what extent the findings observed for WPI-sugar solutions can be extrapolated to quinoa protein. Due to time constraints, it was not possible to test experimentally the effect of HPHT treatment on MR and protein aggregation in quinoa-sugar solutions anymore. Therefore, a prediction of this effect for quinoa protein was made based on preliminary results obtained in this thesis. This provides insights about the influence of protein type on the effect of pressure at high temperature on MR and protein aggregation.

In unpublished work we showed that when treating quinoa protein isolates for 3 min at various pressures, denaturation enthalpy rapidly decreased with increasing pressure (Figure 3). SDS-PAGE gels showed a disappearance of bands at intermediate molecular weight, while more intense bands appeared at low molecular weight at 700 MPa compared to the other pressures. This suggests that quinoa protein is susceptible to pressure-induced denaturation and protein structure changes, especially at high pressures. This behaviour is similar to that of whey proteins under high pressure [6], and may be attributed to the fact that whey and quinoa proteins have globular structures. Therefore, it can be expected that in quinoa protein – sugar solutions, HPHT induces similar changes in protein structure and possibly ion activities as in whey protein – sugar solutions, leading to a pH decrease and thus reduced MR, as well as to reduced protein aggregation. The extent in the pH decrease and protein aggregation may be different compared to whey protein but the overall effect of HPHT on MR and viscosity is expected to be similar. Therefore, among globular proteins the protein type is speculated to not influence the qualitative effect of pressure at high temperature on MR and protein aggregation.

Based on this assumption, on the findings from Chapter 3 and on the relationship between MR and protein digestibility mentioned in section 3.1, the effect of pressure can be extrapolated to a protein property that was not studied: digestibility. In QPI suspensions (5% w/w) at neutral pH, protein aggregation was found to increase, while protein digestibility was reduced after heat treatments, especially at 120°C (Chapter 3). At the same temperature and pH, and at a similar protein concentration (6% w/w), in WPI – sugar solutions, protein aggregation was also shown to increase after heat treatment and compared to HPHT processing. Conversely, protein aggregation was reduced by pressure, which might lead to an increased protein digestibility compared to heat treatment. The lower digestibility of heat-treated QPIs (Chapter 3) supports previous findings of a lower digestibility with increased MR (section 3.1). Thus, it is speculated that the reduced MR found for HPHT-treated WPI – sugar solutions were also responsible for an increased protein digestibility. This hypothesis should be tested in future research. If it is confirmed,

HPHT processing might be better to use than autoclaving to sterilise protein foods for which a high protein digestibility is desired, e.g. in medical, infant or sports nutrition.

Other protein properties that were found to be affected differently by pressure compared to heat are thermal properties and protein fractions of QPI suspensions, as well as texture properties of QPI gels. Figure 3 from this Chapter and Figure 2 and 4 from Chapter 3 were obtained using the same protein concentration (20% w/w), thus they can be compared to each other. It can be noted that the enthalpy decreased more rapidly with increasing pressure (0.1-700 MPa) (this Chapter, Figure 3) than with increasing temperature (20-120°C) (Chapter 3, Figure 2). SDS gels of pressure- and heat-treated QPIs overall showed that the bands became fainter with increasing temperature and pressure. However, for pressure-treated QPIs, the bands at low molecular weight were most intense at 700 MPa. Also, protein material that did not enter the gel, as well as smears at high molecular weight, were visible for pressure-treated QPIs. In contrast, almost no bands were visible for heat-treated QPIs at 120°C. This suggests that in the pressure and temperature range studied, pressure denatured and associated, as well as dissociated, soluble quinoa protein to a higher degree than heat. This finding is in agreement with a study that reported higher denaturation enthalpies and more soluble aggregates at high and low molecular weight for pressure-treated rapeseed protein isolate compared to heat-treated isolate [31].

In a preliminary study, using the same protein concentration and pH as above, pressure-treated suspensions of QPI E9 (Chapter 3) produced self-supporting semi-solid gels at pH 7 and 9 at 3 min processing. Contrary, heat-treated QPI suspensions resulted in weak, soft self-supporting gels but none of them were suitable for texture analysis. At 10 and 15 min processing, the pressure-induced gels were generally harder than the heat-induced gels. The higher gelation ability and gel hardness obtained with pressure may be due to the higher denaturation and protein aggregation mentioned above. The dissociated protein might have been incorporated into the network. For pressure-induced gels from rapeseed protein isolate, a higher gel hardness, springiness and cohesiveness were also associated with an increased formation of high molecular weight proteins and coupled with a higher

degree of hydrophobic interactions found [31]. Our preliminary study thus shows that pressure has a different effect on gel formation from quinoa protein compared to heat. The higher ability of pressure to form quinoa protein gels could be used to design plant-based (semi-)solid dairy products. Treating milk protein solutions using high pressure processing (HPP) has been reported to replace the rennet function to make cheese, to create yoghurt texture with less syneresis and to develop ice-cream texture with less fat or emulsifiers/stabilisers but with improved sensory properties [6].

To conclude, clear differences were found between the effects of pressure and heat on physical-chemical and functional properties of quinoa protein. Pressure at ambient or high temperature can thus have an added value for the possible applications mentioned compared to heat. To better understand the underlying mechanisms and design protein foods with new or improved properties in a more targeted way, the effect of pressure and heat on more protein properties and under conditions closer to application should be studied.

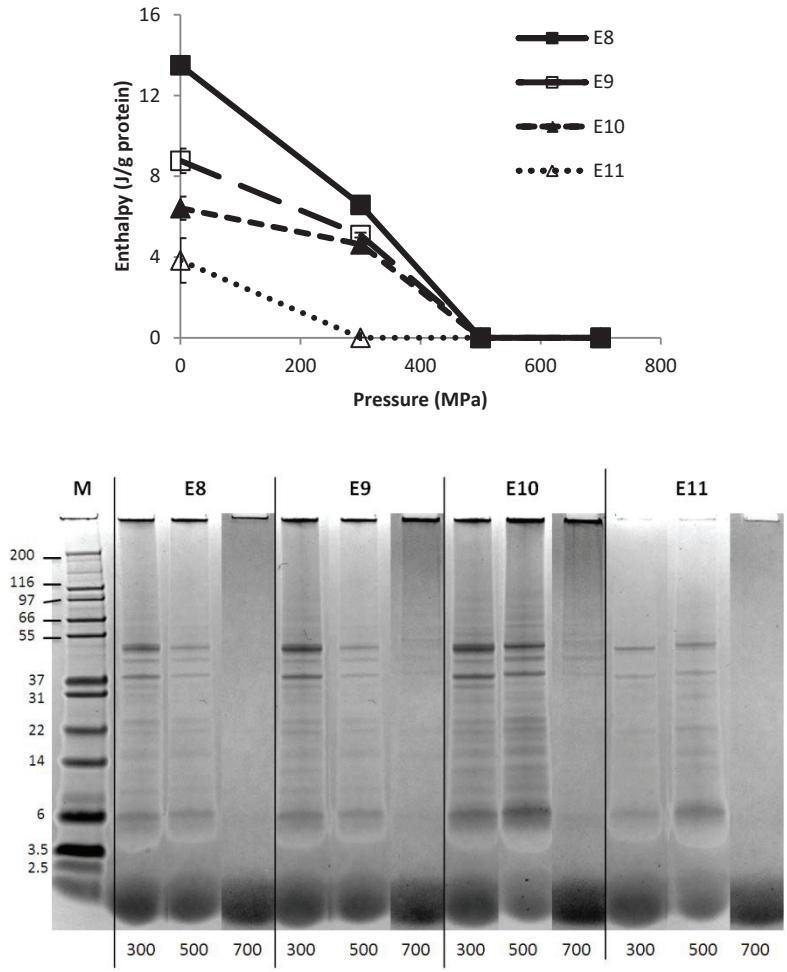


Figure 3. Denaturation enthalpy (above) and SDS-PAGE profile (below) of the QPIs E8, E9, E10 and E11 (Chapter 3) at various pressure levels. Lane M: molecular weight marker.

6. Opportunities and challenges for the future

New insights in the exploration of quinoa protein as a novel food protein and of HPP as a novel food processing technology were added by this thesis. Quinoa protein obtained using the conventional method seems promising to replace current major plant proteins. However, more research should be performed to confirm this, as well as on sensory properties. In this case, quinoa protein should be viewed as one alternative for other plant

proteins in specific food applications, and not as a general replacer. Furthermore, world trends in sustainable industrial production and in "natural" food are stimulating the exchange of highly purified proteins with less purified, more environmental-friendly, minimally processed and more wholesome proteins (containing more fibre, oil and micronutrients). If this also comes with a financial gain (lower processing costs), more research should definitely be performed on quinoa protein-rich fractions obtained with the alternative method. To provide quinoa seed as raw material for an eventual large-scale production of quinoa protein, financial, as well as legal, ethical, environmental, political issues need to be solved. For example, land degradation, socioeconomic disrupts and biodiversity loss in traditional quinoa-producing countries has been associated with the global quinoa expansion [32]. But even if such issues can be solved, quinoa will have to compete for scarce farmland globally, which raises the question as to what extent quinoa can serve as sustainable protein source.

HPP at high or ambient temperature is technically also promising for the development of high-quality protein foods. Vis-a-vis the trends mentioned above, for HPP at ambient temperature a profitable balance seems to have been found, as it has successfully been commercialised. However, for HPP at high temperature, high energy consumption and equipment issues are still to be dealt with. On the other side, due the commercial success of HPP at ambient temperature, legal and ethical issues will be less of a concern for HPP at high temperature. An example of an ethical issue is the labelling of HPP-treated products with the treatment technique. Consumers might feel distrust if HPP-treated products are not labelled or not labelled in a satisfying way, depending on their culture.

7. References

1. Schutyser MAI, van der Goot AJ (2011) The potential of dry fractionation processes for sustainable plant protein production. Trends in Food Science & Technology 22 (4):154-164.
2. Sari YW, Mulder WJ, Sanders JPM, Bruins ME (2015) Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. Biotechnology Journal 10 (8):1138-1157.
3. Valenzuela C, Abugoch L, Tapia C, Gamboa A (2013) Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence

on film formation. International Journal of Food Science & Technology 48 (4):843-849.
4. Lestari D, Mulder W, Sanders J (2010) Improving *Jatropha curcas* seed protein recovery by using counter current multistage extraction. Biochemical Engineering Journal 50 (1-2):16-23.
5. Pelgrom PM, Boom R, Schutyser MI (2015) Method Development to Increase Protein Enrichment During Dry Fractionation of Starch-Rich Legumes. Food and Bioprocess Technology 8 (7):1495-1502.
6. Considine T, Patel HA, Anema SG, Singh H, Creamer LK (2007) Interactions of milk proteins during heat and high hydrostatic pressure treatments — A Review. Innovative Food Science & Emerging Technologies 8 (1):1-23.
7. Jargin SV (2014) Soy and phytoestrogens: possible side effects. GMS German Medical Science 12:Doc18.
8. Roquette Nutralys Pea Protein. http://www.pea-protein.com/. Accessed 17 June 2016
9. Jiang J, Xiong YL, Chen J (2010) pH Shifting Alters Solubility Characteristics and Thermal Stability of Soy Protein Isolate and Its Globulin Fractions in Different pH, Salt Concentration, and Temperature Conditions. Journal of Agricultural and Food Chemistry 58 (13):8035-8042.
10. He T, Spelbrink REJ, Witteman BJ, Giuseppin MLF (2013) Digestion kinetics of potato protein isolates *in vitro* and *in vivo*. International Journal of Food Sciences and Nutrition 64 (7):787-793.
11. Culver CA, Swaisgood HE Changes in the Digestibility of Dried Casein and Glucose Mixtures Occurring During Storage at Different Temperatures and Water Activities¹. Journal of Dairy Science 72 (11):2916-2920.
12. Corzo-Martínez M, Lebrón-Aguilar R, Villamiel M, Quintanilla-López JE, Moreno FJ (2009) Application of liquid chromatography–tandem mass spectrometry for the characterization of galactosylated and tagatosylated β-lactoglobulin peptides derived from in vitro gastrointestinal digestion. Journal of Chromatography A 1216 (43):7205-7212.
13. Ruckenstein E, Shulgin IL (2006) Effect of salts and organic additives on the solubility of proteins in aqueous solutions. Advances in Colloid and Interface Science 123–126:97-103.
14. Ogungbenle HN (2003) Nutritional evaluation and functional properties of quinoa (Chenopodium quinoa) flour. Int J Food Sci Nutr 54 (2):153-158.
15. Bhargava A, Srivastava S (2013) Chemistry. In: Bhargava A, Srivastava S (eds) Quinoa - Botany, Production and Uses. CABI, Oxfordshire, pp 185-239.
16. Jacobs DR, Gross MD, Tapsell LC (2009) Food synergy: an operational concept for understanding nutrition. The American Journal of Clinical Nutrition 89 (5):1543S-1548S.
17. Berghout JAM, Pelgrom PJM, Schutyser MAI, Boom RM, van der Goot AJ (2015) Sustainability assessment of oilseed fractionation processes: A case study on lupin seeds. Journal of Food Engineering 150:117-124.

18. Abugoch LE, Romero N, Tapia CA, Silva J, Rivera M (2008) Study of some physicochemical and functional properties of quinoa (Chenopodium quinoa willd) protein isolates. Journal of Agricultural and Food Chemistry 56 (12):4745-4750.
19. Aora JCC, Alvarado K (2009) Aislados proteínicos de granos altoandinos *chenopodiaceas*; quinua "*chenopodium quinoa*" – cañahua "*chenopodium pallidicaule*" por precipitación isoeléctrica. Revista Boliviana de Química 26 (1):12-20.
20. Martínez EN, Añón MC (1996) Composition and Structural Characterization of Amaranth Protein Isolates. An Electrophoretic and Calorimetric Study. Journal of Agricultural and Food Chemistry 44 (9):2523-2530.
21. Guerreo-Ochoa MR, Pedreschi R, Chirinos R (2015) Optimised methodology for the extraction of protein from quinoa (Chenopodium quinoaWilld.). International Journal of Food Science & Technology:n/a-n/a.
22. Föste M, Elgeti D, Brunner A-K, Jekle M, Becker T (2015) Isolation of quinoa protein by milling fractionation and solvent extraction. Food and Bioproducts Processing 96:20-26.
23. Innova Database (2016) Innova Market Insights. http://www.innovadatabase.com/. Accessed 21 June 2016
24. Mustakas GC (1976) Soy processes, equipment, capital, and processing costs. In: ESTADOS UNIDOS Department of Agriculture Farmer Cooperative Service Edible soy protein: operational aspects of producing and marketing Sl p18-34.
25. Mastebroek HD, van Loo EN, Dolstra O (2002) Combining ability for seed yield traits of *Chenopodium quinoa* breeding lines. Euphytica 125 (3):427-432.
26. van de Velde F (2016) Protein Transition: How Fesible are the Alternatives? The World of Food Ingredients.
27. Mercadero (2016) Annual quinoa imports into European Union exceed 20,000 tons in February 2016. http://mercadero.nl/wp-content/uploads/Quinoa-Trade-EU_May2016.pdf Accessed 22 June 2016
28. Schutyser MAI, Pelgrom PJM, van der Goot AJ, Boom RM (2015) Dry fractionation for sustainable production of functional legume protein concentrates. Trends in Food Science & Technology 45 (2):327-335.
29. Ulrich GD, Vasudevan PT (2006) How to Estimate Utility Costs? Chemical Engineering.
30. ICIS (2008) Indicative Chemical Prices A-Z. http://www.icis.com/chemicals/channel-info-chemicals-a-z/. Accessed 22 June 2016
31. He R, He H-Y, Chao D, Ju X, Aluko R (2014) Effects of High Pressure and Heat Treatments on Physicochemical and Gelation Properties of Rapeseed Protein Isolate. Food and Bioprocess Technology 7 (5):1344-1353.
32. Winkel T, Álvarez-Flores R, Bertero D, Cruz P, del Castillo C, Joffre R, Peredo Parada S, Sáez Tonacca L (2014) Calling for a reappraisal of the impact of quinoa expansion on agricultural sustainability in the Andean highlands. Idesia 32 (4):95-100.

Summary

Foods rich in protein are nowadays high in demand worldwide. To ensure a sustainable supply and a high quality of protein foods, novel food proteins and processing technologies need to be explored to understand whether they can be used for the development of high-quality protein foods. Therefore, the aim of this thesis was to explore the properties of a novel food protein and a novel processing technology for the development of high-quality protein foods. For this, quinoa was chosen as an alternative protein source and high pressure – high temperature (HPHT) processing was chosen as a novel processing technology.

Quinoa protein has been found to have a balanced amino acid profile and to be allergen-free. As this combination is not common among plant proteins, it is worth studying physicochemical and functional protein properties of quinoa further (**Chapter 1**). Extraction and processing conditions can influence protein properties and thus functionality. Therefore, quinoa protein properties were examined at different extraction and processing conditions (**Chapter 2 and 3**). For this, the protein was isolated from the seed using alkaline extraction and subsequent acid precipitation. The quinoa protein isolates (QPIs) obtained were examined in terms of protein purity, yield, solubility, denaturation, aggregation and gelation behaviour, and digestibility.

It was found that when extraction pH increased, protein yield and denaturation increased, which was explained by a higher protein charge, leading to increased unfolding and solubilisation (**Chapter 2**). Protein purity decreased with increasing extraction pH, which was associated with a possible co-extraction of other seed components. QPIs obtained at extraction pH 8 (E8) and 9 (E9) had a higher solubility in the pH range of 3-4.5 (E9 solubility was highest at pH 7) compared to the isolates obtained at extraction pH 10 (E10) and 11 (E11). It was hypothesised that at a higher extraction pH, the larger extent of protein denaturation led to the exposure of hydrophobic groups, thus decreasing surface polarity and solubility. When suspensions of E8 and E9 were heated, protein aggregation increased and semi-solid gels with a dense microstructure were formed. In contrast, suspensions of

E10 and E11 aggregated to a lower degree and did not form self-supporting gels upon heating. The gels obtained with E10 and E11 had furthermore a microstructure showing loose particles. Increased protein aggregation and improved gel formation at lower extraction pH were hypothesised to be due to a higher degree of hydration and swelling of protein particles during heating, leading to increased protein-protein interactions. These findings show that QPI obtained at an extraction pH below 9 might be used to prepare semi-solid gelled foods, while QPI obtained at pH values higher than 10 might be more suitable to be applied in liquid foods.

Heat treatments of QPI suspensions lead to an increased protein denaturation and aggregation but to a decreased *in vitro* gastric protein digestibility, especially at a high temperature (120°C) and extraction pH (11) (**Chapter 3**). It was hypothesised that QPIs obtained at a higher extraction pH and treated at higher temperature were denatured to a greater extent and contained stronger protein crosslinks. Therefore, enzyme action was impaired to a higher degree compared to lower temperatures and extraction pH values. This means that by controlling extraction pH and treatment temperature the digestibility of quinoa protein can be optimised.

The disadvantage of the conventional fractionation method used in Chapter 2 and 3 is that it requires high amounts of energy and water and the solvents used can denature the protein, possibly leading to a loss in functionality. Therefore, recently, a new method has been developed, hybrid dry and aqueous fractionation, which uses less energy and water and has proved successful for obtaining protein-rich fractions from pea. It was not known whether hybrid dry and aqueous fractionation can be used to obtain protein-rich fractions of quinoa (**Chapter 4**). Quinoa seeds were carefully milled to disentangle the protein-rich embryo from the starch-rich perisperm. Using subsequent air-classification, the embryo and perisperm were separated based on size into a protein-rich fraction and a starch-rich fraction, respectively (dry fractionation). The protein-rich fraction was further milled to a smaller particle size and suspended in water. This step was to solubilise the protein (aqueous fractionation), whereby a smaller particle size and adding NaCl optimised the solubilisation efficiency. The addition of salt helped to extract more salt-soluble proteins

from quinoa, next to the water-soluble proteins. After centrifugation, the protein-enriched top aqueous phase was decanted and ultrafiltered for further protein concentration. The process generated a quinoa protein-rich fraction with a protein purity of 59.4 w/dw% and a protein yield of 62.0%. Having used 98% less water compared to conventional protein extraction, this new method is promising for industry to obtain quinoa protein concentrates in a more economic, sustainable and milder way.

Next to exploring novel food proteins for the development of high-quality protein foods, novel processing technologies are also important to study. This is because traditional thermal processing can deteriorate the quality of protein-rich foods and beverages by causing undesired browning or too high viscosities. Therefore, for sterilisation purposes, HPHT processing was investigated for the treatment of protein foods (**Chapter 5**). Model systems, whey protein isolate – sugar solutions, were used to study the effect of pressure at high temperature on Maillard reactions, browning, pH, protein aggregation and viscosity at different pH. It was found that pressure retarded early and advanced Maillard reactions and browning at pH 6, 7 and 9, while it inhibited protein aggregation and, thereby, a high viscosity at pH 7. The mechanism behind this might be that pressure induces a pH drop, possibly via dissociation of ionisable compounds, and thus slows down Maillard reactions. Differences in protein conformation, protein-protein interactions and sensitivity of whey proteins, depending on pH, pressure and heat, might be at the base of the reduced protein aggregation and viscosity observed at pH 7. The results show that HPHT processing can potentially improve the quality of protein-sugar containing foods, for which browning and high viscosities are undesired, such as high-protein beverages.

Finally, the properties of quinoa protein and HPHT processing were discussed in a broader context (**Chapter 6**). It was concluded that QPI obtained at pH 9 is a promising alternative to pea and soy protein isolate from a technical perspective and that QPI protein yields can be optimised. Also, quinoa protein-rich fractions obtained with the hybrid dry and aqueous fractionation method were predicted to have comparable properties to QPI, soy and pea protein isolates. However, from a marketing perspective, the protein-rich fraction was considered more advantageous to be up-scaled compared to QPI. High pressure at

ambient or high temperature was found to have an added value compared to heat, which can be used for the development of high-quality protein food. Lastly, quinoa protein and HPHT processing might become more attractive for industry in the light of current trends, if present predictions can be confirmed and remaining issues can be resolved.

Acknowledgements

Sorted alphabetically by 1) category, and 2) name.

Category	Name	Group WUR	Thanks so much/hartstikke bedankt/herzlichen Dank/muchisimas gracias/merci beaucoup ...
Colleagues involved in PhD	Anja	FPE	Voor je mooie samenwerking voor ons paper en je hulp met de laatste stap van mijn thesis ;)
	Ben	FBR	Voor de kansen die je me binnen de FT groep hebt gegeven. Dat ik aan alle groepsactiviteiten mocht meedoen. Voor je nuttige feedback en steun voor de labhoofdfunctie en PhD-financiering.
	Kasper	FQD	Voor je fijne samenwerking voor het coachen van ons student.
	Maarten	FPE	Voor je mooie samenwerking voor ons paper en persberichtje.
	Mauricio	FPE	Por tu colaboración formidable para nuestro articulo(s) (de revista industrial también), por el tiempo dedicado a mis estudiantes y las buenas platicas que tuvimos.
	Robert	Plant Breed-ing	Voor je input voor mijn papers en project acquisitie m.b.t. de WUR-quinoa en het laten zien van de WUR quinoa velden, dit heeft mijn PhD afgemaakt.
	Vincenzo	FQD	For your nice collaboration for coaching our student and writing a paper, for your support for my PhD financing and for your general "aandacht" (I cannot find a good translation for this, new nice word for you to learn ;))
EMFS 12th Edition	Many		For the great get-togethers we had and for still being kind and welcoming despite the strange behaviour I showed sometimes.
Family	Artus		Fuer deine liebe und lustige Art und vor allem fuer die mentale Untersuetzung fuer meine Mama.
	Axel		Fuer deine Unterstuetzung und deine Muehe, um mir in schwierigen Zeiten beizustehen.
	Familie Avila		Por la bienvenida calorosa y el cariño que

	Familie Ruiz		me han dado. Gracias a ustedes reencontré mis raíces y gané más confianza en mí misma.
	Familie Bouman		Voor het hartelijke en warme welkom van idereen vanaf het begin. Ik ben blij dat ik jullie als schoonfamilie heb/zal hebben ;)
	Jacob		Voor al je steun, motivatie en inspiratie. Ach ja, en voor het omgooien van mijn leven, haha (*Mickey*). Nu heb ik toch nog een goede reden waarom ik een PhD heb gedaan ;)
	Mama		Fuer deine Unterstuetzung und vor allem fuer deine staendige Muehe, damit es mir gut geht. Ohne dich waere ich wahrscheinlich nicht in dieser Position.
	Merlin		Fuer deine Zuneigung, Treue und Stoerungen im Bett ;) Ich weiss, die sind ja lieb gemeint.
	Papa		Por siempre pensar en mi, por el soporte que me has dado y por insistir en contactarme :)
FBR PhDs	Balaji	FBR	For the great lunches and dinners we had. It was a nice time when the chair groups were not in our building yet ;)
	Book	FBR	
	Edwin	BPE	
	Gosia	BPE	
	Lenny	BPE	
	Lonneke	FBR	
	Marit	FCH	
	Noon	FBR	
	Pang	FBR	
	Stefan	FBR	
	Vincent	BPE	
	Yongfu	FBR	
IP/OP-CN	Canan	FBR	For the nice interactions, activities and help you provided. You guys were the main reason I came to the IPOP meetings ;)
	Claire	FCH	
	Grace	FQD	
	Guido	ASG	
	Hsuan	ASG	
	Lonneke	FBR	
	Louise	FBR	
	Sam	ASG	
	Sergio	ASG	

	Tetske	ASG	
Miscellaneous	Daphne	FQD	Voor de super-mooie gesprekken waar we ook waren, voor je aandacht, motivatie en inspiratie, voor de leuke activiteiten samen en voor je hulp as paramimf.
	Elsbeth	FQD	For your dedicated and proactive cooperation in coaching our student.
	Elsbeth	Social worker	For your great support and advice during a very difficult period in my life.
	Marjolein	FBR	Voor het meenemen naar acquisitiegesprekken, het uitleggen van dingen en je cooperatieve aanpak.
	Martijntje	FBR	Voor de warme sfeer die je altijd in onze kamer creeerde en het meeslepen naar de koffiepauze (heeft me altijd goed gedaan) :)
	Matthias	FCH	Fuer die Einfuehrung zu eurem SEC. Hat nichts zu meinem PhD beigetragen, aber sehr wohl zu was anderem: nicht mehr allein in Rhenen! ;)
	Miriam	FBR	Voor de geweldigen wandelingen, voor je aandacht, motivatie en inspiratie, voor het leren/adivseren van allerlei dingen en je hulp als paranimf.
PhD trip committee & Thai people	Many	FBR	For organising an unforgettable lab trip (in the positive way of course!).
Profound talk people	Anika	FPH	For the amazing talks we had and your support in difficult times.
	Daphne	FQD	
	Edita	FCH	
	Grace	FQD	
	Joao	BPE	
	Marine	FQD	
	Miriam	FBR	
	Sara	FQD	
Room mates	Alicja	FBR	Voor de gezellige sfeer op ons kantoor en hulp/tips met allerlei dingen.
	Jaap	FBR	
	Kees	FBR	
	Martijntje	FBR	
	Maurice	FBR	
	Remco	FBR	
Secondary school friends	Chrissy		Fuer die schoenen Treffen in unserer Heimatstadt (oder irgendwo anders) und
	Lena		

	Lydia		dass ihr trotz meiner vielen Absagen frueher immer hinter mir standet.
	Nati		
	Steph		
Secretaries	Julia	FBR	For your dedicated and instant help with all kinds of administrational things I came up with.
	Kimberely	FQD	
	Lea	FBR	
	Lysanne	FQD	
	Marion	FBR	
	Renate	FBR	
	Yvonne	FBR	
Students	All	Mixed	For your dedication, effectiveness, cooperation and nice talks during your internship.
	Anke	FPE	Voor je fijne samenwerking voor ons paper.
	Bingyan	FQD	For coming up with interesting ideas.
	Danmeng	FQD	For being upfront and open.
	Marlon	FQD	For your enthusiasm and emotional moments.
	Radia	FBR	Pour ta fidelité et pour organiser la nuit arabe ;)
	Surabhi	FQD	For helping me connect with the companies.
	Wukai	FQD	For your positivism and funny stories.
Supervisors	Guido	FBR	Voor het toestemmen om (uiteindelijk!) mijn begeleider te worden, voor je proactieve en toegewijde steun, je aandacht, je kritische feedback en de goede gesprekken.
	Jeroen	FBR	Voor de goede start van mijn begeleiding en het snelle actienemen m.b.t. de wisseling van mijn begeleiding.
	Marcel	FBR	Voor de goede gesprekken, dat je er altijd voor mij was, het vertrouwen dat je me gaf en voor je superadvies en hulp om mijn PhD op tijd te kunnen afmaken.
	Markus	HNE/FQD	Fuer deine permanente Unterstuetzung, deine schnellen und nuetzlichen Antworten, das "Voranbringen" von unseren Meetings und fuer dein super-nuetzliches Feedback.
	Tiny	FQD	Voor je toegewijde steun, vooral met je drukke agenda, je nuttige advies en het open zijn voor veranderingen.

Technicians	Arjen	Biophysics	For your dedicated and instant help for any lab-related thing. You contributed a lot to completing my experiments on time and with high-quality results.
	Charlotte	FQD	
	Erik	FQD	
	Frans	FQD	
	Geert	FQD	
	Harry	FPH	
	Jarno	FPE	
	Jos	FPE	
	Martin	FPE	
	Maurice	FPE	
	Miranda	FPH	
	Xandra	FQD	
VLAG PhD council	Many	Mixed	For the great time at the council: supercool meetings in and outside the council.
Whole groups	Many	FBR/FT	Voor jullie aandacht en hulp met allerlei dingen.Ik voelde me altijd thuis bij jullie. Hoogtepunten waren onze groepsoverleggen (de gezelligste overleggen ooit) en koffiepauzes. En natuurlijk hebben jullie het mogelijk gemaakt dat ik nu in het Nederlands kan schrijven ;)
		FQD	For making me feel at home with you as well. For the dedicated help during my PhD, the nice lunch breaks, special occasion-breaks, meetings and the extraordinary PhD trip.
		FPH	For welcoming me warmly every time I came along for all kinds of reasons, including for dating ;) You were unofficially my third group.

Abbreviations:
FBR/FT = Food & Biobased Research/Food Technology
FQD = Food Quality & Design
FPE = Food Process Engineering
FPH = Food Physics
FCH = Food Chemistry
BPE = Bioprocess Engineering
ASG = Animal Science Group

I hope I did not forget anyone. If I did, I'm sorry.

It was a pleasure to work or just be with you guys. You were one of the major reasons I did not quit my PhD. :)

About the author

Geraldine Avila Ruiz was born in Dachau, Germany, in 1986. First she lived in Garching, then in Eching (both near Munich) for most of her childhood. In her teenage years, Geraldine's family moved to Munich, as going out at night became more important to her and her brother. And their new school was located in Munich. When finishing secondary school Geraldine had enough of Germany and went to France (Paris) to study medicine, her long-sought career path in her favourite country back then. However, when battling with thousands of students for one of the few hundred places in the second year of medical studies, she realised that she actually did not want to become a physician. She was more interested in how things work than in treating patients on an assembly line, as it seemed. So, she decided to go to the UK to study Biological Sciences. UK? Just for a change and to learn English. She got the choice between the universities of Birmingham, Cardiff and Edinburgh. From a quick search on Google, the University of Edinburgh looked the nicest. So she went for it. During her studies there, Geraldine googled what she could do as Master's and discovered the world of food science and technology. Being mesmerised, she ran to do internships in the food industry and at the university next-door (Edinburgh University did not have a food science department). On the internet, Geraldine found a super-exciting Master's degree, the European Master in Food Studies, organised by Wageningen University. Although never have heard about this university, she totally went for it. When doing her Master thesis at Cargill in Belgium, the next big thing dawned on her: doing a PhD. After a long time going back and forth and thousands of emails to food professionals (thank you IFT!), she went for it. It was not the smartest choice for her but, in the end, she was glad she did it. After this incredible four-year-journey, she finally found her voice and started a new chapter in her life. Professionally, Geraldine returned to where she started from in the food industry: in the NPD department of a fish company (now: Junior Product Developer at Profish).

For more information about Geraldine Avila Ruiz, you can go to:
https://nl.linkedin.com/in/geraldine-avila-ruiz-30316216

Overview of completed training activities

Activity	Date	Organising institute	City	Country
Reaction Kinetics	15-19 Oct 2012	VLAG	Wageningen	Netherlands
Advanced Food Analysis	28 Jan - 2 Feb 2013	VLAG	Wageningen	Netherlands
Industrial Proteins	30 Sep - 3 Oct 2013	VLAG	Wageningen	Netherlands
iFood Conference	7 - 10 Oct 2013	COST, DIL etc.	Hannover	Germany
Wageningen PhD Council Symposium	10 Dec 2013	WPC	Wageningen	Netherlands
Food Structure & Functionality Symposium	30 Mar - 2 Apr 2014	Elsevier	Amsterdam	Netherlands
High Pressure Biosciences and Biotechnology	15 - 18 Jul 2014	Oniris	Nantes	France
Protein Summit	16-18 Sep 2015	Protein Summit	Rotterdam	Netherlands
Wageningen PhD Council Symposium	6 May 2015	WPC	Wageningen	Netherlands
PhD Week	10-13 Dec 2012	VLAG	Baarlo	Netherlands
Competence Assessment	22 Jan 2013	WGS	Wageningen	Netherlands
Information Literacy	12 - 13 Feb 2013	WU Library	Wageningen	Netherlands
Philosophy and Ethics of Food Science and Technology	22 Jan - 26 Feb 2015	WGS	Wageningen	Netherlands
Scientific Writing	5 Feb - 9 April 2015	Wageningen In'to Languages	Wageningen	Netherlands
Career Perspectives	29 Nov - 10 Dec 2015	WGS	Wageningen	Netherlands
Entrepreneurship in and outside Science	14 Dec 2015 - 7 Jan 2016	WGS	Wageningen	Netherlands
FT overleggen	Aug 2012 - 2016	FBR/FT	Wageningen	Netherlands
Expertise overleggen	Aug 2012 - 2016	FBR/FT/FS&D	Wageningen	Netherlands
Connect4Action	22-23 Apr 2014	EFFoST	Wageningen	Netherlands
PhD colloquia FQD	Sep 2013 - 2016	FQD	Wageningen	Netherlands
Research proposal	2012	FBR/FQD	Wageningen	Netherlands
PhD VLAG council	Jun 2013 - Sep 2014	VLAG	Wageningen	Netherlands
PhD Trip	Oct 2014	FQD	Bangkok, Singapore	Thailand, Singapore

Cover: Geraldine Avila Ruiz, Ridderprint BV, Ridderkerk, the Netherlands
Lay-out: Geraldine Avila Ruiz
Printed by: Ridderprint BV, Ridderkerk, the Netherlands