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Process and Encapsulation Stability

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Process and Encapsulation Stability

Ali Marefati

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Starch Pickering Emulsions: Process and Encapsulation Stability

Abstract

The emulsion stabilization functionality of surfactants and biopolymers can be replaced by solid particles adsorbed at the interface, where they create a category of emulsions known as Pickering emulsions. Recently, health and environmental concerns have led to new market demand for natural, biodegradable and renewable sources of ingredients. Starch granules meet these ingredient requirements and are also good candidates for stabilization of Pickering type emulsions due to their properties such as neutral color, taste, odor and being non-allergic. Starch granules are generally hydrophilic and in order to improve their emulsifying capacity they are modified with hydrophobic groups. The most widely used chemical modification is esterification with acid anhydride, such as octenyl succinic anhydride (OSA). Emulsions stabilized by OSA modified starch have shown to have high stability. Moreover, the application of a heat treatment can induce a partial gelatinization of the starch at the oil-water interface, which leads to changes in interfacial properties of the starch granule stabilized emulsions.

This thesis focuses on the development and application of starch Pickering emulsions and has been carried out along three main lines of investigation where each line has its own section in the thesis. In the first section, the preparation and properties of oil-in-water emulsions stabilized by starch granules with small sizes, including quinoa, rice and amaranth, were investigated. In the second section, the process and encapsulation stabilities of the emulsions were evaluated in the context of simple oil-in-water and double water-in-oil-in-water emulsions, with or without heat treatment. In the third section, the physical and encapsulation stabilities of the emulsions during *in vitro* digestion were evaluated.

Quinoa starch granules showed to have higher emulsifying efficiency both in the initial state and after accelerated stability testing. Moreover, the heat induced gelatinized layer showed to not only contribute to initial general stability, but also increased the process and encapsulation stabilities of the emulsions. Modified starch granules appeared to protect the emulsions against destabilization mechanisms during processing (i.e. freezing and freeze-drying). It was possible to create powders from starch stabilized Pickering emulsions with high oil content (up to 80%). Furthermore, emulsions stabilized by starch granules showed high stability for encapsulation of bioactive compounds during *in vitro* digestion. The results of this work can be used for the development of lipid-based encapsulated systems, in both liquid and powder forms for the delivery of bioactive compounds in food, pharmaceutical and cosmetic applications.

Key words: Starch granules, Pickering emulsions, Encapsulation, Freeze-thaw stability, Freeze-drying, *In vitro* digestion, *in vitro* intestinal lipolysis

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Starch Pickering Emulsions

Process and Encapsulation Stability

Ali Marefati

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Two roads diverged in a wood, and I–
I took the one less traveled by,
And that has made all the difference.

Robert Frost (1874-1963)

Contents

Abstract

The emulsion stabilization functionality of surfactants and biopolymers can be replaced by solid particles adsorbed at the interface, where they create a category of emulsions known as Pickering emulsions. Recently, health and environmental concerns have led to new market demand for natural, biodegradable and renewable sources of ingredients. Starch granules meet these ingredient requirements and are also good candidates for stabilization of Pickering type emulsions due to their properties such as neutral color, taste, odor and being non-allergic. Starch granules are generally hydrophilic and in order to improve their emulsifying capacity they are modified with hydrophobic groups. The most widely used chemical modification is esterification with acid anhydride, such as octenyl succinic anhydride (OSA). Emulsions stabilized by OSA modified starch have shown to have high stability. Moreover, the application of a heat treatment can induce a partial gelatinization of the starch at the oil-water interface, which leads to changes in interfacial properties of the starch granule stabilized emulsions.

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Popular science summary

Many types of food, pharmaceutical and cosmetic products are mixtures of two or more immiscible phases, where one is dispersed in another as spherical droplets. These types of products are called emulsions. Emulsions are found in everyday systems such as mayonnaise, milk and various skin care products, where they mainly consist of water (W) and oil (O). Different types of emulsions can be produced depending on the spatial distribution of different phases. In this way, when droplets of oil are dispersed in water, oil-in-water emulsions (O/W) are formed and when droplets of water are dispersed in oil, water-in-oil (W/O) emulsions are formed. It is also possible to produce different types of multiple emulsions where the dispersed phase contains yet another dispersed phase. From these types of emulsions, water-in-oil-in-water emulsions (W/O/W) and oil-in-water-in-oil emulsions (O/W/O) are the most common.

In order to have stable emulsions and prevent the oil phase separating from the water phase, it is necessary to use other components referred to as stabilizers. Surfactants are commonly used as stabilizers. Health and environmental concerns have led to scrutiny of the extensive use of synthetic surfactants in foods, pharmaceuticals and cosmetics, which in turn has led to new market demand for natural, biodegradable and renewable ingredients and has resulted in a quest for new emulsion stabilizers.

Solid particles have been found to be able to stabilize emulsions. This phenomenon was first observed over a century ago by Ramsden (1903) and was proven experimentally by Pickering (1907). Therefore, emulsions stabilized by solid particles are called Pickering emulsions. These types of emulsions have shown to have advantages over the commonly used ingredients for stabilizing emulsion droplets, namely a higher stability of the emulsion over time. There is a wide range of solid particles that can be used for the formation of emulsions from which only a few are compatible with food systems. One example is starch granules which have been found to be suitable for the assembly of Pickering type emulsions.

Starch is the main component of the daily diet and is therefore something that advantageously can be used in both food and pharmaceuticals, as it is generally considered as safe. Moreover, starch is abundant, tasteless, colorless, odorless, non-allergic and relatively inexpensive. Depending on the botanical source, starch granules also vary in size, shape and composition. Compared to other starch sources, quinoa starch granules with a fairly small size (0.5-2 microns), have a superior

capability to stabilize oil droplets in emulsions. In addition to emulsification properties, the complex structure of starch granules enables them to have unique functional properties, namely the ability to gelatinize to form a fused and cohesive layer, which has been shown to be able to protect the droplets during processing. In order to optimize the emulsifying efficiency of the starch particles, they are chemically modified by hydrophobic groups of octenyl succinic anhydride (OSA). Starch granules produced in this way are approved by the FDA as a food ingredient and pharmaceutical excipient with a degree of modification lower than 3% and with no limitation in use.

Pickering emulsions are particularly attractive in food and pharmaceutical industries due to the possibility of entrapment of the desired material in the internal phase. The act of inclusion of bioactive substances in an emulsion is called encapsulation. Emulsions with encapsulated bioactive ingredients can be used as functional foods or drug carriers, to increase stability and bioavailability, or for controlled release and targeted delivery to specific locations in the body.

The aim of this PhD work was to investigate different aspects of Pickering emulsions stabilized by starch granules. In the first section, physico-chemical and emulsification properties of small starch granules from different botanical origins (i.e. rice, quinoa, and amaranth) in native form (non-modified) and modified with OSA at different levels, were tested. In the second section, the process and encapsulation stabilities of starch granule stabilized emulsions were analyzed in the context of simple and double emulsions, and the effect of a gelatinized and fused starch barrier in preventing destabilization was also evaluated. Finally, in the third section, the fate of starch Pickering emulsions during simulated *in vitro* human digestion was investigated.

The results of this PhD work show that there is good potential for the use of starch granule stabilized Pickering emulsions in food and pharmaceutical formulations. The results show a higher efficiency of quinoa starch granules, which was improved as the chemical modification improved. The Pickering emulsions had high process and encapsulation stabilities that could be improved by heat treatment and creation of a gelatinized barrier. Lastly, the physiological stability showed that the emulsions remained stable during *in vitro* human digestion, making them a useful encapsulation tool for delivery of bioactive compounds to distal parts of the human digestion system.

The higher stability of Pickering emulsions compared to traditional surfactant-stabilized emulsions will create advantages from both industrial and consumer perspectives. These advantages are listed as high storage stability, high process stability, high encapsulation stability and high digestion stability. In this way, Pickering emulsions can stay intact during production and have a longer shelf-life. Moreover, Pickering emulsions can effectively protect encapsulated bioactive

substances that are included in the emulsions. A higher physiological stability makes Pickering emulsions suitable candidates for controlled and targeted release of encapsulated bioactive substances. In addition, if an oil-based formulation can withstand digestive conditions, and reach the distal part of the human digestion system, satiety can be promoted which in turn can result in the prevention of obesity.

List of publications

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

Paper I: Marefati, A., Wiege, B., Haase, N. U., Matos, M., Rayner, M. (2017) Pickering emulsifiers based on hydrophobically modified small granular starches - Part I: Manufacturing and physico-chemical characterization, Carbohydrate Polymers 175, 473-483.

Paper II: Marefati, A., Matos, M., Wiege, B., Haase, N. U., Rayner, M. Pickering emulsifiers based on small hydrophobically modified granular starches - Part II: Effects of the degree of hydrophobic modification on emulsifying capacity and stability, Manuscript submitted to Carbohydrate Polymers.

Paper III: Marefati, A., Rayner, M., Timgren, A., Dejmek, P., Sjöö, M. (2013) Freezing and freeze-drying of Pickering emulsions stabilized by starch granules, Colloids and Surfaces A: Physicochemical and Engineering Aspects 436, 512-520.

Paper IV: Marefati, A., Sjöö, M., Timgren, A., Dejmek, P., Rayner, M. (2015) Fabrication of encapsulated oil powders from starch granule stabilized W/O/W Pickering emulsions by freeze-drying, Food Hydrocolloids 51, 261-271.

Paper V: Marefati, A., Bertrand, M., Sjöö, M., Dejmek, P., Rayner, M. (2017) Storage and digestion stability of encapsulated curcumin in emulsions based on starch granule Pickering stabilization, Food Hydrocolloids 63, 309-320.

Paper VI: Marefati, A., Wiege, B., Abdul Hadi, N., Dejmek, P., Rayner, M. *In vitro* intestinal lipolysis of emulsions based on starch granule Pickering stabilization, Manuscript submitted to Food and Function.

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Additional publication not included in this thesis

Paper VII: Matos, M., Marefati A., Gutiérrez, G., Wahlgren, M., Rayner, M. (2015) Comparative emulsifying properties of OSA-modified starch: granular form vs dissolved state, PloS one 11 (8).

Paper VIII: Semek, S., Ovando-Martinez, M., Marefati, A., Sjöö M., Rayner, M. (2016) Chemical composition, digestibility and emulsification properties of octenyl succinic esters of various starches, Food Research International 75, 41-49.

Paper IX: Matos, M., Marefati, A., Bordes, R., Gutiérrez, G., Rayner, M., (2017) Combined emulsifying capacity of polysaccharide particles of different size and shape, Carbohydrate Polymers 169, 127-138.

Paper X: McNamee, C., Sato, Y., Wiege, B., Marefati, A., Nylander, T., Kappl, M., Rayner, M., Effect of degree of modification of rice starch particles and the presence of salt on the physical properties of a film of rice particles at air/water interfaces, Manuscript submitted to Particles at Fluid Interfaces.

Author's contribution to the publications

Paper I: Ali Marefati and Marilyn Rayner planned the experimental design. Ali Marefati performed the experimental work except for the starch isolation and modification and determination of the degree of modification which was performed by Berthold Wiege. Ali Marefati and co-authors analyzed the results. Ali Marefati wrote the major part of the paper.

Paper II: Ali Marefati planned the experimental design. Ali Marefati and Maria Matos performed the experimental work except BET measurements. Ali Marefati and co-authors analyzed the results except for Turbiscan results that were analyzed by Maria Matos. Ali Marefati wrote the major part of the paper.

Paper III: Ali Marefati planned the experimental design. Ali Marefati performed the experimental work. Ali Marefati and co-authors analyzed the results. Ali Marefati wrote the paper.

Paper IV: Ali Marefati planned the experimental design. Ali Marefati performed the experimental work. Ali Marefati and co-authors analyzed the results. Ali Marefati wrote the paper.

Paper V: Ali Marefati planned the experimental design. Ali Marefati supervised Mariannick Bertrand to perform the experimental work and also performed parts of the experimental work. Ali Marefati and co-authors analyzed the results. Ali Marefati wrote the paper.

Paper VI: Ali Marefati planned the experimental design. Ali Marefati performed the experimental work. Ali Marefati and co-authors analyzed the results. Ali Marefati wrote the paper.

Contribution to conferences and workshops

Marefati, A., Matos, M., Wiege, B., Haase, N., Rayner, M. (Poster presentation), The effect of degree of modification on the emulsifying capacity of small granular starches, AACCI 2017, October 8-11th, 2017, San Diego, USA.

Marefati, A., Matos, M., Wiege, B., Haase, N., Rayner, M. (Poster presentation), The effect of degree of modification on the emulsifying capacity of small granular starches, Starch Round Table 2017, October 4-7th, 2017, San Diego, USA.

Marefati, A., Matos, M., Wiege, B., Haase, N., Rayner, M. (Poster presentation), Pickering emulsifiers based on hydrophobically modified small granular starches, effect of the degree of modification on the emulsification capacity and stability, 7th International Colloid Conference, June 18- 21st, 2017, Sitges, Spain.

Wiege, B., Hasse, N., Marefati, A., Rayner, M., Matos, M. (Poster presentation), Pickering emulsifiers based on small granular starches, manufacturing and physico-chemical characterization, 12th International Conference on Polysaccharide-Glycoscience, October 19-21st, 2016, Prague, Czech Republic.

Marefati, A., Rayner, M., Dejmek, P., Sjöö, M. (Oral presentation), Structural approaches of Pickering emulsions for encapsulation of hydrophobic and hydrophilic substances, American Association of Cereal Chemistry International Annual Meeting, September 29th - October 2nd, 2013, Albuquerque, USA.

Marefati, A., Sjöö, M., Dejmek, P., Rayner, M. (Oral presentation), Structural Approaches of Starch Pickering Emulsions Drying Starch Pickering Double Emulsions, Topical Science Cluster Meeting 2013, June 19th, 2013, Biofilms-Research Center for Bioinformatics, Malmö University, Hergård, Sweden.

Marefati, A., Matos, M., Timgren, A., Sjöö, M., Dejmek, P., Rayner, M. (Oral presentation), Starch Pickering Double Emulsions and the Production of Oil-Containing Powders Nordic Starch Network 2012, November 22nd, 2012, Carlsberg Research Centre, Copenhagen, Denmark.

Marefati, A., Matos, M., Timgren, A., Sjöö, M., Dejmek, P., Rayner, M. (Oral presentation), Drying Starch Pickering Double Emulsions, SAPS and CODIRECT joint meeting on Drug Processing and Delivery, November 12-14th, 2012, Södertälje, Sweden.

Marefati, A., Sjöö, M., Rayner, M., Timgren, A., Dejmek P. (Poster presentation), Oil filled powders produced from starch stabilized emulsions, P65:11th International Hydrocolloid Conference. May 11-14th, 2012, West Lafayette, Indiana, USA.

Marefati, A., Rayner, M., Timgren, A., Sjöö, M., Dejmek, P. (Poster presentation), Drying of starch Pickering emulsions, P-190, 2011 European Federation of Food Science and Technology (EFFoST) Annual Meeting - Process - Structure - Function Relationships, November 9-11th, 2011, Berlin, Germany.

Aim and objectives

The overall aim of this research was to develop Pickering emulsion systems stabilized by starch granules and understand their functional properties with respect to processing and encapsulation stability.

This overall aim is divided into the following specific objectives of this project:

- Carry-out a detailed physico-chemical characterization of starch granules and the subsequent emulsions.

- Evaluate emulsifying capacity of small granular starches with respect to granule type and level of hydrophobic modification in a systematic way.

- Create and modify the starch barrier at the oil-water interface through gelatinization by the careful application of heat treatment, and elucidate the effect of gelatinized barrier on overall properties of the emulsions.

- Verify the process stability with respect to freezing and freeze-drying.

- Develop encapsulation systems in liquid and dry forms for hydrophobic and hydrophilic bioactive compounds.

- Assess the physiological stability towards simulated *in vitro* human gastrointestinal conditions.

Introduction

Emulsions are basis of many food, pharmaceutical and cosmetic products; such as milk and salad dressing and mayonnaise in food, creams and lotion in pharmaceutical and cosmetic products where one phase of oil or water is dispersed in another phase of water or oil. These mixtures are thermodynamically unfavorable and tend to separate, and thereby, lowering the free energy by minimizing the contact area between phases. Therefore, stabilizers are needed to counterbalance the interfacial tension that exist between the two phases and thus, create stable multi-phase systems. Commonly, emulsion stabilizers are categorized to two groups. The first group of emulsion stabilizers include texture modifiers or thickening agents that increase the viscosity of the continuous phase and thereby, decrease the rate of separation. Hydrocolloids such as xanthan gum are the best example of this category. The second group of emulsions stabilizers are amphiphilic molecules that are known as emulsifiers. The amphiphilic nature of emulsifiers make them able to be adsorbed by both polar and non-polar media and such as oil-water interfaces of emulsions and decrease the interfacial energy and free energy of the system [1]. The physico-chemical properties of this adsorbed layer such as attractive/repulsive interaction, thickness, etc. determines the properties of resulting emulsions. There are two main types of emulsifiers including: low molecular weight emulsifiers and amphiphilic biopolymers. Low molecular weight emulsifiers are also known as surfactants and are consisted of a hydrophilic head and hydrophobic tail and can be natural or synthetic. The amphiphilic biopolymers mainly includes proteins especially the dairy proteins but the plant-based proteins such as soy proteins are becoming more popular [1]. Surface active polysaccharides have also been used as emulsifiers [2].

Particles can also be used to stabilize emulsions. Particle stabilized emulsions were first described independently by Ramsden [3] and Pickering [4] in early twentieth century, however, these type of emulsions are named after the second scientist. Although, these types of emulsions are known for over a century, considerable interest has only devoted to them during the past decades. The renewed interest in Pickering emulsions is due to their long term stability [5]. Many different particle types have been proposed for the stabilization of Pickering emulsions and although only a few number are applicable for food, there has been a raising interest in

natural, bio-based, food grade sources [1, 5]. The food-based Pickering particles include: carbohydrate-based, protein-based and lipid-based particles [1].

Starch particles have been used as Pickering agent extensively. Starch is the second most abundant polymer in the nature after cellulose, and depending on the botanical source it comes in different sizes, shapes and compositions. In addition, starch is colorless, odorless, tasteless, non-allergic and relatively inexpensive [6]. Starch is generally hydrophilic and the desired hydrophobicity can be achieved by chemical modification with octenyl succinic anhydride (OSA). The OSA modified starch is acceptable food ingredient and pharmaceutical excipient with modification level lower than 3% based on the dry weight (INS1450, E1450) [6].

Overview of design of the thesis work

This thesis, tries to investigate suitability of small starch granules for stabilization of emulsions. The process and encapsulation stability of Pickering emulsions stabilized by OSA modified starch granules as stabilizing particles are evaluated in the next steps. The physico-chemical stability of emulsions has been characterized in the initial state and after freezing and freeze-drying. The physico-chemical and encapsulation stability of emulsions have been characterized in the initial state and after simulated *in vitro* digestion. The design of this thesis work can be organized into three main sections. In the first section (paper I and II), the emulsifying capacity of small granular starches with respect to starch type and the level of hydrophobic modification was evaluated. In the second section, (paper III and IV) the process and encapsulation stabilities of emulsions towards the freezing and freeze-drying process was evaluated for starches in granular form and heat treated and therefore gelatinized at the interface. Lastly, in the third section (paper V and VI), the physical and encapsulation stabilities of the Pickering emulsions investigated during an *in vitro* digestion of emulsions stabilized by starch in granular form compared to heat treated emulsions. In addition, the barrier effect of starch at the oil-water interface was evaluated by lipolysis and compared to a protein stabilized emulsion.

Background

Emulsions

Many products in the food, pharmaceutical, and cosmetic industries, are formulations based on emulsions. Emulsions are mixtures of two immiscible phases where one phase is dispersed in the other in the form of small spherical droplets. In food materials, emulsions consisting of oil and water are more common, where the mean diameter of the droplets is normally in the range of 0.1-100 µm [7]. Due to the large interfacial area between the finely dispersed phase droplets and the continuous phase, emulsions are generally not thermodynamically stable, as there is a reduction in free energy of the system if dispersed phase droplets coalesce, thereby minimizing the interfacial or contact area between the phases. To prevent coalescence and stabilize the droplets, emulsifiers are used which act by decreasing the interfacial tension between the phases, increasing the steric hindrances and/or electrostatic repulsion between the droplets [8]. Typical examples include low molecular weight emulsifiers and amphiphilic biopolymers [1, 7].

Emulsions are categorized by the relative spatial distribution of their constituent phases. In this way, they can be water continuous in the case of oil-in-water emulsions (O/W) such as milk, cream, ice-cream, dressing, mayonnaise, beverages, soups, dips and sauces, or oil continuous in water-in-oil emulsions (W/O) such as butter, margarine and some spreads (Fig. 1). The material that forms the droplets are referred to as dispersed phase, discontinuous phase or internal phase while the surrounding material is called dispersing phase, continuous phase or external phase [7]. In this thesis we use the terms dispersed and continuous phase.
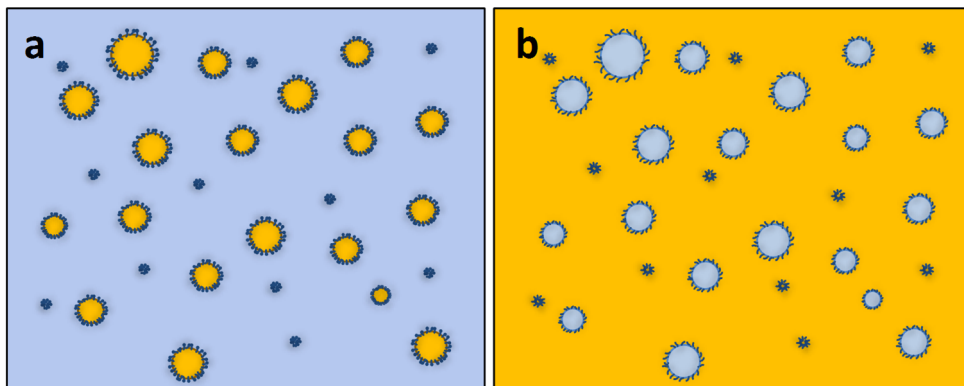
Fig. 1.
Schematic representation of emulsions stabilized by surfactant (a) oil-in-water (O/W) and (b) water-in-oil (W/O) emulsions, where the excess of surfactants is present as micelles.

In addition to dispersed phase, continuous phase and emulsifier, mechanical energy is required to convert the bulk immiscible phases to an emulsion. This process, which is referred to as homogenization, is achieved by creating and reducing the size of the droplets using a homogenizer. Examples of homogenizers include: rotor-stator high shear homogenizer, high pressure valve homogenizer, colloidal mill, micro-fluidizer and ultrasonic homogenizer. The droplet size of emulsions is mainly dependent on the intensity of the homogenizer (energy) and the concentration of emulsifier per amount of dispersed phase [5]. Emulsion-based systems, have been widely investigated for utilization as delivery systems in food and pharmaceutical fields as these systems are ideal for protection of the ingredients, increasing bioavailability and controlled delivery of bioactive components [9].

Multiple emulsions

Multiple emulsions are a special class of emulsions where the dispersed phase contains even smaller dispersed droplets as its own dispersed phase (Fig. 2). These types of emulsions were first described by Seifriz in 1925 [10]. From these types of emulsions water-in-oil-in-water double emulsions (W/O/W) and oil-in-water-in-oil double emulsions (O/W/O) are the most common [7]. Due to their ability for the entrapment of desired materials in an inner phase during the primary emulsification procedure, multiple emulsions can act as vehicles for encapsulation and delivery of nutrients (vitamins and minerals) and active substances [11-13]. Furthermore, by the addition of water as internal phase, reduced fat foods can also be formulated

[14]. Encapsulation of flavors, taste masking and prevention of oxidation are other uses of double emulsions [15-17]. Encapsulation of probiotic bacteria within double emulsions in order to preserve them in a viable form during production, storage and gastro-intestinal digestion has been reported in previous studies [18-20].
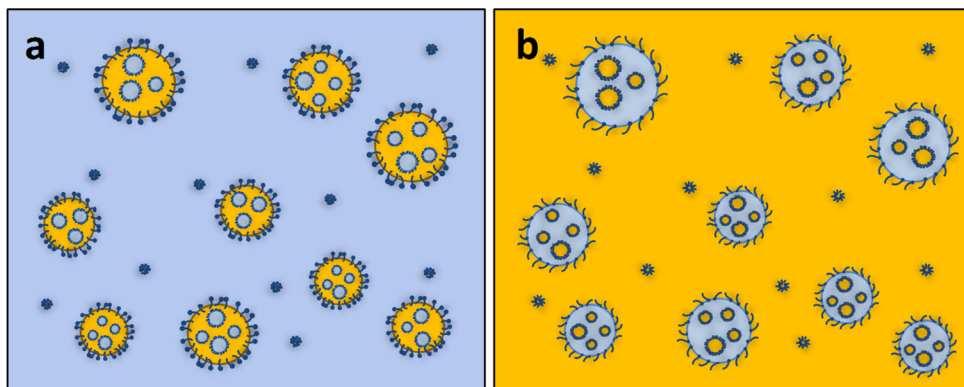
Fig. 2.
Schematic representation of double emulsions (a) water-in-oil-in-water (W/O/W) and (b) oil-in-water-in-oil (O/W/O) emulsions stabilized by surfactant.

Instability mechanisms

Food emulsions may change over the course of time due to a variety of different instability promoting mechanisms. These physico-chemical mechanisms include: gravitational separation (creaming/sedimentation), flocculation, coalescence, partial coalescence, Ostwald ripening and phase inversion (Fig. 3). Gravitational separation is the process where the droplets move upwards resulting in creaming or downwards resulting in sedimentation due to lower or higher density compared to the continuous phase respectively. Gravitation separation (especially at low dispersed phase concentration) can be described by Stokes' law and predicted from the following equation:

$$v = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta} \qquad\qquad Equation\ (1)$$

where v is the gravitational separation rate (m/s), g is the gravitational acceleration (m/s^2), r is the droplet radius (m), ρ_1 is the density of continuous phase and ρ_2 is the density of the dispersed phase and η is the viscosity of the continuous phase. From the equation above, it can be said that the rate of gravitational separation can be controlled by reduction of droplet size or promotion of the viscosity of the continuous phase [1].

Flocculation is a process where two or more droplets stick together and form an aggregate while maintaining their individual integrity. Coalescence is a process where two or more droplets merge together and form a larger droplet. Partial coalescence is a process where two or more partially crystalline droplets merge together and form irregularly shaped aggregate. Partial coalescence is the main cause of destabilization of emulsions during the oil crystallization. Collision of semi-crystalline oil droplets can result in penetration of crystals from one droplet into the interface of the other which will cause the remaining oil content of the droplets to flow out and wet the solid fat, creating a linkage between droplets, which can eventually result in true coalescence when the oil melts [21-23]. Ostwald ripening is a process where larger droplets grow at the expense of smaller droplets as a result of mass transport of dispersed phase material through the continuous phase. Finally, phase inversion is a phenomenon where oil-in-water emulsion turns to water-in-oil emulsion and vice versa. It should be noted that these phenomena are often interrelated. For instance, flocculation, coalescence and Ostwald ripening usually leads to gravitational separation or closely connected or flocculated droplets are more prone to coalescence [7].
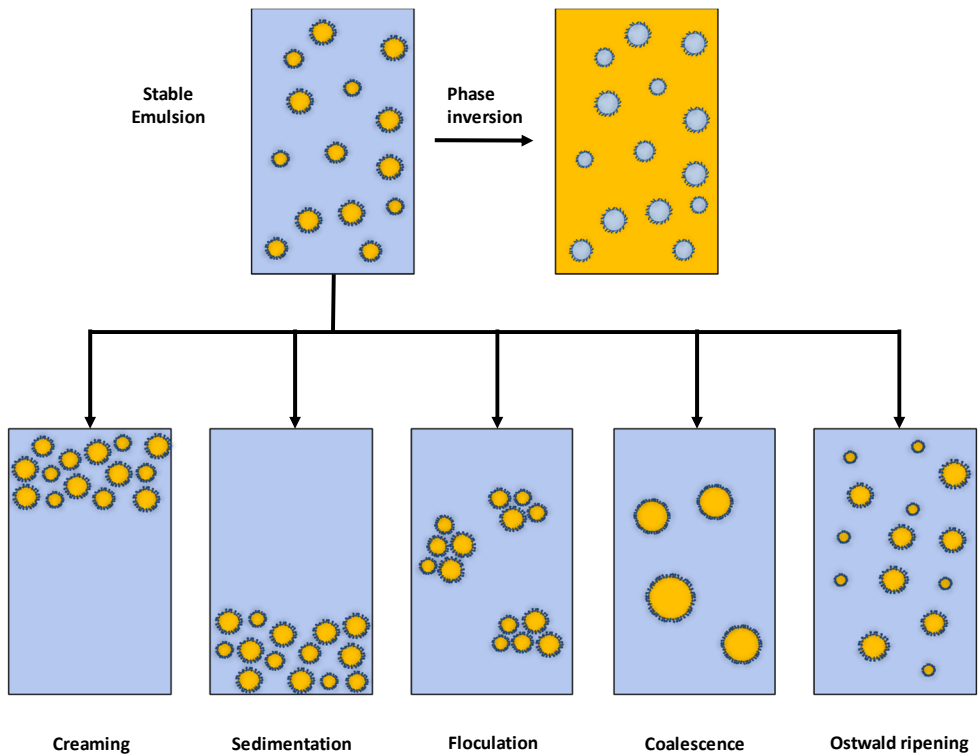
Fig. 3.
Schematic diagram of the most common instability mechanisms in food emulsions, adapted from McClements 2007 [7].

Emulsion characteristics

The characteristics of the droplets strongly influence the physicochemical properties of the emulsions. The major contributing characteristics are concentration, size, charge, interfacial properties and interactions, from which the first two are relevant to this thesis and discussed in more details in the following sections.

Droplet concentration

Droplet concentration is an influential attribute which influences the texture, stability, appearance, sensory and nutritional quality [24]. Droplet concentration is usually expressed in terms of dispersed phase volume fraction (ϕ) which can be obtained from the following equation:

$$\phi = \frac{V_D}{V_E} \times 100 \hspace{4cm} \textit{Equation (2)}$$

where V_D is the volume of the emulsion droplets and V_E is the total volume of the emulsion.

Droplet size

The size and size distribution of droplets is one of the major characteristics that strongly influences emulsions stability towards gravitational separation, flocculation and coalescence, as well as its rheological and sensorial properties [24]. As a result, there is a strong emphasis on accurately measuring the size of the droplets on emulsions in the scientific literature. The droplet size is usually reported by its diameter. If all droplets in an emulsion have the same size there are referred to as "monodisperse" whereas, if the droplets have a wide range of sizes, there are referred to as "polydisperse". Food emulsions fall in the polydisperse category [12]. A polydisperse emulsion is characterized by its particle size distribution, which defines the frequency of droplets in different size classes. Due to the statistically large number of droplets in a food emulsion, the resulting particle size distribution can be treated as continuous, however, it is more convenient to present the particle size distribution as a histogram or table where the concentration of droplets is recorded over a distribution that is divided into a number of size intervals [7, 24].

In addition to particle size, the particle concentration (frequency of a given size class) can be presented as volume percent (volume %) or number percent (number %) which reflects the volume or number of droplets in each different size class [13]. Depending on the appearance, polydisperse particle size distributions can appear as monomodal, bimodal, or multimodal when they have one, two or multiple peaks. In most cases it is crucial to study the full particle size distribution since it not only reflects the information about all particles present in an emulsion, but also indicates the origin and nature of any instability in the system. In most cases it is more

convenient to report the central tendency i.e. mean, median, mode and spread (standard deviation and span) of the distribution [7, 25]. These three characteristic values coincide in symmetric particle size distributions (Fig. 4), but are different in slightly skewed distributions. In a relative percentage frequency curve, mode represents the value at which frequency density is maximum and passes through the peak of the curve. Median and mean are other values where median is the value at which 50% of particles are larger or smaller from and mean is the arithmetic mean of area under the particle size distribution curve [26]. It is important to know that there are a number of different mean values derived from a particle size distribution that should be interpreted according to their definition. The most commonly used mean particle sizes are number weighted mean diameter (D_{10}), surface weighted mean diameter ($D_{3,2}$) and volume weighted mean diameter ($D_{4,3}$). In addition, Mode of $D_{4,3}$ (peak of volume weighted size distribution) is commonly used as it is the most frequently occurring size in the distribution. Therefore, one must be careful when reporting or interpreting a particle size distribution to identify which values are being used [7].

In general mean (D_{ab}) and standard deviation ($STDV$) can be defined by the following equation:

$$D_{ab} = \left(\frac{\sum_{i=1}^{N} n_i d_i^a}{\sum_{i=1}^{N} n_i d_i^b}\right)^{1/(a-b)} \qquad \text{Equation (3a)}$$

$$STDV = \left(\frac{(\sum_{i=1}^{N} n_i d_i^b)(\sum_{i=1}^{N} n_i d_i^{b+2})}{(\sum_{i=1}^{N} n_i d_i^{b+1})}\right)^{1/2} \qquad \text{Equation (3b)}$$

where a and b are integers, n_i is the number of droplets with size di and N is the total number of size categories present. In this way, the number weighted mean diameter (D_{10}), and the surface weighted mean diameter ($D_{3,2}$) and volume weighted mean diameter ($D_{4,3}$) can be calculated as follows:

$$D_{10} = \sum n_i d_i / \sum n_i \qquad \text{Equation (4a)}$$

$$D_{3,2} = \sum n_i d_i^3 / \sum n_i\, d_i^2 \qquad \text{Equation (4b)}$$

$$D_{4,3} = \sum n_i d_i^4 / \sum n_i\, d_i^3 \qquad \text{Equation (4c)}$$

A cumulative distribution is a convenient way of plotting particle size distribution, where the horizontal axis represents the particle size and the vertical axis represents the percentage that is smaller and larger than a given size. The advantage of this method is that the median size and the percentage between any two sizes can be read directly. However, for more details and comparison of similar sizes the relative percentage frequency distribution may be preferred. For a large range of particle

sizes, especially if the intervals are in a geometric progression, a logarithmic scale should be used [26].
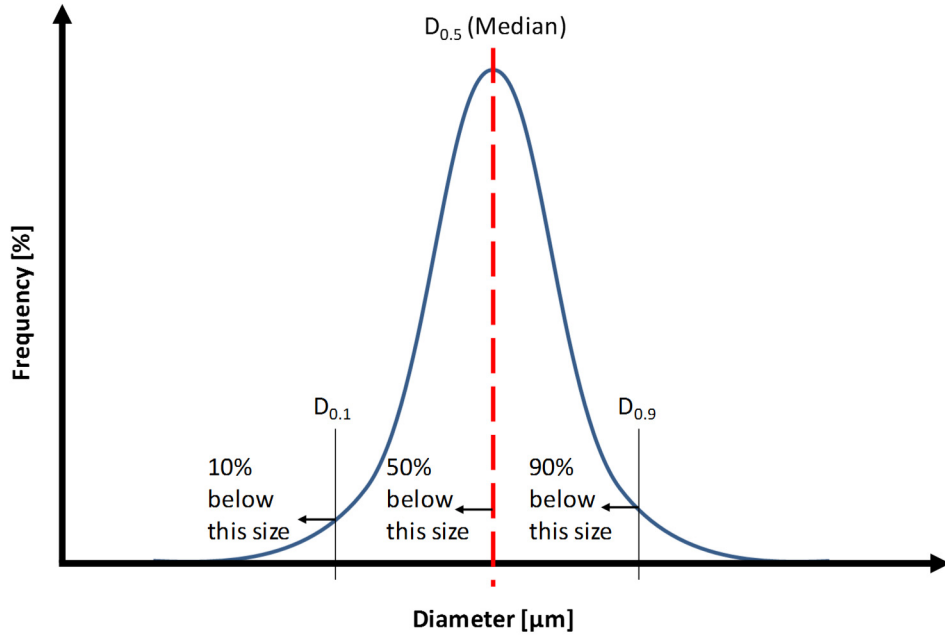
Fig. 4.
A representation of frequency percent particle size distribution with different parameters.

One common value to describe the width of the particle size distribution is span which can be defined by the following equation:

$$Span = \frac{D_{0,9} - D_{0,1}}{D_{0,5}}$$ *Equation (5)*

Pickering Emulsions

Solid colloidal particles can be used to achieve emulsion droplet stabilization, in addition to low molecular mass and polymeric emulsifiers (Fig. 5). Emulsions stabilized by particles that were first proposed by Ramsden [3] in 1903, and proven a few years later by Pickering [4], are known as Pickering emulsions. Despite a substantial amount of work on understanding the underlying mechanism of Pickering stabilization, the potential applications of Pickering emulsions in food and pharmaceutical products have only been considered in the past decades, although with a fast growing pace [1]. This is mainly d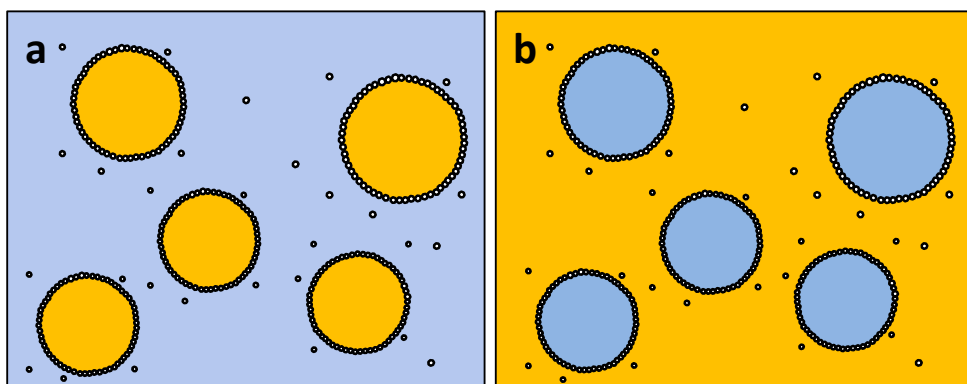ue to the market demand for highly stable emulsions combined with the growing awareness towards biocompatibility, surfactant free, and clean label ingredients for food, pharmaceutical and cosmetic industries [27].

Fig. 5.
Schematic representation of Pickering emulsions (a) oil-in-water (O/W) and (b) water-in-oil (W/O) Pickering emulsions stabilized by solid particles.

In many ways, solid particles can act as surfactants. The oil or water affinity that is referred as hydrophile-lipophile balance (HLB) in surfactants is analogous to particle wettability characterized via the contact angle [28, 29]. Nevertheless, compared to conventional emulsifiers, the stabilization mechanisms involved in Pickering emulsions is fundamentally different. Pickering particles achieve droplet stabilization by dual wettability towards both phases which can be characterized by contact angle θ. The adsorbed particle will cause the interface to bend towards the phase with lower affinity and therefore particles that are more hydrophilic are more suitable for O/W emulsions and particles with more hydrophobic traits are more suitable for W/O emulsions [1]. The comparison of surfactant and particles is summarized in Table 1.
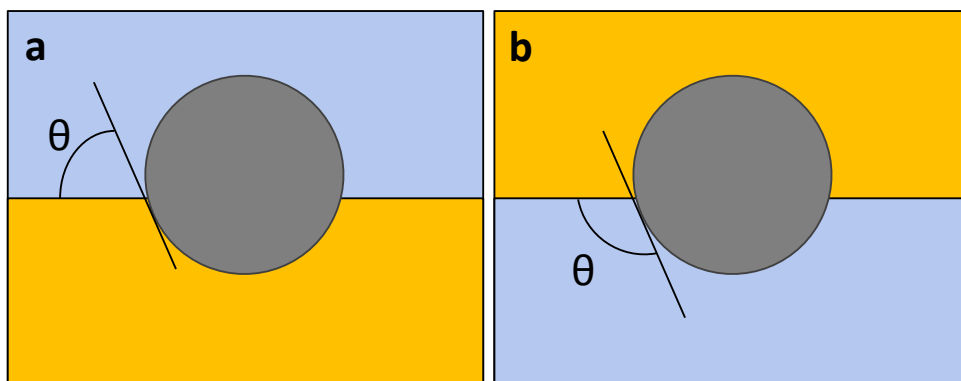
Fig. 6.
Schematic representation of contact angle for (a) when lower than 90°, oil-in-water emulsions are formed and (b) when higher than 90°, water-in-oil emulsions are formed.

Table. 1.
The differences between surfactants and particles [5].

	Surfactant Stabilized	Particle Stabilized
Size of stabilizer	Surfactants ~ 0.4 to 1 nm	Particles 10 nm to 10 µm or more
Surface active?	Yes	Yes – via partial dual wettability
Amphiphilic?	Yes	Generally no (except Janus particles)
O/W vs. W/O	Bancroft's Rule – the phase the surfactant is most soluble in is likely to be the continuous phase. Determined by HLB value. HLB 7 to 11 → W/O emulsions HLB 12 to 16 → O/W emulsions	Finkle's rule – the phase the majority of the particle protrudes out into is likely to be the continuous phase. Determined by contact angle θ at oil-water-particle interface. Contact angle θ > 90º → W/O emulsions (Fig. 6a) Contact angle θ < 90º → O/W emulsions ((Fig. 6b)
Adsorption kinetics	Fast in dynamic equilibrium	Slow but essentially irreversible
Desorption Energy	Low < 10 kT	Exceptionally high > several thousand kT

Compared to other stabilization mechanisms, Pickering emulsions are usually more stable against coalescence and Ostwald ripening [29, 30]. This higher stability is due to the large particle sizes (> 10 nm) that provide a steric hindrance/barrier against close contacts of emulsion droplets, which result in prevention of

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coalescence [31]. Besides, due to higher energy of detachment of the particles thanks to large particle sizes, once these large particles are adsorbed at the oil-water interface at the appropriate contact angle (is not too close to 0º or 180º), the energy needed to remove them is several thousand *kT*. In addition, the interfacial pressure formed due to the irreversibly adsorbed particle can prevent mass transfer across the interface [32]. The energy of detachment per particle can be calculated by the following equation:

$$\Delta G = r^2 \pi \gamma_{OW} (1 - |\cos\theta|)^2 \qquad\qquad \textit{Equation (6)}$$

where *ΔG* is the energy of detachment, r is the particle radius (m), γ_{OW} is the interfacial tension between oil and water (N/m), and θ is the particle-oil-water contact angle measured through the water phase [1, 28, 29]. As a result, the presence of a thick and irreversibly adsorbed barrier provides highly stable emulsions [23, 30, 31]. Particle-particle interactions play a key role in stability of Pickering emulsions through formation of three-dimensional network of particles that support the stability of the emulsions, or at the interface where attractive particle-particle interactions provides a cohesive interfacial layer due to capillary forces [32]. Unlike traditional emulsifiers (surfactants, proteins and polymers) and nanoparticles (sizes < 2 nm) where the Brownian motion is responsible for the overcoming the adsorption barrier and transportation of emulsifier to the interface, transfer of particles (with sizes > 2 nm) to the oil-water interface from the bulk continuous phase is governed by convective transport [33]. Therefore, adsorption is only possible if the kinetic energy of a particle approaching an interface is at least of the same order of magnitudes as the energy barrier to adsorption generated by repulsive and hydrodynamic forces [1].

The physical and mechanical properties of the interface in Pickering emulsions are different from those of conventional emulsifiers. The thickness of particle-stabilized interface is much larger which is at least equal to the particle size for a particle monolayer. Hence, the surface load (mg/mL) is higher compared to conventional emulsifiers. The mechanical properties of the interfacial layer in Pickering emulsions are related to interactions between the particles and the immiscible phases as well as particle-to-particle interactions within a film which can contribute to mechanical strength of adsorbed layer [1]. In addition to the physical stability of Pickering emulsions, the rheological properties of Pickering emulsions are different from conventional emulsions due to its unique interfacial properties. The difference in the rheological properties can be due to different factors. First, the stronger attachment of the particles at the interface gives it a more elastic behavior. The excess non-adsorbed particles can form a network in the continuous phase that can modify the rheological properties of the emulsions giving them a gel like structure [31]. The adsorbed particles can also form bridges between droplets which can also

contribute to these gel-like structures and give the emulsions a more elastic nature [5].

One of the main challenges for food industry for application of Pickering formulations is to create food-grade, effective Pickering emulsifiers [1]. Although Pickering emulsions are known for over a century, their application in food is limited since relatively low number of a particles are permissible for food applications as reflected in the number of research conducted in this area (Fig. 7). Recently, there has been increasing interest in use of biomass-based, edible and food grade Pickering emulsifiers [1, 23, 34]. While some inorganic particles such as silica derivatives are generally recognized as safe by American and European food authorities, their application can be criticized because of their origin [1]. Therefore, there is an increasing interest towards developing Pickering stabilizer for food applications based on materials such as carbohydrates, protein and lipid-based particles. One such material is the focus of this thesis: starch.
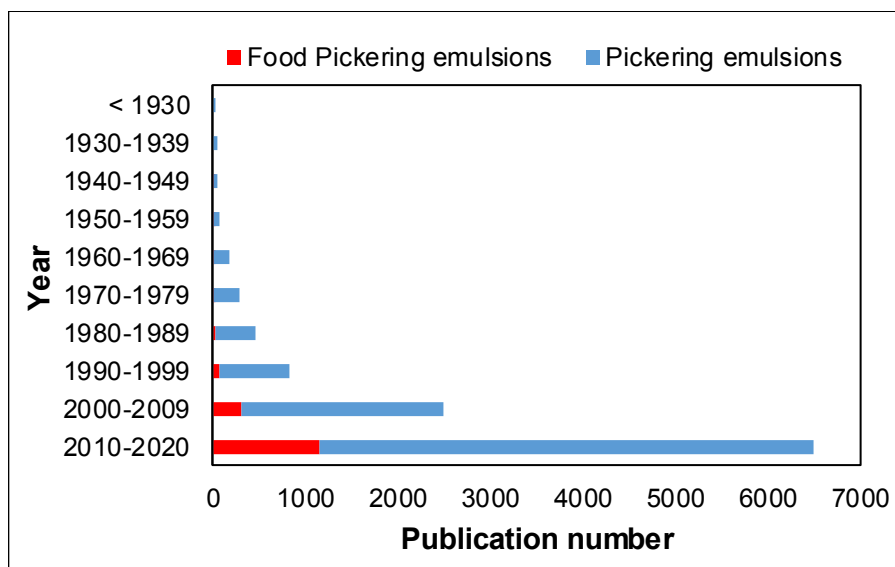
Fig. 7.
The number publications in the area of Pickering emulsions using the search term of "Pickering emulsions or "particle stabilized emulsions" compared to that of food Pickering emulsions. Data obtained from Sci-finder until the end of year 2017.

Starch

Starch formation is one of the main methods of energy storage in plants, and one of the most abundant carbohydrates in the biosphere. Starch serves as an important energy source for the human diet in the form of cereal grains, and tubers. In addition, due to biodegradability and good functional properties, starch has a great potential for various material applications such as paper, bio-plastic, bio-fuel, etc. [35].

Size, shape and morphology of starch granules

In plants, starch is synthesized and stored in the form of granules in amyloplasts [36]. The sizes of starch granules are intrinsic to the botanical source they are isolated from, however, the shape and size may also vary in the same tissue during the development of the tissue [35, 37]. Starches have been classified in large granule (30-100 μm) including tubers such as potato and canna; medium granule (5-30 μm) including starches such as tapioca, barley, maize, sorghum [37, 38]; small granule (2-10 μm) including rice, oat, buckwheat [37, 39]; and extremely small starch granules (0.3-2 μm) such as quinoa, amaranth, cow cockle and pig weed [37, 39, 40]. Some types of starch have bimodal sized starch granules including some species of rice, barley, sorghum and wheat [37, 38, 41-44]. In addition to size, the shape and morphology of starch granules are specific to their botanical origin and can have smooth or rough surface and spherical, polygonal irregular, ellipsoidal or disc-shaped. In general, the morphology of starches can be sorted as small and polyhedral for cereal starches, elliptical for tubular starches and ovoidal for legume starches [36, 45]. A great range of native and modified starches with various functionalities are available on the market today.

Small granular starches

Since small particles can cover more surface area/unit mass of particle, smaller emulsions droplets with better coverage and therefore higher stability can be produced. The theoretical maximum coverage, Γ (mg/m^2), can be estimated from the following equation:

$$\Gamma = \rho_p \frac{2}{3} D_P \varphi . 10^6 \qquad \qquad Equation\ (8)$$

where ρ_p is the density of the particle (kg/m^3), D_P is the surface mean diameter of particle (μm) and φ is the packing density. Specific surface area of an emulsion per volume of dispersed phase (S) can be calculated from surface mean diameter ($D_{3,2}$) using following equation:

$$S = \frac{6}{D_{3,2}}$$
<div align="right">*Equation (9)*</div>

Several studies have investigated the emulsifying properties of different types of starches in native and modified forms granules [30, 46-48]. There has also been considerable amount of work on development characterization and physical and physiological stability of emulsions stabilized by OSA modified quinoa, maize, tapioca, and rice starch granules [6, 30, 46, 47, 49-52]. However, a comparison of emulsifying properties of small granular starches in native and different OSA modification levels in defined intervals (i.e. 0.6, 1.2, 1.8, 2.4, 3.0%) has not been studied. In this work, three starches of different botanical origin with small granules (i.e. quinoa, amaranth and rice) have been evaluated as potential candidates as Pickering emulsifiers. The emulsifying properties of small granular starches were compared with constant amount of starch with varying the modification level and also varying the amount of starch with different modification level.

Quinoa (*Chenopodium quinoa Willd*) is a native pseudocereal from Andes in South America which has been cultivated for 3000-4000 years and constituted an important component in the diet of the Incan civilization [53, 54]. Quinoa has recently attracted interest due to its unique characteristics including: high nutritional value due to the quality of protein and fatty acids and its ability to grow under extreme conditions such as salinity, acidity, drought, flooding and frost [54-56]. Starch is a major component of quinoa seeds which comprises approximately 55-60% of the dry matter [53, 57]. The starch is present in the form of small polygonal granules in diameter 0.6-3 μm with mean diameter of 1.5 μm [53, 58-60]. The amylose content of quinoa is reported to vary between 3.5-27% [53, 58, 61, 62] and the gelatinization temperature range (T_o-T_c) was reported to be 50-74.9 °C where T_o is the gelatinization onset temperature T_c is the gelatinization conclusion temperature [54, 59, 61].

Amaranth (*Amaranthus*) is another ancient pseudocereal domesticated in South America [57]. In addition to South America, amaranth currently constituents a large part of diet in Asia and Africa [61]. The total starch content in amaranth grain has been reported to be around 67% [57]. The starch has small polygonal granules with a mean diameter around 0.8-1.3 μm among different amaranth cultivars [63, 64]. The amylose content of amaranth has reported to be in the range of 0-28% [61-63] and the gelatinization temperature ranges from 63.4-86.9 °C [61-63].

Rice (*Oryza Sativa*) is a cereal grain that is the staple food in Asian countries [65]. The total starch content of rice grain is 78-83% [66-68]. Rice has small and polygonal granules between 3-9 μm [69]. The reported amylose content ranges from 0.0-33.0% and the gelatinization temperature range of 55-84.6 °C [69, 70].

Structure of starch granules

Starch granules consist of two polymers, i.e. amylose and amylopectin. The mainly linear polymer is amylose, having of 840-22000 units of α-D-glucopyranosyl residues that are linked by α-(1-4) bonds and to a small extant of branched linkages (1 per 170-500) with α-(1-6) bonds as can be seen in Fig. 8. The amylose content of most starches is about 20-30% [71, 72]. The number of the glucose units widely varies among different plant species and the stage of development. The highly branched polymer is amylopectin, which comprises a major part of the starch granules (usually about 70%) and is composed of higher amount (~5%) of α-(1-6) as glucosidic linkage as shown in Fig. 8 [35, 71]. Other minor components of starch include: proteins (0.1-0.7%) that are found on the starch surface and the interior of the granules, and lipids (up to 1%) in the form of starch-lipid complexes [41, 73, 74].
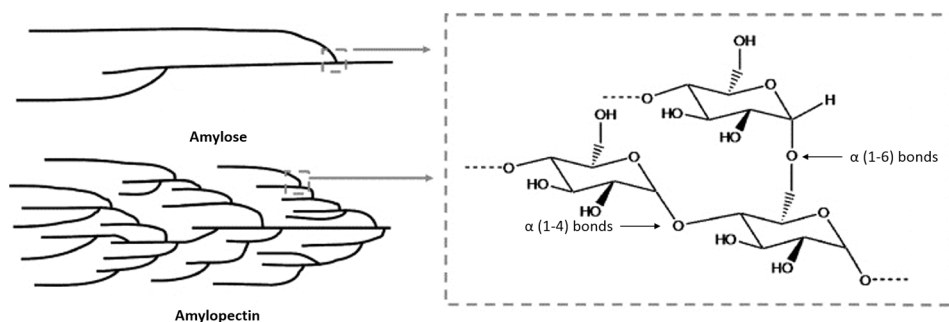
Fig. 8.
Chemical structure of amylose and amylopectin. Fig. from Sweedman, Tizzotti et al. 2013 [75].

Amylopectin is composed of three different unit chains. The A-chain is the outermost and shortest which is unsubstituted. The B-chains are substituted by one or many A and/or B-chains by α-(1-6) bonds are the inner chains. The C-chain is the backbone of the amylopectin that contains the only reducing sugar in the molecule [41, 76] as can be seen in Fig. 9. Native starches have semi-crystalline structures. The alternating crystalline and amorphous growth rings (that appear as concentric rings) and the central amorphous region represent the different structural regions of starch granules [77]. The center (hilum) which is the nucleus or starting point from which the granule grows, is usually less organized than the rest of the granule and contains a large proportion of reducing ends of starch molecules and the non-reducing ends are radiated toward the surface of the granules [77]. The internal model of starch granules contains a radial growth of amylopectin with 16 cluster per growth ring and a thickness of 120-400 nm [78]. The growth rings are built up by alternating layers of semi-crystalline and amorphous lamellas. The

crystallinity is related to the interlinking outer chains of amylopectin that form double helices in cluster structure. The cluster arrangement of amylopectin branches is based on alternating regions of ordered or tightly packed and parallel glucan chains (crystalline lamella) and less ordered regions that are composed of branching points (amorphous lamella). Native starch contains between 15% to 45% of crystallite material [36, 79]. Fig. 9 represents different structural levels of starch granules.

The semi-crystalline growth rings are surrounded by another amorphous structure i.e. amorphous growth rings. The composition and structure of amorphous growth rings is unknown, however, the role of it is important in the properties of starch. The structure of amorphous growth rings is composed of amylose, lipids and amylopectin with a higher water content compared to the semi-crystalline regions [80].
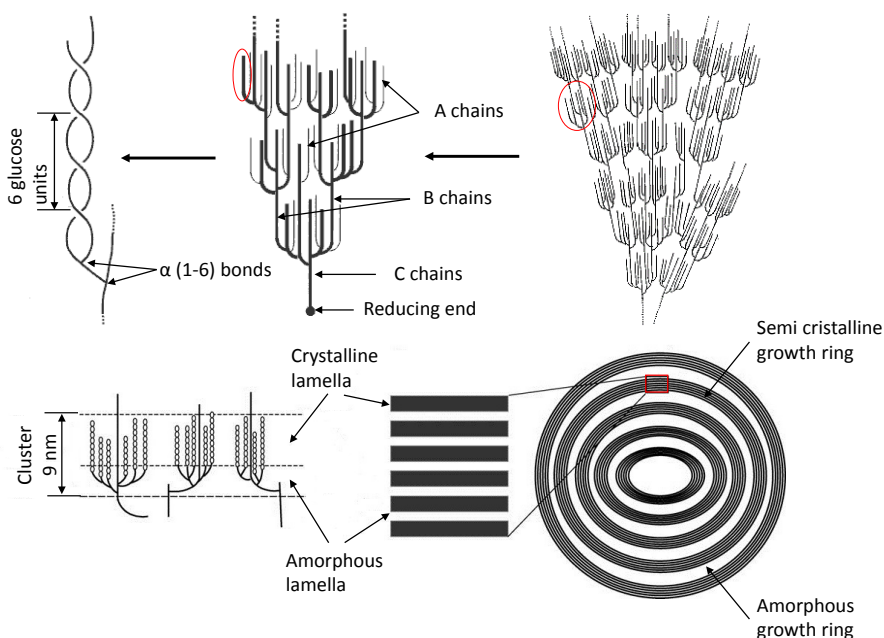
Fig. 9.
Different structural levels of starch granules, adapted from Tester, Karkalaset al. 2004 [78] and Parker and Ring, 2001 [81]. The alternating crystalline and amorphous layers containing amylopectin double helices and branch respectively.

The interrelationship between amylose and amylopectin or the exact location of amylose in the granule is not known. Amylose molecules are thought to occur in the granules as individual molecules or in complex with lipids in both crystalline and amorphous regions, however, the location depends on the botanical origin [36, 79].

Bioavailability/digestibility

From digestion point of view, starches are classified into readily digestible, slowly digestible and resistant starches. Readily digestible starches, are digested and absorbed rapidly, while, slowly digestible starch, is digested slowly but completely by pancreatic amylases in the small intestine. This type of starch is usually protected within the plant structure such as legumes or densely packed such as spaghetti. In contrast, resistant starch (RS) is resistant towards pancreatic amylases and therefore passes through small intestine. This type of starch is subcategorized to four subclasses: physically inaccessible such as partly milled grains (RS$_1$), intact resistant starch granules such as granules in raw potato and banana (RS$_2$) and retrograded starch which is re-associated macromolecules following gelatinization (RS$_3$) [79] and lastly chemically modified starch (RS$_4$) to achieve indigestibility [82]. Several studies have shown RS have the potential to improve health, with one of the primary benefits being maintenance of healthy blood sugar levels [83] as well as lowering inflammatory markers, and increasing satiety [84].

Gelatinization

When starch granules are heated up to certain temperature in presence of adequate amount of water, they undergo an irreversible order-disorder transition called gelatinization [35]. This phenomenon which is initiated through water absorption by the amorphous regions of starch granules and swelling and dissociation of crystalline structure and double helixes (i.e. loss birefringence), results in loss of crystalline structure of starch granules and diffusion of amylose to the surrounding aqueous phase [35, 72, 77]. During the gelatinization process, water absorption and swelling of the amorphous regions will impose a stress to the amylopectin crystallites and cause the amylopectin double helices to dissociate, since amylopectin molecules at the edge of crystalline lamellar stack are inter-connected to amorphous region through bonds [35].

Gelatinization is an important feature of starch which contributes to its functionality and is widely utilized in the food industry such as pasting behavior and rheological properties of the starch dispersions [85]. A wide range of techniques including differential scanning calorimetry (DSC), X-ray diffraction, small angle neutron scattering and hot stage microscopy with Köhler illumination has been used to study gelatinization behavior of starch granules [86-89].

DSC is possibly the best technique to investigate the phase transition of starch/water dispersions due to opportunity to both study of the gelatinization over a wide range of starch/water ratios, as well as the determination of gelatinization temperature in high temperatures (> 100 °C), and estimation of enthalpies [90]. The gelatinization

characteristics of starch in a DSC thermogram can be presented as T_o the onset temperature, T_p the peak temperature, T_c the conclusion temperature (Fig. 10).
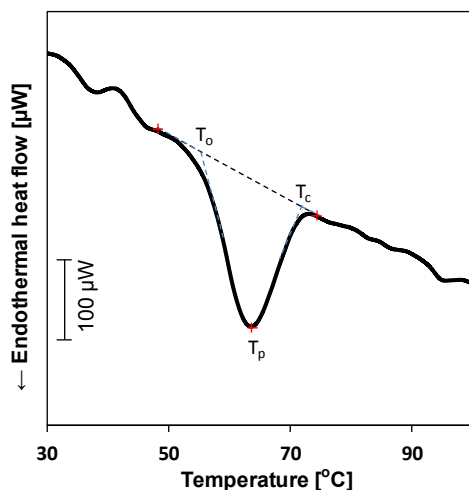
Fig. 10.
DSC thermogram for gelatinization temperature of quinoa starch in phosphate buffer (5 mM, 0.2 M NaCl, PH 7). Fig. from Marefati, Bertrand et al. 2017 [51].

The enthalpy of gelatinization can be calculated using the equation below:

$$\Delta H = \frac{A \cdot 60 \cdot B \cdot E \cdot \Delta qs}{M \cdot C}$$ *Equation (7)*

where A is area of thermogram (in^2), B is time base setting (min/in), C is sample concentration (% w/w), E is cell calibration coefficient (no units), M is sample mass (mg) and Δqs is the range (mcal/s/in) that is used to calculate the enthalpy (endothermal heat flow), ΔH, as shown in Fig. 10 [90].

Biliaderis [90] showed that starch gelatinization is also influenced by the amount of water present in the system during gelatinization. High amounts of water causes extensive hydration and swelling and facilitates loss of crystallinity. Whereas the temperature range increases with decreasing the amount of water. In a lower amounts of water, destabilization of amorphous regions is limited and only partial loss of crystallinity is possible. Subsequent distribution of water will cause melting of the unmelted regions on further heating at higher temperatures. Moreover, starch crystallinity increases with the amylopectin content, and hence, starches with higher amylopectin content (i.e. lower amylose content) would expect to have higher onset (T_o), peak (T_p) and conclusion (T_c) temperatures [72].

Formation of gelatinized Barrier at oil-water interface

Application of heat treatment can induce partial gelatinization of the starch at the oil-water interface (Fig. 11) which leads to modification of interfacial structure with enhanced barrier properties to develop so called "structured emulsions". In this way a sealed or fused barrier will be created that may reduce the effect of physical and chemical destabilization mechanisms for both emulsions and encapsulated compounds. Previous studies on Pickering emulsions stabilized by quinoa starch granules showed that the barrier created by heat treatment decreased the rate of *in vitro* lipolysis with lipase and co-lipase up to 60% compared to original non-heat treated emulsions [6].
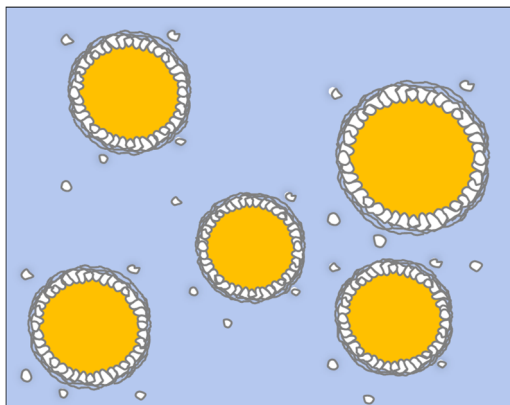
Fig. 11.
Formation of a partially gelatinized barrier at the oil water interface.

Retrogradation

The term retrogradation describes the changes that occurs upon cooling and storage of gelatinized starch, from an amorphous state to more ordered structure or crystalline state, resulting in a drop in quality of some starch based foods [53, 72, 91]. The most commonly mentioned representation of the effect of retrogradation in food products is bread staling. Retrogradation occurs as gelatinized starch is not in a thermodynamic equilibrium and therefore, not stable [91]. These changes are attributed to the re-association of starch chains to double helices and semi-crystalline arrays of these helices [92]. During retrogradation, the amylose fraction recover crystallinity to some extent in short term [93, 94]. Retrograded amylose is indigestible and heat stable (120 °C) starch fraction [95, 96]. On the other hand, the amylopectin fraction, contributes to the long term changes that happens during retrogradation [97]. In other words, amylose reaches the crystallization limit after 2 days while it takes 30-40 days for amylopectin to reach this limit [80].

There are various methods which have been used in characterization of retrogradation from which X-ray diffraction analysis, DSC and rheological techniques are more common [91]. Retrogradation has been reported to be positively correlated with amylose content. Higher content of amylose results in greater association of starch molecules and a higher degree of retrogradation [53]. On the other hand, extremely short chain amylopectin content has reported to have inhibiting effect on retrogradation of starch [98]. Though, factors other than amylose content and amylopectin structure seems to affect retrogradation of starch including: short-term development of crystallinity, size and shape of granules and lipid content [93, 94, 99]. Other factors including: starch source and concentration, storage temperature, and other components present have been reported to be influential as well [91].

Modified starch

There has been a lot of research interest for utilization of starch granules as Pickering particles and starch granules from different botanical origins have been used as Pickering emulsifiers [1]. In many applications the functional properties of native starch are not optimal and hence, in order to improve its performance, starch is modified. Since starch granules are generally hydrophilic The emulsifying capacities of native starch granules is low [48, 100] and hydrophobicity can be increased by chemical or physical modification. The chemical modification is performed by addition of hydrophobic side chains to the surface of starch granules [75]. The most widely used chemical modification is esterification with acid anhydride, such as octenyl succinic anhydride (OSA) under mild alkaline conditions (Fig. 12). OSA modified starch is widely used in emulsion and encapsulation applications [64]. The modified starch produced in this way is approved food additive (E1450) and excipient with a degree of modification lower than 3% based on the dry weight of starch, with no limit on application [6, 64]. Other chemical modification methods include esterification with acetic anhydride or phthalic anhydride [101]. The physical modification of starch is performed by physical treatment of starch granules leading to an alteration of the surface properties of particles. Dry heating is one of the methods reported for physical modification of starch granules [49, 102].
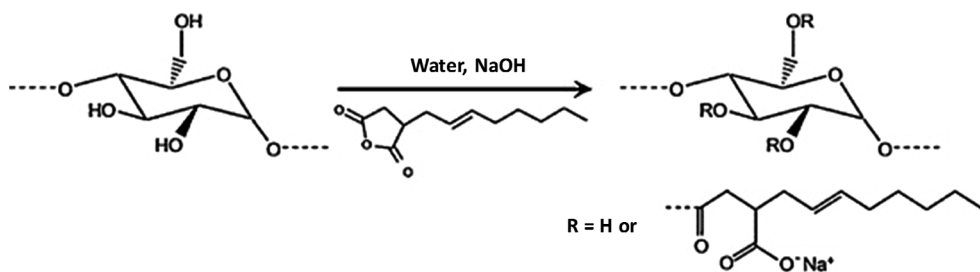
Fig. 12.
Chemical modification of starch with octenyl succinic anhydride. Figure from Sweedman, Tizzotti et al. 2013 [75].

Starch as emulsion stabilizer

Starch in different structural forms i.e. molecules and particles have been used to stabilize emulsions. Regardless of structural form, in most cases starch is chemically modified to increase hydrophobicity and therefore is able to be adsorbed at the oil-water interfaces and thereby stabilize emulsions. Chemical modification with OSA is a common way to increase hydrophobicity of starch as described in earlier.

Molecular starch

Chemically modified molecular starch, is obtained through dissolution of OSA modified starch using high temperature [103, 104]. This types of starches due to high molar mass and branched structure, can be adsorbed at the interface and provide steric stabilization of emulsions. OSA modified starches in molecular state, have shown to be able to produce stable emulsions [105-107]. The stability of resulting emulsions have reported to be independent of pH and ionic strength [103, 105]. The botanical origin of these starches have reported to be different including waxy barley [103], amaranth [64], quinoa [104], waxy maize [64, 107, 108].

Starch particles

Several studies have been conducted on utilization of starch particles from various botanical origin as Pickering emulsifiers. Most types of starch granules are large compared to the size of the emulsion droplets which they are intended to stabilize, and thus unfit to stabilize emulsions. Therefore, there has been different techniques for developing small-sized particles such as starch nano-particles and starch nano-spheres or starch nano-crystals for use as Pickering emulsifier [101, 109-114].

Utilization starch particles in granular form has also attracted research interest [6, 30, 39, 46, 47, 49-52, 100]. Previous studies on emulsifying capacity of starches of different botanical origin showed that small granular starches, had better emulsification potential [39, 48, 100]. Besides, in most cases, starch is commonly modified with OSA or (in a few occasions) dry heat treatment [30, 47, 100]. The starch granules that have successfully been used as Pickering emulsifiers are from rice [30, 39], quinoa [6], amaranth [39].

Functionality and application of Pickering emulsions

The functionality of Pickering emulsions has been considered with respect to process stability, encapsulation stability and physiological stability.

Process stability

Freeze-thaw stability

Emulsion systems may undergo different processes. These processes may alter the emulsions which could result in their destabilization. Freezing is a process to increase the shelf-life of foods. In the case of emulsions, freezing can cause destabilization of the droplets in a number of ways. Ice formation may reduce the amount of water needed for hydration of the emulsifier. In addition, formation of ice in the continuous phase may result in droplet-droplet interaction. Elevation of the concentration of the solutes in the remaining unfrozen continuous phase will alter the ionic strength and pH which may result in disruption of electro-static repulsion between the droplets [115, 116]. Furthermore, as a result of the volumetric expansion of water due to freezing, ice crystals may penetrate oil droplets and rupture the interfacial layer which can make oil-oil contact possible, resulting in coalescence [117]. Crystallization of the oil phase in an oil-in-water emulsion may result in partial coalescence, if the lipid crystal of one oil droplet penetrates the liquid region of another droplet, it will have a negative impact on the stability of the emulsions [116]. Incorporation of cryo-protectants such as sucrose or creating multiple layers of emulsifiers around the oil droplets is proposed to avoid destabilization of emulsions due to water crystallization [118]. Moreover, the rate of coalescence during freezing have been correlated with the thickness of stabilizer and droplets with a thick barrier at the interface have better protection against coalescence [21, 22, 117, 118]. Thus, the large particles stabilizing Pickering emulsions not only create a high energy barrier to remove them, but can also provide high resistance against crystal penetration [29].

Freeze-drying stability

Another process that is common in the industry is drying or dehydration. Dehydration can be used to increase shelf-life, improve application or facilitate transportation [46]. There are several ways of drying, from which freeze-drying is known to impose the lowest degree of damage to sensitive structures and therefore is utilized for sensitive materials (especially those sensitive to heat) as well as of other biological products such as enzymes and bacteria [119]. Freeze-drying is a process in which the solvent content of the material is crystalized in the low temperature and then sublimated directly from the frozen state into vapor by decreasing the pressure around the product. Therefore, freezing is the precursor to freeze-drying process. There are several approaches to maintain the stability of the emulsions during drying among which a common way is addition of solid hydrophilic carriers such as lactose, glucose, maltodextrin, and cellulose [120, 121], however, addition of these substances may cause other complications. As an alternative, multi-layer or layer-by-layer deposition (LBL) of polyelectrolytes that crosslink on the droplet surface, crosslinking of protein-stabilized interfaces, and protein-polysaccharide conjugates have also been applied [122-124].

Encapsulation

Incorporation of bioactive ingredients, enzymes and cells in another phase is referred to as encapsulation [125]. In the past century, encapsulation systems have evolved from inclusion and protection of certain entities during storage (such as antioxidants and colorants) to development of formulations for controlled and targeted delivery of specific compounds (such as vitamins, drugs, and probiotics) in human digestive systems in the past decade [126]. Utilization of emulsions as delivery systems for active compounds is rather common. Emulsion-based delivery systems are often used to protect, encapsulate, modulate, and increase bioavailability and/or targeted delivery of drugs in pharmaceutical products or bioactive compounds in food products.

The potential of OSA modified quinoa starch granules in stabilization of O/W and external phase of W/O/W makes encapsulation of both hydrophobic and hydrophilic compounds feasible [6, 50]. In addition to high physical stability, Pickering emulsions have demonstrated other merits such as high oxidative and encapsulation stability and controlled and targeted release of encapsulated compounds [127-129]. Besides, due to the large size of the particles and irreversible adsorption of them at the interfaces, particles make a thick and rigid but porous shell structure with reduced mobility at the droplet interfaces [29, 130-132].

In order to quantify the encapsulation efficiency and stability of starch Pickering emulsion formulations, two tracers have been used; carmine and curcumin.

The hydrophilic tracer, carmine, is a common food coloring agent (E120) that is both stable to heat and light, and at neutral pH it has a bright red color [133]. Carmine is obtained from the dried bodies of cochineal insects shown in Fig. 13 [134]. Carmine is used as a food colorant in juices, ice cream, yogurt, dessert and candy, and also as a dye in cosmetic products such as eye shadow and lipstick [135], however, it is reported to cause allergic reactions [136, 137].

Curcumin is a natural polyphenol present in the rhizomes of the turmeric plant as shown in Fig. 13 [137], which in addition to being used as a popular spice in south and central Asia, has been used in Indian and Chinese traditional medicine for many centuries [138]. Curcumin,1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5 dione, is the main curcuminoid present in the turmeric [139]. Curcumin has been suggested to induce wide range of biological and pharmacological activities including anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, antialzheimer's, as well as hypotensive and hypocholesteremic activities [139-143]. In addition, *in vivo* studies on both humans and animals has been shown that curcumin is safe even in high doses [137]. Curcumin has low solubility in neutral and acidic pH which makes the food application somewhat limited [144, 145]. There have been numerous approaches proposed for improving the bioavailability of curcumin such as incorporation of curcumin in novel formulation of nano-particles, liposomes, micelle, phospholipid complexes and emulsions [26, 137].
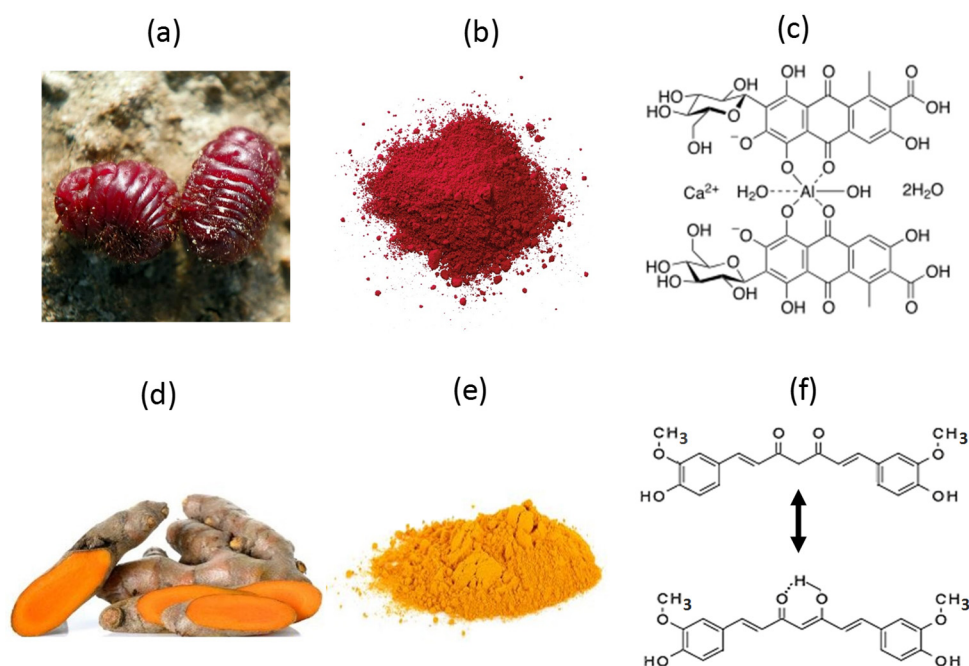
Fig. 13.
Female cochineal insects by Vahe Martirosyan C.C. 2011 (a), carmine red (b), the chemical structure of carmine (c), rhizomes of tumeric plant (d), tumeric spice (e), chemical structure of tumeric in different pH conditions i.e. in acidic and neutral conditions on the top and basic conditions on the bottom.

Lipolysis inhibition

In the context of lipid encapsulation, Pickering stabilization is expected to regulate lipolysis [126]. Lipid digestion is an interfacial process which is governed by lipase-colipase binding to the interface, therefore modification of the interfacial properties through Pickering emulsification can control this lipase-colipase interaction to the lipid interface. This can ultimately affect satiety which results in a more balanced caloric intake and potentially reduce obesity [27]. If intact dietary triglycerols reach ileum, they promote a natural feedback to induce satiety. Due to high efficiency of human digestive system, development of formulations that are orally ingested and released in ileum has shown to be challenging. One way to tackle this challenge can be through structured emulsions that delay lipolysis and thereby promote satiety [126]. During digestion, emulsifiers are displaced by bio-surfactants (i.e. bile salts) to allow lipase-colipase binding. Therefore, it is proposed that lipid digestion can be reduced by formation of a rigid barrier at the interface [138]. To achieve this barrier, layer-by-layer deposition or highly surface active surfactants are proposed [138]. In

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the same way, emulsions that are highly stable against severe environmental conditions such as Pickering emulsions, can potentially resist the lipid digestion process [138].

Pickering emulsions provides opportunities to combine the knowledge of soft matter structuring with physiological processes of digestion, through which the kinetics and extent of lipid digestion can be altered by modification of the interfacial structure. Since the initial interfacial structure of an emulsion is not retained as the emulsifier are eventually displaced by bile salts, it can be expected that highly stable and irreversibly adsorbed particles in Pickering emulsions can inhibit or control the displacement by the bile salts and therefore regulate lipid digestion [27].

General Methods

Isolation, modification and characterization of starch granules

Isolation of starch

Quinoa grain was milled in a laboratory mill (Perten Instruments, Sweden). This whole grain quinoa flour contained seed coatings and large fibers in addition to the starch granules. To isolate the starch, a sedimentation method based on Stokes' law was adapted from Dhital, Shrestha [139]. First, the flour was dispersed in water at 30% (w/w) for 2 min using an overhead mixer (Ytron, Germany). The suspension was then transferred to a rectangular tank 78 cm by 56 cm by 43 cm and water was added to reach a final concentration of 5% (w/w) and thoroughly mixed.

The sedimentation was performed in two steps. Initially, the suspension was left to sediment for 1 h (to sediment the fraction > 10 µm) and the supernatant was transferred to another tank for a second sedimentation step, in which the suspension was left to sediment for 4 h (to sediment the fraction > 5 µm).

The sedimentation times for the two cut-off sizes were calculated by Stokes' low using the following equation:

$$t = \frac{18\eta h}{g(\rho_2 - \rho_1)d^2}$$ *Equation (10)*

where η is the viscosity of the water (1.003×10^{-3} Pa s), h is the sedimentation height (m), g is acceleration due to gravity (9.8 m/s^2), ρ_2 the density of impurities (1500 kg/m^3), ρ_1 the density of water (998.23 kg/m^3) and d is the particle diameter (m) [139].

After the second settling time, the supernatant (containing the fine starch granules) was carefully removed, and centrifuged (X-15, Beckman coulter, USA) at 3000 g for 10 min to make a compact pellet. The pellet was then mixed with NaOH solution (0.3% wt) and centrifuged at 3000 g for 10 min and the protein residues were scraped off in multiple steps until the pellet was white. Thereafter, the pellet was mixed with citric acid (pH ~ 4.5) to neutralize the pH and centrifuged at 3000 g for

10 min. The pellet was then re-suspended in Milli-Q water and centrifuged for at least 2 times. Finally, the pellet was re-suspended in Milli-Q water and frozen using liquid nitrogen and freeze-dried (CD 12, Hetosicc, Denmark), where the temperature of the drying chamber and the cooling unit were 20 and -50 ºC respectively and the vacuum was 10^{-2} mbar. The dried powder was removed from freeze dryer and ground using a mortar and pestle.

OSA modification

The dry weight of starch was determined using an IR-Balance (MA 30, Sartorius, Germany) at 135°C in duplicates. Then the starch to be modified was weighed (based on dry-weight) and dispersed in 1.5-2 v/v of water by stirring. The pH was adjusted to 7.4-7.8 by addition of either of HCl or NaOH. Thereafter, using an automatic titration machine the pH was set to 7.6 and 3% of OSA (based on the dry weight of starch) was added in 4 portions while maintaining the pH to 7.6. The process was finished when the pH was stable at 7.6 at least 15 min. The starch was then centrifuged at 3000 g for 10 min and the excess water was poured out. The starch pellet was then re-dispersed in water for at least 2 more times and washed with water. Then the starch was washed with citric acid (pH 4.5-5) and then with water for at least 2 times. Finally, the starch pellet was spread in a tray and dried in room temperature before it was ground using a mortar and pastel.

Determination of the degree of modification

The dry weight of starch was determined with IR-balance in 135 °C. Thereafter 5 g of modified and native starch (as control) based on dry weight was transferred to a beaker and was wet with some drops of ethanol. Then, 50 mL of 0.1 M HCl was added to the beaker and the dispersion was mixed for 30 min using a magnetic bar. The slurry was then centrifuged and the supernatant was discarded. The starch was then washed with 50 mL of ethanol and distilled water. The starch was then dispersed in 300 mL of water and the mixture was heated in 95 °C for 10 min. the mixture was then cooled to 25 °C in ice bath. The pH was then titrated to 8.3 with 0.1 M NaOH using an automatic titration apparatus. The volume of 0.1 M NaOH was used in calculation of the degree of modification using the equation below:

$$OSA\% = \frac{(V_{sample} - V_{control}) \times M \times 210}{W} \times 100 \qquad \textit{Equation (11)}$$

where V_{sample} (mL) is the volume of 0.1 M NaOH for the modified starch and $V_{control}$ (mL) is the volume of 0.1 M NaOH for the native samples, M is molarity of NaOH (M), W is dry weight of starch (g) and 210 is molecular weight (g/M) of OSA.

Characterization of starch granules

Size

Particle size is one of the most important traits of a particle that influences properties such as stability and performance. Therefore, measurement of particle size is critical for research and industry [26]. The particle size distribution of starch granules was determined using a laser diffraction particle size analyzer (Mastersizer 2000 Ver. 5.60, Malvern, UK). 70 mg of starch was dispersed in a 7 mL of phosphate buffer (95%, 5 mM, pH 7, 0.2 M NaCl) using a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s. The sample was added to the flow system containing Milli-Q water and was pumped through the optical chamber at a pump velocity of 2000 rpm. The refractive index (RI) of the starch was set to 1.54 [140] and the RI of the continuous phase was set to 1.33 (water) and the obscuration was between 10 and 20%. For each starch dispersion added to the system, three measurements were performed, and all samples were made in duplicates.

SEM

The microstructure (shape and morphology) of the particles is an important characteristic of particles. Therefore, the microstructure of starch granules was screened by scanning electron microscopy (SEM). The dried samples were coated with gold and examined under SEM (field emission SEM, JSM-6700F, JEOL, Japan) operated at 5 kV with a working distance of 8 mm. Lower detection imaging mode (LEI) was used to give clear three-dimensional images of the sample surface. The LEI detector combines both signals secondary and back scattered electrons during operation.

Characterization of gelatinization properties of starch

The gelatinization properties of starch granules were analyzed using a differential scanning calorimeter (DSC, Seiko 6200, Seiko Instruments Inc., Japan), calibrated with indium (M_p = 156.6 °C). Starch dispersions were prepared and weighed into coated aluminum pans (TA Instruments, USA) at a ratio of 1:10 and gelatinization transition enthalpy (ΔH, J/g dry matter), gelatinization onset temperature (°C), gelatinization peak temperature (°C) and gelatinization conclusion temperature (°C) were determined. The scanning rate was 10 °C/min from 10 to 120 °C.

Protein content

There is a trace amount of proteins in the starch granules. Proteins are known for having surface activity and can be affecting in adsorption of starch granules at the oil-water interfaces. Therefore, the protein level of starches was determined using a nitrogen/protein analyzer (Flash EA 1112 Series, Thermo Scientific, USA).

Amylose/amylopectin

The amylopectin level of starch is known to have influence on the crystallinity and consequently the gelatinization/retrogradation of starch. The amylose level (%, w/w) determined using a lectin Concanavalin A assay (Megazyme International, Ireland) which is a modified version of the method developed by Yun and Matheson, 1990 [141].

Emulsification and characterization of emulsions

Emulsification

Oil-in-water starch granules stabilized emulsions were prepared using corresponding amount of Miglyol 812 (Caesar & Loretz GmbH, Germany) as dispersed phase and phosphate buffer (5 mM, pH 7, 0.2 M NaCl) as continuous phase and starch granules as stabilizer.

7 mL emulsions were prepared in a glass test tubes. Starch based on mL of oil was added to the test tube, where the amount of oil varied between 5 and 20% depending on the particular study. All the emulsions were homogenized using a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s and were prepared in at least duplicate for analysis. The appearance of these emulsions are presented in (Fig. 14). Thereafter, the emulsions were characterized as described below in the upcoming sections. The emulsion index (*EI%*) was then calculated from the equation below:

$$EI\% = \frac{H_{cream}}{H_{total}} \times 100 \hspace{3cm} \textit{Equation (12)}$$
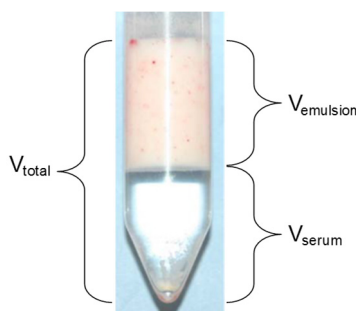
Fig. 14.
OSA modified quinoa starch granule stabilized Pickering emulsion.

Accelerated stability test

The stability of the emulsions to coalescence subjected to external stress was evaluated according to a method previously developed by Tcholakova, Denkov et al. 2002 and 2006 [142, 143]. The emulsion is added to a Turbiscan cell and loaded into a centrifuge (Allegera X-15, Beckman Coulter). This emulsion is then subjected to centrifugal acceleration at defined intensity and time. The oil droplets will tend to move towards the axis of rotation (z direction in Fig. 15) due to their relatively lower density. Emulsion droplets first form a cream layer where they are forced into close proximity due to buoyancy forces but are not significantly deformed. As the centrifugal force is increased they are pressed tighter and tighter together and eventually the interfacial layer surrounding and stabilizing the droplets will rupture releasing a layer of oil on the top of the emulsion column in the tube (see Fig. 15). After centrifugation the height of the creamed layer, H_C and oil released H_{Rel} was measured using a static multiple light scattering (Turbiscan, Formulation Co., France) and the emulsion indices (*EI%*) were calculated and compared before and after centrifugation as well as among different samples.
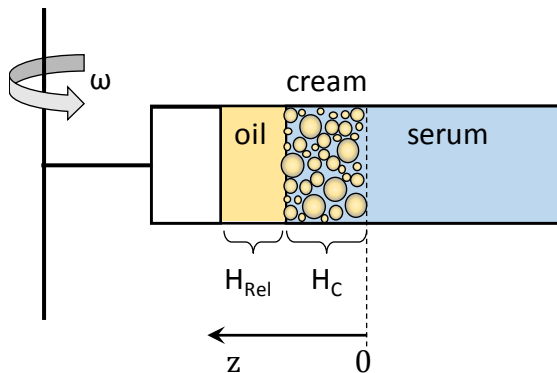
Fig. 15.
Schematic representation of accelerated stability test.

Application of heat treatment

Heat treatment was performed in two different ways in this research work. In the initial step, emulsions were heat treated after making them in a glass test tube, by holding in a water bath in 70 °C while monitoring the temperature using a thermocouple. In this method, in addition to formation of gelatinized barriers around the droplets, the gelatinized layer in the neighboring droplets merged and created a network of gelatinized starch in which oil droplets were observed in the form of

clusters as can be seen in papers III and IV. Therefore, in order to create a gelatinized barrier on individually existing droplets and to avoid formation of large clusters, another method was developed. In the second method, a gentle mixing (500 rpm) was applied during the heat treatment using an overhead stirrer to keep the droplets apart, while avoiding additional homogenization. The emulsions using this method were characterized in papers V and VI.

Emulsions droplet size characterization

The stability of emulsions is strongly correlated to its droplet size distribution and therefore, there is a range of analytical methods that can be used to obtain the information about particle size within an emulsion. These methods include: microscopy, light scattering, particle counting and sedimentation methods and the collected data is dependent on the method used. In some cases, the particle size distribution is obtained by direct measurement (such as microscopy) and in some cases it is calculated from measurements of some size dependent physical property of the system by fitting the data with a mathematical model such as light scattering [24].

Static light scattering

Light scattering or laser diffraction technique use the scattering pattern produced as a result of a directed laser beam through an emulsion. Thereafter, the particle size distribution is predicted using a software that utilize mathematical models. Commercial static light scattering instruments are able to verify particle sizes within the range of 100 nm to 100 μm [7].

The particle size distributions of the starch granule stabilized emulsions were characterized with a laser diffraction particle size analyzer, Mastersizer 2000 (Malvern Instruments, UK). Each emulsion was added to the flow system (Hydro SM small volume wet dispersion unit) containing Milli-Q water and was then pumped through the optical chamber where it was measured. The refractive index of starch particles was set to 1.54 [140] and the refractive index of the continuous phase was set to 1.33 which is the refractive index of the water and the obscuration was between 10 and 20%. For each emulsion sample added to the flow system, three measurements were performed, and all emulsions were prepared in duplicates.

Microscopy

Since the components of emulsions are in micron and submicron scale, microscopy is one of widely used techniques to study emulsions. The different microscopy techniques including optical microscopy, atomic force microscopy have different principles but they all provide information about the microstructure, particle size

distribution and morphology of emulsions in the form of images that can be easily studied and comprehended by individuals [7]. These images facilitate the study of trends, changes and possible instability processes occurring in the emulsion systems. Most modern microscopes are attached to a computer to store and process data using various processing programs. In some cases, in order to increase the contrast between the major components in an emulsion, chemical stains that partition into either of the oil or aqueous phase are used. These stains absorb the light in the visible region for bright field microscopy or as in fluorescent and confocal microscopy they are fluorescent [7].

The emulsions were characterized by light microscopy using a camera (DFK 41AF02, The Imaging Source, Germany) that was attached to a light microscope (Olympus BX50, Japan) and both were connected to a computer. The emulsions were diluted 5 times with Milli-Q water and then one drop was placed on a glass microscopic slide. In order to prevent deformation of droplets no cover glass was used. The microscopic images were taken using objective magnifications of 20× and 50×.

Assessment of functional properties of starch Pickering emulsions

Process stability

We have studied the possibility to produce novel powder materials based on chemically modified starch granule stabilized Pickering oil-in-water (O/W) and water-in-oil-in-water emulsions (W/O/W) by freeze-drying. This study also investigated the effect of *in situ* partial starch gelatinization, dispersed phase type (two oil types with different melting points), freeze-thaw cycling, and freeze-drying on the physical properties of the emulsions. The effect of the freeze-thaw and freeze-drying processes on the stability of the encapsulated substance in the context of double emulsions was also investigated.

Freeze-thaw stability

Some food emulsions are experiencing some processes that may alter or destabilize them during the production. One common process that may alter the stability of the emulsions is freezing. Therefore, there has been numerous research to study the effect of freeze-thaw cycling on the stability of the emulsions. The effect of freeze-thaw cycling on the overall stability (paper III and IV) and encapsulation stability (paper IV) of starch granules stabilized emulsions was investigated in this study.

In paper III, two different freezing method i.e. using a laboratory freezer at -18 °C as slow freezing method or liquid nitrogen at -196 °C as the rapid freezing method was used while, in paper IV, only the slow freezing method was used. Approximately 10 mL of samples was transferred to a stainless steel container and covered by an aluminum foil. Thereafter, the samples were transferred to a freezer, at -18 °C and left there over the night. The samples were taken out of the freezer after 8 h and thawed for 4 h in room temperature. In order to see the effect of the rate of freezing on emulsions some samples were frozen using liquid nitrogen at -196 °C. These samples were also kept in the freezer over the night to be characterized in the following day.

Freeze-drying stability

Another common process that may alter the stability of the emulsions is freeze-drying. The effect of freeze-drying of starch granules stabilized emulsions on the physical stability (paper III and IV) and encapsulation stability (paper IV) was investigated in this work.

Some samples were frozen using the same method above followed by freeze-drying. After freezing, the aluminum cover of the samples were punctured so that the water vapor be removed from the samples and collected in the condenser. Frozen samples were then transferred to a laboratory freeze-dryer (CD 12, Hetosicc, Denmark) with 20 °C in the drying chamber and -50 °C in the cooling unit and a vacuum of 10^{-2} mbar. The freeze-dried samples were restored to the same emulsion concentration with Milli-Q water in and Eppendorf tube using a vortex in a low mixing rate.

The frozen and freeze-dried and restored samples were characterized using a particle size analyzer and light microscopy.

Encapsulation stability

The encapsulation stability of the Pickering emulsions for hydrophobic and hydrophilic substances were evaluated in the context of simple oil-in-water (O/W) and double water-in-oil-in-water (W/O/W) emulsions. Curcumin was chosen as the hydrophobic tracer in O/W emulsions and for hydrophilic tracer, carmine was used in context of W/O/W emulsions. The encapsulation properties were evaluated as encapsulation efficiency (*EE%*) of newly formed emulsions and encapsulation stability (*ES%*) during storage and after being exposed to processing or physiological conditions using a spectrophotometer (Cary 50, Varian, Australia), using the equations below:

$$EE\% = \frac{C_{measured}}{C_{recoverable}} \times 100 \qquad\qquad Equation\ (13a)$$

$$ES\% = \frac{C_{measured}}{C_{recoverable}} \times 100 \qquad\qquad \textit{Equation (13b)}$$

For both tracers a standard curve was developed. In the case of curcumin and O/W emulsions, the $C_{measured}$ was the amount of the curcumin measured by dissolving the emulsions in methanol and detected with spectrophotometer and $C_{recoverable}$ was the amount of curcumin initially added. With respect to carmine as some of the tracer found to be absorbed by the starch, $C_{recoverable}$ was calculated from an O/W_2 emulsions where the same amount of tracer to be encapsulated was added to the external water phase (W_2). Thereafter for each double emulsion, $C_{measured}$ was calculated from the subtracting concentration of the marker detected in the external aqueous phase from the $C_{recoverable}$.

Simple Emulsions

Curcumin was added to Mygliol 812 (125 µg/mL). The combination was mixed at 22 000 rpm for 20 min with rotor-stator high shear homogenizer (Ystral. D79828, Ballrechten-Dottingen Germany). Then, the undissolved curcumin was separated by ultra-centrifuge (LE-80K, Beckman, USA) at 14 000 rpm for 10 min. The supernatant was collected and used as dispersed phase as described above in the emulsification section.

Double emulsions

Initially carmine was dissolved in the internal water phase (W_1). Polyglycerol polyricinoleate (PGPR 90, Danisco, Denmark) was added to the oil as stabilizer for primary emulsions (W_1/O) at 5% w/v and mixed for 45 min using a magnetic plate and stir bar. Primary emulsions (W_1/O) were prepared by 20% v/v of W_1 in 80% v/v oil phase including PGPR 90 using the Ystral mixer at 24 000 rpm for 10 min. The primary emulsion was then dispersed into the external aqueous phase (W_2) as described above in the emulsification section.

Physiological stability

The physiological stability was evaluated with respect to the physical stability of droplets as well as stability of the encapsulated tracer (i.e. retention of curcumin) during *in vitro* digestion (Fig. 16). The physiological stability of emulsions was also evaluated during an *in vitro* intestinal lipolysis. In order to investigate the effect of partial gelatinization of starch at the oil-water interfaces, the barrier properties of emulsions were evaluated for non-heat treated and heat treated emulsions.

Simulated oral digestion

Simulated salivary fluid (SSF) was prepared according to a previously used method by Hur, Decker et al. 2009 [144]. Briefly, SSF was prepared by mixing 10 mL of KCl solution (1.2 M), 10 mL of KSCN solution (0.2 M), 10 mL of NaH_2PO_4 solution (0.74 M), 1.7 mL of NaCl solution (1 M), 20 mL of $NaHCO_3$ solution (1 M) and 8 mL of urea solution (0.42 M). Thereafter, 290 mg a-amylase, 15 mg uric acid and 25 mg of mucin were added and the pH was adjusted to 6.8. SSF was freshly made each day of experimentation and stored refrigerated until use.

4 mL of SSF was mixed with 4 mL of emulsion. The temperature was set to 37 °C in a water bath. After 5 min, the test tube was taken out of the water bath and the enzymatic reaction was stopped by addition of 2 mL of 1 M HCl.
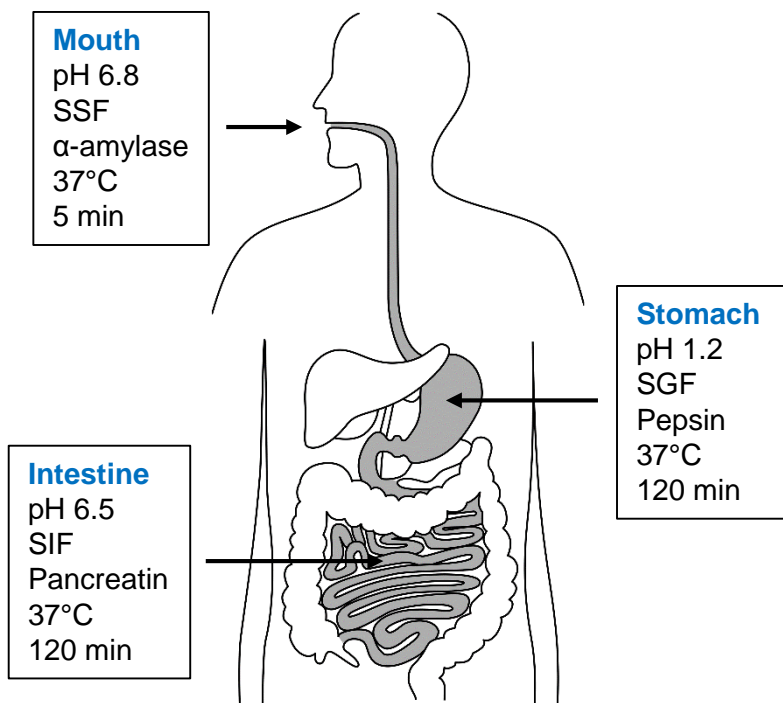
Simulated gastric digestion

The simulated gastric digestion was performed based on a method described by Tikekar, Pan et al. 2013 [129] with minor modification based on a method described by United State Pharmacopeia. The simulated gastric fluid (SGF) was prepared by addition of 2 g of NaCl in 7 mL of 1 M HCl. The volume of the solution was then adjusted to 1000 mL using Milli-Q water. The pH was then adjusted to 1.2 with 1 M HCl and 1 M NaOH.

Subsequently, 0.032 g pepsin was dissolved in 1 mL SGF and together with 9 mL emulsion were put in a dialysis cassette. The content of the cassette was mixed and then immersed in a 2.5 L beaker of SGF at 37 °C under stirred condition using a magnetic stirring plate and a stir bar. After definite time intervals (10 min, 30 min, 60 min, 90 min and 120 min), samples were taken out of the dialysis cassette and the enzymatic reaction was stopped through adjusting the pH to 6.5 with 1 M $NaHCO_3$.

Simulated intestinal digestion

The simulated intestinal fluid (SIF) was also adopted from a previous method by Tikekar, Pan et al. 2013 [129] with some modification based on the United States Pharmacopeia. Briefly, SIF was prepared by addition of 10 mg/mL of pancreatin mix into 0.05 M KH_2PO_4 at pH 6.5. Then 10 mL of emulsion were adjusted to pH 6.5 with 0.2 M HCl or 0.2 M NaOH.

The emulsions were mixed with 10 mL SIF which contained 0.5 g bile extract. A set of control samples were also prepared without bile salts. Mixtures were then incubated at 37 °C. 200 mL of samples were pipetted out at definite time intervals (10 min, 30 min, 60 min, 90 min and 120 min) and the enzymatic reaction was stopped by cooling down the samples on ice for 10 min.

Mouth
pH 6.8
SSF
α-amylase
37°C
5 min

Stomach
pH 1.2
SGF
Pepsin
37°C
120 min

Intestine
pH 6.5
SIF
Pancreatin
37°C
120 min

Fig. 16.
Specifications of *in vitro* digestion steps.

In vitro lipolysis

The lipid digestion was monitored by measurement of the rate of lipid hydrolysis through the volume of NaOH 0.1 M needed to neutralize the pH drop caused by production of free fatty acids using a pH-stat (Titrando 902, Metrohm, Switzerland). In brief, 12 mL of the 25 mM Tris buffer together with 200 µL $CaCl_2$ 0.3 M and 2.5 mL of bile salt solution (80 mM sodium cholate and sodium deoxycholate bile salts 50:50 w/w) were transferred to a pH-stat vessel with thermo-stat jacket that kept the temperature on 37 °C. The concentration of $CaCl_2$ and bile salts were chosen in a way to achieve 0.3 mM and 10 mM in the final digestion mixture respectively. Thereafter, pancreatin was added to achieve 800 USP/mL in the final digestion mixture [145] and the pH was carefully set to 7.0 using 1.0 M NaOH. Subsequently, 5 mL of emulsion was added and the process started. The amount of NaOH 0.1 M needed to neutralize the FFA in a 3 h period was used to calculate the concentration of free fatty acids (FFA) generated in the reaction vessel during digestion of the emulsified lipids and can be interpreted as availability of the oil for lipolysis which

in turn gives a measure for comparison of barrier properties of the different samples. The percentage of free fatty acids (FFA%) was calculated from the number of moles of NaOH 0.1 M required to neutralize the FFA released from triglycerols, considering 2 FFA can be produced per triglycerol, using the equation below:

$$FFA\% = \left(\frac{V_{NaOH} \times M_{NaOH} \times M_{W\ lipid}}{2 \times W_{lipid}}\right) \qquad Equation\ (14)$$

where, V_{NaOH} is the volume (mL) of NaOH consumed, M_{NaOH} is the molarity of NaOH (0.1 M), M_{Wlipid}, is the average molecular weight of the Miglyol 812 (0.520 kg/M) and W_{lipid} is the weight of lipid initially inserted to the reaction vessel (0.47 g) [27].

Since lipolysis is an interfacial phenomena and in order to have a fair comparison, the FFA% was normalized against the droplet surface area of the different emulsions using equations 15a and 15b:

$$S = \frac{6 \emptyset V_{emulsion}}{d_{3,2}} \qquad Equation\ (15a)$$

$$\frac{FFA\%}{S} = \left(\frac{FFA\%}{\frac{6 \times \emptyset \times V_{emulsion}}{d_{3,2}}}\right) \qquad Equation\ (15b)$$

where S is the specific surface area (m^2), $\emptyset$ is the volume fraction of the oil phase (0.1%), $V_{emulsion}$ is the volume of the emulsions analyzed (5 mL).

Summary of the main results

The main results of the six papers included in this thesis are summarized in the three sections as follows:
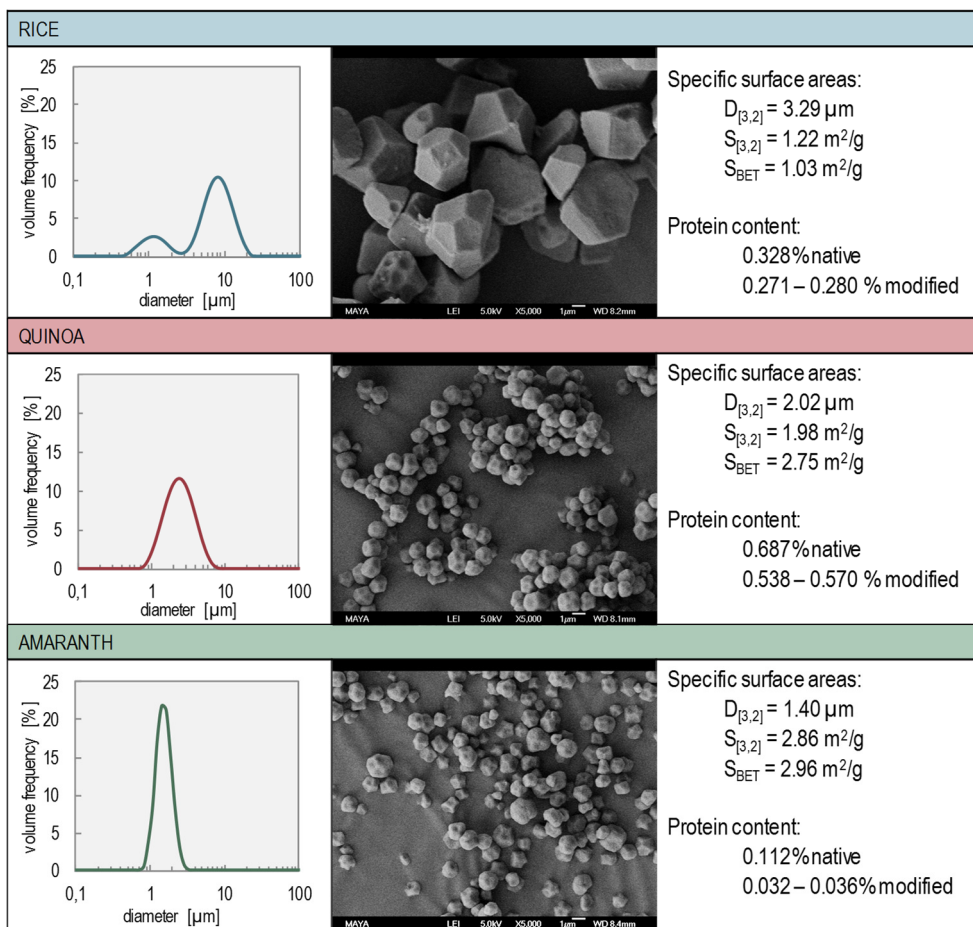
Section I (papers I and II)

The detailed physico-chemical characterization of small starch granules of different botanical origins (i.e. rice, quinoa and amaranth) and different OSA modification levels with respect to suitability for use as Pickering emulsifier was performed in paper I. To establish the effect of different types of starches and modification levels, the emulsifying capacity and stability of the corresponding emulsions was then evaluated in paper I and continued in paper II. The main findings of this section of the thesis are as follows:

Proximate analysis of starch granules: Proximate analysis was performed to quantify the protein and amylose content. At the preparation conditions used, the protein content was the highest for quinoa with 0.54-0.69% followed by rice with 0.27-0.33% and the lowest amount was for amaranth with 0.032-0.11%, for the modified and native samples respectively (Fig. 17). This difference in protein levels can be due to different isolation methods. Moreover, it was shown that the protein content for the native samples were higher than the modified samples, which was deemed due to alkali exposure and acetone washes during modification. The amylose content was the lowest for rice 4.43% ± 0.78 and somewhat similar for quinoa and amaranth with 20.95% ± 0.45 and 20.90% ± 0.98 respectively.

Gelatinization of starch: The gelatinization temperature range (T_o-T_c) was the highest for rice 72.2-85.0 °C and somewhat similar for quinoa and amaranth with 64.1-75.5 °C and 63.8-76.8 °C respectively. The higher gelatinization range for rice can be attributed to the higher level of amylopectin. Since according to Fredriksson, Silverio et al. 1998 [72] starch crystallinity has a direct correlation with the amylopectin content and hence, starches with higher amylopectin content (lower amylose content) would expect to have higher onset, peak and conclusion temperatures.

Size and morphology of starch granules: The size of the different types of starch granules showed the highest for rice with 7.7 ± 0.1 μm, followed by quinoa 2.2 ± 0.2 μm and amaranth 1.4 ± 0.03 μm (Fig. 17). Additionally, the particle size distribution was bimodal for rice starch granules with a small peak around 1 μm and

RICE

Specific surface areas:
$D_{[3,2]}$ = 3.29 μm
$S_{[3,2]}$ = 1.22 m²/g
S_{BET} = 1.03 m²/g

Protein content:
0.328% native
0.271 – 0.280 % modified

QUINOA

Specific surface areas:
$D_{[3,2]}$ = 2.02 μm
$S_{[3,2]}$ = 1.98 m²/g
S_{BET} = 2.75 m²/g

Protein content:
0.687% native
0.538 – 0.570 % modified

AMARANTH

Specific surface areas:
$D_{[3,2]}$ = 1.40 μm
$S_{[3,2]}$ = 2.86 m²/g
S_{BET} = 2.96 m²/g

Protein content:
0.112% native
0.032 – 0.036% modified

larger peak around 7.6 μm (Fig. 16). Comparing with the results in the literature unimodal and bimodal size distributions can be found [146, 147].

Fig. 17.
Particle size distributions, SEM images, specific surface data, and protein content of rice, quinoa and amaranth starches, data from Marefati, Wiege et al. 2017 [39].

The effect of the degree of modification on the emulsifying capacity: For a given starch type and formulation (mg starch/mL oil), higher levels of modification resulted in smaller emulsion droplet sizes (for particle size distribution see Fig. 18 and for morphology of emulsions with 3% OSA starches see Fig. 19). Additionally,

increasing the level of modification resulted in decreasing the amount of free starch. Moreover, the degree of modification was more influential on the emulsifying capacity of amaranth especially at higher levels of modification where the emulsifying capacity increased considerably. The particle size distribution of emulsions stabilized by quinoa showed that these emulsions were less sensitive to the level of modification which can be due to the higher level of proteins of these starch granules that can provide more hydrophobicity and better adsorption to the oil-water interface. Similarly, the thickness of the emulsion layer increased, as the modification level increased which may be due to the inter particle interactions as proposed by Schröder, Sprakel et al. 2017 [32]. The overall emulsifying capacity was higher for quinoa at native and lower levels of OSA modification levels at the tested concentration (100 mg starch/mL oil). The better performance of native and modified quinoa could be attributed to the higher protein content which can provide additional hydrophobic groups and better interfacial affinity, which in turn results in presence of less free starch in the emulsion systems. According to Baldwin 2001 [148], a substantial amount of starch granule associated proteins are located at the surface of the starch granules. Due to large surface areas of the starches tested in this study (1.22, 1.98 and 2.86 m^2/g for rice, quinoa and amaranth respectively), the presence of proteins may significantly affect overall surface properties of starch granules. From the proximate analysis, it was shown that the amount of protein in quinoa was higher than rice (by factor of 2 for both native and modified) and amaranth (by a factor of 6 and 17 for native and modified respectively). If we assume that all the proteins are located at the surface of the starch granules, the amount of protein per unit surface area would be 2.7×10^{-3}, 3.5×10^{-3} and 0.4×10^{-3} g/m^2 for native rice, quinoa and amaranth respectively and 2.2×10^{-3}, 2.7×10^{-3} and 0.1×10^{-3} g/m^2 for modified rice, quinoa and amaranth respectively. Considering the intermediate size of quinoa, with more similarity in size and shape to amaranth, the higher emulsifying capacity can only be explained by this difference in protein content which is further manifested in the case of native starches where the trace amount of protein in amaranth did not create stable emulsions. The higher hydrophobicity caused by protein may also explain the lower amounts of free starch observed in the particle size distribution of emulsions stabilized by quinoa, compared to amaranth as can be seen in Fig. 18.
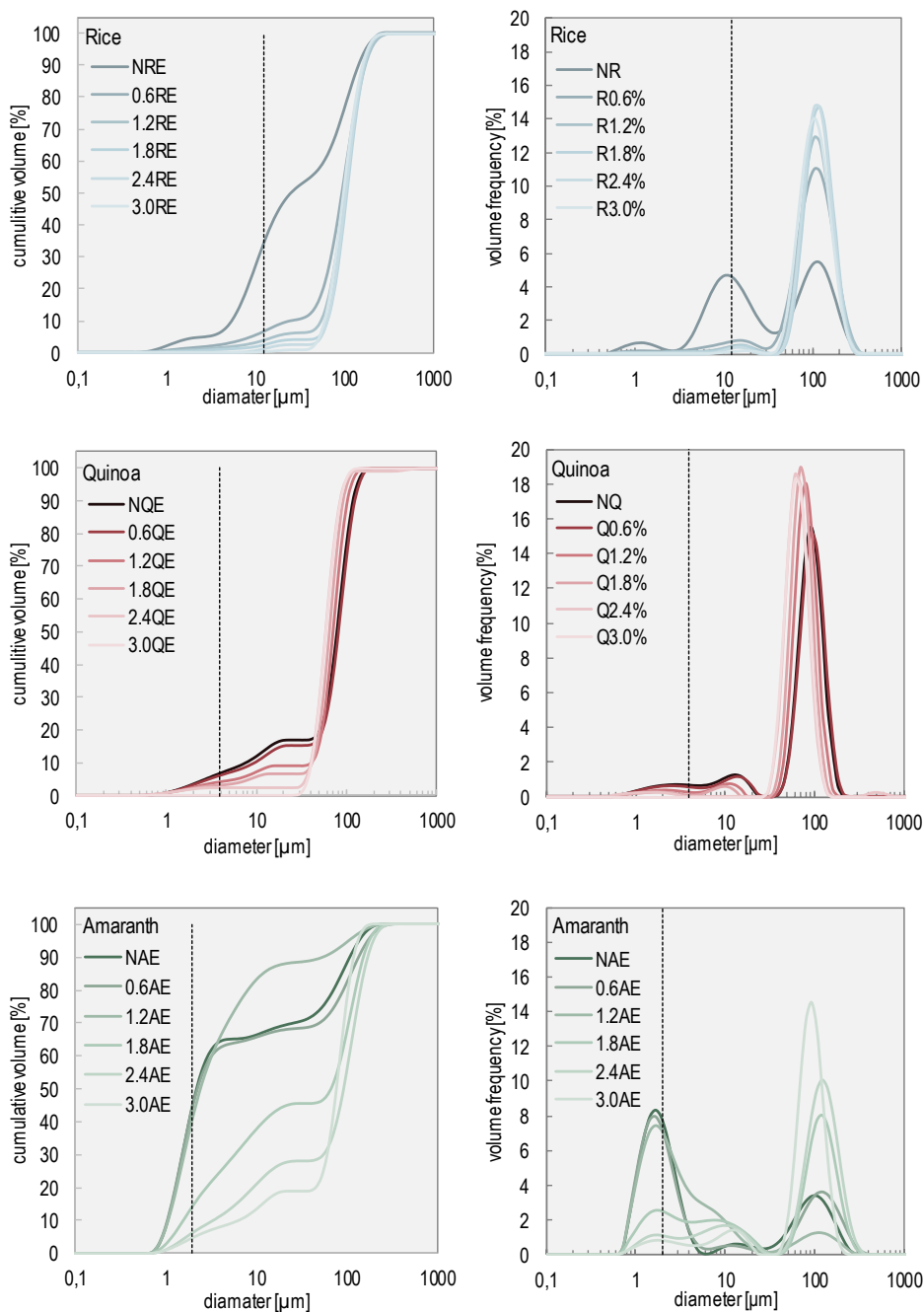
Fig. 18.
Particle size distributions (left cumulative and right frequency) for starch granule stabilized emulsions with 20% oil fraction and 100 mg starch/mL oil for various levels of OSA modification: native, 0.6%, 1.2%, 1.8%, 2.4%, 3.0% OSA. Vertical dashed lines are D_{90} of the granules size Rice: 12.3 µm, Quinoa 3.93 µm, and Amaranth 1.97 µm respectively.
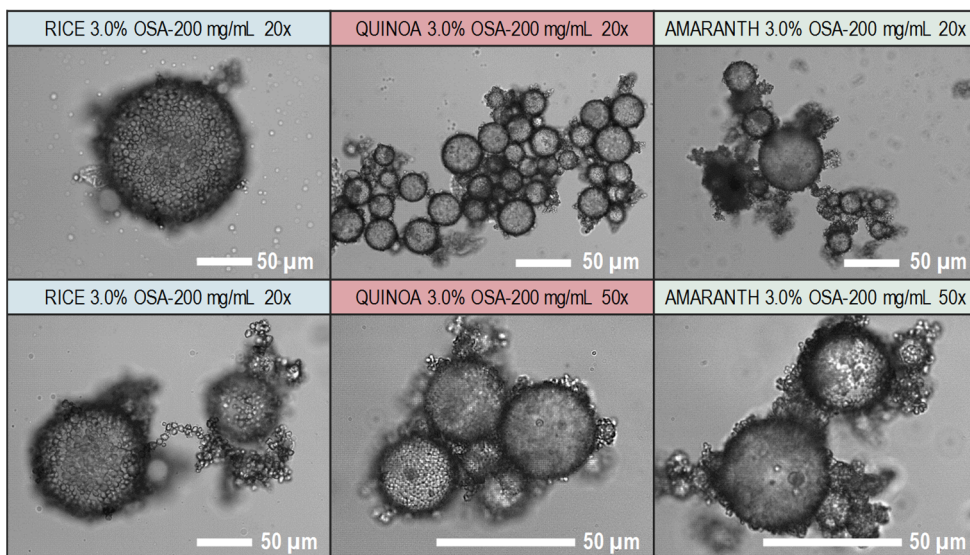
Fig. 19.
Micrographs of emulsions stabilized by different types of starches with OSA modification level of 3%, reported from Marefati, Wiege et al. 2017 [39].

The effect of starch concentration on emulsion droplet size: Increasing the starch to oil ratio, decreased the emulsion droplet size which was expected. Quinoa demonstrated a good overall emulsifying capacity, and the best emulsifying capacity at lower starch concentrations among the three starch types tested. Additionally, at higher amount of starch (> 400 mg/mL) and sufficient level of hydrophobicity (3% OSA modification), amaranth had the highest emulsifying capacity which can be due to their small size (see Fig. 20).
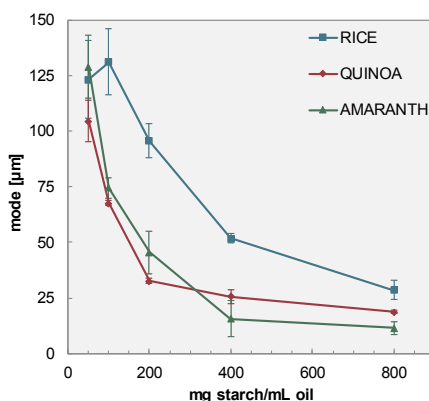

Fig. 20.
The effect of starch type, concentration and level of OSA modification on emulsions' droplet size (mode of $D_{4,3}$). Data reported from Marefati, Wiege et al. 2017 [39].

The emulsification index: The emulsification index of fresh emulsions (*EI%*) was highest for quinoa, followed by rice and amaranth (Fig. 21). The *EI%* had positive correlation with the level of modification. The modification level seems to be more influential on the *EI%* of amaranth and less for the quinoa stabilized emulsions. This can again be described by the higher level of protein in the quinoa starches, making the modification playing a less decisive role.

Stability towards accelerated stability test: For a given starch type, the emulsions' stability towards centrifugation stress test increased with the modification level (Fig. 21). The emulsions obtained from starches with higher level of modification (regardless of the starch type) showed higher stability in both initial and after accelerated stability test. Among all the samples tested, quinoa showed the best stability during the accelerated stability test which can be attributed to the higher surface hydrophobicity of quinoa starch granules due to higher protein content as described previously [39].
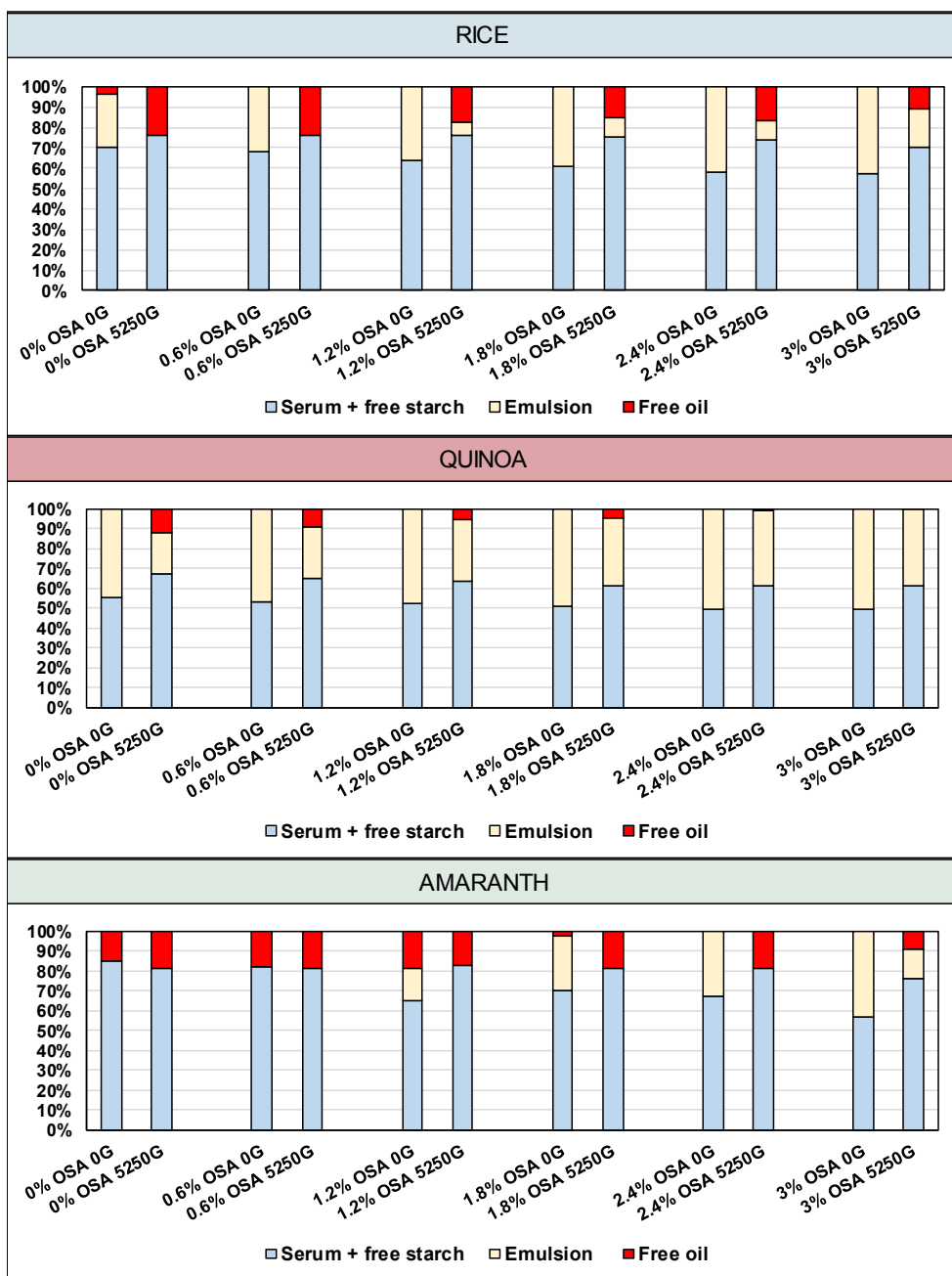
Fig. 21.
Representation of emulsions before and after accelerated stability test, where the blue part represents the water (serum) phase, the yellow part represents the cream phase and the red part represents the free oil as measured by multiple light scattering, n=2.

Section II (papers III and IV)

In section II, the effect of gelatinization on morphological properties of starch and the effect of a gelatinized barrier on the process and encapsulation stability was studied in paper III and IV. The process stability included freezing-thaw stability and freeze-drying stability. In this section, development of encapsulation systems for hydrophilic and hydrophobic bio-active compounds in liquid and dried forms were evaluated. Paper III considered a single emulsion system, while Paper IV extended this result to a double emulsion system. The main results are summarized below:

Effect of heat treatment: Heat treated samples with liquid oil were slightly larger than non-heat treated samples with a shoulder on the right of the mode of $D_{4,3}$ indicating some aggregation. However, the position of major peak of mode of $D_{4,3}$ (50 ± 2 μm) remained similar to that of the non-heat treated samples (49.2 ± 2 μm) with no statistically significant change ($p > 0.05$). The larger sizes were due to aggregation of droplets or rather formation of gelatinized network of starch with entrapped dispersed droplets. These networks showed large sizes sometimes exceeding 200 μm which was confirmed by the microscopy. Heat treated samples with solid oil behaved in the same manner, however, they were smaller than the liquid oil-based emulsions. The major peak raised from 36 ± 1 μm to 40 ± 3 μm (Fig. 22).
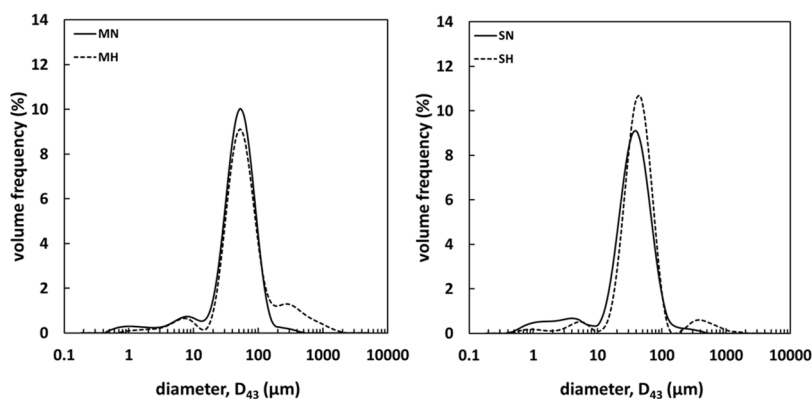
Fig. 22.
Particle size distribution $D_{4,3}$ of non-heat treated (MN) and heat treated (MH) emulsions with liquid oil as dispersed phase (left),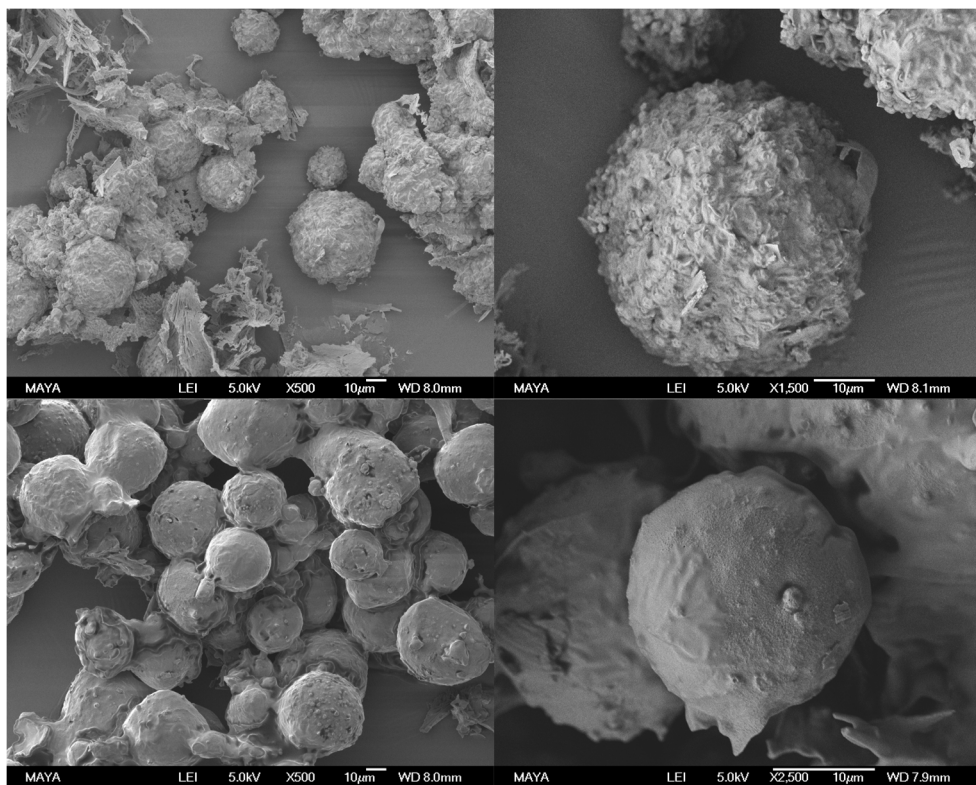 Particle size distribution $D_{4,3}$ of non-heat treated (SN) and heat treated (SH) emulsions with solid oil as dispersed phase (right), n=6. Data reported from Marefati, Rayner et al. 2013 [46].

Effect of freezing: Slow freezing at -18 °C generally did not alter the particle size distribution significantly compared to the fresh emulsions for the both types of the

oils tested. Flash freezing with liquid nitrogen at -196 °C appears to detach starch granules from the oil-water interface and facilitate a higher degree of coalescence and thereby created larger mean droplet sizes which was seen as reduced number of small droplets and an increase in amount of free starch. For liquid oil the mode of $D_{4,3}$ after slow freezing remained 40 ± 2 μm while for the flash frozen samples the mode of $D_{4,3}$ increased to 106 ± 11 μm. With respect to solid oil-based emulsions, a similar trend was observed when samples were flash frozen from the liquid state at 40 °C but not to the same extent (from 36 ± 1 μm to 45 ± 2 μm). However, for the solid oil that was already cooled down to the room temperature after emulsification, no significant changes in droplet sizes was observed upon flash freezing. This results were somewhat unexpected as it was assumed that rapid freezing will impose lower extant of changes to the morphology of an emulsion due to smaller ice crystals formed in the aqueous phase. Faster cooling rate and the lower temperatures reached using liquid nitrogen (-196 °C) for emulsions with liquid oil droplets led to the displacement of a fraction of the starch from the oil-water interfaces which in turn resulted in coalescence of droplets and created larger sizes. This observation was not made in the solid oil phase. This may be due to higher shrinkage of the liquid oil compared to the solid oil due to freezing which will affect the protrusion of crystals. According to Boode and Walstra, 1993 [22], the thermal expansion coefficient of triglycerides is 10^{-6} m^{3}kg^{-1}K^{-1} for oil and 3.9×10^{-7} m^{3}kg^{-1}K^{-1} for triglyceride crystals which is about 2.5 times less. Therefore, when an oil droplet is cooled, the higher volume shrinkage of liquid oil compared to fat crystals causes the crystals to protrude into the aqueous phase. The higher degree of cooling in flash frozen samples compared to slow frozen samples (to -196 °C for liquid nitrogen vs. -18 °C in the freezer) will cause more oil to crystallize with more protrusion, and therefore partial coalescence becomes inevitable [22]. Finally, despite the different freezing methods used, the emulsion characteristics remained and the emulsions did not collapse. This can be attributed to the relatively large starch particles that provide a stronger and thicker barrier between droplets and protect their integrity during the freeze-thaw process.

Effect of drying: Upon freeze-drying, the emulsions formulated with solid oil in both heat treated and non-heat treated samples created powders. This was attributed to higher physical stability of solid oil-based emulsions. In the case of the less stable, liquid oil-based emulsions, only heat treated samples resulted in dried emulsions. This was due to the stability improvement created by the gelatinized barrier. When powders were rehydrated, a well dispersed emulsion was created. For heat treated samples, association of emulsion droplets in the form of clusters consisting of several small droplets with relatively even surfaces of smooth gelatinized starch layers (Fig. 23) were observed with mode of $D_{4,3}$ of 324 ± 90 μm for liquid oil-based and 193 ± 60 μm for the solid oil-based formulations (Fig. 24). In the non-heated sample dried from solid oil, individual droplets with rough surfaces (Fig. 23) were

observed with mode of $D_{4,3}$ of $54 \pm 3\mu m$ (Fig. 24). This increase in size could have been due to aggregation. The results of these studies showed that oil containing powders can be produced with relatively high oil content (80%) from starch granule stabilized Pickering emulsions where adsorption and partial gelatinization of starch granules at the oil-water interface protects the integrity of droplets during freezing and freeze-drying (Fig. 23).

Fig. 23.
Freeze-dried emulsions from solid oil for non-heat treated (top row) and heat treated emulsions (bottom row). Reported from Marefati, Rayner et al. 2013 [46].
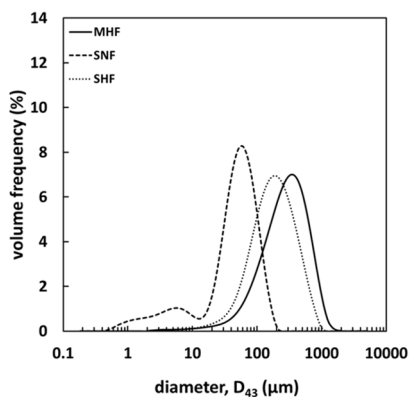
Fig. 24.
Particle size distribution $D_{4,3}$ of freeze-dried and rehydrated emulsions for heat treated liquid oil (MHF), and non-heat treated solid oil-based (SNF) and heat treated solid oil-based (SHF) emulsions, n=6. Data from Marefati, Rayner et al. 2013 [46].

Multiple emulsions are generally less stable than single emulsions due to the extra interface created by the internal aqueous phase. Thus, creating dried multiple emulsions was a result in itself. Below are summarized the steps in the development of this system.

Effect of heat treatment of W/O/W emulsions: In situ heat treatment created a gelatinized barrier at the oil-water interface. Heat treatment caused the droplet sizes to increase for liquid oil from 27.6 ± 0.3 µm to 32.2 ± 2.0 µm with some aggregation as right shoulder and for solid oil from 48.2 ± 0.1 µm to 118 ± 3 µm due to formation of gelatinized clusters (Table 3). The difference between the droplet sizes of fresh emulsions of liquid oil (melting point 15 °C) and solid oil (melting point 33 °C) can be due to the difference in viscosity of the oil phase at the homogenization temperature of 40 °C which was 49 mPa s and 59 mPa s for liquid and solid oils respectively. Viscosity of the oil phase is known to affect the droplet size developed at least in part when using a high shear homogenizer.

Table 2.

Different size parameters of W/OW emulsions with liquid and shea nut oil in initial state, after heat treatment, after freeze-thaw stability test and after freeze-drying, n=6. Data reported from Marefati, Sjöö et al. 2015 [149].

Sample	D [3,2] [µm]	D [4,3] [µm]	Span of D [4,3]	Mode of D [4,3] [µm]
Freshly prepared initial liquid W1/O/W2	11.3±0.7	44.2±4.9	1.6±0.3	27.6±2.3
Heat treated liquid W1/O/W2	27.4±1.4	155.6±8.1	10.0±1.8	32.0±2.0
Freeze thawed liquid W1/O/W2	7.3±0.1	80.0±2.1	3.2±0.3	120.0±0.7
Heat treated freeze thawed liquid W1/O/W2	48.6±3.3	105.8±8.0	1.8±0.1	90.2±6.5
Freshly prepared initial solid W1/O/W2	11.8±0.0	41.8±0.3	1.9±0.1	48.2±0.1
Heat treated solid W1/O/W2	42.7±1.1	129±7	2.9±0.1	118±3
Freeze thawed Solid W1/O/W2	11.2±0.7	44.7±7.5	1.9±0.3	39.8±0.1
Heat treated freeze thawed solid W1/O/W2	20.7±0.3	59.2±5.0	3.6±0.2	33.0±0.2
Reconstituted dried non-heated liquid W1/O/W2	Emulsion collapsed			
Reconstituted dried heat treated liquid W1/O/W2	104±0.1	240±5	2.1±0.2	210±11
Reconstituted dried non-heat treated solid W1/O/W2	10.6±1.2	60.2±19.4	2.7±0.3	62.4±3.2
Reconstituted dried heated solid W1/O/W2	47.1±3.5	114±16	2.5±0.1	85.2±11.1

Effect of freeze-thaw cycling of W/O/W emulsions: The droplet size of emulsions increased to 120 ± 1 µm when liquid oil was used as a result of freeze-thaw cycling. This size increase could have been caused by several mechanisms. As the aqueous phase of the emulsions crystalize, the oil droplets are pushed to a closer proximity of each other which can cause deformation of the droplets. This may result in oil-oil contact and cause coalescence. Moreover, formation and development of ice crystals can result in displacement of the stabilizing particles which can be seen as higher amount of free starch in the particle size distribution. Similar trends have been observed by Thanasukarn, Pongsawatmanit et al. 2004 [118]. This observation also agrees with the previous results where liquid oil droplets were frozen and formation and shrinkage and protrusion of fat crystals caused displacement of starch from oil-water interfaces and as a result of this displacement, partial coalescence occurred [46]. In the heat treated samples with liquid oil droplets, particle size distribution showed an increase which can be due to the formation of gelatinized starch clusters. When solid oil was used, freeze-thaw cycling showed a lower impact on particle size distribution (mode of $D_{4,3}$ of 39.8 ± 0.1 µm). According to previous

observations in paper III, when the oil phase of the emulsions crystalizes before the aqueous phase, the susceptibility of the oil droplets to compression due to crystallization of the water phase is decreased. The combination of heating and freeze-thaw cycling resulted in a particle size distribution with a larger span and mode of $D_{4,3}$ of 33 ± 0.2 μm (Table 2).

Effect of drying on W/O/W emulsions: Only some of the samples resulted in powders. Due to lower freezing stability of liquid oil, only heat treat samples created powder thanks to the formation of the gelatinized barrier that could prevent the collapse of emulsion droplets. In contrast, samples with solid oil as dispersed phase, for both non-heat treated and heat treated emulsions created powders (Fig. 25). Upon rehydration, some degree of aggregation was observed. For heat treated samples, this was as high as 210 ± 11 μm for liquid oil samples and 85.2 ± 11.1 μm for solid oil emulsions. However, in non-heat treated samples with solid oil droplets as dispersed phase, individually existing droplets with mode of $D_{4,3}$ of 62.4 ± 3.2 μm were observed (Table 2). Moreover, comparing the size distribution of original samples and rehydrated powders, it was concluded that the increase in sizes where more likely related to aggregation rather than coalescence.
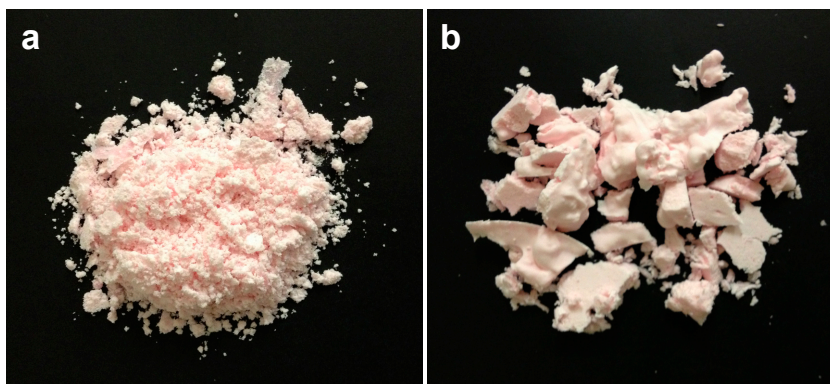
Fig. 25.
Dried emulsions from solid shea nut oil: (a) non heat treated freeze-dried double emulsion, (b) heat treated freeze-dried double emulsion, from Marefati, Sjöö et al. 2015 [149].

The encapsulation properties of W/O/W emulsions: The encapsulation efficiency (EE%) of the initial emulsions immediately after the emulsification, was $96.6\% \pm 4.2$ for liquid and $98.5\% \pm 0.2$ for solid oil-based double emulsions. Furthermore, the encapsulation stability (ES%) in all cases remained higher than 97.3% (Table 3).

Table 3.
Encapsulation properties of starch granules stabilized W/OW emulsions with liquid and solid oils, n=4.
Data reported from Marefati, Sjöö et al. 2015 [149].

Sample	EE % liquid	EE % solid
Freshly prepared initial W1/O/W2	96.6±4.2	98.5±0.2
Heat-treated W1/O/W2	97.6±2.0	99.4±0.2
Freeze-thawed W1/O/W2	98.8±0.1	98.5±0.4
Non-heat treated Freeze-dried W1/O/W2	Emulsion collapsed	97.3±0.6
Heat treated Freeze-dried W1/O/W2	97.5±0.4	97.3±0.2

Section III (papers V and VI)

In section III, the knowledge of encapsulation and process stability was extended to the stability of emulsions and encapsulated bioactive compound during an *in vitro* human gastro-intestinal conditions. The effect of the starch barrier of non-heat treated (intact) and heat treated (gelatinized) starch Pickering emulsions were evaluated during an *in vitro* oral, gastric and intestinal digestion in paper V and *in vitro* intestinal lipolysis in paper VI.

The effect of heat treatment on the morphology starch granules and emulsion droplets: In order to see the effect of heat treatment on the size of the starch granules, dispersions of starch in buffer solution were heated. Particle size distribution of starch granules showed a mode of $D_{4,3}$ of 1.8 ± 0.1 μm which was increased to 3.8 ± 0.1 μm as a result of heat treatment (Fig. 26). This increase was due to absorption of water and swelling of the starch granules as a result of gelatinization. In addition, in this section we used a modified version of heat treatment with moderate mixing, which prevented the formation of clusters of gelatinized starch and emulsion droplets (Fig. 27).
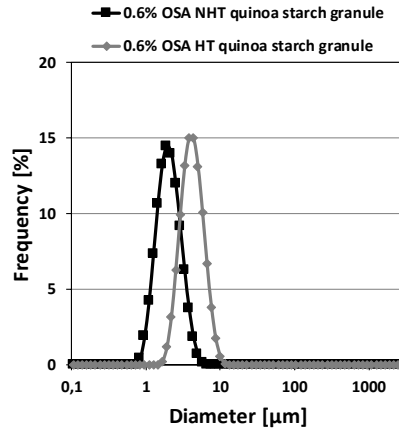
Fig. 26.
Particle size distribution $D_{4,3}$ for non-heat treated and heat treated 0.6% OSA modified quinoa starch granules, n=6. Data from Marefati, Bertrand et al. 2017 [51].
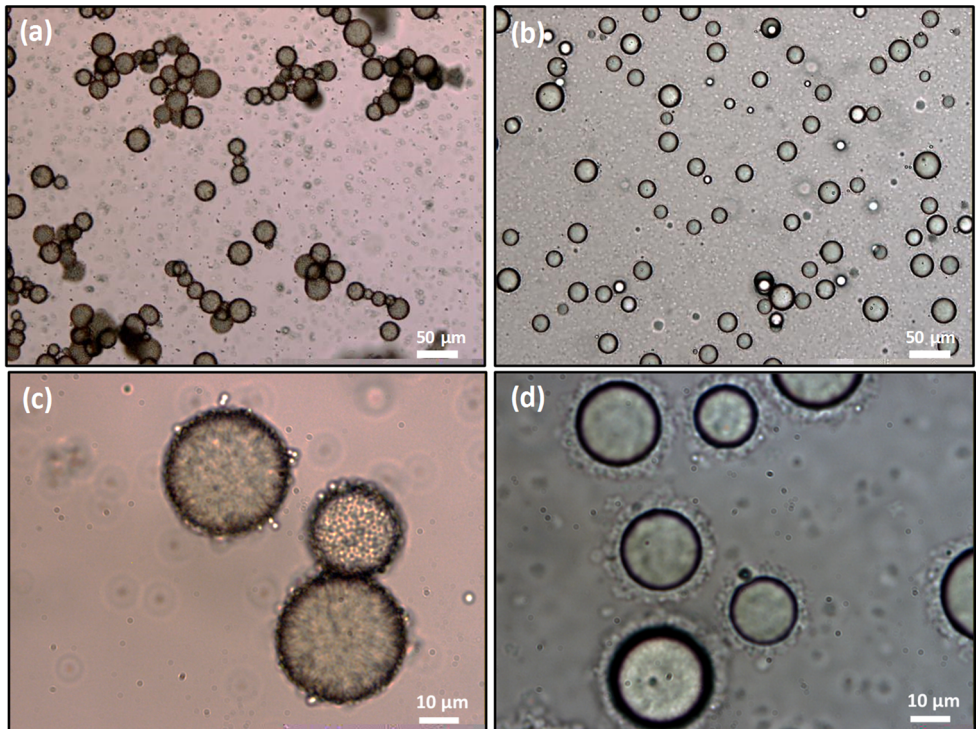
Fig. 27.
Light microscopy images of non-heat treated (a) and (c) and heat treated emulsions (b) and (d). Data from Marefati, Bertrand et al. 2017 [51]

Encapsulation efficiency (EE%): The encapsulation efficiency (*EE%*) for the curcumin was determined for the non-heat treated emulsions and found to be 79.3% ± 4.3.

The effect of heat treatment and storage on droplet size and encapsulation efficiency: Particle size distribution of the heat treated samples showed mode of $D_{4,3}$ of 28.0 ± 0.6 μm which was significantly lower than the initial size in non-heat treated samples 30.6 ± 0.3 μm. This reduction in size can be due to separation of the neighboring droplets as a result of gentle mixing during heat treatment which also resulted in release of some free starch granules that were entrapped in the aggregates. To verify this hypothesis, freshly produces non-heat treated emulsions were gently mixed (in the same manner as for the heat treated emulsions) and it was confirmed that the mixing is the cause for this difference. Moreover, the particle size distribution of the non-heat treated and heat treated samples remained unchanged during a 24 h storage stability test with the sizes of 31.1 ± 0.7 μm and 28.7 ± 0.7 μm respectively ($p < 0.05$). Heat treated emulsions retained more encapsulated curcumin during the storage stability test in excess of water having 73.2% ± 1.6 compared to non-heat treated samples 38.3% ± 0.6, while similar results for silica nano-particle stabilized emulsions or tween 20 stabilized emulsions were approximately 40% and 50% respectively. The difference between the non-heat treated and heat treated samples was due to formation of the gelatinized and therefore fused barrier of gelatinized starch at the oil-water interface that could have improved the encapsulation stability of curcumin. It was suggested that increasing the thickness of the interfacial barrier by techniques such as layer-by-later adsorption of positively charged biopolymers could also be used to increase the stability of encapsulated material [129]. The higher loss of curcumin in the non-heat treated emulsions may be due to presence of a porous interfacial layer which left a large amount of oil in contact with water and facilitated the release or destabilization of the encapsulated substance. For the same reason, creation of a fused barrier through gelatinization at the oil water interface can create a more cohesive barrier and preserve the encapsulated substance.

The stability during the in vitro oral digestion: Particle size distribution for non-heat treated samples before and after oral digestion showed that there was a decline in the mode of $D_{4,3}$ from 29.5 ± 1.4 μm to 23.0 ± 1.8 μm ($p < 0.05$) which could be due to dissociation of the aggregated droplets as a result of mixing during the oral digestion which also resulted in release of some free starch (Fig. 28). In the heat treated samples, no significant changes in the mode of $D_{4,3}$ before (30.2 ± 0.7 μm) and after oral digestion (28.4 ± 1.8 μm) was observed ($p > 0.05$). The decrease in the sizes of the emulsion droplets in heat treated samples could be due to the higher susceptibility of gelatinized starch at the oil-water interfaces to amylolytic hydrolysis [150]. However, the partially gelatinized starch barrier could protect the emulsion droplets from destabilization even during longer exposure (60 min) to the

66

in vitro simulated oral digestion conditions. The curcumin content after the *in vitro* simulated oral digestion showed that heat treated samples could retain more curcumin (95.3% ± 5.5) compared to non-heat treated samples (69.6% ± 9.0).

The stability during the in vitro gastric digestion: Comparison of the droplet size distribution for non-heat treated samples before and after *in vitro* simulated gastric digestion showed that there was a slight shift in the mode of $D_{4,3}$ (from 32.1 ± 1.4 μm to 26.9 ± 0.4, $p < 0.05$), which could be due to the mixing during the gastric digestion. Heat treated emulsions did not show any statistically significant changes in mode of $D_{4,3}$ after *in vitro* simulated gastric digestion (from 30.4 ± 0.3 μm to 28.9 ± 0.8 μm, $p > 0.05$) as can be seen in Fig. 28. Moreover, gastric digestion had a trivial effect on the encapsulated curcumin for both non-heat treated and heat treated samples with 86.2% ± 1.6 and 82.4% ± 7.0 respectively where the difference among the samples were not statistically significant. The result stability of curcumin in the gastric is in agreement with the previous results of Tikekar, Pan et al. 2013 [129], which was approximately 78%.
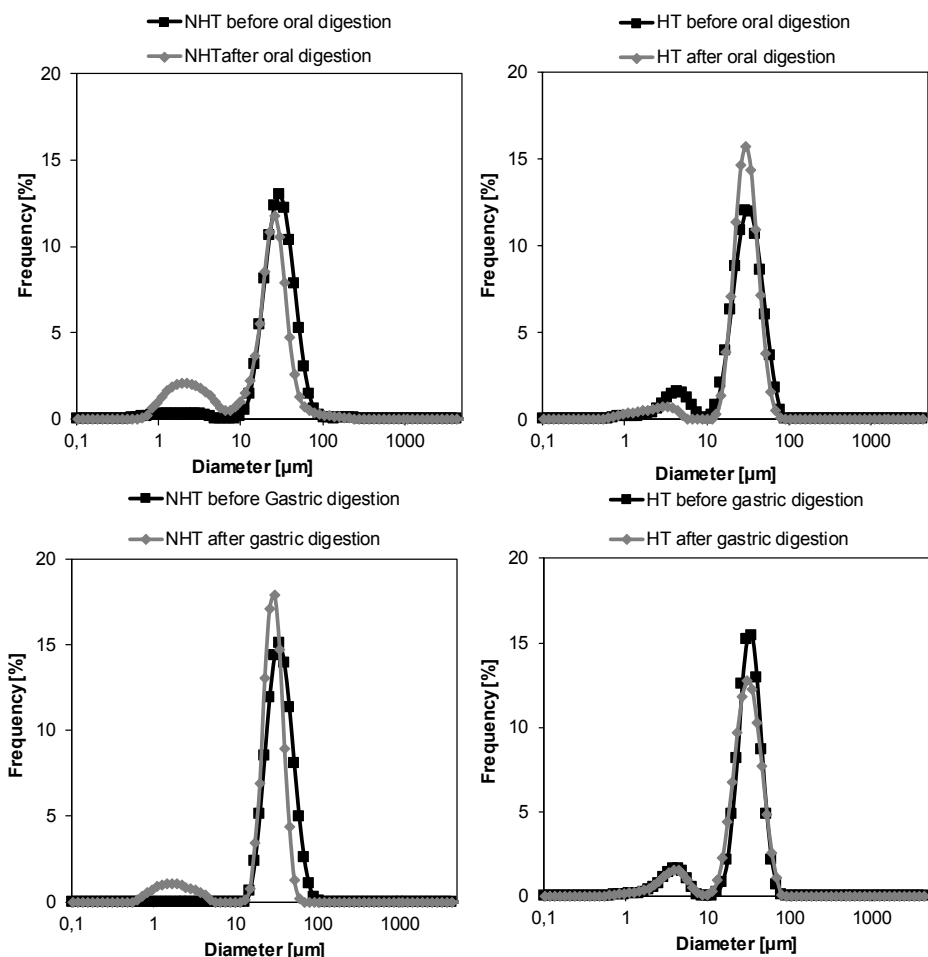
Fig. 28.
Particle size distribution ($D_{4,3}$) for non-heat treated (NHT) and heat treated (HT) emulsions before and after *in vitro* oral digestion (top row), Particle size distribution ($D_{4,3}$) for non-heat treated (NHT) and heat treated (HT) emulsions before and after *in vitro* gastric digestion (bottom row), n=6. Data from Marefati, Bertrand et al. 2017 [51].

The stability during the in vitro intestinal digestion: In order to separate the effect of biological surfactants from that of the digestive conditions, the *in vitro* intestinal digestion was performed with and without the presence of the bile salts. When bile salts were not present it took 30 min for the *in vitro* intestinal conditions to destabilize emulsions for non-heat treated emulsions, while it took 120 for the heat treated emulsions to destabilize. The destabilization was expected to happen faster when the bile salts were present, however, the heat treated emulsions remained stable during the first 30 min of *in vitro* intestinal digestion (Fig .29). This result illustrated that, although bile salts are effective in displacing material from the oil

droplet surfaces [151], the protective barrier created by the gelatinized starch at the intestinal condition is of interest. The preservative effect was lower for the non-heat treated emulsions which can be due to presence of gaps between the starch granules at the oil-water interface which allows the bile salts to reach the oil droplets and facilitate the lipase-co-lipase adsorption at the oil droplet surfaces, which in turn enables lipid digestion. This process seemed to be somewhat delayed in the case of the heat treated emulsions. Despite the known higher susceptibility of gelatinized starch to the amylase present in the intestinal condition, we postulated that the starch towards the droplets may have less digestibility due to incomplete gelatinization. The encapsulated properties of samples after 2 h exposure to *in vitro* intestinal conditions showed that there was higher amount of the curcumin retained in the heat treated emulsions (86.3% ± 13.5) compared to non-heat treated emulsions (40.2% ± 3.4). The amount of retained curcumin in the non-heat treated emulsions were in a close agreement with the previous results of Tikekar, Pan et al. 2013 [129] for silica nano-particle stabilized emulsions with ~ 40%.
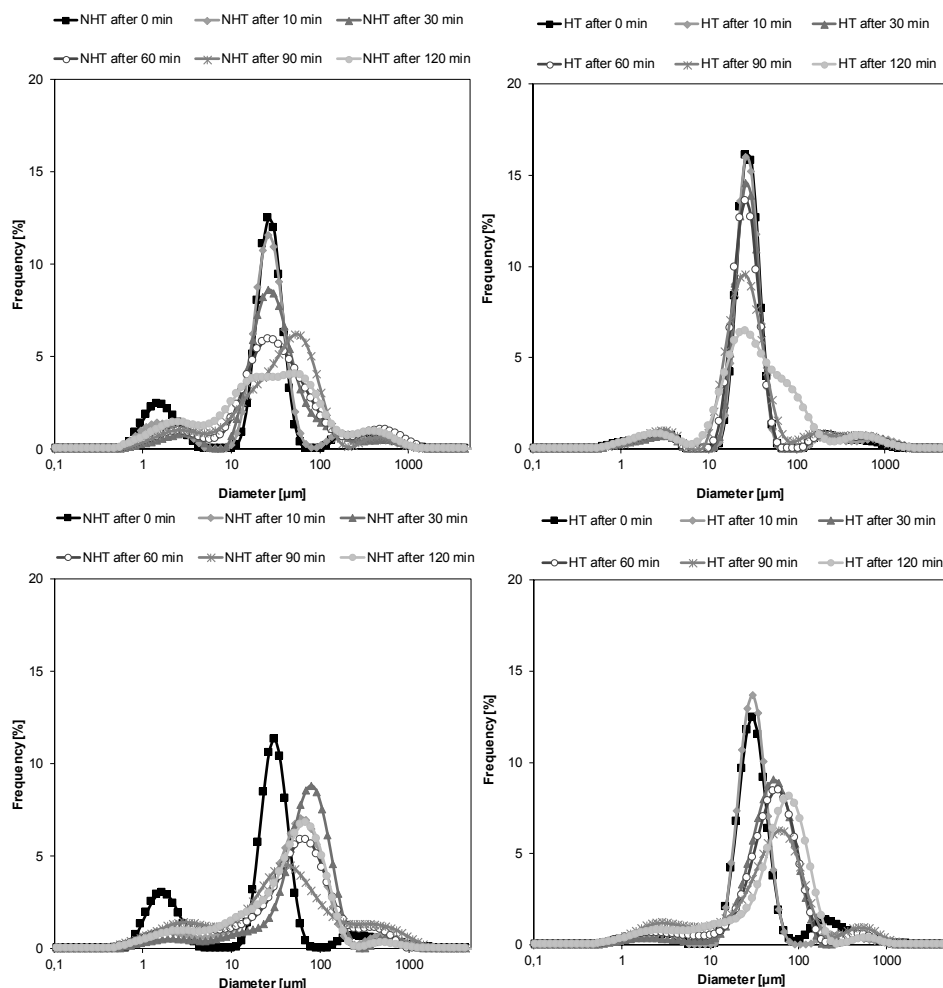
Fig. 29.
Particle size distribution ($D_{4,3}$) for non-heat treated NHT and heat treated (HT) before and after *in vitro* intestinal digestion without addition of bile salts (top row) with addition of bile salts (bottom row), n=6. Data from Marefati, Bertrand et al. 2017 [51].

In the final study of section III, the rate and extent of lipolysis of starch Pickering emulsions was compared to a protein stabilized emulsions using the pH-stat method. Here, the effect of heat treatment and subsequent storage of heat treated emulsions, on the starch barrier was also considered.

Characterization of emulsions before digestion: The particle size distribution of the non-heated samples had a mode of $D_{4,3}$ of 35.4 ± 1.3 μm which was significantly different from the heat treated and heat treated and stored emulsion with 38.9 ± 1.3 μm and 38.8 ± 0.4 μm. As the protein stabilized emulsions with sodium caseinate

(Na-Cas) were formulated in a way to have similar sizes as of the Pickering emulsions the mode of $D_{4,3}$ these emulsions were 37.0 ± 0.1 μm (Fig. 30).
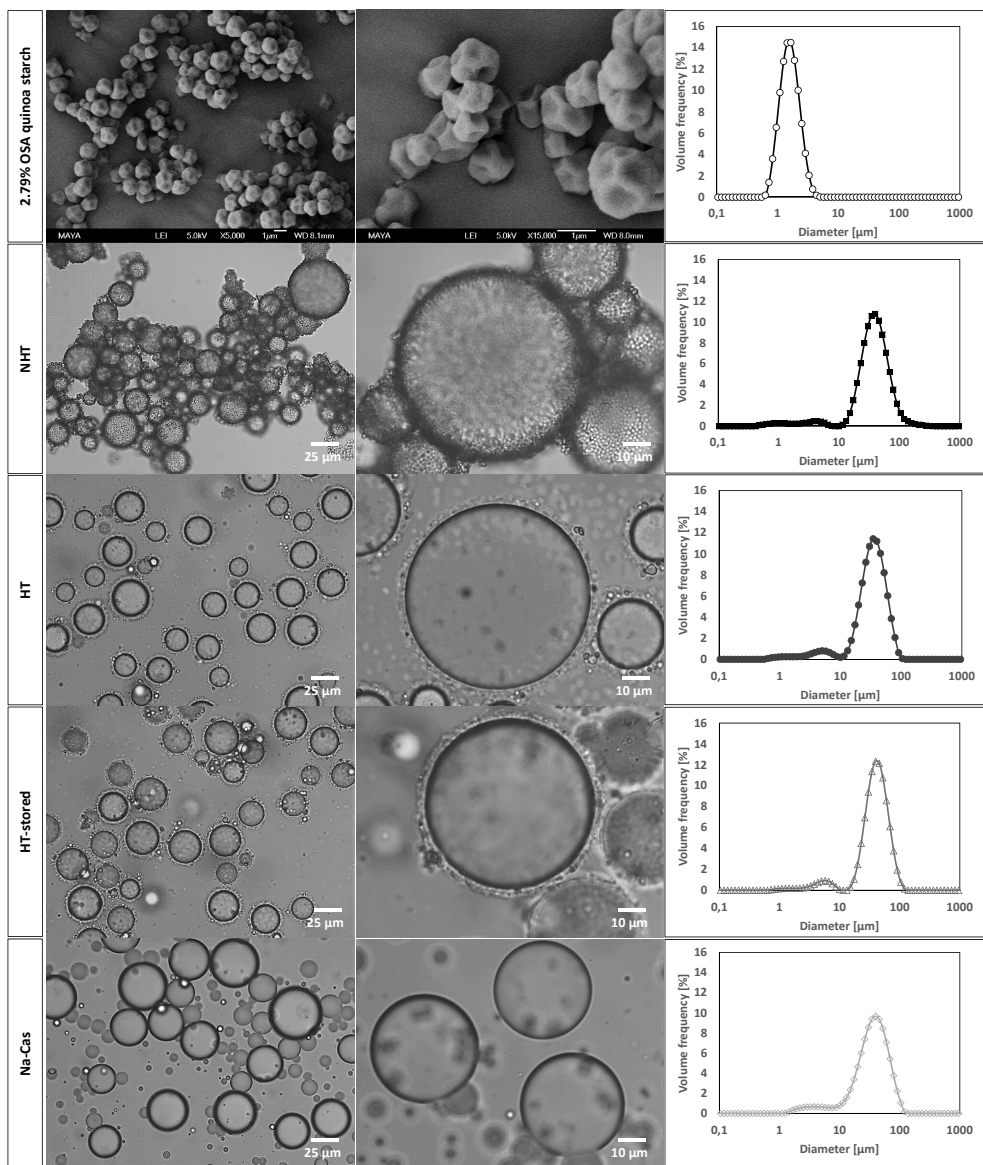
Fig. 29.
SEM micrographs of quinoa starch granules and particle size distribution ($D_{4,3}$) of starch granules (top row), light microscopy micrographs and particle size distribution of non-heat treated emulsions (2nd row), light microscopy micrographs and particle size distribution of heat treated emulsions (3rd row), light microscopy micrographs and particle size distribution of heat treated and stored emulsions, (4th row) and light microscopy micrographs and particle size distribution of protein stabilized emulsions (bottom row), n=6.

The effect of bile salts and mixing: The particle size distribution of the non-heat treated control samples showed that although there was an increase in the amount of free starch, the addition of bile salts and mixing did not affect the mode of $D_{4,3}$ during the first hour. However, after 2 and 3 h, there were more particle displacement from the oil-water interface which resulted in lower surface coverage that may have led to some degree coalescence.

Simulated in vitro lipolysis: The rate and the extent of the lipolysis from the amount of free fatty acids released (FFA%) was evaluated during 3 h (Fig. 31, left). The results showed that the rate of lipolysis was lower for the all of the Pickering-based emulsions compared to protein stabilized emulsion. Moreover, the lipolysis rate during the first 5 min was lower for the non-heat treated samples compared to heat treated and heat treated and stored samples. However, this difference was not statistically significant until after 15 min. With respect to the extent of the release of the FFA during 180 min, the lowest rate was observed for non-heat treated samples with $25.1\% \pm 1.2$ followed by $29.0\% \pm 0.8$ and $28.9\% \pm 0.3$ for heat treated and heat treated and stored emulsions and lastly $32.2\% \pm 0.2$ for protein stabilized emulsions (Fig. 31, left). This relatively low FFA release during the simulated lipolysis, is due to the large droplet sizes of these emulsions. Mcclements and Li, 2010 [152] showed that the extent of FFA release of triglyceride with medium chain fatty acids (MCT) decreased from ~ 70% to ~ 45% when the droplet size increased from 178 nm to 756 nm. Similar observations about the effect of the size have been made in other works [27, 153, 154]. In addition, accumulation of the digestive products (FFA and mono glycerides) at the oil-water interfaces is proposed to inhibit lipase activity [155]. Mcclements and Li, 2010 [152] have also shown that the concentration of the lipids in the digestion cell has an inverse effect on the rate and extent of digestion and when the concentration of the oil increased from 1% to 2.5% the amount of FFA released decreased by ~ 10%. Since lipolysis is an interfacial phenomenon and in order to have a fair comparison, the FFA released was normalized against the specific surface area of the emulsion droplets (Fig. 31, right). The results showed that non-heat treated samples presented a lower rate and extent of lipolysis compared to the other samples. While heat treated and stored samples had a similar rate and extent of lipolysis per unit area to that of the Na-Cas emulsions. This could be attributed to the fact that heat treatment causes the starch granules to lose their crystallinity and swell which in turn make them more accessible to amylase present in the pancreatin mix used. As a result, a faster elimination of the starch barrier by amylase may have resulted in higher lipolytic activity. It was also shown that unlike expectations, in heat treated and stored samples the expected increase in crystallinity did not emerge in slowed digestion of the starch barrier. Finally, protein stabilized emulsions showed the lowest resistance to lipolysis.
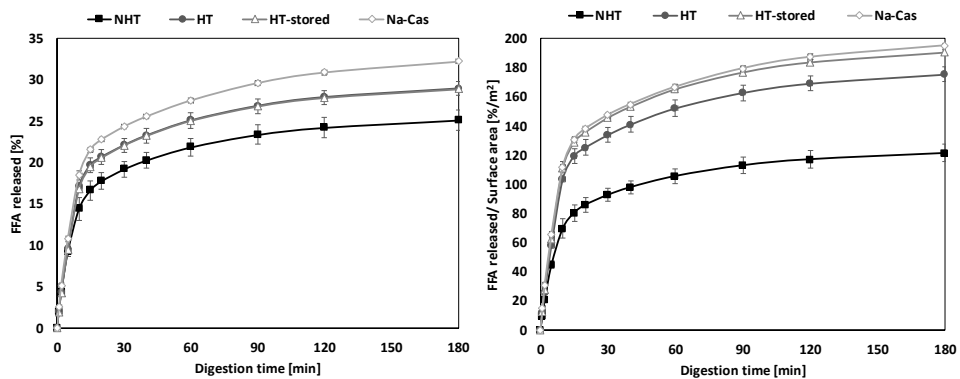
Fig. 31.
The amount of FFA% released for different types of emulsions during a 3 h *in vitro* intestinal lipolysis (left), the amount of FFA released for different types of emulsions during a 3 h *in vitro* intestinal lipolysis normalized against the surface area, error bars represent standard deviation of the mean, n=3.

Conclusions

The results of this research demonstrated the feasibility of development of Pickering emulsions from small granular starches in native and modified forms. Moreover, it was shown that, gelatinization of the starch at the oil-water interface, not only contributes to the general structural stability of emulsions but also it contributes to the perseverance of structure and encapsulation stability during processing and *in vitro* digestion. The result of this work revealed the possibility of application such as encapsulated emulsions in liquid and solid forms for development of functional foods for controlled release or targeted delivery of bioactive substances.

Section I (papers I and II)

In the first section the emulsifying capacity of small granular starches with respect to starch type and the level of hydrophobic modification was evaluated. From these studies, it could be concluded that starch granules from the small granule botanical origins have the capacity to stabilize emulsions. Moreover, starch granules form quinoa showed a good emulsifying capacity and especially better emulsifying capacity in lower starch concentrations or lower modification levels. Native starches from quinoa and rice were also able to stabilize emulsion droplets. This could be due to the higher protein content of quinoa and rice starch granules which can contribute to the hydrophobicity. It was also shown that there was a positive correlation between the level of modification and the stability of the emulsions for different starches used. In addition, in the case of modified starches, when enough starch was available (concentrations > 400 mg/mL oil), the smaller size of amaranth granules seemed to be optimum with respect to making small emulsion droplets.

The emulsion index indicated that there was a positive correlation between the stability of the emulsions and the modification level in both initial and after the accelerated stability test. Moreover, starches from quinoa showed higher stability in both initial state and after accelerated stability test at the same level of modification. Lastly, the bimodal nature of rice starches could be the subject of future studies for exploring the effect of size on the emulsifying capacity of the starch granules from the same botanical source.

Section II (papers III and IV)

The second section considered the process and encapsulation stability of single and double emulsions towards the freezing and freeze-drying process. From this work, it was proven it was possible to create re-dispersible, food-grade, oil-filled powders from OSA modified starch Pickering emulsions by freeze-drying without addition of hydrophobic carriers with 80% oil content. Pickering emulsions with the solid oil as dispersed phase showed a higher stability towards freezing and freeze-drying processes. *In situ* heat treatment leading to the gelatinization of the starch layer enhanced the stability of liquid oil emulsions and prevented from destabilization during freeze-drying. With regard to the stability against freezing and freeze-drying, starch granule-based Pickering emulsions, could have versatile application in food, pharmaceutical and cosmetic emulsions.

Furthermore, it was also demonstrated to be possible to create powders from starch stabilized Pickering double emulsions (W/O/W) with high encapsulation stability using solid and liquid oils as the intermediate phase with high encapsulation stability (> 97.3%) and high oil content (~ 70%). The stability of solid oil-based emulsions during freezing and freeze-drying was found to be higher compared to the liquid oil-based emulsions. The lower stability of the liquid oil-based emulsions was attributed to higher susceptibility of the emulsions towards the crystallization of the external aqueous phase. Finally, the stability of the liquid oil-based double emulsions could be enhanced by application of heat treatment which results in formation of gelatinized barrier at the oil-water interface that can protect the oil droplets against coalescence. This system can be applied for versatile food and pharmaceutical formulations where encapsulation of an internal aqueous phase is desirable.

Section II (papers V and VI)

Lastly, in the third section, the physiological and encapsulation stabilities of the Pickering emulsions and the effects of heat treatments and cold storage were evaluated during *in vitro* digestion. It was verified that starch granules could successfully encapsulate curcumin with high encapsulation efficiency (~ 80%) and high storage stability (with 38.3% ± 0.6 for non-heat treated and 73.2% ± 1.6 for heat treated emulsions). It was also shown that emulsions based on starch granule stabilization could successfully preserve curcumin during *in vitro* oral (69.6% vs. 95.3% for non-heat treated and heat treated samples respectively), gastric (86.2% vs. 82.4% for non-heat treated and heat treated samples respectively) and intestinal (40.2% vs. 86.3% for non-heat treated and heat treated samples respectively) digestion. The significantly higher encapsulation stability in the heat treated

emulsions during storage and simulated *in vitro* conditions is due to formation of the fused barrier at the oil-water interface which can preserve the encapsulated curcumin.

Pickering emulsions stabilized by quinoa starch granules, irrespective of heat treatment or storage had a lower rate and extent of lipolysis compared to protein stabilized emulsions. In addition, compared to heat treated samples, intact or non-heat treated emulsions presented lower rate and extent of lipolysis. Thus, in conclusion, the results of this study demonstrate the possibility of development of functional foods in the form of emulsions based on starch granule stabilized Pickering emulsions for controlled and targeted delivery of bioactive compounds.

Future perspectives

The work presented in this thesis has investigated several aspects of Pickering emulsions stabilized by starch granules from physico-chemical characterization of starch granules, to process, encapsulation and physiological stability of emulsions stabilized by starch granules. However, there are some questions which remain to be answered, and are left for future works. The main recommendations which could be worthy of investigation are summarized as follows:

- Physico-chemical characterization of starch granules:
  - o Since the higher emulsifying capacity of quinoa starch granules were attributed to the higher protein level, the development of a standard isolation technique for all different types of starches that result in the similar protein content is suggested.
  - o Isolation and characterization of proteins from quinoa starch that may be the determining factor in the higher emulsifying capacity of quinoa starch is a recommended topic for further study.
  - o Since OSA modification may alter the crystallinity of starch granules, a more in detailed study of crystallinity of modified starch granules is proposed.
- Evaluation of emulsifying properties of starch granules
  - o The effect of different modification levels on rheological properties of both starch dispersions and emulsions to evaluate the effect of the modification level on aggregation and inter-droplet interactions can be interesting for a future study.
- Processing
  - o Feasibility of utilization of other drying techniques such as spray-drying can be a subject to a future study.
- Encapsulation and *in vitro* digestion
  - o The digestive fate of encapsulated bioactive compounds *in vivo* can verify the presented results in this PhD work and therefore could be an interesting topic for the future.
  - o Feasibility of encapsulation of health promoting microorganisms (i.e. probiotics) with respect to initial encapsulation efficiency (*EE%*) and encapsulation stability (*ES%*) during storage and

processing, as well as the physiological fate of those probiotics could be evaluated *in vitro* and *in vivo*.

- o The effect of *in vitro* intestinal lipolysis for emulsions obtained with different types of starches and different levels of modification.
- o The effect of *in vitro* intestinal lipolysis on Pickering emulsions stabilized with starch granules composed of different triglyceride oils of different chain length and different concentration of oils.
- o The effect of *in vitro* intestinal lipolysis on Pickering emulsions stabilized with starch granules with different concentrations of starch that results in different droplet sizes can also be an interesting topic for a future work.

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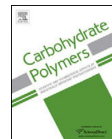
Paper I

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Pickering emulsifiers based on hydrophobically modified small granular starches – Part I: Manufacturing and physico-chemical characterization

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ARTICLE INFO

ABSTRACT

Small granular starches from rice, quinoa and amaranth were hydrophobized by esterification with octenyl succinic anhydride (OSA) in an aqueous alkaline slurry to obtain series of modified starches at defined intervals (i.e. 0.6, 1.2, 1.8, 2.4, 3.0%). The physical and the physico-chemical properties of the starch particles were characterized by proximate analysis including protein level, amylose level and dry matter. The shape and size of the starch granules were characterized by scanning electron microscopy and light scattering. The gelatinization properties were characterized by differential scanning calorimetry. The degree of modification was determined by titration with NaOH. With regard to the emulsion formulation and in order to assess the emulsifying capacity of the small granular starches, the effect of starch type, degree of modification and starch concentration on the resulting emulsion droplet size were evaluated by light scattering and optical microscopy.

Emulsifying properties were found to depend on the degree of substitution, size of the granules and the starch to oil ratio of the formulation. Quinoa starch granules, in general, had the best emulsifying capacity followed by amaranth and rice. However, in higher starch concentrations (>400 mg/mL oil) and adequate levels of OSA (3.0%) amaranth performed best, having the smallest size of starches studied.

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1. Introduction

Many food, pharmaceutical, and cosmetic products are formulations based on emulsions. Emulsions are mixtures of two immiscible phases where one phase is dispersed in the other in the form of small droplets. They can be water continuous in the case of oil-in-water emulsions (o/w) or oil continuous in water-in-oil emulsions (w/o). Due to the large interfacial area between the finely dispersed phase droplets and the continuous phase, emulsions are generally not thermodynamically stable, as there is a reduction in free energy if dispersed phase droplets coalesce, thereby minimizing the interfacial area. To prevent coalescence and stabilize the droplets, emulsifiers are used which act by decreasing the interfacial tension between the phases, increasing the steric hindrances and/or electrostatic repulsion between the droplets (Bergenstahl,

2015). Typical examples include small molecular surfactants, proteins and hydrocolloids. In addition to low molecular mass and polymeric emulsifiers, particles can also be used to achieve droplet stabilization. Emulsions stabilized by particles are known as Pickering emulsions named after Pickering (1907). Pickering particles achieve droplet stabilization by dual wettability towards both phases. The adsorbed particles provide a steric barrier amongst the newly formed droplets which result in prevention of coalescence (Sjöö, Rayner, & Wahlgren, 2015). Compared to other stabilization mechanisms, Pickering emulsions are usually more stable against coalescence and Ostwald ripening (Aveyard, Binks, & Clint, 2003; Yusoff & Murray, 2011). This higher stability is due to higher energy of detachment of the particles thanks to the large particle sizes >10 nm). Once these large particles are adsorbed at the oil-water interface, the energy needed to remove them is several thousand kT as long as the contact angle is not too close to 0° or 180°. As a result, presence of thick and irreversibly adsorbed barrier provides highly stable emulsions (Yusoff & Murray 2011; Rayner et al.,

2014). The energy of detachment per particle can be calculated by the following equation:

$$\Delta G = r^2 \pi \gamma (1 - |\cos\theta|)^2 \qquad (1)$$

Where ΔG is the energy of detachment, r is the particle radius (m), γ is the interfacial tension between oil and water (N/m), and θ is the particle-oil-water contact angle measured through the water phase (Berton-Carabin & Schroën, 2015).

In recent years there has been a push towards "green label" and "clean label" in many industries, especially in food, cosmetics and consumer products (Frost, 2013). This trend has two main drivers, centering on the increase in consumer concerns for health and the environment. With respect to health, low molecular weight emulsifiers and surfactants have come under scrutiny in topical cream due to skin irritation (Wahlgren, Engblom, Sjoo, & Rayner, 2013), as well as in food where there is a proposed negative impact on gut health and inflammation (Chassaing et al., 2015). Furthermore, many surfactants have an unknown fate in the aquatic environment. For these reasons, as well as an increasing interest in using ingredients that are biodegradable, based on renewable resources, and perceived as being natural and such, Pickering stabilizers based on biomass have been receiving increased interest. For recent and comprehensive reviews the interested reader is directed to Berton-Carabin and Schroën (2015), Dickinson (2010), Rayner (2015), Rayner et al. (2014). Examples of Pickering particles include clay, silica, alumina, titanium oxide, latex and starch (Ashby & Binks, 2000; Binks & Lumsdon, 1999; Binks & Lumsdon, 2000; Binks & Lumsdon, 2001; Chen et al., 2011; Stiller et al., 2004; Timgren, Rayner, Sjöö, & Dejmek, 2011; Yusoff & Murray, 2011).

Starch is an interesting material as it is one of our major food constituents, a carbohydrate produced by most green plants as an energy store consisting of a large number of glucose units joined by glycosidic bonds. Starch consists of two different polymers, amylose and amylopectin. Amylose is a mainly linear polymer consisting of units of α-D-glucopyranose with (1–4) glycosidic links and a few branches attached in (1–6) position. Amylopectin has a similar structure as amylose except that the polymer is more branched, and hence it is much larger. In native starches, amylose and amylopectin molecules, along with small amounts of water, are densely packed into partially crystalline, water-insoluble granules (Eliasson, 2004).

Although the emulsifying capacities of native starch granules have been observed to be low (Li, Li, Sun, & Yang, 2013; Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013), the hydrophobicity can be increased by chemical or physical modification. Starch can be chemically modified by treatment with different alkenyl succinyl anhydrides, for example 2-octen-L-yl succinic anhydride (OSA). The substitution with OSA can occur at the OH- group of carbon 2, 3 and 6 in the glucose molecule. The most widely described synthesis pathway is a reaction in aqueous medium under mild alkaline conditions with starch in its granular form (Trubiano, 1986). In addition, modified starch produced through esterification is tasteless, colorless, odorless, inexpensive, non-allergic and approved food additive (E1450) and excipient with degree of modification lower than 3% based on the dry weight of starch, with no limit on application (Timgren et al., 2011).

Generating Pickering particles based on starch can be achieved in 3 main ways. By dissolving and precipitation, size reduction of large granules by physical or chemical means, or isolating native starch granules from botanical sources which have small granules (Saari, Heravifar, Rayner, Wahlgren, & Sjöö, 2016). Small particles (or granules) are of interest as the larger the particles, the larger the mass required to stabilize droplets of a given size. The sizes of starch granules are intrinsic to the botanical source they are isolated from. Starches have been classified in large granule (30–100 μm) including in tubers such as potato and canna; medium granule (5–30 μm)

including starches such as tapioca, barley, maize, sorghum, small granule (2–10 μm) including rice, oat, buckwheat, and extremely small starch granules (0.3–2 μm) such as quinoa, amaranth, cow cockle and pig weed. Some types of starch have bimodal sized starch granules including some species of rice, barley, sorghum and wheat (French, 1973; Hall & Sayre, 1970; Hall & Sayre, 1971; Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994; Lindeboom, Chang, & Tyler, 2004; Pérez & Bertoft, 2010).

In this work 3 small granule starches of different botanical origin (i.e. rice, quinoa, amaranth) have been considered as potential candidates as Pickering emulsifiers.

Rice (*Oryza Sativa*) is a cereal grain which is the staple food for Asian countries (Singh, Okadome, Toyoshima, Isobe, & Ohtsub, 2000). Rice has small and polygonal granule between 3 and 9 μm (Juliano, 1992; Wani et al., 2012). The total starch content of rice grain is 78–83% (Ahmed et al., 2015; Yadav, Sharma, & Yadav, 2010; Tran et al., 2011). The reported amylose content ranges from 0.0–33.0% and the gelatinization temperature range (T_o-T_c) of 55–84.6 °C where temperature and T_o is the gelatinization onset temperature T_c is the gelatinization conclusion (Juliano, 1992; Singh, Kaur, Sandhu, Kaur, & Nishinari, 2006).

Quinoa (*Chenopodium quinoa Willd*) is a native pseudocereal of Andes in South America which has been cultivated for 3000–4000 years and constituted an important component in the diet of the Incan civilization (Li, Wang, & Zhu, 2016; Lindeboom, Chang, Falk, & Tyler, 2005). Recently quinoa has attracted interest due to its unique characteristics including: high nutritional value due to the quality of protein and fatty acids and its ability to grow under extreme conditions such as salinity, acidity, drought, flooding and frost (Gonzalez, Roldan, Gallardo, Escudero, & Prado, 1989; Li et al., 2016; Przybylski, Chauhan, & Eskin, 1994). Starch is a major component of quinoa seed which comprises approximately 55–60% of the dry matter (Mundigler, 1998; Lindeboom et al., 2005). The starch is present in the form of small polygonal granules in diameter 0.6–3 μm with mean diameter of 1.5 μm (Atwell, Patrick, Johnson, & Glass, 1983; Lorenz, 1990; Lindeboom et al., 2005; Tang, Watanabe, & Mitsunaga, 2002). The amylose content of quinoa is reported to vary between 3.5–27% (Inouchi et al., 1999; Lindeboom et al., 2005; Qian & Kuhn, 1999; Tang et al., 2002) and the gelatinization temperature ranges from 50 to 74.9 °C (Atwell et al., 1983; Li et al., 2016; Qian & Kuhn, 1999).

Amaranth (*Amaranthus*) is another ancient pseudocereal domesticated in South America (Mundigler, 1998) which currently constituents a large part of diet in Asia and Africa in addition to South America (Qian & Kuhn, 1999). The total starch content has been reported to be 67.2% (Mundigler, 1998). The starch has small polygonal granules with a mean diameter around 0.8–1.3 μm among different amaranth cultivars (Bhosale & Singhal, 2006; Kong, Bao, & Corke, 2009). The amylose content of amaranth has reported to be in the range of 0–28% (Inouchi et al., 1999; Kong et al., 2009; Qian & Kuhn, 1999) and the gelatinization temperature ranges from 63.4–86.9 °C (Inouchi et al., 1999; Kong et al., 2009; Qian & Kuhn, 1999).

Several reports have been published on emulsifying properties of different types of OSA modified starch granules (Timgren et al., 2013; Simsek, Ovando-Martinez, Marefati, Sjöö, & Rayner, 2015; Yusoff & Murray, 2011). In addition, there has been a considerable amount of work on development, characterization and physical and physiological stability of emulsions stabilized by OSA modified quinoa, maize, tapioca, and rice starch granules (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013; Marefati, Bertrand, Sjöö, Dejmek, & Rayner, 2017; Marku, Wahlgren, Rayner, Sjöö, & Timgren, 2012; Matos, Timgren, Sjöö, Dejmek, & Rayner, 2013; Rayner, Sjöö, Timgren, & Dejmek, 2012a; Simsek et al., 2015; Song, Pei, Zhu, Fu, & Ren, 2014; Timgren et al., 2011; Timgren et al., 2013; Yusoff and Murray, 2011). Though, a comparison of small

starch granules with varying OSA level in incremental steps has not been investigated. In addition, although Bhosale and Singhal (2006) have carried out some research on manufacturing and characterization of OSA modified amaranth where they investigated the emulsification capacity of those starches in molecular form, to the best of authors' knowledge, OSA modified amaranth starch granules have not previously been utilized to stabilize Pickering type emulsions. In addition, there are several studies on physicochemical characterization of rice starch, however, application of different conditions in them makes a direct comparison difficult. Therefore, we investigated the three different starches in the same conditions to be able to compare their performance as Pickering emulsifiers as well as document the properties of the granules used.

2. Materials & methods

2.1. Isolation of starch granules

2.1.1. Rice

Rice starch was isolated in a semitechnical scale. 8 kg of rice were steeped in 16 kg of a 0.4% NaOH-solution for 16 h at 4 °C to soften the endosperm and enhance protein solubilization. Then the supernatant was separated, 30 kg of fresh water added and the rice wet milled with a colloid-mill (150 μm). Afterwards, the protein and fiber were separated from the starch by repeated centrifugation (decanter) and wet-sieving (vibration sieve) steps. Finally, the starch suspension was neutralized and spray dried using a spray dryer (type Minor Production, Niro A/S, Denmark) at an inlet and outlet temperature of 180 °C and 80 °C, respectively. At this point it should be noted that spray drying is widely used in the food industry as a gentle drying process which is even used for heat sensitive enzymes (You et al., 2017). In this work, despite the high inlet and outlet temperatures of the air in the spray dyer, moist droplets never reach the air temperature and experience a much lower temperature than the air during drying that is the wet bulb temperature due to evaporative cooling during the constant rate period, Singh and Heldman (2001) and thus gelatinization is avoided. Only at the end of the drying time (3–5 s) does the temperature begin to rise in the now almost dry particles. The temperature within the water droplets and thus the starch particles during the drying process is always below the outlet temperature of 80 °C and after separation in the cyclone the temperature decreases rapidly to about 50 °C (below the peak temperature of gelatinization measured in excess water). Furthermore the peak temperature of gelatinization depends on the mass fraction of water in relation to starch (BeMiller & Whistler, 2009). For example, the dried starch with a mass fraction of water of 0.12 gelatinizes at temperatures above 150 °C. Since the mass fraction of water during spray drying is quickly reduced from 0.75 to 0.12 no gelatinization occurs. The absence of gelatinization was verified by the SEM photographs of the 3 starches and DSC thermographs described in Sections 2.4.2 and 2.4.3 below.

2.1.2. Quinoa/Amaranth

Quinoa and amaranth starch were separated according to the semitechnical process of Wilhelm et al. (1998). Raw materials were *Amaranthus hypochondriacus* from Mexico and *Chenopodium quinoa* from Bolivia. All raw materials were procured as import products. In brief, the grains were dry-milled and the flour was suspended in water and mixed. Thereafter, for improvement of the protein separation from the starch suspension, the slurry went through enzymatic hydrolysis using a commercially available enzyme (Alcalase 2.4 L FG, Novozymes A/S, Bagsvaerd, Denmark) and then mixed with a screw loop mixer (type 50, DMT, Germany) and a high-pressure homogenizer (type 317HD4-3TBS, APV Gaulin, Germany). The starch and fiber were then separated by sieving. The

proteins were separated from the starch in two steps, first using a decanter and then the remaining protein residues were manually removed by centrifugation. Finally, the starch was dried using a spray dryer (type Minor Production, Niro A/S, Denmark) at an input and output temperature of 180 °C to 80 °C respectively.

2.2. OSA modification of starch granules

2.2.1. OSA modification reaction

50.0 g of the starch was suspended in 200.0 g distilled water. The pH was adjusted to 8.2-8.4 by titration with a 0.5 N NaOH solution, and maintained constant during the reaction. Then a solution of OSA in acetone (100 mg OSA/mL solution) was added within 5–40 min and the temperature was kept constant (32.0 ± 0.5 °C). The total amount of added OSA was varied (0.6, 1.2, 1.8, 2.4 and 3.0% OSA) in relation to the dry matter of the starches. The reaction finished after 90–120 min. When the pH-value was constant at 8.3, no further addition of NaOH solution was necessary.

2.2.2. Isolation of the product

To the reaction slurry 190 g distilled water was added and the slurry was centrifuged (7 min, 5000 rpm). The sediment (89–92 g) was suspended again in 350 g distilled water and centrifuged. The second sediment was then suspended in 300 mL acetone stirred for 5 min and again centrifuged. The third sediment was first dried at room temperature over the night and then in a laboratory convection dryer, WTB binder (Type MB6, Binder GmbH, Germany) at 30 °C for 4 h. At this conditions the acetone was quantitatively evaporated and the starch was dried below its equilibrium water content. Finally, in order for the starches to reach their equilibrium moisture content, samples conditioned at room temperature for 2 days. All yields varied between 50.1 and 51.1 g.

2.3. Determination of the degree of modification

2000 ± 0.5 mg of the modified starches were weighed in a 100 mL Erlenmeyer flask. Then 60 mL of distilled water was added. The suspension was stirred with a magnetic bar and the pH-value (about 8.4) was adjusted exactly to pH = 7.0 ± 0.1 by addition of 0.1 N H_2SO_4 (Quinoa: 0.20–0.45 mL; Amaranth: 0.06–0.36 mL; Rice: 0.27–0.46 mL) until the pH-value was constant at the end of addition for at least 3 min. Then 20.00 ± 0.03 mL of a 0.1 N NaOH solution were added and the Erlenmeyer flask was quickly closed with a stopper to minimize the adsorption of carbon dioxide from the air. The suspension was then stirred in a water bath at 35.0 ± 0.5 °C for 24 h. The minimum time of 24 h required for a quantitative hydrolysis of the ester was determined by kinetic studies. After 24 h, the suspension was cooled to room temperature and the excess of 0.1 N NaOH solution was back titrated to pH = 7.0 ± 0.1 with an 0.1 N H_2SO_4 solution and a pH-meter. All samples were investigated in triplicate. The blank volume of 0.1 N H_2SO_4 was determined by a linear or quadratic extrapolation of the mean values of the titration function. The blank values were 19.786 mL, 19.712 mL and 19.836 mL for rice, quinoa and amaranth starch, respectively.

2.4. Characterization of starch granules

2.4.1. Proximate analysis (protein, amylose/amylopectin content, dry matter)

The protein level of quinoa starch granules was determined using a nitrogen/protein analyzer (Flash EA 1112 Series, Thermo Scientific, USA). The amylose level (%, w/w) determined using a lectin Concanavalin A assay (Megazyme International, Ireland) which is a modified version of the method developed by (Yun & Matheson, 1990). The dry matter of the isolated and OSA-modified

starches was determined according to a modified version of previous method (Amtliche Sammlung von Untersuchungsverfahren nach, 2008). Approximately 1 g of each sample was weighed with an accuracy of ± 0.2 mg in a dry matter glass and dried at $130 \pm 1\,^\circ C$ for 90 min in a non-convection oven. The dry matter glass was then closed and cooled for 45 min in a desiccator to room temperature and weighed.

2.4.2. Scanning electron microscopy

Starch granules were characterized by scanning electron microscopy (SEM). The dried samples were coated with gold and examined under SEM (field emission SEM, JSM-6700F, JEOL, Japan) operated at 5 kV with a working distance of 8 mm. Lower detection imaging mode (LEI) was used to give clear three-dimensional images of the sample surface. The LEI detector combines both signals secondary and back scattered electrons during operation.

2.4.3. Characterization of gelatinization properties of starch

The gelatinization properties of starch granules were analyzed using a differential scanning calorimeter (DSC, Seiko 6200, Seiko instruments Inc., Japan), calibrated with indium ($M_p = 156.6\,^\circ C$). Starch dispersions were prepared and weighed into coated aluminum pans (TA Instruments, USA) at a ratio of 1:10 and gelatinization transition enthalpy (ΔH, J/g dry matter), gelatinization onset temperature ($^\circ C$), gelatinization peak temperature ($^\circ C$) and gelatinization conclusion temperature ($^\circ C$) were determined. The scanning rate was $10\,^\circ C$/min from 10 to $120\,^\circ C$.

2.4.4. Particle size of starch granules

The particle size distribution of starch granules was determined using a laser diffraction particle size analyzer (Mastersizer 2000 Ver. 5.60, Malvern, Worcestershire UK). 70 mg of starch was dispersed in a 7 mL of phosphate buffer (95%, 5 mM, pH 7, 0.2 M NaCl) using a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s. The sample was added to the flow system containing MilliQ-water and was pumped through the optical chamber at a pump velocity of 2000 rpm. The refractive index (RI) of the starch was set to 1.54 (Bromley & Hopkinson, 2002) and the RI of the continuous phase was set to 1.33 (water) and the obscuration was between 10 and 20%. This is referred as starch buffer mix (SBM) throughout the text.

2.5. Formulations and emulsification

2.5.1. Formulation with varying levels of OSA

5% v/v oil-in-water starch granules stabilized emulsions were prepared using Miglyol 812 (Caesar & Loretz GmbH, Germany) as dispersed phase, phosphate buffer (95%, 5 mM, pH 7, 0.2 M NaCl) as continuous phase and starch granules from 3 different botanical origins (i.e. rice, quinoa and amaranth) in native and different modification levels from 0.6–3.0% OSA to stabilize the emulsions.

7 mL emulsions were prepared in a glass test tube using 5% v/v of the dispersed phase and 95% of aqueous phase. 200 mg of starch/mL oil was used to stabilize the emulsions. The emulsions were homogenized using a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s. The samples were prepared in duplicates. The appearance of these emulsions are presented in Fig. 3. Thereafter the emulsions were characterized as described below in Section 2.6.

2.5.2. Formulation with varying levels of starch

5% v/v oil-in-water starch granules stabilized emulsions were prepared using Miglyol 812 (Caesar & Loretz GmbH, Germany) as dispersed phase, phosphate buffer (95%, 5 mM, pH 7, 0.2 M NaCl)

and starch granules from the 3 different botanical origin (i.e. rice, quinoa and amaranth) with 3.0% OSA modification to stabilize the emulsions (this is described in Section 2.4.4).

7 mL of emulsions were prepared in a glass test tube using 5% v/v of the dispersed phase and 95% of aqueous phase. Different amounts of starch namely 50, 100, 200, 400, 800 mg/mL oil were added to stabilize the emulsions. The emulsions and the starch dispersions were homogenized using a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s. Thereafter, the emulsions were characterized as described below in Section 2.6.

2.6. Emulsion characterization

2.6.1. Particle size distributions of starch pickering emulsions

The particle size distributions of the starch granule stabilized emulsions were characterized with a laser diffraction particle size analyzer, Mastersizer 2000 (Malvern Instruments, UK). Each emulsion was added to the flow system (Hydro SM small volume wet dispersion unit) containing MilliQ-water and was then pumped through the optical chamber where it was measured. The refractive index of starch particles was set to 1.54 (Bromley & Hopkinson, 2002) and the refractive index of the continuous phase was set to 1.33 which is the refractive index of the water and the obscuration was between 10 and 20%. For each emulsion sample added to the flow system three measurements were performed, and all emulsions were prepared in duplicates and analyzed 3 times.

2.6.2. Microscopy

The emulsions were characterized by light microscopy using a camera (DFK 41AF02, The Imaging Source, Germany) that was attached to a light microscope (Olympus BX50, Japan) and both were connected to a computer. The emulsions were diluted 5 times with MilliQ water and then one drop was placed on a glass microscopic slide. In order to prevent deformation of droplets no cover glass was used. The microscopic images were taken using objective magnifications of $20\times$ and $50\times$.

3. Results and discussion

3.1. Granules

3.1.1. Proximate analysis and degree of modification

The protein level of starches was highest for quinoa 0.538–0.687 followed by rice 0.271–0.328% and the lowest was amaranth with 0.032–0.112% (Table 1). Moreover, the protein content of native samples showed higher values which may be due to solubilization of proteins during modification as a result of exposure to alkali solution or being washed away by acetone at the end of modification process which could also result in further removal of proteins.

The amylose content of starches was lower for rice $4.43\% \pm 0.78$ and somewhat similar for quinoa and amaranth with $20.95\% \pm 0.45$ and $20.90\% \pm 0.98$ which fall within the range of previous results in the literature (Inouchi et al., 1999; Juliano, 1992; Kong et al., 2009; Lindeboom et al., 2005; Qian & Kuhn, 1999; Singh et al., 2006; Tanget al., 2002).

3.1.2. Gelatinization properties of starch

The gelatinization temperature range (T_o–T_c) of different starches was the highest for rice 72.2–$85.0\,^\circ C$ which was similar to the previously reported values in the literature and lower for quinoa and amaranth with 64.1–$75.5\,^\circ C$ and 63.8–$76.8\,^\circ C$ respectively which again was similar previously reported values (Atwell et al., 1983; Inouchi et al., 1999; Kong et al., 2009; Li et al., 2016; Qian & Kuhn, 1999; Singh et al., 2006) (Fig. 1).

Table 1
Proximate Analysis and OSA levels.

Rice

Sample	Dry matter (%)	OSA (%)	Degree of substitution (DS)	Reaction efficiency	Protein content (%)
R-Native	89.3 ± 0.00	0	0	–	0.328 ± 0.000
R-OSA-0.6	87.5 ± 0.12	0.46 ± 0.01	0.0036	0.783	0.278 ± 0.000
R-OSA-1.2	87.4 ± 0.00	0.97 ± 0.03	0.0077	0.840	0.274 ± 0.005
R-OSA-1.8	87.7 ± 0.04	1.40 ± 0.05	0.0108	0.808	0.272 ± 0.005
R-OSA-2.4	87.7 ± 0.11	1.90 ± 0.05	0.0149	0.828	0.274 ± 0.001
R-OSA-3.0	87.5 ± 0.01	2.36 ± 0.02	0.0186	0.827	0.271 ± 0.001

Quinoa

Sample	Dry matter (%)	OSA (%)	Degree of substitution (DS)	Reaction efficiency	Protein content (%)
Q- Native	88.6 ± 0.04	0	0	–	0.687 ± 0.001
Q-OSA-0.6	87.7 ± 0.20	0.58 ± 0.01	0.0045	0.987	0.570 ± 0.006
Q-OSA-1.2	87.6 ± 0.25	1.14 ± 0.04	0.0091	0.979	0.548 ± 0.003
Q-OSA-1.8	87.3 ± 0.01	1.67 ± 0.02	0.0130	0.958	0.538 ± 0.004
Q-OSA-2.4	87.7 ± 0.06	2.13 ± 0.04	0.0168	0.923	0.547 ± 0.002
Q-OSA-3.0	87.7 ± 0.59	2.59 ± 0.02	0.0205	0.903	0.539 ± 0.025

Amaranth

Sample	Dry matter (%)	OSA (%)	Degree of substitution (DS)	Reaction efficiency	Protein content (%)
A-native	88.8 ± 0.08	0	0	–	0.112 ± 0.030
A-OSA-0.6	87.6 ± 0.14	0.53 ± 0.02	0.0041	0.908	0.036 ± 0.001
A-OSA-1.2	87.8 ± 0.21	1.07 ± 0.01	0.0084	0.918	0.033 ± 0.001
A-OSA-1.8	87.9 ± 0.13	1.50 ± 0.02	0.0117	0.862	0.032 ± 0.001
A-OSA-2.4	88.2 ± 0.18	2.06 ± 0.03	0.0161	0.893	0.032 ± 0.004
A-OSA-3.0	88.3 ± 0.14	2.62 ± 0.02	0.0208	0.915	0.032 ± 0.004

	RICE	QUINOA	AMARANTH
Onset temp [°C]	72.2 ± 0.2	64.1 ± 0.2	63.8 ± 0.7
Peak temp [°C]	79.4 ± 0.3	69.8 ± 0.2	70.3 ± 0.2
Conclusion temp [°C]	85.0 ± 0.2	75.5 ± 0.3	76.8 ± 0.9
Enthalpy [mJ/mg]	13.9 ± 3.1	15.4 ± 0.8	19.0 ± 2.9
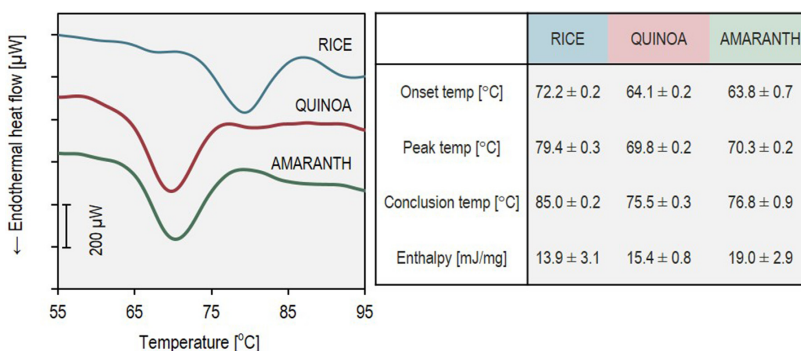
Fig. 1. DSC thermogram and thermal properties data for gelatinization of starches in buffer.

The higher gelatinization range for rice can be attributed to the higher amylopectin content since according to Fredriksson et al. (1998) starch crystallinity increases with amylopectin content, and hence, starches with higher amylopectin content (i.e. lower amylose content) would expect to have higher onset, peak and conclusion temperature. In the same way both similarity and lower gelatinization range of quinoa and amaranth compared to rice can be described by similar and lower amylopectin content of those corresponding starches.

3.2. OSA modification

Different amount of OSA was bond to the starches at the same level of added OSA (i.e. 0.6, 1.2, 1.8, 2.4, 3.0) for different starches with different botanical origin (Table 1). Table 1 shows that the OSA reaction efficiency (RE) was the highest for quinoa, followed

by amaranth and it was lowest for rice modified starches. The RE values for rice, quinoa and amaranth varied between 0.783–0.840, 0.903–0.987 and 0.862–0.918 and among all added OSA points respectively.

3.3. Size and morphology of starch granules

The size distribution and granule morphology of native and 3.0% OSA modified rice, quinoa, and amaranth can be observed in Fig. 2. The details of particle size values for native starches can be found in Table 2. The volume mean diameter (D[4,3]) was consistent with what was expected with rice being the largest (6.92 μm) followed by quinoa (2.44 μm) and amaranth (1.48 μm) and comply with former results in the literature. The particle size distribution graph for the native rice granules showed a bimodal size distribution with small peak around 1 μm and a large peak around 7.6 μm. Compar-
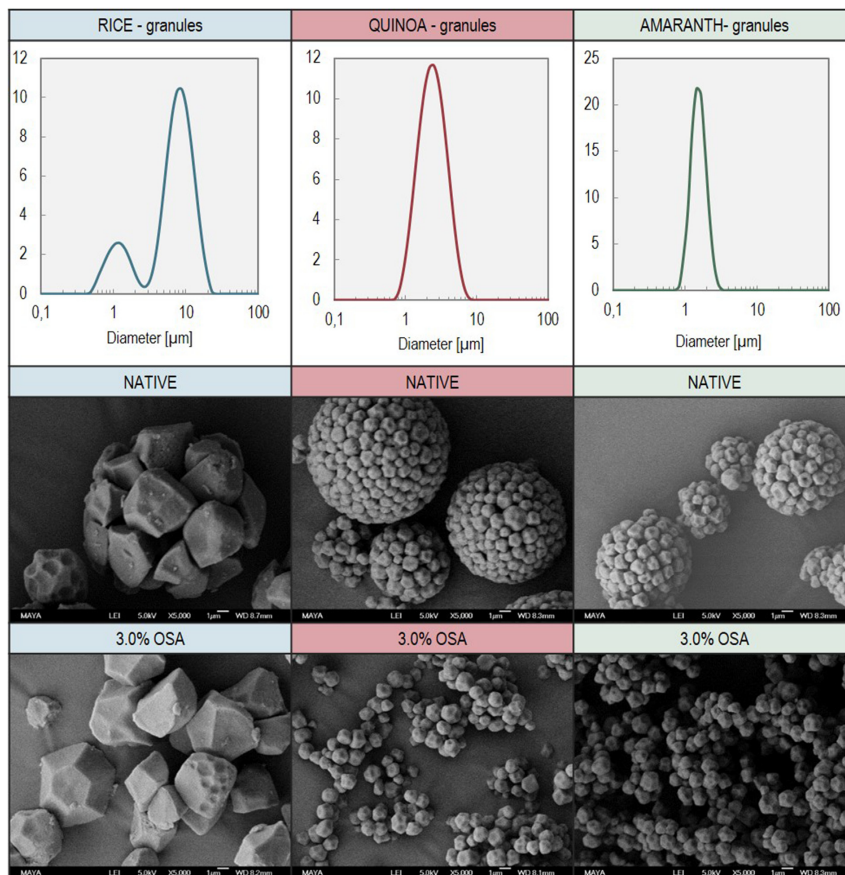
Fig. 2. Size and morphology of granules. Top row: Particle size distribution of starch in buffer dispersions for Rice, Quinoa, and Amaranth. Middle row: SEM images of the various native starch granules. Bottom row: SEM images of the various starch granules after 3.0% OSA modification.

ing these results with previous results in the literature, unimodal and bimodal size distribution could be found (Wani et al., 2012; Zuo, Knoerzer, Mawson, Kentish, & Ashokkumar, 2009). The amount of the small granules could depend on botanical source, as well as the isolation process as in some industrial processes the fine granules are lost in the separation step. In addition, depending on the measuring technique, the small peak may not be resolved and instead a wider peak is seen.

3.4. Effect of the degree of OSA modification on the emulsifying capacity

The appearance of the emulsions at all OSA modification levels and the morphology of the emulsions' droplets produced with 200 mg/mL oil of different starches at 3.0% is presented in Fig. 3. The starch particles can be seen on the surface of the emulsions' droplet which is the characteristic trait of Pickering emulsion. As can be seen in Fig. 3, these emulsions were not space filling and the droplets formed a sediment in the bottom of the test tubes due low oil fraction and high density of starch compared to the continuous phase respectively which agrees with the previous results for quinoa (Rayner, Timgren, Sjöö, & Dejmek, 2012b). The cumulative and volume frequency particle size distribution of emulsions produced from native and modified starches at all OSA modification levels (i.e. 0.6, 1.2, 1.8, 2.4, 3.0%) at the same oil/starch ratios (200 mg/mL oil) are presented in Fig. 4 and Table 2. There seems to be a negative correlation between the level of modification and droplet size and the amount of free starch at the same oil/starch ratios meaning that the greater the degree of OSA the smaller the resulting emulsions droplet were at the same starch to oil ratio and starch type as can be seen in the right column of Fig. 4. Moreover, the degree of modification appears to be more influential on emulsifying capacity of amaranth. In addition, the thickness of the emulsions layer increased as the modification level increased. According to

Table 2

Different size measurements for: native starch granules dispersed in buffer, native emulsion, 0.6% OSA emulsion, 1.2% OSA emulsion, 1.8% OSA emulsion, 2.4% OSA emulsion, 3.0% OSA emulsion for rice, quinoa and amaranth respectively.

Rice

Sample	Mode [μm]	D [4,3] [μm]	Span	D [3,2] [μm]	d (0.5) [μm]
NRSBM	7.69 ± 0.12	6.92 ± 0.17	1.66 ± 0.01	3.29 ± 0.15	6.77 ± 0.14
NRE	90.2 ± 12.2	52.9 ± 4.95	3.58 ± 0.53	7.88 ± 0.49	34.7 ± 2.39
0.6RE	94.4 ± 11.5	66.3 ± 12.3	2.50 ± 0.71	10.5 ± 2.66	56.3 ± 17.7
1.2RE	36.0 ± 8.68	23.9 ± 6.50	1.50 ± 0.19	11.7 ± 1.75	65.1 ± 5.10
1.8RE	42.2 ± 5.00	27.8 ± 2.35	1.50 ± 0.28	9.27 ± 0.67	50.3 ± 3.45
2.4RE	87.8 ± 12.9	80.8 ± 12.8	1.73 ± 0.06	15.6 ± 1.70	74.9 ± 10.9
3.0RE	86.8 ± 6.84	76.6 ± 5.99	1.77 ± 0.10	15.2 ± 1.79	72.2 ± 4.56

Quinoa

Sample	Mode[μm]	D [4,3] [μm]	Span	D [3,2] [μm]	d (0.5) [μm]
NQSBM	2.22 ± 0.15	2.44 ± 0.10	1.21 ± 0.06	2.02 ± 0.09	2.22 ± 0.11
NQE	47.8 ± 5.33	32.6 ± 3.39	2.61 ± 0.13	7.36 ± 0.41	26.9 ± 3.79
0.6QE	46.1 ± 7.82	34.3 ± 4.07	2.40 ± 0.11	7.86 ± 0.64	29.6 ± 4.43
1.2QE	39.2 ± 2.13	35.2 ± 2.26	1.91 ± 0.05	9.86 ± 0.63	32.6 ± 2.39
1.8QE	31.8 ± 0.66	34.5 ± 3.59	1.83 ± 0.07	10.3 ± 0.39	29.3 ± 0.90
2.4QE	31.8 ± 1.32	62.1 ± 4.75	2.67 ± 1.47	13.4 ± 0.85	32.5 ± 2.11
3.0QE	36.4 ± 4.18	48.2 ± 8.19	1.88 ± 0.08	13.6 ± 2.04	36.7 ± 4.17

Amaranth

| Sample	Mode[μm]	D [4,3] [μm]	Span	D [3,2] [μm]	d (0.5) [μm]
NASBM	1.42 ± 0.03	1.48 ± 0.03	0.63 ± 0.01	1.40 ± 0.03	1.43 ± 0.03
NAE	No emulsion	–	–	–	–
0.6AE	1.52 ± 2.08	18.9 ± 0.12	36.5 ± 4.92	2.05 ± 0.10	1.99 ± 0.09
1.2AE	1.50 ± 0.10	26.3 ± 6.72	29.0 ± 1.22	2.57 ± 0.22	3.30 ± 0.73
1.8AE	95.0 ± 9.48	42.6 ± 3.09	10.6 ± 2.53	4.47 ± 0.35	11.7 ± 2.31
2.4AE	66.1 ± 1.46	43.0 ± 0.92	2.44 ± 0.06	7.15 ± 0.18	38.3 ± 0.32
3.0AE	49.8 ± 0.28	37.9 ± 1.02	2.09 ± 0.06	8.19 ± 0.40	35.7 ± 1.18

(Schröder, Sprakel, Schroën, & Berton-Carabin, 2017), inter-particle interactions that are key factor to control the stability of the Pickering emulsions results in formation of three-dimensional network of aggregated droplets in continuous phase as can be confirmed by the micrographs in Fig. 3. The emulsifying capacity was observed to be higher for quinoa and rice and lower for amaranth at lower OSA levels and this concentration of starch (200 mg/mL). In addition, among different native starches, native quinoa showed to have better emulsifying capacity (Fig. 4, Table 2). This higher emulsifying capacity in native quinoa (as well as modified quinoa starches) could be attributed to higher protein level that may provide additional hydrophobic groups and results in higher hydrophobicity and better interfacial affinity which in turn can also result in lower amount of free starch as well. The amount of protein present as a minor component in starch granules is dependent on the botanical origin and purification method. According to Baldwin (2001), a substantial part of starch granule associated proteins are located at the surface of the starch granules. Due to the considerable surface area/g of the starches used in this study, which is 1.22, 1.98 and 2.86 m²/g for native rice, quinoa and amaranth respectively, the presence of these proteins may significantly influence the overall surface properties of starch granules. From the data presented in Table 1, the amount of protein was found to be higher in quinoa than rice (by a factor of 2) and amaranth (by a factor of approximately 6 and 17 for native and modified starches respectively). If we assume that all the proteins are at the surface of the starch granules, the amount of protein/unit area for native rice, quinoa and amaranth will be 2.7×10^{-3}, 3.5×10^{-3}, 0.4×10^{-3} g/m² and for the modified rice, quinoa and amaranth we will have 2.2×10^{-3}, 2.7×10^{-3}, 0.1×10^{-3} g/m². Considering the intermediate size of quinoa starch granules with more similarity in size and shape to amaranth, the better hydrophobicity and the higher emulsification capacity of quinoa starch could be explained by this protein level difference. This is further manifested in the case of the native starches where

there was no chemical hydrophobization and the trace amount of protein present in amaranth did not create a stable emulsion. It is also due to this higher hydrophobicity that lower amounts of free (non-adsorbed) starches can be seen in particle size measurements for quinoa compared to other starches (see Fig. 4). Vertical dashed lines in Fig. 4 are the d(90) of the size distribution of starch granules dispersed in buffer. This gives an indication of the degree of free starch in the system. For amaranth in particular, there is a very high amount of free starch observed in the particle size distribution seen as a peak in the 1–2 μm range. By looking at the cumulative plot we can note that for native amaranth, there was no effective droplet stabilization as the majority of the oil was phase separated and 80% of the starch remained free in the continuous phase. Furthermore, between 30% and 50% of the cumulative volume of particles measured in the samples for 0.6% OSA, and 1.2% OSA amaranth starch stabilized emulsions were present as free starch and not adsorbed at the oil-water interface. For similar degrees of modification in quinoa the volume of free starch is observed to be in the range of 8–12% (see left column of Fig. 4). Therefore, the precise influence of the surface proteins of starch granules with respect to the optimization of emulsification capacity of starch granules could be topic of further investigations.

3.5. Effect of starch concentration on emulsion droplet size

The particle size distribution values and particle size distribution as a function of starch concentration for 3.0% OSA level can be found in Table 3 and Fig. 5. Except in very low concentration for rice and amaranth modified starches, there was a negative correlation between the amount of starch and the particle size. It was shown that modified quinoa starch had a good overall emulsifying capacity and it was the best emulsifier in the lower starch concentrations among the starch varieties tested. Furthermore, it was also shown that, in higher concentrations of starch and adequate level
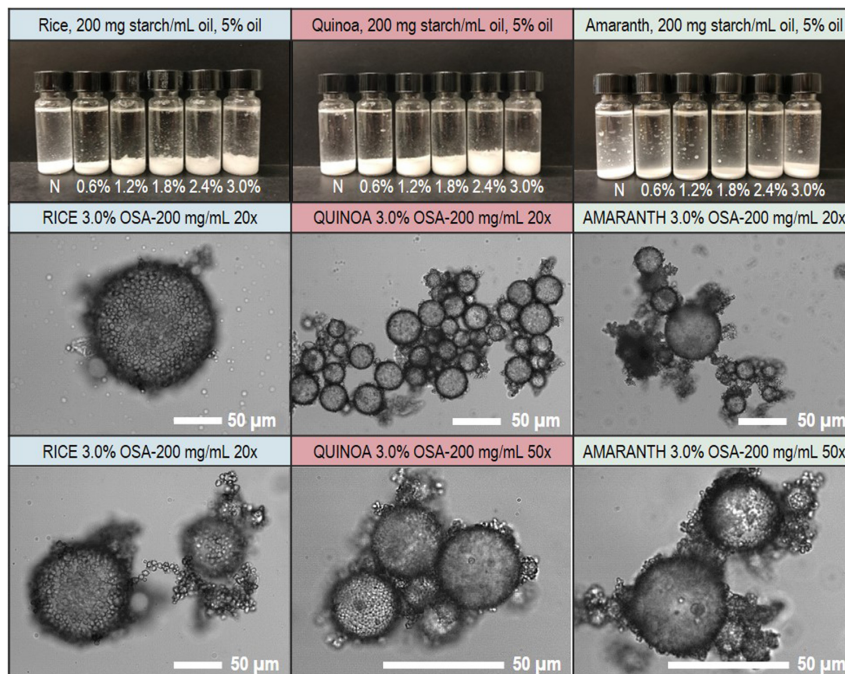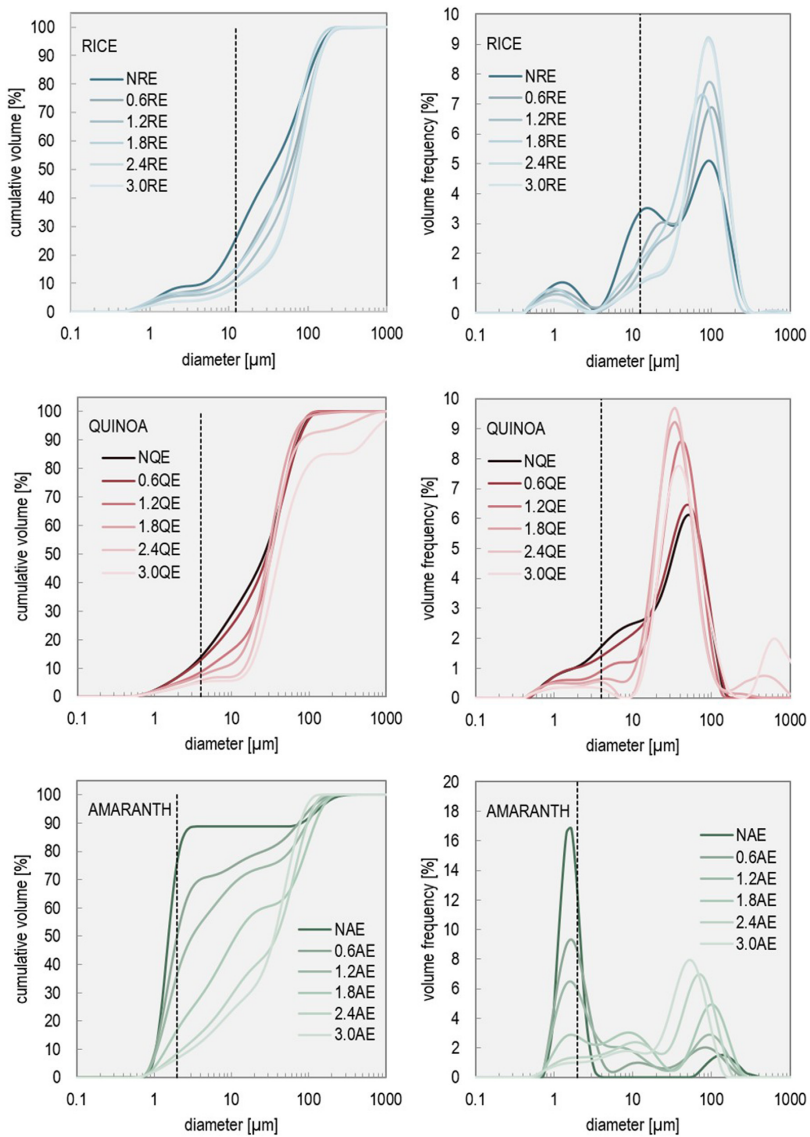
Fig. 3. Top row: Images of starch granule stabilized emulsions, Two bottom rows: Optical micrographs of 3.0% OSA modified starch granule stabilized emulsions (200 mg starch/mL oil).

Table 3
Different size measurements for: 3.0% OSA modified starch emulsions, with 50, 100, 200, 400, 800 mg starch/mL of oil for rice, quinoa, and amaranth respectively.

Rice

Sample	Mode [μm]	D [4,3] [μm]	Span	D [3,2] [μm]	d (0.5) [μm]
50 mg/mL	123.2 ± 17.5	101.6 ± 11.79	1.86 ± 0.09	19.8 ± 2.83	97.4 ± 10.44
100 mg/mL	131.0 ± 15.0	107.8 ± 9.67	1.79 ± 0.06	24.1 ± 0.88	103.9 ± 8.76
200 mg/mL	95.6 ± 7.59	80.6 ± 6.45	1.89 ± 0.15	18.8 ± 1.60	74.6 ± 5.25
400 mg/mL	51.9 ± 2.31	49.9 ± 4.04	1.90 ± 0.08	13.7 ± 0.74	44.2 ± 2.45
800 mg/mL	28.6 ± 4.29	34.0 ± 2.78	2.06 ± 0.20	9.34 ± 1.01	26.0 ± 3.74

Quinoa

Sample	Mode [μm]	D [4,3] [μm]	Span	D [3,2] [μm]	d (0.5) [μm]
50 mg/mL	104.5 ± 9.29	104.80 ± 7.66	1.16 ± 0.18	35.0 ± 4.28	99.9 ± 6.47
100 mg/mL	67.6 ± 1.29	69.6 ± 2.91	1.12 ± 0.10	24.9 ± 0.57	66.0 ± 1.78
200 mg/mL	32.7 ± 1.09	48.8 ± 3.13	2.49 ± 0.29	14.9 ± 0.57	34.9 ± 1.17
400 mg/mL	25.6 ± 3.13	35.8 ± 2.58	2.34 ± 0.78	10.7 ± 1.29	25.7 ± 2.49
800 mg/mL	18.7 ± 0.71	20.7 ± 2.68	1.55 ± 0.04	8.34 ± 0.42	17.6 ± 0.89

Amaranth

Sample	Mode [μm]	D [4,3] [μm]	Span [μm]	D [3,2] [μm]	d (0.5) [μm]
50 mg/mL	128.9 ± 14.0	100.3 ± 9.77	1.89 ± 0.03	13.4 ± 0.48	100.6 ± 9.61
100 mg/mL	74.5 ± 4.57	46.7 ± 7.10	3.11 ± 0.83	7.21 ± 0.81	36.9 ± 12.81
200 mg/mL	45.4 ± 9.54	38.2 ± 2.24	3.34 ± 0.76	6.89 ± 0.27	27.1 ± 3.56
400 mg/mL	15.6 ± 8.15	23.5 ± 1.70	5.05 ± 2.22	4.94 ± 0.69	12.2 ± 3.31
800 mg/mL	11.6 ± 2.85	12.0 ± 0.29	3.28 ± 0.36	3.78 ± 0.09	8.15 ± 0.96

Fig. 4. Particle size distributions (left cumulative and right frequency) of starch granule stabilized emulsions (200 mg starch/mL oil) for various levels of OSA modification: native emulsion, 0.6% OSA, 1.2%, 1.8%, 2.4%, 3.0%. Vertical dashed lines are d_{90} of the granules size Rice: 12.3 μm, Quinoa 3.93 μm, and Amaranth 1.97 μm respectively.
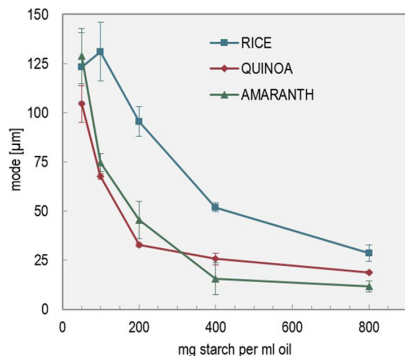
Fig. 5. Particle size (mode of D[4,3]) for: 3.0% OSA modified starch emulsions.

of OSA modification, (>400 mg/mL oil) amaranth had the highest emulsification capacity among the different starches.

By taking a mass balance over the amount of particles available for stabilizing the emulsions droplets assuming no free starch in the limited coalescence regime the theoretical droplet diameter of emulsion droplets can be estimated by:

$$\frac{1}{D} = \frac{m_p}{\varphi 4 d_p \rho_p V_{disp}} \qquad (2)$$

Where the emulsions droplet size is D, the mass m_p and density ρ_p of particles, and the volume of dispersed phase V_d and φ is the packing density assumed to be $\varphi \approx 0.907$, i.e. hexagonal close packing of spheres in a plane (Arditty, Schmitt, Giermanska-Kahn, & Leal-Calderon, 2004). If we compare the theoretical droplet size for a formulation with a certain starch granule size and amount of starch we find that in the case of quinoa and amaranth the experimental droplet sizes to be 1.7–5 times and 3.4–4.9 times larger than the predicted theoretical dimeter respectively. However, in the case of rice, the measured droplet sizes we closed to be predicted values being 0.6–2.2 times larger. This suggests that the rice granules are performing better than the quinoa and amaranth if we adjust for the effect of their size. This could be attributed to rice's bimodal particle size distribution (Fig. 2) with the smaller fraction contributing more to the apparent emulsifying capacity.

4. Conclusions

This study showed that starch granules from small granule botanical sources have the capacity to stabilize emulsions. In addition, starch granules from quinoa have good emulsifying capacity in both native and OSA modified form and especially better emulsifying capacity in the lower starch concentrations compared to the rice and amaranth. Native rice was also able to stabilize emulsion droplets. This may be due to higher protein contents of quinoa and rice starch granules in the native form that can optimize the hydrophobicity, which could be the topic of further investigations.

In the case of modified starches and when enough starch is available for stabilization (starch concentrations >400 mg/mL oil), smaller size of amaranth granules seems to be optimum.

Lastly, the bimodal nature of rice starches could be subjected to future studies for exploring the effect of size on the emulsifying capacity of starch granules from the same plant.

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Paper II

Pickering emulsifiers based on small hydrophobically modified granular starches Part II – Effects of the degree of hydrophobic modification on emulsifying capacity and stability

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Abstract

The effect of starch type and modification level of small granular starches from rice, quinoa and amaranth both in native and chemically modified with octenyl succinic anhydride at defined interval (i.e. 0.6, 1.2, 1.8, 2.4 and 3.0% based on the dry weight) on emulsifying capacity and stability of emulsions were investigated. The surface area and surface hydrophobicity of the starch granules were characterized by BET and contact angle measurements respectively. The emulsifying capacity of the emulsions was characterized by emulsion index using static multiple light scattering (Turbiscan) and light scattering particle size analyzer. The stability of the emulsions to environmental stress was characterized by accelerated stability test using a centrifuge followed by static multiple light scattering.

The surface hydrophobicity and the emulsifying capacity and stability were found to be correlated with the starch type and then the modification level. The starch granules from quinoa and rice showed higher hydrophobicity. Furthermore, the emulsions form quinoa showed higher initial stability (higher emulsion index and smaller droplet sizes) followed by rice and amaranth showed the lowest values. Finally, emulsions from quinoa exhibited highest stability towards accelerated stability test in native and the modified levels. The higher emulsifying efficiency and stability of quinoa starch granules may be due to higher protein content.

Key words:

Rice, Quinoa, Amaranth, Starch granules, OSA, Pickering emulsions

1. Introduction

Emulsions stabilized by particles are known as Pickering emulsions named after Pickering (1907). Particle stabilized emulsions have gained a lot of research interest for having unique physico-chemical and functional properties. Compared to conventional stabilizers, Pickering particles have shown to have merits such as improved stability, lower toxicity and contamination for the environment (Qi et al., 2014). One major advantage of Pickering emulsions is high resistance to destabilization mechanisms such as coalescence and Ostwald ripening (Aveyard, Binks, & Clint, 2003; Yusoff & Murray, 2011). Due to large sizes of particles as stabilizer (> 10 nm), formation of a layer of solid particles at the surface of the droplets that sterically hinders close contact of droplets results in higher resistance to destabilization. Furthermore, due to the large size of Pickering particles, once they are adsorbed to the interface at the appropriate contact angle (not too close to 0 or 180°), the energy needed to desorb them is several times larger than kT. In addition, owing to problems associated with application of surfactants including air entrapment, foaming, irritancy and interaction with living matter, the surfactant free character of Pickering emulsions makes this type of formulations appealing for several applications such as food, pharmaceutical and cosmetic applications (Frelichowska, Bolzinger, & Chevalier, 2009, 2010). Although Pickering emulsions are known for over a century, their application in food is limited since relatively low number of a particles are permissible for food application. As a result, there has been increasing interest in use of biomass-based Pickering emulsifiers (Rayner et al., 2014).

There has been a great deal of research interest for utilization of starch granules as Pickering particles (Berton-Carabin & Schroën, 2015). Starch granules are good candidates for Pickering emulsions since they are readily available, non-allergic, non-toxic, tasteless, odorless, colorless and relatively inexpensive (Timgren, Rayner, Sjöö, & Dejmek, 2011). The emulsifying capacities of native starch granules have been reported to be low (Li, Li, Sun, & Yang, 2013; Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013), however, the hydrophobicity can be increased by chemical or physical modification. The chemical modification is performed by addition of hydrophilic chains to the surface of starch granules. The most widely used chemical modification is esterification with acid anhydride such as octenyl succinic anhydride (OSA) under mild alkaline conditions (Bhosale & Singhal, 2006). The modified starch produced this way is approved food additive (E1450) and excipient with degree of modification lower than 3% based on the dry weight of starch, with no limit on application (Bhosale & Singhal, 2006; Timgren et al., 2011). Other chemical modification methods include esterification with acetic anhydride or phthalic anhydride (Tan et al., 2014). The physical modification of starch is performed by physical treatment of starch granules to alter the surface

properties of the granules. Dry heating is one of the physical modification of starch granules (Rayner, Sjöö, Timgren, & Dejmek, 2012; Seguchi, 1984).

A large number of studies have been published on emulsifying properties of OSA starch granules and there has been a considerable amount of work on development, characterization of physical and physiological stability of emulsions stabilized by OSA modified quinoa, maize, tapioca, and rice starch granules (Ali Marefati, Bertrand, Sjöö, Dejmek, & Rayner, 2017; Ali Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013; Marku, Wahlgren, Rayner, Sjöö, & Timgren, 2012; Matos, Timgren, Sjöö, Dejmek, & Rayner, 2013; Rayner, Sjöö, et al., 2012; Simsek, Ovando-Martinez, Marefati, Sjöö, & Rayner, 2015; Song, Pei, Zhu, Fu, & Ren, 2014; Timgren et al., 2013; Timgren et al., 2011; Yusoff & Murray, 2011). Though, a comparison of small starch granules with varying OSA level in incremental steps to see the effectiveness of the degree of modification on emulsions stability has not been systematically investigated. This study is a follow up on our previous study (A Marefati, Wiege, Haase, Matos, & Rayner, 2017) that investigated the manufacturing and physico-chemical characterization of starch granules from rice, quinoa and amaranth in native and varying OSA modified levels (0.6-3.0%) which is now extended to study of the effect of different degrees of modification on increasing the hydrophobicity of starch granules and the resulting impact of that on emulsifying capacity and stability in more details. With respect to starch, the contact angles of sessile oil droplets on starch pellet surfaces with different modification levels was measured and the specific surface area of starches was characterized, using Brunauer–Emmett–Teller (BET) method. These results were then compared and related to results from emulsification. With respect to emulsions, the particle size distribution of emulsions stabilized by starch granules in native and different modification levels were evaluated using a light scattering particle size analyzer and the emulsion index of freshly made emulsions and stability towards centrifugal stress was examined using multiple light scattering.

2. Material & Methods
2.1 Materials

Starches from three different botanical origins (namely: rice, quinoa and amaranth) were chosen for this study. The isolation, modification, and determination OSA of starches has been described in part I of this study (A Marefati et al., 2017). The starches in native and OSA modified form (i.e. 0.6, 1.2, 1.8, 2.4, 3.0%) were used to stabilize oil-in-water emulsions and the effect of chemical modification was investigated as follows.

3

2.2 Contact angle

Initially 300 mg of different types of starches were pressed into pellet with 13 mm in diameter using a hydraulic press (Specac, UK) under the pressure of 10 metric tons. The measurement of contact angle of oil on starch in air was performed at the room temperature using Tracker drop tensiometer device (Telics Scientific, France) equipped with digital camera and the WINDROP software. All measurements were performed in duplicates.

2.3 Specific surface area

Measurements of specific surface area, and pore size distributions of starch granules were carried out using low-temperature nitrogen adsorption in a 3 Flex Micrometrics Physisorption apparatus (Micrometric Instrument Corporation, USA) running software version 4.01. The specific surface area, S_{BET}, was calculated based on the monolayer capacity using the Brunauer-Emmett-Teller (BET adsorption isotherm) equation applied to nitrogen adsorption data (77.3 K) in the relative pressure (P/P0) range of 0.05–0.35 (Brunauer, Emmett, & Teller 1938). Prior to measurements, all samples were degassed under vacuum. The specific surface area of the starch granules was calculated from based on the surface mean diameter of the granules D[3,2] of the particle size distribution obtained by light scattering (described below) using equation 1:

$$S_{[3,2]} = \frac{6}{\rho D_{32}}$$
(Eq. 1)

where ρ is the solid density of starch (1500 kg/m³).

2.4 Formulation and Emulsification with varying levels of OSA

To study the effect of starch type and modification level on accelerated stability test emulsions with 20 % v/v oil and 100 mg starch/mL of oil were used. The reason for choosing this oil fraction and starch concentration were to have a partially filled Turbiscan cells and creating an emulsion layer that floats rather than sinks for further stability characterization. In previous work it has been shown that for higher starch to oil rations the droplets can become buoyancy neutral or even sink (Rayner, Timgren, Sjöö, & Dejmek, 2012). The oil-in-water starch granules stabilized emulsions were prepared using Miglyol 812, density 945 kg m⁻³ at 20 °C (Caesar & Loretz GmbH, Germany) as dispersed phase, phosphate buffer (0.5 mM, pH 7, 0.2 M NaCl) as the continuous phase and starch granules from three different botanical origins (namely: rice, quinoa and amaranth) in native and different modification levels from 0.6-3.0% to stabilize the emulsions. Starch dispersions in phosphate buffer were also prepared to characterize the granule sizes. The emulsions and the starch dispersions were homogenized using a rotor-stator high shear homogenizer

(Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, at 22 000 rpm for 30 s. The samples were prepared in duplicates. Thereafter the emulsions were characterized as described below.

2.5 Particle size distributions of emulsion droplets and starch granules

The particle size distributions of the starch granule stabilized emulsions were characterized with a light scattering particle size analyzer, Mastersizer 2000 (Malvern Instruments, UK). Each emulsion was added to the flow system (Hydro SM small volume wet dispersion unit) containing MilliQ water and was then pumped through the optical chamber where it was measured. The refractive index of starch particles was set to 1.54 (Bromley & Hopkinson, 2002) and the refractive index of the continuous phase was set to 1.33 which is the refractive index of the water and the obscuration was between 10 and 20%. For each emulsion sample added to the flow system three measurements were performed, and all emulsions were prepared in duplicates. In the similar fashion, the particle size distributions starch granules were measured after performing the same preparation procedure in buffer, without the oil phase.

2.6 Emulsion Index and accelerated stability test

The emulsion index *EI* is a measure of the volume of emulsion layer formed relative to the total volume (Fig. 1. a) which was characterized by a static multiple light scattering using a Turbiscan (Lab Expert, Formulation Co.) and calculated by the following equation:

$$EI = \frac{V_{cream}}{V_{total}} \times 100 = \frac{H_{cream}}{H_{total}} \times 100 \qquad\qquad Eq.\ (2)$$

where V_{emuls} is the volume of the observed emulsion (i.e. the non-clear fraction) after emulsification, V_{total} is the volume of the system, i.e. the sum of oil, water, and particles together. In the case of a cylindrical cell, these are proportional to the observed height.
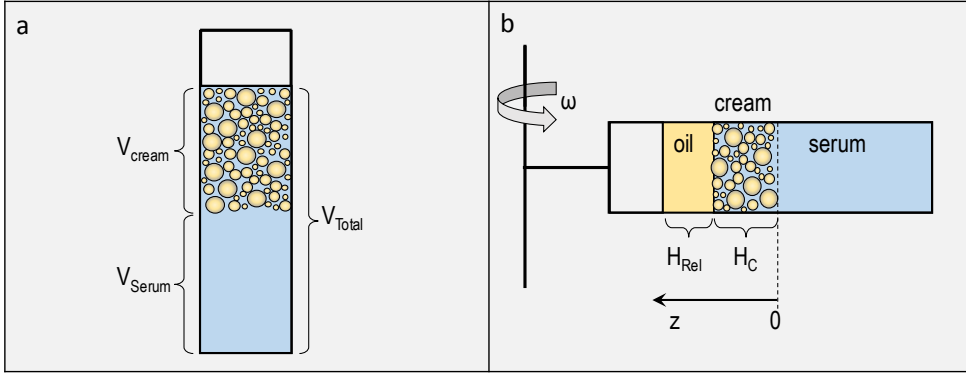
Fig. 1. a) Schematic representation of emulsion sample used for estimation emulsion index and **b)** Schematic image of the thickness of the oil layer released during a forced accelerated stability test.

Tcholakova and co-workers, have developed a centrifugal method that can provide quantitative data about the stability of oil-in-water emulsions to coalescence subjected to external stress (Slavka Tcholakova, Denkov, Ivanov, & Campbell, 2002, 2006). This technique was initially established for assessing the coalescence stability of different types of protein stabilized emulsion (Slavka Tcholakova et al., 2002, 2006; S. Tcholakova, Denkov, Sidzhakova, Ivanov, & Campbell, 2003). The emulsion is added to a Turbiscan cell and loaded into a centrifuge (Allegera X-15, Beckman Coulter). This emulsion is then subjected to centrifugal acceleration defined intensity intervals (i.e. 1050, 2100, 3150, 4200, 5250 g) and time (10 min). The oils droplets will tend to move towards the axis of rotation (z direction in Fig. 1. b) due to their relatively lower density. Emulsion droplets first form a cream layer where they are forced into close proximity due to buoyancy forces but are not significantly deformed. As the centrifugal force is increased they are pressed tighter and tighter together and eventually the interfacial layer surrounding and stabilizing the droplets will rupture releasing a layer of oil on the top of the emulsion column in the tube (see Fig. 1. b).

The critical pressure that the emulsion can withstand before oil is release when the film ruptures during droplet coalescence is described as a critical capillary pressure, P_C^{crit}, which is equivalent to the pressure jump across the oil-water meniscus compressing the oil drops, at the moment of drop coalescence with large oil-water interface (Slavka Tcholakova et al., 2006). For a full derivation refer to Slavka Tcholakova et al. (2002, 2006) and references therein.

$$P_C^{crit} = \Delta\rho g_k \int_0^{H_c} \phi(z)dz = \Delta\rho g_k (H_{cream} - H_{oil\,rel}) \qquad Eq.\ (3)$$

where $\Delta\rho$ is the density difference between the oil and aqueous phases, g_k is the centrifugal acceleration, $\phi(z)$ is the local volume fraction of oil along the z direction along the centrifugal field. After centrifugation the height of the creamed layer, H_C and oil released $H_{oil\ rel}$ was measured using a static multiple light scattering using a Turbiscan. In this experiment g_k is taken directly from the centrifuge as the number of g applied, i.e. $g_k = n\ g$ where g is the standard gravity constant of 9.81 m/s². In this work we have developed this technique by employing multiple light scattering to more accurately monitor (40 μm resolution) the cream and oil layers. Here we centrifuge the glass Turbiscan cells in a centrifuge (Beckman Coulter, Allegera X-15, USA) over a range of 0-5250 g. By measuring the height of the oil released, we can estimate the critical capillary pressure when coalescence taking place during exposure to a centrifugal stress, in addition to the emulsion index.

3. Results & Discussion

In order to have a better understanding of emulsifying properties of small granular starches in native and varying OSA modified levels, the surface properties were investigated in more detail. In our previous paper (Ali Marefati et al., 2017), we had come to the conclusion that that the higher emulsifying efficiency of quinoa starch may attributed to having a higher protein content. In this study, we will provide better understanding of the surface properties in relationship to the emulsion stability of different types of starches with varying levels of modification.

3.1 Size and specific surface area of starch granules

The surface area obtained from the BET measurements (S_{BET}) is presented in Fig. 2 and compared to the surface area calculated based on $S_{[3,2]}$. The S_{BET} data agrees with literature data for rice, 1.27 m²/g (Sujka & Jamroz, 2010). Based on the sizes of the granules, the measured S_{BET} follows the same pattern, rice having the lowest surface area, followed by quinoa, and amaranth. For amaranth and rice starches, the surface area measured by physisoption was similar to previously calculated values based on the measured D[3,2]. However, in the case of quinoa the BET surface area is approximately 40% higher than what was calculated based on the D[3,2]. The difference can be due to how the size of individual particles are detected with the particle size analyzer. If we consider size of individual granules obtained in SEM imaging, we see that there is no observable difference between amaranth and quinoa in terms of the size of individual granules. Yet, in the particles size distributions, it is observed that amaranth has a much narrower size distribution than quinoa.
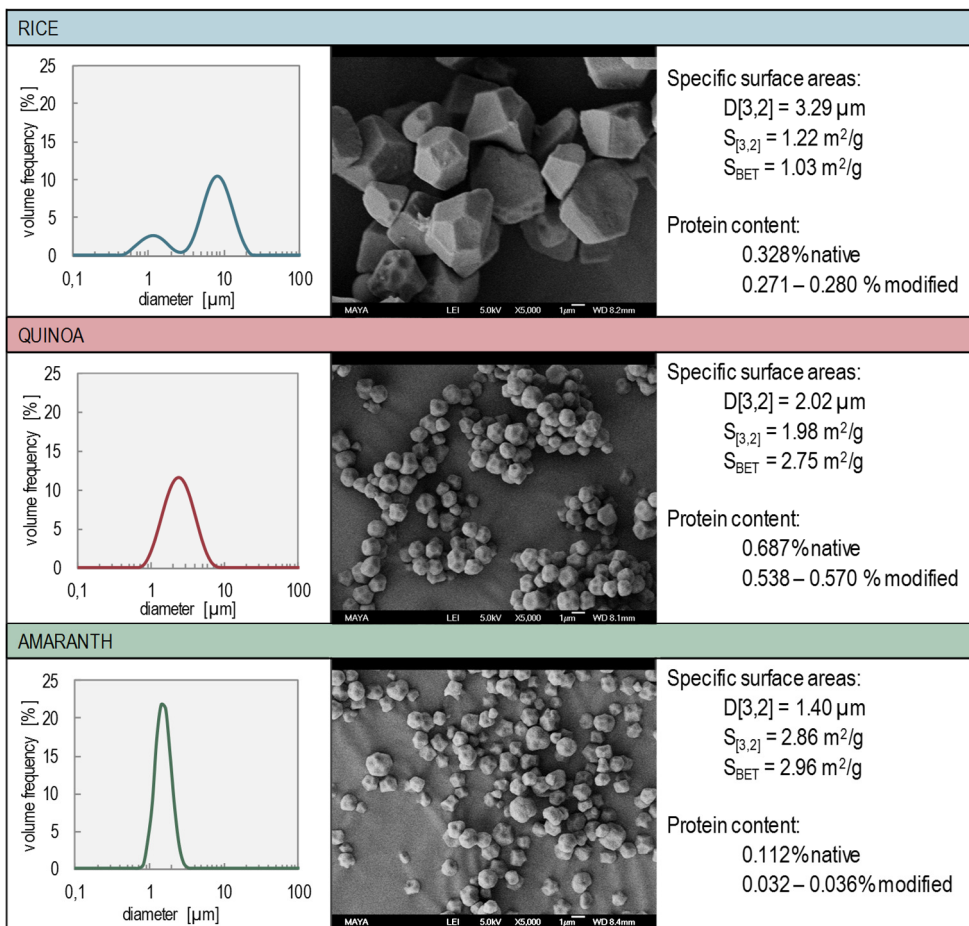
Fig 2. Particle size distributions, SEM images, specific surface data, and protein content of rice, quinoa and amaranth starches. Particle size data and images from Marefati, Wiege et al. 2017.

3.2 Contact angle

The contact angle measurements showed that there was an overall trend of a negative correlation between the level of modification and contact angle (Fig. 3). In other words, the tendency of the oil to wet the surface of the pellet increased with increasing hydrophobicity. Though, it should be mentioned that the macroscopic contact angle measured in this way is not a good indicator for functionality as an emulsions stabilizer as it is not a direct reflection of the particle at the interface. Nonetheless, what is clearly seen is a distinct difference between amaranth and the other two starches, quinoa and rice.
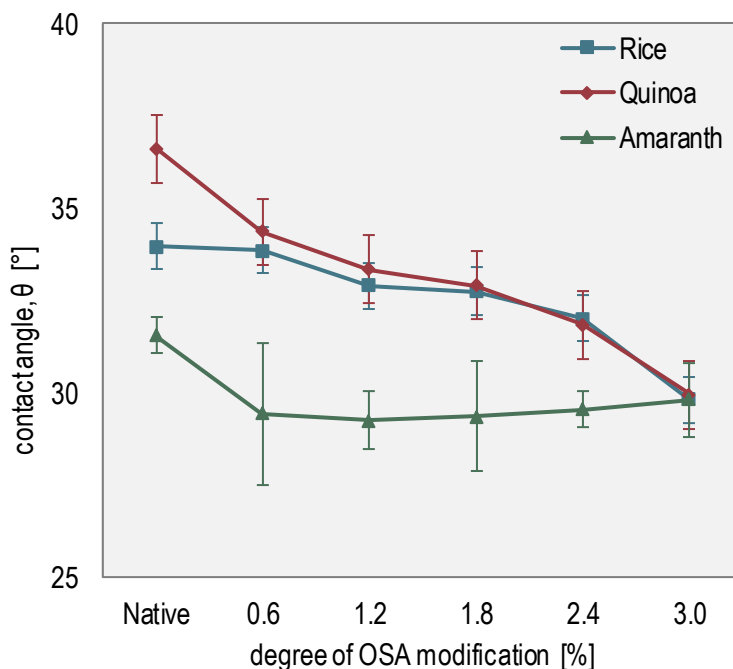
Fig. 3. Contact angle results for different starches.

3.3 Emulsions droplet size

The cumulative and volume frequency particle size distribution of emulsions produced from native and modified starches at all OSA modification levels (i.e. 0.6, 1.2, 1.8, 2.4, 3.0%) at the same oil/starch ratios (100 mg/mL oil) are presented in Fig. 4 and the mean values in Table 1. At this concentration of starch, the emulsifying capacity was observed to be higher for quinoa followed by rice and the lowest for amaranth. There also seems to be a negative correlation between the level of modification and droplet sized and the amount of free starch. These results are in line with the previous findings of Ali Marefati et al. (2017). In addition, particle size distribution of quinoa stabilized emulsions seems to be less affected by increasing the level of modification compared to amaranth, which can be due to the higher level of protein in the quinoa samples that provides sufficient natural hydrophobic groups.
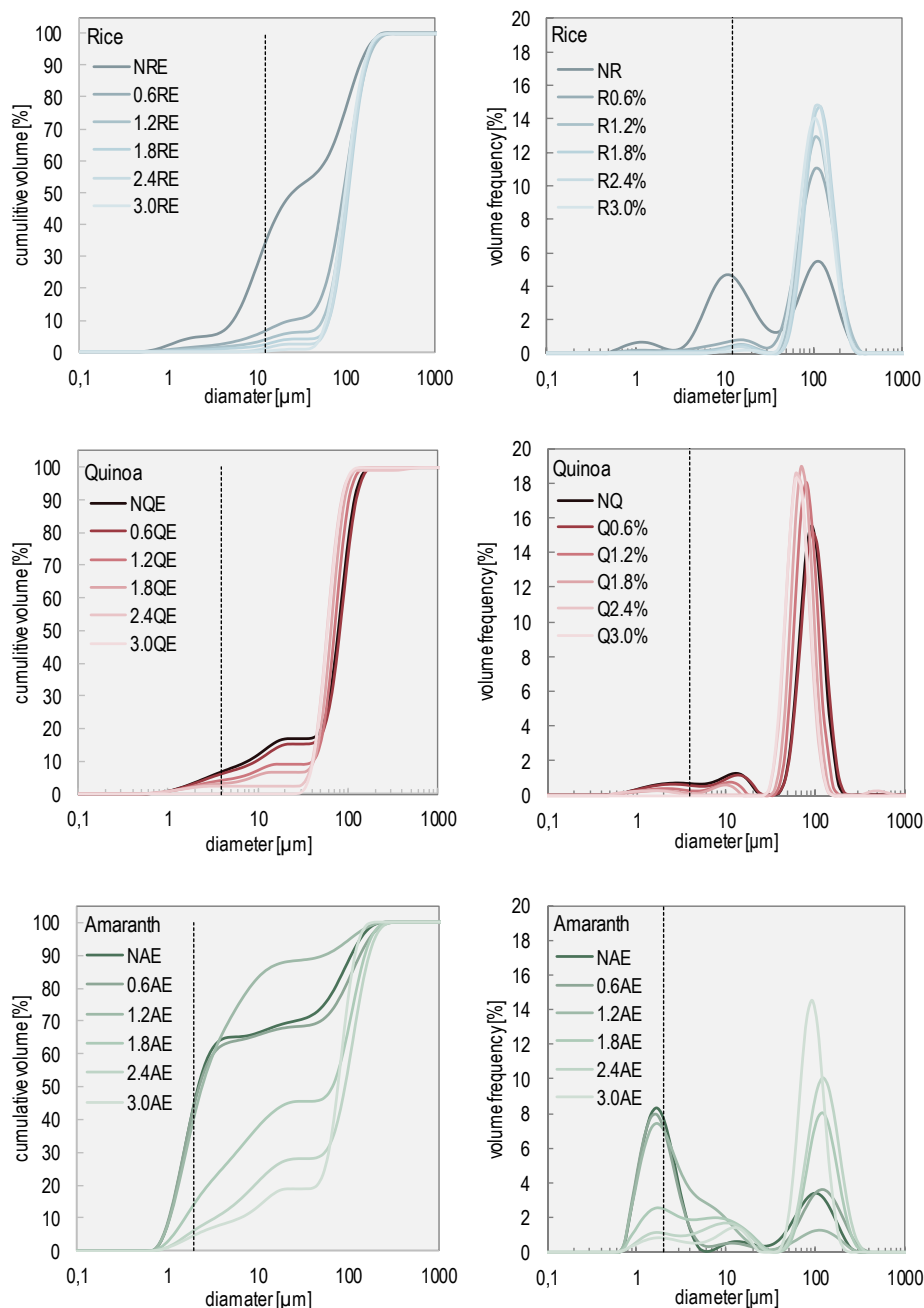
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Fig. 4. Particle size distributions (left cumulative and right frequency) of starch granule stabilized emulsions with 20% oil fraction and 100 mg starch/mL oil for various levels of OSA modification: native, 0.6%, 1.2%, 1.8%, 2.4%, 3.0% OSA. Vertical dashed lines are d_{90} of the granules size Rice: 12.3 µm, Quinoa 3.93 µm, and Amaranth 1.97 µm respectively.

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Table 1. Particle size and emulsion index data for starch granule stabilized emulsions having varying degrees of modification (native, 0.6%, 1.2%, 1.8%, 2.4%, 3.0% OSA) for rice, quinoa and amaranth respectively, with 20% oil fraction and 100 mg starch/mL oil.

Rice						
Sample	Mode [μm]	D [4, 3] [μm]	Span	D [3, 2] [μm]	d (0.5) [μm]	EI (%)
NRE	107.4±17.0	56.6±7.91	5.49±0.45	9.53±0.41	24.9±2.05	27.6±0.07
0.6RE	103.3±12.2	97.4±10.2	1.47±0.05	27.8±2.35	94.3±10.6	31.8±0.17
1.2RE	107.7±20.2	106.1±19.2	1.07±0.14	39.2±6.21	102.8±17.4	36.2±0.21
1.8RE	109.9±13.1	111.0±13.3	0.94±0.04	57.4±18.1	107.5±12.4	39.2±0.16
2.4RE	105.8±10.9	108.7±11.0	0.92±0.05	84.7±7.97	104.2±103	41.4±0.16
3.0RE	99.8±20.6	104.3±19.6	0.92±0.02	88.4±8.96	99.1±19.4	42.5±0.20
Quinoa						
Sample	Mode [μm]	D[4, 3] [μm]	Span	D [3, 2] [μm]	d (0.5) [μm]	EI (%)
NQE	85.0±4.57	74.2±4.25	1.40±0.01	16.0±0.85	78.6±4.43	45.1±0.0
0.6QE	87.8±5.25	78.2±4.84	1.39±0.04	17.4±1.40	81.7±5.39	46.7±0.3
1.2QE	73.9±2.40	70.1±2.62	0.87±0.03	21.4±0.96	71.3±2.22	47.6±0.5
1.8QE	66.5±1.54	64.5±1.63	0.77±0.01	23.2±0.78	64.9±1.54	49.2±1.3
2.4QE	59.0±1.09	63.7±3.96	0.76±0.03	26.3±0.88	58.9±1.15	50.6±2.0
3.0QE	59.2±1.05	61.8±1.23	0.76±0.04	57.0±0.72	59.3±0.99	50.8±2.1
Amaranth						
Sample	Mode [μm]	D [4, 3] [μm]	Span	D [3, 2] [μm]	d (0.5) [μm]	EI (%)
NAE	No emulsion	-	-	-	-	0
0.6AE	No emulsion	-	-	-	-	0
1.2AE	No emulsion	-	-	-	-	0
1.8AE	111.9±17.0	67.3±13.2	2.17±0.11	5.42±0.17	68.9±8.61	27.9±0.13
2.4AE	114.9±21.8	88.6±15.8	1.73±0.02	10.4±0.44	94.8±17.2	32.8±0.18
3.0AE	84.1±3.71	72.5±3.15	1.46±0.04	13.5±0.41	76.9±2.69	42.9±0.25

3.4 Emulsion Index (EI)

The *EI* values were shown to be correlated to the type of starch and the level of modification of starch used as emulsifier (Table 1 and Fig. 5, top row). The *EI%* was the highest for quinoa emulsions, followed by rice and finally amaranth. In addition, *EI%* showed a positive correlation with degree of modification. OSA modification seems to be more influential on *EI* of amaranth stabilized emulsions compared to rice and quinoa stabilized emulsions and least influential for quinoa stabilized emulsions. This effect can be described by the difference in protein level of the starches, making OSA modification playing a more effective role in emulsion formation in the case of amaranth where the protein level is low. These results are in line with particle size distribution in section 3.3.
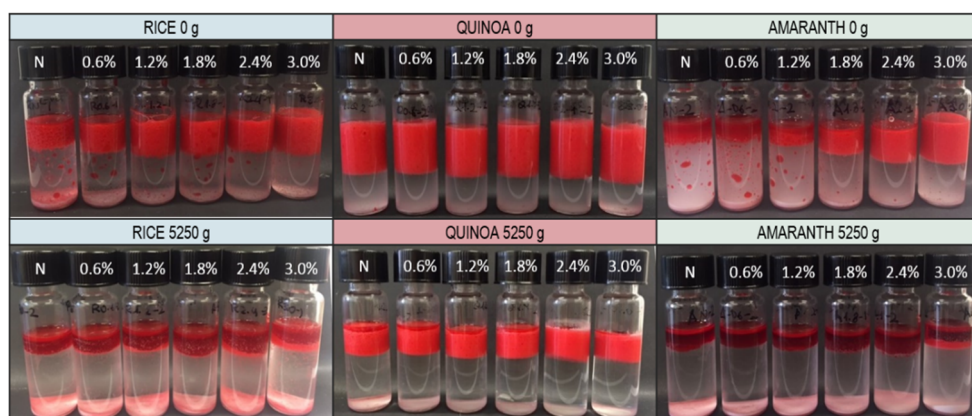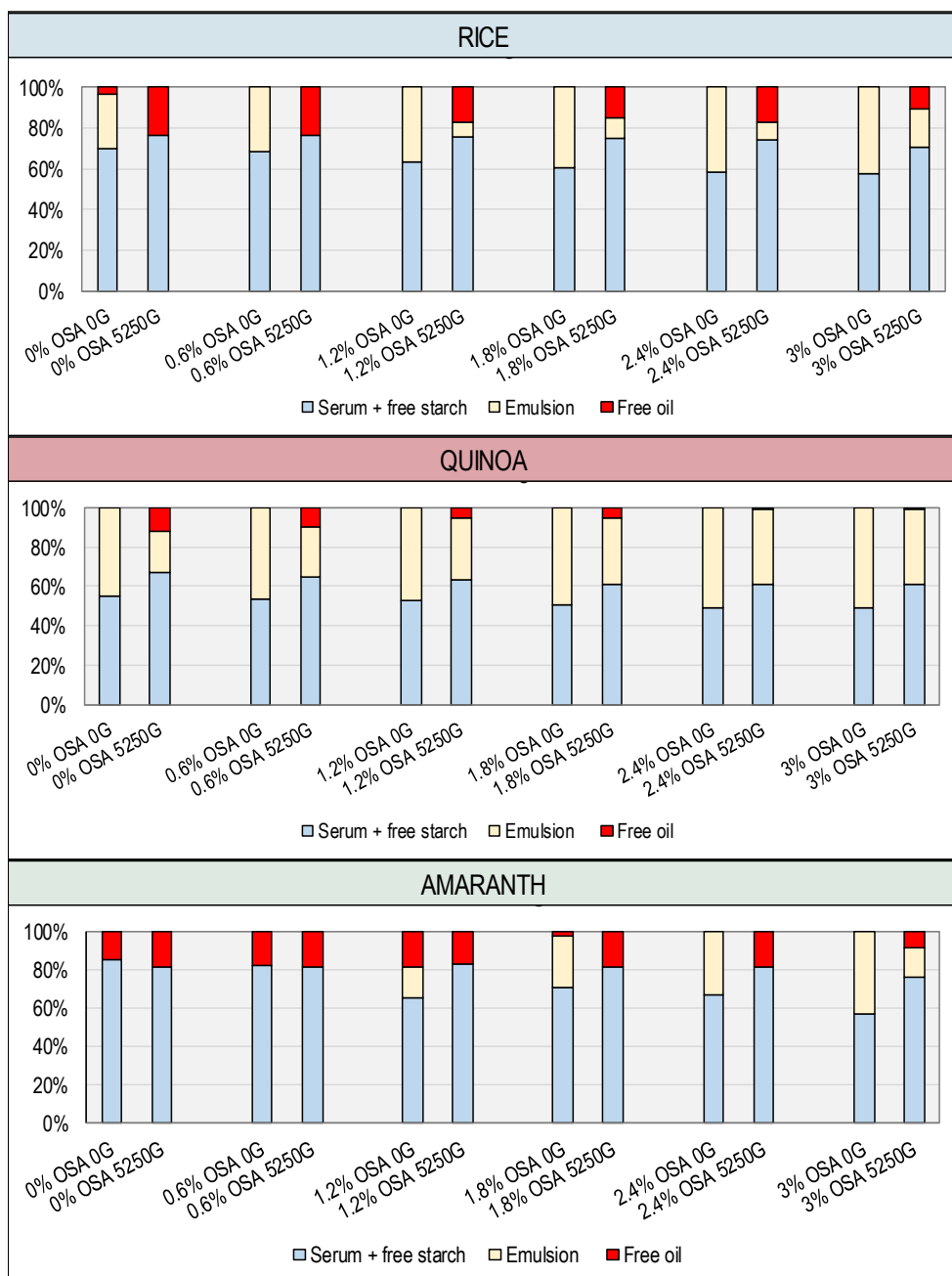
Fig. 5. Representation of emulsions before and after accelerated stability test.

3.5 Accelerated stability test

The stability of emulsions before and after accelerated stability test showed a direct correlation to the type of starch and the level of modification of starch used as emulsifier. Emulsions obtained with starches with higher level of modification showed higher stability both in initial state (the initial emulsion index, can also be seen in Fig. 5 and Fig. 6 as the thickness of the cream layer) and after accelerated stability test (Fig. 5 and Fig. 6). In addition, quinoa had the best performance followed by rice and amaranth showed the lowest stability before and after stability test. Quinoa and rice starch granules showed ability to make emulsions in the native form which may be due to higher protein content of those starches that can increase hydrophobicity as discussed previously (Ali Marefati et al., 2017). Besides, quinoa stabilized emulsions remained stable after centrifugation (with only trace amount of oil release in the lower modification levels); whereas, the emulsions stabilized with rice and amaranth were destabilized in all modification levels. Furthermore, the amount of free (unattached or desorbed) starch was higher for amaranth and rice compared to quinoa indicating a higher affinity of quinoa starch granule for the oil-water interfaces.

Fig. 6. Representation of emulsions before and after accelerated stability test, where the blue part represents the water (serum) phase, the yellow part represents the cream phase and the red part represents the free oil as measured by multiple light scattering, n=2.
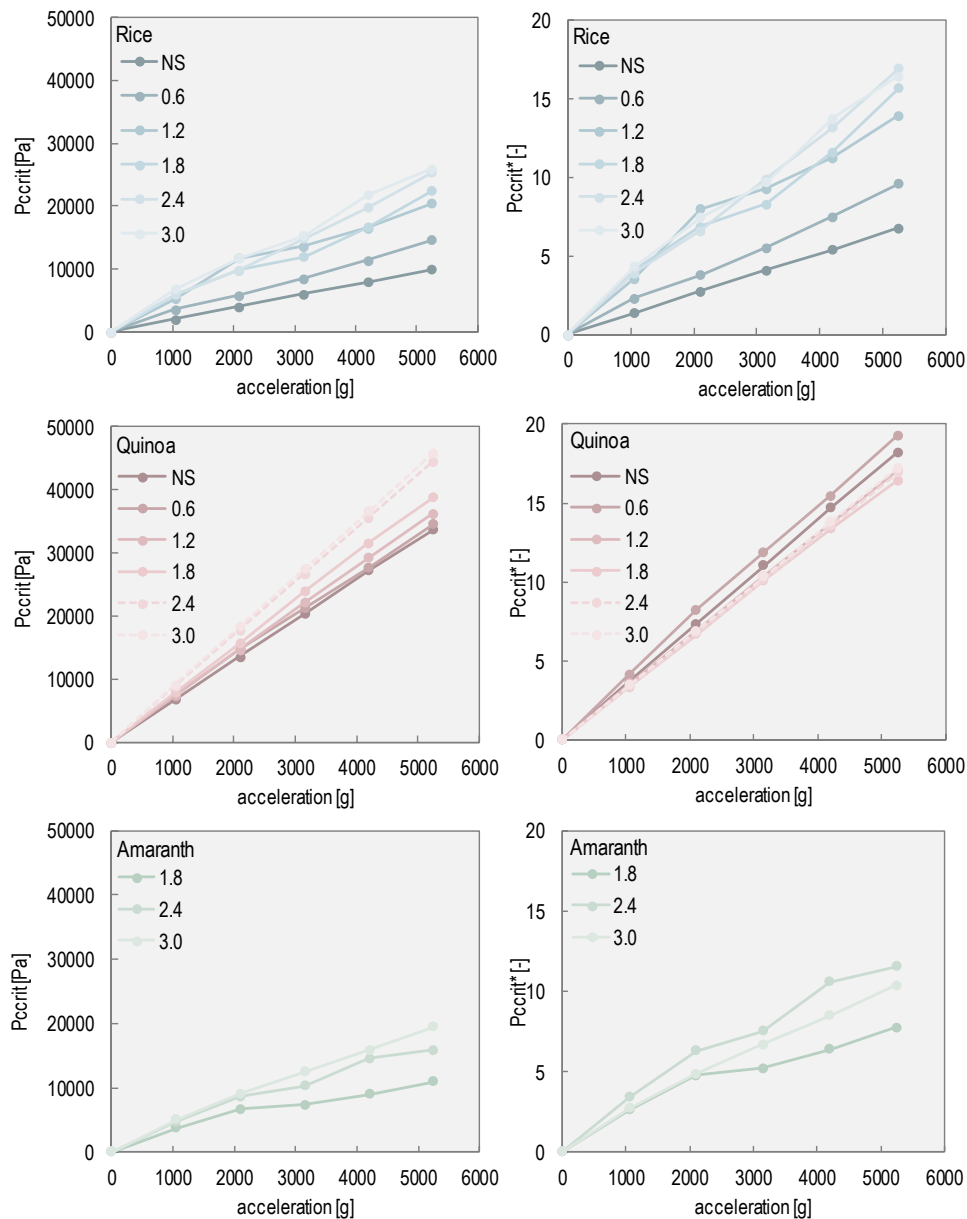
Fig. 7. Stability analysis results for emulsions before and after centrifugation over a range of centrifugal accelerations. Left: Plots of critical pressure calculated by equation 3 as a function of applied centrifugal field. Right: Critical pressure normalized by the Laplace pressure of the drops. Note dotted lines indicate "at least" this pressure as no oil was released.

In Fig. 7, left column, the critical pressure is plotted for the three types of starches. It was shown that the critical capillary pressure (P_C^{Crit}) that droplets can withstand before destabilization was dependent on the starch type and the level of modification, where quinoa showed the highest values and amaranth appeared as the lowest. Moreover, as the level of modification increased, the P_C^{Crit} increased.

In order to isolate the effect of the stabilizing layer, the critical pressure values were normalized by the Laplace Pressure of the droplets:

$$P_{lap} = \frac{4\gamma_{ow}}{D_{[4,3]}} \qquad\qquad (Eq.\ 4)$$

where γ_{ow} is the oil water interfacial tension of Miglyol 812 (39.4 mN/m) (Rowe, Sheskey, & Owen, 2006) and D[4,3] is the droplet size. The reason for this scaling is to take into account for the fact that depending on the formulation, droplets in the emulsions layer experiencing the centrifugal stress are starting at different sizes. Smaller droplets are more difficult to deform than larger ones due to their larger curvature. The volume mean diameter was used as this better represents the size of the droplets the majority of the volume of oil is contained within. The result of this scaling is plotted in Fig. 7, right column. When the sizes were taken into account, P_C^{Crit*} values were more or less similar for quinoa and rice starches (at least in the modification levels above 0.6% of rice starch) and higher than amaranth. In addition, the P_C^{Crit*} appeared to be dependent on the modification level, except for quinoa where the P_C^{Crit*} values were more similar and independent of the modification level. This may be due to higher protein content of quinoa starch that provides hydrophobic domains for the required strong adsorption of the starch to the oil-water interfaces.

3.6 General discussion

The reaction efficiency OSA modification reported in our previous work (A Marefati et al., 2017) was significantly higher for quinoa than amaranth (p < 0.05), specially at the lower modification levels 0.6 to 1.8%, where the efficiency was 9 to 11% higher. Furthermore, the specific surface area of the amaranth is larger than that of quinoa. This means that in amaranth there was less OSA attached per mass, and had a larger surface area to react on compared to quinoa. The question arises if this could have a large enough impact on the surface properties of the granules in the context of emulsion stabilization? To make a first quantitate attempt we have made the following analysis.

An independent estimation if the amount of OSA (by weight) which could be bound as a monolayer on the surface of a starch granule could be made based on the cross-sectional area of OSA alkyl chain, the measured/estimated surface area of a starch granule, and the molar mass of OSA. The cross sectional area of an alkyl chain is

about 0.22 nm^2 per molecule (2.2×10^{-19} m^2). The molar mass of OSA is M = 210.27 g/mol, dividing by the Avogadro constant, 3.491×10^{-22} g per OSA molecule. If each OSA molecule needs 2.2×10^{-19} m^2 and weighs 3.49×10^{-22} g, it covers 630 m^2 per g of OSA. Taking the specific surface areas (SBET) of quinoa 2.75 m^2/g starch and amaranth 2.96 m^2/g we find a degree of modification (mass %) to be 0.44% and 0.49% respectively. Even if we include the additional effect of the lower reaction efficiency for amaranth, the combined effect would be at most 10% more OSA on the surface of the granules. Since the OSA modifications are all more than 0.5% (by weight), we conclude that these differences could be due to different surface properties of quinoa and amaranth starch granules such as presence of protein or other groups with that provide higher binding affinity for OSA in the case of quinoa or other factors inhibiting binding of OSA to the amaranth granule surfaces.

The question still remains, what is causing the differences emulsion stability between quinoa and amaranth? The only major difference between the modified starches (apart from their size) is the protein content. Quinoa had an average protein content of 0.55%, while amaranth had only 0.033%, and rice was in middle with 0.27%. Despite the fact that the magnitude of protein content is small in all cases (less than 1%) it appears to play a critical role independent of OSA modification, which could be interpreted through the differences in macroscopic contact angle and emulsion stability. Since rice has an intermediate protein content, roughly half as much as quinoa, but still ten times more than amaranth, it also exhibits intermediate emulsifying properties. Furthermore, it we account for the size difference of the granules which result in larger droplets at the same starch to oil ratio, we find that it is even more similar to quinoa. This phenomenon can also be observed in comparing rice and quinoa in Fig. 7, right column, where the stability evaluated by the capillary pressure is scaled by the droplet size. In this case quinoa and rice have a distinctively better performance than amaranth.

4. Conclusions

The surface area measurements performed by physisoption showed that the surface area calculated from light scattering was underestimated for the quinoa starch granules, and confirmed by scanning electron microscope images.

Based on measurements of a sessile drop of Miglyol on a starch pellet, the surface hydrophobicity of starch granules was affected by the starch type and the level of modification. Starches from rice and quinoa presented higher contact angles in the native state, and contact angle decreased with increasing modification level. Although amaranth starch had lower contact angle in both the native and corresponding modified levels, there was no decreasing trend in the contact angle for amaranth as the OSA modification level increased. The emulsifying capacity of starch granules had a correlation with the starch type and a positive correlation the

level of OSA modification, where the starch granules from quinoa, displayed higher emulsifying capacity in native and modified levels followed by rice. Amaranth presented a significantly lower emulsifying capacity at all levels of modification. The accelerated stability test showed that the stability of emulsions from quinoa starch granules in native and various modification levels was higher, even after exposure to acceleration as high as 5250 g. The higher initial and accelerated stability of quinoa starch is related to the high protein content in addition to suitable granular size. The high protein content increases the hydrophobicity and makes quinoa granules adsorb stronger to the oil-water interfaces. This role of the protein should be considered when designing starch isolation processes as excessive washing in alkaline solutions will reduce protein content to such a degree that it may be detrimental to the starches' performance as a Pickering type emulsifier.

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Paper III

Freezing and freeze-drying of Pickering emulsions stabilized by starch granules

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HIGHLIGHTS

- Oil powders with 80% oil were produced from starch granule stabilized emulsions by freeze-drying.
- The oil powders could be reconstituted to stable emulsions.
- Partial gelatinization of starch increased freeze-drying stability of emulsions.
- Dispersed phase melting point affected freeze-drying stability of the emulsions.
- Different freezing methods imposed different changes on stability of the droplets.

GRAPHICAL ABSTRACT

Oil containing powders can be produced with relatively high oil content (80%) from starch granule stabilized Pickering emulsions where adsorption and partial gelatinization of starch granules at the oil–water interface protects the integrity of droplets during freezing and freeze-drying.

ARTICLE INFO

ABSTRACT
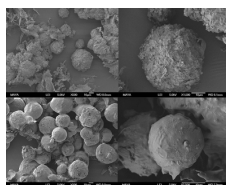
The aim of this study was to investigate the possibility to produce novel powder materials based on chemically modified starch granule stabilized Pickering oil-in-water (O/W) emulsions. This study also investigated the effect of partial starch gelatinization *in situ*, dispersed phase type (two oil types with different melting points), freezing method and thawing, and freeze-drying and rehydrating on the overall properties of the emulsions. The emulsions showed high freeze–thaw stability. The results of this study demonstrated the feasibility of the production of oil containing hydrocolloid-based powders, through combination of heat treated or even non-heat treated starch Pickering emulsions and freeze-drying. The final powders comprised high weight percentage of oil (over 80%, w/w). Upon rehydration of powders, the starch stabilized oil drops were found to be only moderately affected by the process with some aggregation observed.

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1. Introduction

Emulsions are mixtures of two immiscible liquid phases where one is dispersed in the other as spherical droplets. These systems are not thermodynamically stable, thus stabilizers are required to prolong their stability [1]. Surfactants, proteins and hydrocolloids are the main types of stabilizers in food emulsion systems

[2] and function by enhancing droplet stability by lowering the interfacial tension, increasing steric hindrance, and/or electrostatic repulsion between droplets [3]. In Pickering emulsions, solid particles stabilize emulsions by being essentially irreversibly adsorbed at the oil–water interface, creating a thick physical/mechanical barrier [4]. Pickering emulsions demonstrate long-term stability [5–7]. Starch granules have been shown suitable for Pickering type stabilization after chemical modification with octenyl succinic anhydride (OSA) to adjust their wetting behaviour [8]. Previously, emulsions stabilized by quinoa starch granules were found to remain stable over a two-year storage period with no phase separation or change in droplet size [9]. Depending on the botanical

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source, starch granules vary in size, shape and composition. For Pickering emulsions, small and uni-modal granules such as quinoa starch were found preferable [9,10]. The physicochemical properties of starch enable the adjustment of the Pickering emulsion droplet barrier by careful application of heat treatment causing partial *in situ* gelatinization of the starch granules adsorbed at the oil–water interface [11].

Dehydration of emulsion systems could be used to increase shelf life, improve their use, and facilitate transportation [12–14]. However, dehydration may alter the interfacial properties and lead to disruption [15–17]. There are several approaches to maintain the stability of emulsions during drying and subsequent storage. A common way is to add a solid hydrophilic carrier to the aqueous phase in amounts ranging between 30% and 80% of the total weight of the final powder [18,19]. Examples of such carrier compounds include lactose, glucose, maltodextrin, and cellulose [13,20]. As an alternative and to avoid carrier compounds, multiple or layer by layer (LBL) deposition of polyelectrolytes that crosslink on the droplet surface, crosslinking of protein-stabilized interfaces, and protein–polysaccharide conjugates have also been applied [21–23].

Freeze-drying is a process where the solvent (usually water) is crystallized at low temperature and then sublimated directly from the frozen state into vapor by decreasing the pressure around the product. Compared to other drying methods, freeze-drying causes less damage to sensitive structures and thus useful for preservation of heat sensitive food materials as well as of other biological products [24].

Upon freezing of emulsions, the water and oil phases start to crystallize which introduces a number of destabilization mechanisms [25,26]. Ice formation in the continuous phase increases droplet-droplet interaction and less water is available to hydrate the emulsifier on the droplet surface. The formation of ice crystals also results in an increased concentration of solutes in the unfrozen aqueous phase, causing a change in ionic strength and pH. This can lead to disruption of electrostatic repulsion between the droplets [14,27]. Notably, Pickering emulsions stabilized by quinoa starch granules were previously shown to be highly stable towards changes in ionic strength (in the range 0.2–2 M NaCl) of the continuous phase [8]. When an oil-in-water emulsion is cooled to temperatures where the oil phase starts to crystallize, partial coalescence may occur since lipid crystals of one droplet can penetrate into the liquid region of another droplet upon collision. Complete or partial crystallization of oil in droplets during the production or storage of emulsions may have a large negative impact on the emulsion stability [14,28]. Additionally, due to the volumetric expansion of water upon freezing, ice crystals may penetrate oil droplets and possibly rupture the interfacial layer, which enables oil-to-oil contact [16,31]. Droplets covered by a thick film have been found better protected against crystal penetration and partial coalescence [29,30]. The rate of coalescence during freezing has been correlated to the size of the stabilizing agent [31,32]. The large particles used in starch Pickering emulsions form a dense layer around droplets and therefore provide higher resistance against crystal penetration. The reasons are mainly the large size relative to the ice crystals and the high energy required to remove a micron sized particle from the oil–water interface [4].

The objective of this work was to produce oil-filled powders by freeze-drying without the need of additional carrier compounds. Pickering emulsions stabilized by OSA modified quinoa starch were used as initial emulsions; with the aims to further study the effect of *in situ* heat treatment to induce a partial gelatinization of the starch granules adsorbed at the oil–water interface prior drying, and to evaluate the influence of the dispersed phase oils with different melting temperatures. Furthermore, the freezing step, as a prerequisite of freeze-drying, was studied. Overall properties of initial

emulsions, frozen and thawed emulsions, and dried and rehydrated powders were analyzed.

2. Materials and methods

2.1. Materials

The materials used were hydrophobically modified quinoa starch granules with 1.8% OSA isolated and modified as described previously [8], phosphate buffer (5 mM, pH 7, 0.2 M NaCl), and two different dispersed phases: Miglyol 812 (density 945 kg m^{-3}, melting point $-12.5\,°C$, Sasol GmbH, Germany) or shea nut oil (density 910 kg m^{-3}, melting point 32–34 °C, a kind gift from AAK, Karlshamn, Sweden), respectively.

2.2. Methods

2.2.1. Emulsion preparation

Oil (7%, v/v), buffer (93%, v/v) and starch (214 mg/mL oil) in a total volume of 7 mL were weighed into a glass test tube and emulsified first using a vortex mixer (10 s) and then homogenized using a high-shear homogenizer Ystral (D-79 282; Ystral GmbH Ballrechten-Dottingen, Germany) at 22 000 rpm for 30 s. Since the melting point of shea nut oil is above room temperature ($\sim$20 °C) it was kept in a water bath at 40 °C before emulsification. After emulsification, partial *in situ* gelatinization was induced in half of the samples by heating the emulsions in a water bath (*i.e.* maintaining the emulsions at 70 °C for 1 min, with a total heat treatment time of approximately 3 min including warm up time monitored using a type K, 0.1 mm thermocouple). All samples were produced in duplicate.

2.2.2. Freeze–thaw cycling

The freeze–thaw cycling experiment was performed on non-heat treated samples to see the effect of the freezing step. A total volume of 10 mL of each emulsion type was transferred to stainless steel trays, covered by aluminium foil, and thereafter frozen overnight using a freezer room (-18 °C). Thereafter, the emulsions (previously frozen in -18 °C) thawed during 4 h in room temperature. For comparison, and in order to see the effect of the rate of freezing on overall properties of emulsions, an additional set of emulsions were produced and flash frozen by dipping the trays into liquid nitrogen (-196 °C) and kept in the freezer overnight before thawing. Miglyol emulsions were flash frozen from its liquid state at room temperature. Shea nut oil emulsions were flash frozen either directly from the liquid state at 40 °C or after the dispersed phase was solidified at room temperature for 3 h.

2.2.3. Freeze-drying

Samples were frozen in freezer room as described in Section 2.2.2. All samples were then kept in the freezer before being transferred to the freeze dryer. The emulsions were freeze-dried during 5 days using a laboratory freeze dryer (CD 12, Hetosicc, Denmark) with 20 °C in the drying chamber, -50 °C in the cooling unit, and a vacuum of 10^{-2} mbar. The aluminium foil covering the containers of frozen emulsion was punctured before freeze-drying.

2.2.4. Characterization of emulsions and powders

The microstructure of the initial emulsions, freeze–thawed emulsions, and re-hydrated powders (restored to the original emulsion concentration using MilliQ water) were characterized by light microscopy (Olympus BX50, Japan) with 100–500 times magnification and using a digital camera (DFK 41AF02, Imaging source, Germany) together with the software ImageJ (NIH, Version 1.42 m). Each emulsion drop was diluted with 5 drops MilliQ water. Dried
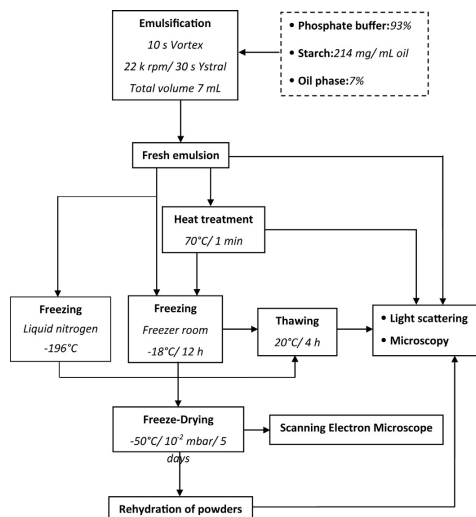
Fig. 1. Illustration of the preparation and characterization methods.

a clear three-dimensional images of the surface of the samples, the lower electron imaging (LEI) detection mode was used where signals from both secondary electrons and back scattered electrons were combined. Fig. 1 illustrates the preparation and characterization methods used. Significance of results were determined using Student's *t*-test and 95% confidence interval.

3. Results and discussion

3.1. Effect of heat treatment and oil phase on initial emulsions

3.1.1. Liquid Miglyol 812 emulsions

Intact starch granules covering the surface of droplets are shown in Fig. 2a (light microscopy) and drawn schematically in Fig. 2c. *In situ* heat treatment prior to drying resulted in formation of a layer of partially gelatinized starch at the oil–water interface (Fig. 2b and d). Particle size distributions of non-heated samples showed distributions with a major peak (or mode of droplet mean diameter D_{43}) of $49 \pm 2\,\mu m$ before heat treatment, see Fig. 3. Free granular starch was found in both samples, quinoa starch granules have a natural size of 0.5–3 μm but OSA modified granules may also form aggregates of approximately 10 μm and appeared as the minor peak to the left. The swelling of free starch in heated samples was also observed. The particle size distribution shown in Fig. 3 together with the overall microstructure observed in the light microscope indicated that heat treated emulsion droplets were slightly larger; this may be due to swelling of the starch granules during gelatinization. Therefore the heated samples exhibited a trimodal particle size distribution, however, no significant change in the position of the main peak ($50 \pm 2\,\mu m$) before and after heat treatment was observed (Student's *t*-test *p* value = 0.51). Aggregation of droplets, or even formation of a gelatinized network of starch where dispersed droplets were entrapped was measured as large sizes (>200 μm) and confirmed under the microscope. This would occur when starch granules around neighbouring droplets gelatinize and/or together with free starch in the surrounding aqueous phase form a three-dimensional network of gelatinized starch. This was also evident from the larger difference in mean droplet

emulsions were restored to the same concentration as the initial emulsions prior to dilution for microscopy characterization. Laser light scattering (Malvern Mastersizer 2000, UK) was used to determine the droplet size distributions of the emulsions and rehydrated powders. Refractive indexes of 1.54 and 1.33 for emulsion droplets coated with starch and continuous phase were used for light scattering analysis [33]. Duplicate samples were measured 3 times each (*i.e. n* = 6). Additionally, the powders were characterized by scanning electron microscopy (FegSEM, JEOL JSM-6700F, Japan) operated at 5 kV and a working distance at 8 mm. In order to have
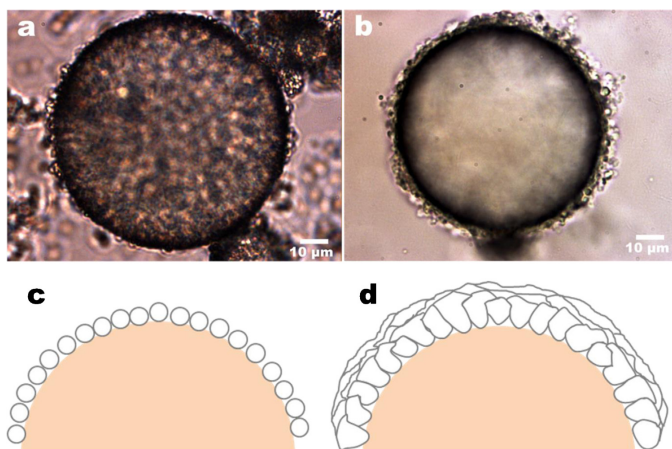

Fig. 2. Light microscopy images (a and b) and schematic pictures (c and d) of starch Pickering emulsions. Non-heat treated (a and c) and heat treated (b and d) emulsions with Miglyol 812 as dispersed phase.
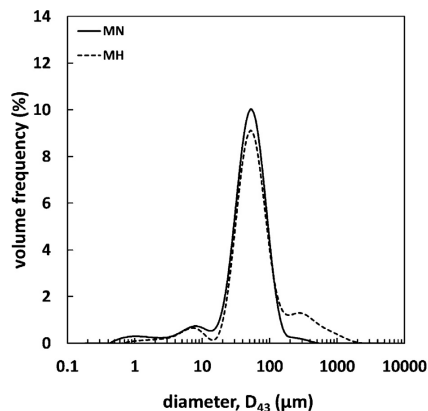
Fig. 3. Particle size distribution ($D_{4,3}$) graph of non-heat treated (MN) and heat treated (MH) emulsions using Miglyol 812 as dispersed phase.

Table 1
Different size parameters of starch Pickering emulsions varying in dispersed phase type before and after heat treatment. The values are presented as mean ± standard deviation.

Sample ($n=6$)	D[4,3] [µm]	Span of D[4,3]	Mode of D[4,3] [µm]	D[3,2] [µm]
MN	52 ± 5	1.65 ± 0.05	49 ± 2	16 ± 1
MH	103 ± 10	3.91 ± 0.4	50 ± 2	27 ± 2
SN	40 ± 8	1.84 ± 0.1	36 ± 1	12 ± 4
SH	65 ± 15	1.53 ± 0.3	40 ± 3	21 ± 3

MN: Miglyol 812 emulsion non-heat treated.
MH: Miglyol 812 emulsion heat treated.
SN: Shea nut oil emulsion non-heat treated.
SH: Shea nut oil emulsion heated treated.

size D_{43} of 103 ± 10 µm compared to non-heated samples with a mean diameter size D_{43} of 52 ± 5 µm (Table 1).

3.1.2. Solid shea nut oil emulsions

Micrographs of emulsions produced using shea nut oil as dispersed phase are shown in Fig. 4 before (a) and after heat treatment (b). Particle size distributions of emulsions (Fig. 5) with shea nut oil as dispersed phase were represented by a bimodal curve with a major peak of 36 ± 1 and a tri-modal curve with a slightly larger peak of 40 ± 3 for non-heated and heated emulsions respectively, which was significantly different (Student's *t*-test *p* value = 0.01). As
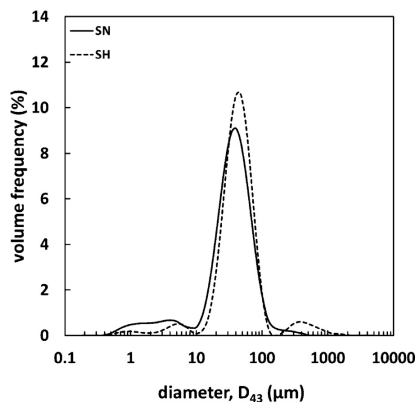

Fig. 5. Particle size distribution ($D_{4,3}$) graph of non-heat treated (SN) and heat treated (SF) starch Pickering emulsions with shea nut oil as dispersed phase.

in Fig. 3, the minor peaks in Fig. 5 were interpreted as representing free starch granules (left of major peak) and aggregation of starch granule covered droplets caused by starch gelatinization (right of major peak), and a slight shift in the position of the major peak towards larger droplet sizes. This was more evident when comparing mean droplet sizes (D_{43}) of the regarded samples (40 ± 8 µm for fresh compared to 65 ± 15 µm for heated samples).

3.2. Effect of different types of freezing treatments and oil phase on emulsions

The starch stabilized emulsions showed excellent freeze–thaw stability. Characterization of samples after thawing in room temperature showed that slow freezing at −18 °C generally did not alter the particle size distribution significantly compared to the fresh emulsions for the two oils tested (Tables 1 and 2). Flash freezing appeared to cause detachment of more starch granules from drop surfaces and facilitate a higher degree of coalescence and thereby larger mean droplet size (Figs. 6 and 7). This can be seen as a reduced number of small droplets and an increased amount of free starch granules. The particle size distribution for Miglyol-based frozen emulsions using a freezer room showed a major peak of 49 ± 2 µm compared to liquid nitrogen flash frozen samples with a mode of D_{43} of 106 ± 11 µm (Table 2). The minor peak in the particle size distribution of flash frozen samples (Fig. 6) can be attributed to free starch. Less attached starch indicates that less total oil–water
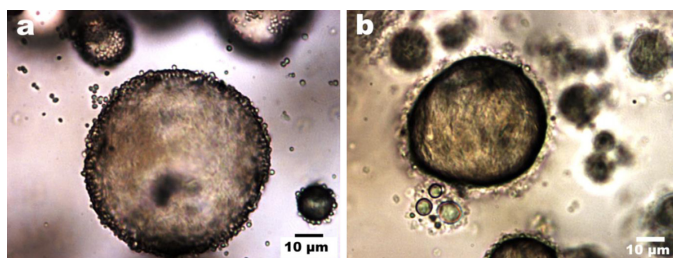

Fig. 4. Light microscopy images of non-heat treated (a) and heat treated (b) starch Pickering emulsions with shea nut oil as dispersed phase.
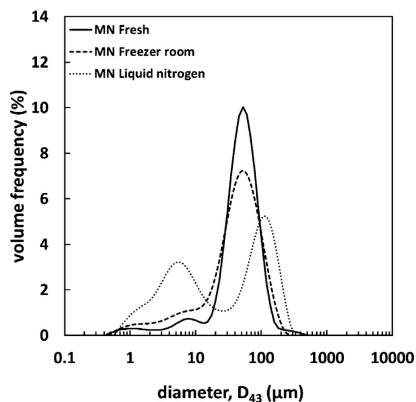
Fig. 6. Particle size distribution ($D_{4,3}$) graph of thawed starch Pickering emulsions with Miglyol 812 as dispersed phase, frozen using freezer room (MN Freezer room) or liquid nitrogen (MN Liquid nitrogen) and the corresponding fresh emulsion (MN Fresh).
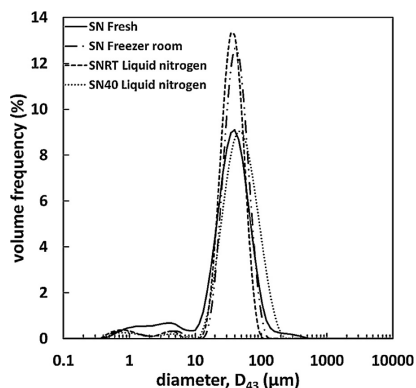

Fig. 7. Particle size distribution ($D_{4,3}$) graph of starch Pickering emulsions with shea nut oil as dispersed phase before and after freeze and thaw treatment using different methods. Fresh emulsion (SN Fresh), emulsion frozen in freezing room at −18 °C (SN Freezer room), flash frozen at −196 °C from room temperature (SNRT Liquid nitrogen) or flash frozen at −196 °C from 40 °C (SN40 Liquid nitrogen).

interfacial area was stabilized [9,10], which was also confirmed by the significantly larger emulsion droplet sizes (Student's t-test p value = 0.00005). This data complies with the results of Choi et al. [34] where faster freezing rates and lower overall temperatures was found to cause more aggregation in Miglyol containing emulsions.

A similar result was obtained in shea nut oil emulsions when flash frozen from the liquid state at 40 °C. The mode of $D_{4,3}$ increased from 36 ± 1 to 45 ± 2 μm upon flash freezing and thawing (Table 2). However, in the case of shea nut oil emulsions that were primarily cooled to room temperature before flash freezing (*i.e.* the shea nut oil was already solidified) no significant difference in mean droplet size ($D_{4,3}$) and mode of $D_{4,3}$ before and after the freeze–thaw cycle was found.

This result was somewhat surprising as we expected the rapid freezing using liquid nitrogen to quench the sample, creating small and less disruptive crystals. There are some contradictory results reported in the literature in terms of whether a fast freezing or slow freezing is preferred for emulsions stability. For example according to Tippetts and Martini, a slow cooling rate results in larger and more stable crystals compared to a fast cooling rate, as was observed in oil-in-water emulsions with soybean and anhydrous milk fat (50:50) as dispersed phase where slow cooling (0.2 °C/min compared to 30 °C/min) resulted in higher stability, especially for samples with lower oil contents (20%, v/v) [35]. However, an opposite result was observed in a study by Vanapalli et al. for oil-in-water emulsions with confectionary coating fat (with similar physical properties as cocoa butter) with similar oil content of 20% as dispersed phase [36]. In the latter case slower cooling rate destabilized emulsions, while the system remained stable in cases where a faster cooling rate was used.

In the present work using starch granules as stabilizing particles, it was observed that the faster cooling rate and the lower temperatures reached using liquid nitrogen led to the displacement of a fraction of the starch from the oil–water interface, and thereby to larger emulsion droplet sizes. However, this result was observed only for samples that were flash frozen from a temperature in which the dispersed phase was liquid (room temperature for Miglyol and 40 °C for shea nut oil). Lowering the temperature causes shrinkage of the dispersed phase, where liquid oil shrinks more than solid oil. This will affect the protrusion of crystals, for example according to Boode and Walstra the thermal expansion coefficient of triglycerides is $\cdot 10^{-6}$ m^3 kg^{-1} K^{-1} for oil and $3.9 \cdot 10^{-7}$ m^3 kg^{-1} K^{-1} for triglyceride crystals [30], *i.e.* about 2.5 times less. When an oil droplet is cooled, the higher volume shrinkage of liquid oil compared to fat crystals causes the crystals to protrude into the aqueous phase. Higher degree of cooling (to −196 °C for liquid nitrogen *versus* to −18 °C in the freezer) will cause more fat to crystallize with more protrusion, and therefore partial coalescence is inevitable [30]. Partial coalescence is the driving force that causes destabilization of emulsions as oil crystallization occurs. Collision of semi-crystalline oil droplets can result in penetration of crystals

Table 2
Different size parameters of starch Pickering emulsions varying in dispersed phase type before and after freezing treatment. The values are presented as mean ± standard deviation.

Sample ($n = 6$)	D[4,3] [μm]	Span of D[4,3]	Mode of D[4,3] [μm]	D[3,2] [μm]
MN freezing room	49 ± 2	2.26 ± 0.03	49 ± 2	12 ± 1
MN liquid nitrogen	56 ± 5	6.07 ± 1.6	106 ± 11	6.2 ± 0.3
SN freezing room	40 ± 2	1.14 ± 0.06	39 ± 2	15 ± 1
SNRT liquid nitrogen	35 ± 2	1.06 ± 0.02	34 ± 2	13 ± 1
SN40 liquid nitrogen	51 ± 2	1.66 ± 0.06	45 ± 2	17 ± 1

MN freezing room: Miglyol 812 emulsion non-heat treated- frozen in freezing room and thawed.
MN liquid nitrogen: Miglyol 812 non-heat treated- frozen in liquid nitrogen and thawed.
SN freezing room: Shea nut oil non-heat treated- kept in room temp, frozen in freezing room and thawed.
SNRT liquid nitrogen: Shea nut oil non-heat treated- kept in room temp (as SN), frozen in liquid nitrogen and thawed.
SN40 liquid nitrogen: Shea nut oil non-heat treated- kept in 40 °C frozen in liquid nitrogen and thawed.
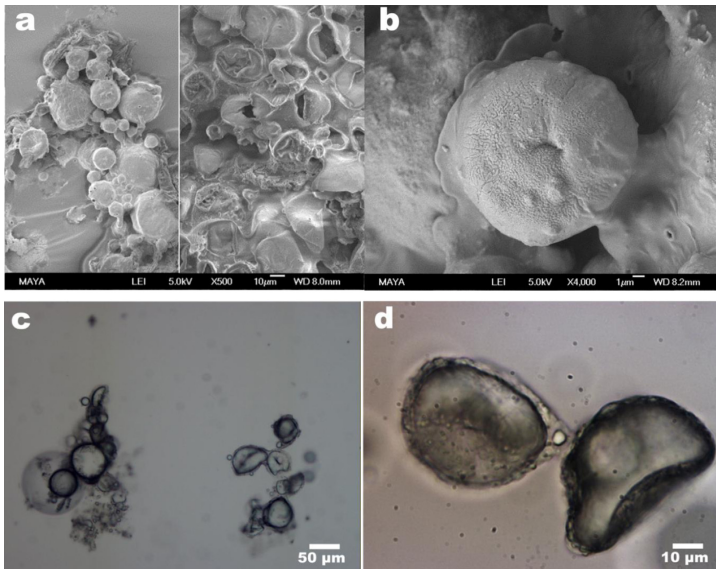
Fig. 8. SEM micrographs of freeze-dried starch Pickering emulsion (a and b) and light microscopy images of rehydrated powders (c and d) with Miglyol 812 as dispersed phase. Emulsions were heat treated before the drying process.
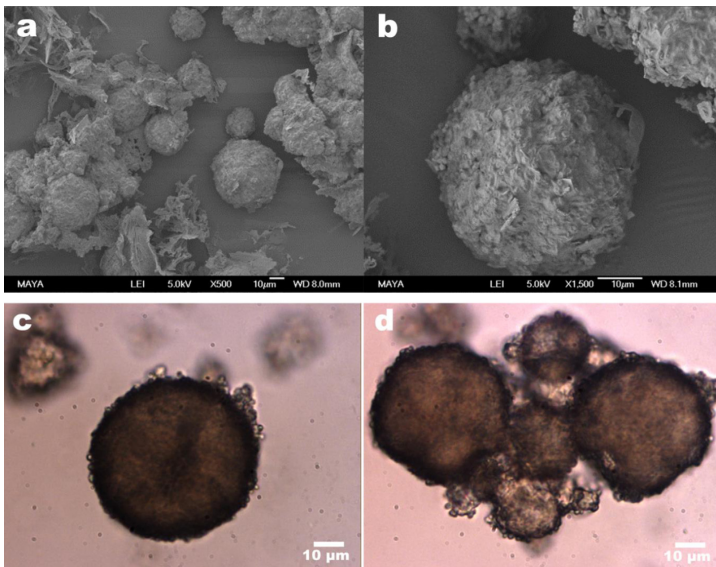
Fig. 9. SEM micrographs of freeze-dried starch Pickering emulsion (a and b) and light microscopy images of rehydrated powders (c and d) with shea nut oil as dispersed phase. Emulsions were not heat treated before the drying process.

from one droplet into the interface of the other. This will cause the remaining oil content of the droplets to flow out and wet the solid fat and thereby form a linkage between droplets, which can result in true coalescence when the oil melts. Triglyceride composition, oil additives and/or impurities, together with freezing conditions can affect the amount, size, and morphology of the solid fat formed in the droplet and thus affect the destabilization [30,37]. The composition of oil is further known to have great impact on freeze–thaw stability of oil-in-water emulsions [25].

The present results revealed that a faster cooling rate and lower cooling temperature caused greater loss of emulsion stability when flash frozen from a temperature where the oil was in a liquid state. We postulate that if the drops are already solid at the point of flash freezing, there will be no super cooling effect and the dispersed phase will shrink relatively less during the actual freezing step than an oil flash frozen from its liquid state. Also, in the case of freezing in the freezing room, the freezing step takes place much more slowly (hours *vs.* seconds) and the final temperature is much higher ($-18\,^{\circ}$C *vs.* $-196\,^{\circ}$C) compared to flash freezing with liquid nitrogen. This mechanism in combination with the speed of cooling may cause flash frozen samples to experience a significantly different environmental stress which could lead to emulsion destabilization and coalescence upon thawing which is not observed in the slowly and less deeply frozen samples kept in the freezer room. Still, despite the different freezing methods used, the emulsion characteristics remained and the emulsions did not break. This can be attributed to the starch particles at the interface. The relatively large starch particles provide a stronger and thicker barrier between droplets and protect their integrity during a freeze–thaw process.

3.3. Effect of freeze-drying on emulsions

The adsorption of a closely-packed particle layer surrounding each droplet may contribute to structural rigidity during the freeze-drying process, resulting in oil filled powders, Figs. 8 and 10. Due to the higher physical stability of emulsions made from shea nut oil (*i.e.* solid at room temperature), these types of emulsions in both cases, heat treated and non-heat treated, resulted in powders. For the less physically stable Miglyol-based emulsions (*i.e.* liquid at room temperature), only heat treated samples resulted in dried emulsions. The partially gelatinized starch layer was then crucial during drying. Figs. 8a and b, 9a and b, and 10a and b demonstrate SEM micrographs of emulsion powders.

Upon rehydration of the powders, well-dispersed emulsions were created. Figs. 8c and d, 9c and d, and 10c and d show light microscopy images of dried and rehydrated emulsions. Corresponding particle size distributions can be seen in Fig. 11. For heat-treated samples, association of emulsion droplets as clusters consisting of several small droplets with relatively even surfaces of smooth gelatinized starch layers were observed. This resulted in a major size population of $324 \pm 90\,\mu$m for Miglyol samples (Fig. 8a) and for shea nut oil (Fig. 10a) in a major population of $193 \pm 60\,\mu$m (Table 3). In contrast, in non-heat treated samples (Fig. 9) individually existing droplets with uneven surfaces were observed with the granules recognizable, and a lower size of the major population peak of $54 \pm 3\,\mu$m (Fig. 9) was obtained. Moreover, micrographs of original and rehydrated dried emulsions revealed a reduced presence of larger droplets in the latter, probably caused by destabilization during the drying process. This was most likely due to reduced mechanical stability of larger droplets.
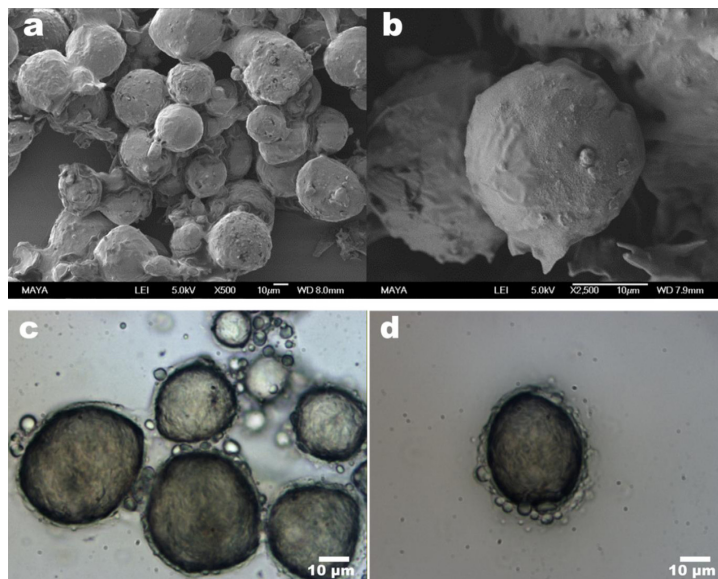
Fig. 10. SEM micrographs of freeze-dried starch Pickering emulsion (a and b) and light microscopy images of rehydrated powders (c and d) with shea nut oil as dispersed phase. Emulsions were heat treated before the drying process.
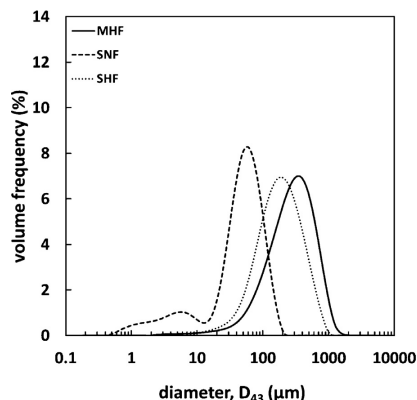
Fig. 11. Particle size distribution ($D_{4,3}$) for freeze-dried and rehydrated starch Pickering emulsion from Miglyol 812 heat treated emulsion (MHF), shea nut oil non-heat treated emulsion (SNF) and shea nut oil heat treated emulsion (SHF).

Table 3
Different size parameters of rehydrated dried starch Pickering emulsions. The values are presented as mean ± standard deviation.

Sample ($n = 6$)	D[4,3] [μm]	Span of D[4,3]	Mode of D[4,3] [μm]	D[3,2] [μm]
MHF	319 ± 70	2.01 ± 0.11	324 ± 90	128 ± 20
SNF	51 ± 2	1.97 ± 0.10	54 ± 3	12 ± 3
SHF	210 ± 40	2.08 ± 0.12	193 ± 60	104 ± 20

MHF: Miglyol 812 emulsion heat treated and freeze-dried.
SNF: Shea nut oil emulsion non-heat treated and freeze-dried.
SHF: Shea nut oil emulsion heat treated and freeze-dried.

Comparison of size distribution results for original *vs* dried and rehydrated emulsions (Tables 1 and 3) showed an increase in overall size distribution which was confirmed by micrographs to be due to aggregation. The starch in heat treated emulsions is expected to undergo retrogradation prior to drying and potentially also after rehydration. Retrogradation of starch in the partially gelatinized layer is not expected to have any negative impact on the droplet stability during drying, but rather to increase stability; however, this was beyond the scope of the present study.

The results of this study showed that it was possible to produce oil-filled powders with a high weight percentage of oil (over 80%, w/w). Further studies will be needed to show the limits of the technique in terms of trade-offs between droplet size and oil percentage.

4. Conclusion

It was feasible to develop well re-dispersible, food-grade, oil-filled powders from OSA modified starch Pickering emulsions by freeze-drying. Since no additional hydrophilic carrier compounds were required, powders of at least 80% oil content were easily achievable. Pickering emulsions containing oil which was partially solid at the freeze-drying temperature were inherently stable to freeze-drying. For liquid oil emulsions, *in situ* heat treatment leading to partial gelatinization of starch granules prevented collapse during freeze-drying. It remains unclear why slow freezing at −18 °C had less impact on the stability of emulsions than fast freezing in liquid nitrogen. Starch granule-base Pickering emulsions could have versatile applications for different food, pharmaceutical and cosmetic emulsion types which need to be dried or withstand freezing.

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Paper IV
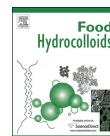
Fabrication of encapsulated oil powders from starch granule stabilized W/O/W Pickering emulsions by freeze-drying

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ABSTRACT

The process stability of water-in-oil-in-water (W/O/W) double emulsions stabilized with food-grade OSA modified quinoa starch granules was investigated. The effect of oils with differing melting points, as well as the effect of *in situ* partial gelatinization of the granules, on stability of emulsions was also investigated. The physical stability and release of a tracer (carmine) from the internal aqueous phase of double emulsions were characterized after each process stage using particle size analysis, light microscopy and spectrophotometry.

When liquid shea oil was used the particle sizes varied in mode of D[4,3] from 28 ± 2 μm for initial fresh double emulsions to 90 ± 7 μm for heat treated and 210 ± 11 μm for heat treated, freeze-dried and reconstituted emulsions. Non-heat treated emulsions collapsed on freeze-drying due to high susceptibility of liquid oil droplets towards destabilization, when the external aqueous phase is crystalized.

When solid shea oil was used the mode of D[4,3] varied from 48 ± 0 μm in initial emulsions to 118 ± 3 μm for heat treated emulsions and the freeze-dried and reconstituted emulsions showed 62 ± 3 μm or 85 ± 11 μm for non-heat treated and heat treated samples respectively. Reconstituted freeze-dried emulsion retained the encapsulated marker to over 97%. Overall, application of oil phase solid at room temperature and *in situ* heat treatment had a positive impact on process stability towards freezing and freeze drying.

The result of this study revealed the feasibility to develop food-grade oil filled powders from OSA modified starch Pickering emulsions with approximately 70 wt% oil content by freeze-drying.

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1. Introduction

Emulsions are the mixture of two immiscible liquid phases where one phase is dispersed in the other as droplets. In food materials, emulsions consisting of oil and water are more common, where the mean diameter of the droplets is normally in the range of 0.1–100 μm (McClements, 2007). Since emulsions are thermodynamically unstable systems, other components are used to prolong their stability over the time. Surfactants, proteins and hydrocolloids are the main types of stabilizers in food emulsion systems (Fig. 1a) (McClements, 2007). It is also possible to use solid particles to stabilize emulsions. Emulsions stabilized by solid particles, so called Pickering emulsions (Fig. 1b), demonstrate long-term stability compared to surfactant additives which is particularly important in food as well as pharmaceutical and personal care

products (Akartuna, Studart, Tervoort, Gonzenbach, & Gauckler, 2008; Binks, 2002; Tcholakova, Denkov, Sidzhakova, Ivanov, & Campbell, 2003).

Conventionally, emulsions are categorized by the relative spatial distribution of their constituent phases. Emulsions where oil droplets are dispersed in water as continuous phase are called oil-in-water emulsions (O/W). On the other hand the emulsions where the dispersed phase is water and the continuous phase is oil are called water-in-oil emulsions (W/O). It is also possible to produce different types of multiple systems where the dispersed phase contains even smaller dispersed droplets as its own dispersed phase (Fig. 1c). These types of emulsions first described by Seifriz (1925), are therefore called multiple emulsions. From these types of emulsions water-in-oil-in-water double emulsions (W/O/W) and oil-in-water-in-oil double emulsions (O/W/O) are the most common (McClements, 2007). However in food industry, since most of the food emulsions have an aqueous continuous phase and there are more food grade hydrophobic stabilizers available, W/O/W double emulsions are more common (O'Regan
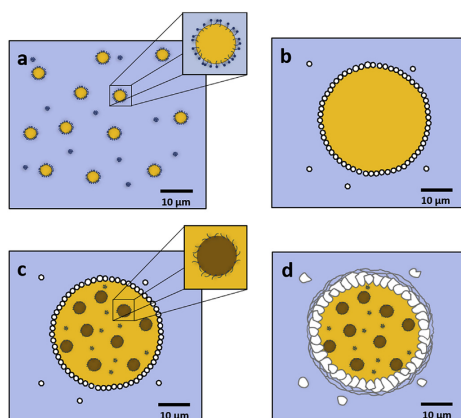
Fig. 1. Schematic picture of different types of emulsions, a. surfactant stabilized emulsion, b. particle stabilized emulsion, c. Starch granule stabilized double emulsion and d. heat treated starch granule stabilized double emulsion.

& Mulvihill, 2010). What makes multiple emulsions attractive is their ability to entrap desired materials in an inner phase during a primary emulsification procedure which is beneficial for food, pharmaceutical and cosmetic purposes, such as vehicles for encapsulation and delivery nutrients (vitamins and minerals) and active substances (Bonnet et al., 2009; Dickinson, 2011; Giroux, Constantineau et al. 2013), furthermore by the addition of water as internal phase, reduced fat foods can also be formulated (Norton & Norton, 2010). Encapsulation of flavours, taste masking, and prevention of oxidation are other uses of double emulsions (van der Graaf, Schroën, & Boom, 2005; Muschiolik, 2007; O'Regan & Mulvihill, 2010). Encapsulation of probiotic bacteria within double emulsions in order to preserve them in a viable form during production, storage and gastro-intestinal digestion has been reported in previous studies (Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter, 2009; Rodríguez-Huezo, Estrada-Fernández et al., 2014; Shima, Morita, Yamashita, & Adachi, 2006). One of the early applications of encapsulation of drugs using double emulsion was reported by Herbert (1965) who investigated possibility of optimization of encapsulated antigen for influenza vaccines through a W/O/W emulsion. Several attempts for applications of double emulsions in skin care products have also been reported (Akhtar & Yazan, 2008; Yener & Öke, 2009). Despite the extensive recognition of the value of double emulsions, due to difficulty of creation and characterization of stable double emulsions within the constraints of food industry, the potential capabilities of double emulsions have yet to be fully developed (Dickinson, 2011).

In addition to the destabilization mechanisms for simple emulsions including coalescence, Ostwald ripening, aggregation, etc., there are four commonly observed pathways that result in destabilization of the internal phase of W/O/W double emulsions including: *i.* Coalescence of the internal aqueous droplets, *ii.* Rupture of the oil film that separates the internal and external aqueous phases, *iii.* Passage of water to and from internal droplets through the oil phase, *iv.* Water transport through the oil phase in an inversed surfactant micelles (Florence & Whitehill, 1981).

Osmotic pressure and Laplace pressure driven diffusion of the entrapped molecules through the oil phase, are the main factors involving release of the encapsulated water content. Diffusion is attenuated when solid oil is used due to minimal solubility of water in the oil crystals. In this case if the melting point of the oil is between room temperature and body temperature, the release is facilitated upon application on skin or ingestion (Magdassi & Garti, 1987). According to Florence and Whitehill (1982) water can move to the aqueous phase with higher osmotic pressure. If the osmotic pressure is higher in the internal aqueous phase, the transport of water from the external phase will cause the internal aqueous phase to swell and eventually burst. Similarly, higher osmotic pressure of external phase will cause the water to transfer to the external aqueous phase causing the internal aqueous phase to shrink. Due to Laplace pressure and smaller size of the internal aqueous phase droplets, there is a greater pressure on the internal aqueous phase to pass out and promote Ostwald ripening. However, if the internal aqueous phase contains salts, since small ions are insoluble in the oil phase, further water transport will be quickly arrested (Dickinson, 1992).

Traditionally small-molecular weight surfactants have been used as stabilizing agent for both the inner and the outer interfaces of double emulsions. In such a system, instability of encapsulated inner phase due to coalescence as well as fast release rates are the main drawbacks of surfactant stabilized double emulsions. The fast release maybe due to diffusion of internal phase content or migration of emulsifiers from both inner and outer interfaces where they aggregate and form reverse micelles that can transport the internal phase content out of the intermediate phase (Benichou, Aserin, & Garti, 2004; Garti & Aserin, 1996). High molecular weight synthetic emulsifiers such as PGPR are effective at stabilizing the internal W/O drops in double emulsions, however the use of these types of emulsifiers is highly regulated in many food applications (Dickinson, 2011). Continued progress in the formulation and application of double emulsions will require new means to improve their long-term stability while at the same time replacing or reducing the use of small-molecular weight surfactants and synthetic polymeric emulsifiers with food-compatible components (Dickinson, 2011). One approach to improve stability and achieve a more attractive formulation is through the use of food-compatible solid particles as Pickering type stabilizers. In many ways, solid particles can act as surfactants. The oil or water affinity that is referred as hydrophile-lipophile balance (HLB) in surfactants is analogous to particle wettability characterized via the contact angle (Binks, 2002).

Compared to surfactant stabilized systems (Fig. 1a), Pickering emulsions (Fig. 1b) are usually more stable against coalescence and Ostwald ripening (Aveyard, Binks, & Clint, 2003; Yusoff & Murray, 2011). The stability of Pickering emulsions can be attributed to their large particles (above 10 nm in diameter), and the fact that the particles tend to adsorb strongly (irreversibly) at the oil-water interface. Once these particles are above certain size and with an intermediate contact angle (not too close to 0 or 180°) they form a mechanical protective barrier with very high desorption energy per particle (several thousand kT) which results in a high energy barrier to droplet shrinkage and drop–drop coalescence (Rayner et al., 2014; Yusoff & Murray, 2011). The high resistance of particle stabilized emulsions to coalescence allows preparation of highly concentrated emulsions and stable emulsions even with coarse droplet size distributions (Frelichowska, Bolzinger, Pelletier, Valour, & Chevalier, 2014). There are several types of solid particles applicable for the stabilization of emulsions such as silica particles, waxes, cellulose, protein assemblies and starch particles. However, according to Dickinson (2010), compared to the total number of emulsion stabilizing solid particles, a relatively low number of

them are permissible in food. Among all insoluble particles which are used as emulsion stabilizing agent, starch particles have shown promising properties making them a good candidate in food grade Pickering emulsions (Rayner et al., 2014; Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013; Timgren, Rayner, Sjöö, & Dejmek, 2011; Yusoff & Murray, 2011). Starch particles are generally hydrophilic and the favourable dual wettability is enhanced through chemical modification and inclusions of hydrophobic groups of octenyl succinic anhydride (OSA) to increase their affinity to the O/W interface. Modified starches produced through OSA esterification are tasteless, colourless, odourless, non-allergic and inexpensive. OSA modified starch (E1450) with a degree of modification of lower than 3% is well established food ingredient with no limit on its application (Timgren et al., 2011).

Quinoa was selected as the starch granule source in this study, based on the findings of a previous screening study on 7 different starches of various botanical origin, amylose/amylopectin content and granule size, where it was found that quinoa starch, due to its shape, small (~1 μm) and uni-modal particle size, had the highest stabilizing capacity especially after hydrophobic modification (Timgren et al., 2013). In addition to emulsification properties of OSA modified starch, the physicochemical characteristics of starch granules allows further modification of barrier properties by careful application of heat treatment causing *in situ* partial gelatinization of starch granules at the interfaces (Fig. 1d). This technique been used to improve the process stability of starch granule stabilized emulsions during freeze-drying (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013) as well as for reduction of lipolysis rates *in vitro* (Timgren et al., 2011).

Solid particles have been used in development of multiple emulsions as either or as both of the internal phase or external phase stabilizers. Primarily solid particles were used in combination with surfactants to improve shelf life of surfactant stabilized double emulsions. Oza and Frank (1989) used microcrystalline cellulose particles as external phase hydrophilic stabilizer while Garti, Aserin, Tiunova, and Binyamin (1999) used fat crystals as hydrophobic inner interface stabilizer. Multiple emulsions stabilized solely by silica particles than are slightly different in their level of SiOH modification were successfully developed by Aveyard et al. (2003). Later Garrec, Frasch-Melnik, Henry, Spyropoulos, and Norton (2012) developed W/O/W double emulsion using fat crystals and silica particles as stabilizing particles for inner and outer phases respectively. Pickering emulsification has also been shown to have high encapsulation efficiency for development of double emulsions useful for food and pharmaceutical industries. Previous study by Matos, Timgren, Sjöö, Dejmek, and Rayner (2013) showed possibility of fabrication of starch granule stabilized double emulsions with high initial encapsulation efficiency (EE > 98.5%) which remained over 90% after 3 week of storage at room temperature (ES > 90%).

Dehydration of emulsion systems can be used to increase shelf life to improve their functionality, or facilitate transportation. Freeze-drying has been reported as an efficient method for drying emulsions (Adelmann, Binks, & Mezzenga, 2012; McClements, 2004). Freeze-drying is a method where initially the liquid phase of the material is crystallized at low temperatures and thereafter, sublimated directly from the frozen state into the vapour at low pressure (Liu, Zhao, & Feng, 2008). Retaining stability of double emulsions is a challenge in general and even more so when exposing double emulsions to external stresses such as freezing and drying. When a food emulsion is being cooled and eventually frozen, a variety of physicochemical processes may occur including fat crystallization, ice formation, interfacial phase transitions and other conformational changes (Walstra, 2003). When the O/W emulsions are cooled to temperatures where only the oil phase

becomes partially crystallized, a phenomenon known as partial coalescence is likely to happen due to penetration of oil crystals from one droplet into the liquid region of another partially crystalline oil droplet. On the other hand, when O/W emulsions are cooled to the temperature where the water phase crystallizes, a number of additional physicochemical processes are likely to happen. *i.* Formation of ice crystals can force the oil droplets to closer proximity to each other. *ii.* The hydration of emulsifier at droplet surface can be disturbed. *iii.* Formation of ice crystals will result in an increase in concentration of solutes in the unfrozen aqueous phase which may alter the electrostatic repulsion between the droplets. *iv.* Penetration of ice crystals into the oil droplet may disturb the interfacial membranes and make them prone to destabilization upon thawing. *v.* Emulsifiers may absorb to the surface of ice crystals. *vi.* Emulsifiers may lose their functionality (Thanasukarn, Pongsawatmanit, & McClements, 2004). When emulsions are being dried, their stability may be disrupted due to altered interfacial properties. In order to prevent coalescence during the drying of emulsions, one approach is to add hydrophobic carriers to the formulation. As a result the final powder ends up containing between 30 and 80 wt% of such compounds (Adelmann et al., 2012). Due to interfacial rigidity of Pickering emulsions and elimination of the need for hydrophobic carriers it is possible to produce emulsion powders with high oil content (80%) and therefore compared to surfactant stabilized emulsions, particle stabilized emulsions are more attractive when creating dried formulations (Marefati et al., 2013). Silica nanoparticles and cellulose nanocrystals have been previously used in the development of dry emulsion powders (Adelmann et al., 2012; Aveyard et al., 2003; Tasset, Cathala, Bizot, & Capron, 2014). Aveyard et al. (2003) showed stabilization of emulsions with particles results in a more controlled evaporation of the continuous phase compared to surfactant stabilized emulsions. In addition subsequent evaporation of the dispersed phase results in macro-porous or sponge like solid materials with unique characteristics useful as bio-scaffolds, low weight structures and separation membranes. (Akartuna et al., 2008; Aveyard et al., 2003).

The aim of this study was to develop a novel dried material from starch granule stabilized Pickering double emulsions for food and pharmaceutical purposes. The idea originated from an earlier study on drying of starch granule stabilized simple O/W emulsions (Marefati et al., 2013) and is now extended to multiple emulsions. Structural and encapsulation properties of the W/O/W emulsions and the resulting powders are also investigated to characterize the quality of the emulsion. The effect of the melting point of the oil phase used in the formulations was also studied. Lastly, the effect of *in situ* gelatinization of starch coating layer on overall process stability of double emulsions was also investigated.

2. Materials

In order to facilitate the understanding of the process and materials, we used the terms primary (W_1/O) and secondary emulsion (W_1/O/W_2), where the primary emulsion is the dispersed phase of the secondary emulsion. Phosphate buffer (5 mM, pH 7, 0.2 M NaCl) was used as water phase for both internal (W_1) and continuous phases (W_2). Two different types of shea nut oil (a kind gift from AAK, Karlshamn, Sweden) were used for oil phases differing in melting points. According to the supplier, the liquid shea nut oil (Lipex 205) has a melting point at 15 °C and viscosity at 40 °C of 49 mPa s. The solid shea nut oil (Lipex shea) has a melting point at 33 °C and a viscosity at 40 °C of 59 mPa s. The fatty acid composition and solid fat content of the different oils as a function of temperature are presented in Table 1 and Table 2 respectively. In order to evaluate encapsulation properties, carmine (Procordia Food AB,

Table 1
Fatty acid composition of liquid shea nut oil and solid shea nut oil.

Fatty acid	Liquid Shea nut oil [%]	Solid Shea nut oil [%]
a $\leq$ C14	<1	<1
C16	4–7	5–7
C18:0	24–28	26–28
C18:1	55–58	59–63
C18:2	8–10	2–5
C18:3	<0.5	<0.5
$\geq$C20	<2.5	<2

Table 2
Solid fat content of liquid shea nut oil and solid shea nut oil as a function of temperature.

Temperature [°C]	Liquid Shea nut oil [%]	Solid Shea nut oil [%]
10	0 solid fat content above 10	44–51
20		32–37
30		7–12
35		0.5–3.5
40		<0.5

Sweden) was used. Carmine is a common food colouring agent (E120) that is both stable to heat and light, and at neutral pH it has a bright red colour with maximum light absorption of 520 nm (Matos et al., 2013). Primary emulsions consisted of 20% v/v internal aqueous phase and 80% v/v oil phase. Polyglycerol polyrcinoleate (PGPR 90, Danisco, Denmark) which was previously added to the oil phase at 5% w/v was used as stabilizing agent of primary emulsions. The final ratios were chosen so that the final double emulsions should contain 33% v/v of oil phase. For that 41.25% v/v of dispersed phase (W$_1$/O) was emulsified in 58.75% v/v external water phase (W$_1$). Starch isolated from quinoa (Biofood-Biolivs AB, Sweden) and OSA-modified to 1.8% as described in Rayner, Sjoo, Timgren, and Dejmek (2012) was added at 214 mg starch/ml of dispersed phase (W$_1$/O) as stabilizing agent for Pickering double emulsions. The amylose content given as % of total starch has been reported to be 12 ± 1% for the Bolivian quinoa (Perez, 2014), however this value can be different between the different quinoa cultivars. In this study, the starch is used in granular form and therefore the amylose/amylopectin ratio is not expected to have any substantial impact on stabilizing mechanism, however, may impact the starch gelatinization behaviour during heat treatment. The gelatinization of quinoa starch used here was characterized previously by differential scanning calorimetry (see Section 3.2.) (Timgren et al., 2011).

3. Methods

3.1. Crystallization behaviour of the different oils

The different oils were investigated by differential scanning calorimetry (DSC) (Seiko DSC 6200, Seiko Instruments, Tokyo, Japan). 5–10 mg of samples, previously heated up to 50 °C, were transferred to the DSC aluminium pans and sealed. The samples were loaded to the DSC with empty pans as reference. The temperature intervals were −30 to +50 for melting and +50 to −30 for crystallization with the cooling and heating rate of 5 °C/min. Samples for DSC measurements were prepared in triplicates.

3.2. Preparation and characterization of emulsions

Fig. 2 illustrates the preparation and characterization methods used in this study. Double emulsions were produced in a glass test

tube and with a tooth rim rotor-stator system, Ystral (D-79 282, Ystral GmbH Ballrechten-Dottingen, Germany) with 6 mm dispersing tool, using a two-step emulsification method as follows:

3.2.1. Preparation of primary (W1/O) emulsions

During the first step, primary (W$_1$/O) emulsions were produced by 20% v/v of W$_1$ in 80% v/v oil phase previously mixed with 5% w/v PGPR 90 for 1 h in 45 °C using a magnet stirrer. The W$_1$/O emulsions were prepared by the Ystral mixer at 24 000 rpm for 10 min at 40 °C.

3.2.2. Preparation of double (W1/O/W2) emulsions

Initially, modified starch granules were dispersed in the external water phase (W$_2$) as the stabilizer of double emulsions using a vortex for 10 s. Thereafter, double emulsions were produced by incorporation of primary emulsion (W$_1$/O) into the external water phase (W$_2$) by the Ystral mixer at 22 000 rpm for 30 s at 40 °C. Partial gelatinization was performed in heat treated samples by heating the emulsions in a glass test tube of diameter 14 mm using a water bath up to 70 °C holding for 1 min. The selection of heat treatment temperature was based on previous measurement of gelatinization temperature of quinoa starch granules by DSC. This study showed that the gelatinization onset (T$_o$), peak (T$_p$) and conclusion (T$_c$) temperatures were 46.1 °C, 60.0 °C and 70.4 °C respectively (Timgren et al., 2011). After preparation the samples were transferred to stainless steel dishes of diameter 60 mm to cool down. All samples were produced in duplicates.

3.3. Freeze thaw cycling

Freeze-thaw cycling was performed on non-heated and heated samples to see the effect of the freezing step on double emulsions (W$_1$/O/W$_2$). A total volume of 10 mL of emulsions were transferred to the stainless steel dishes of 62 mm diameter, covered by aluminium foil and placed in a laboratory freezer (−24 °C). The frozen samples were left in the room temperature (20 °C) for 4 h to be thawed.

3.4. Freeze-drying

Samples were frozen using the same method as described in Section 3.3. The aluminium covers were punctured and the frozen emulsions were dried using laboratory freeze-dryer (CD 12, Heto-sicc, Denmark). The temperature in the drying chamber was 20 °C, while the cooling unit had the temperature of −50 °C and the vacuum was 10^{-2} mbar.

3.5. Structural characterization of emulsions and oil-filled powders

In order to investigate the effect of different treatments on structural and encapsulation properties of double emulsions (namely: heat treatment, freeze-thaw cycling and combination of both), duplicates of double emulsions were produced and fresh emulsions were compared with treated emulsions. In addition, freeze-dried double emulsions were reconstituted to the same composition by addition of the same amount of MilliQ water removed by drying. Double emulsions were diluted 5 times with MilliQ water and the starting and treated emulsions and re-hydrated powders were characterized by microscopy (Olympus BX50 and JEOL JSM-6700F, Japan) with 200–1000× magnification and using a digital camera (8DFK 41AF02, Imaging source, Germany) operating on ImageJ software (NIH Ver. 1.42 m). Particle size distributions of emulsion droplets were obtained by laser light scattering (Malvern Mastersizer 2000, UK). MilliQ water was used as carrier media in the sample dispersion unit for the water continuous double emulsions. The particle and dispersant refractive indices of 1.54 and 1.33 were used as proposed for starch and
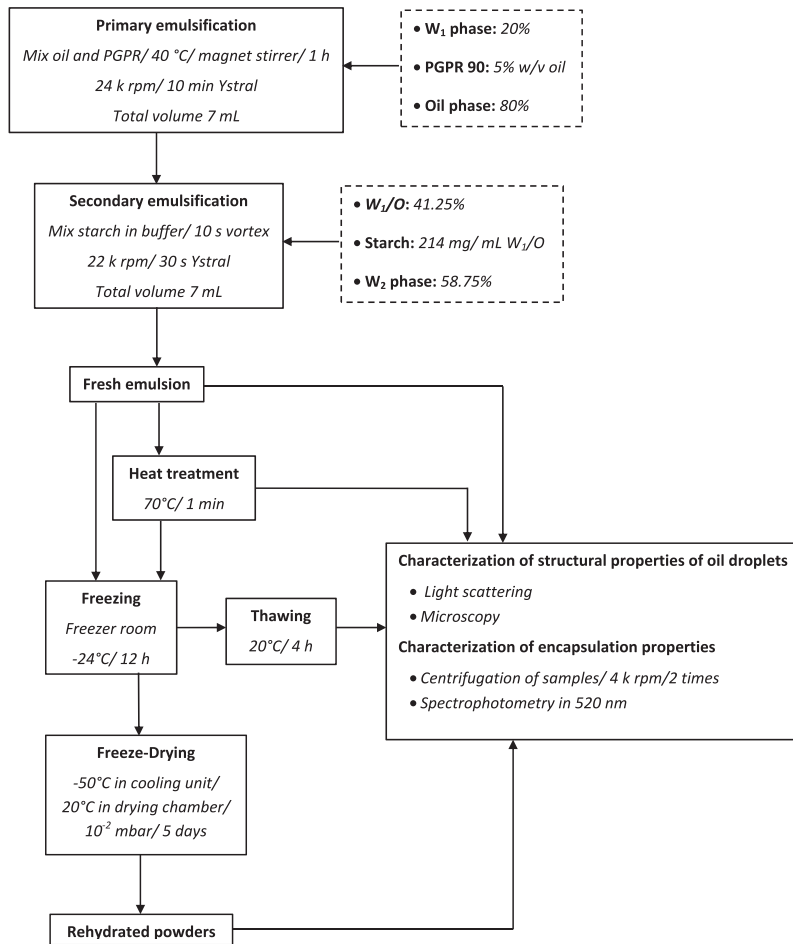
Fig. 2. Illustration of the preparation and characterization methods.

water (Bromley & Hopkinson, 2002). The measurements were done 3 times for each of the duplicates. The time in the flow cell was short (matter of minutes) and did not cause any significant changes in the droplet size repeated measurements on the same sample. The particle size values expressed as mode of volume-weighted diameter D[4,3] and the volume-weighted particle size distributions are presented in figures. Additionally, the other values (including D[3,2], D[4,3], Span of D[4,3]) are presented in Table 4.

3.6. Determination of encapsulation properties of double emulsions

Encapsulation properties of emulsions were characterized using the method that was previously developed by O'Regan and Mulvihill (2009) with some modification. The encapsulation properties of the double emulsions were characterized by centrifugation (Beckman Coulter, Allegera X-15, USA) in 2 steps, 4000 rpm, 10 min followed by spectrophotometry (Carry 50, USA). Since removal of all particles from the external aqueous phase was impossible the reading was corrected for scattering. In order to avoid baseline errors the difference between the absorbance of marker in the major peak (520 nm) with the following valley (545 nm), Δ_{ABS} was used in all the measurements in this study. All readings were at total absorbance <1.5. Different concentrations of internal aqueous phase marker were prepared to develop a calibration curve (Fig. 3).

The encapsulation properties of double emulsions are expressed by encapsulation efficiency (EE%) and encapsulation stability (ES%).
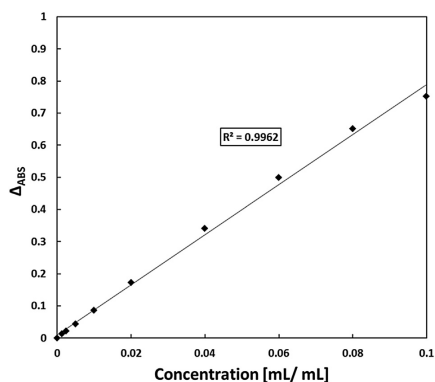
Fig. 3. Calibration curve for carmine as internal aqueous phase marker calculated from Δ_{ABS} 520 and 545 nm as a function of marker concentration.

Encapsulation efficiency (EE%) or yield of a double emulsion is defined as the amount of aqueous phase marker that is retained in the inner aqueous phase (W_1) after the second emulsification step. The encapsulation efficiency (EE%) was determined based on measuring the concentration of the dye found in the external aqueous water phase (W_2). The encapsulation stability (ES%) can be defined as the amount of aqueous phase marker which remains entrapped in the inner phase (W_1) on storage or exposure to processing stresses.

A complication arises from the fact that a certain amount of the internal phase marker can be absorbed to the starch. To correct for this we have quantified the maximum recoverable concentration ($C_{recoverable}$) defined as the relative concentration of marker that could be measured in the external aqueous phase (W_2) when an emulsion has lost all of its internal aqueous phase (W_1 including the marker) to the external aqueous phase. Therefore, O/W_2 emulsions were developed using the same components and with the internal aqueous phase W_1, including marker (at the same concentration as in the double emulsions), added to the O/W_2 emulsions. Using the same method the reference blank was prepared with elimination of the marker.

If C_0 is the expected concentration of the marker based on the initial added amount, the maximum recoverable (expressed as a
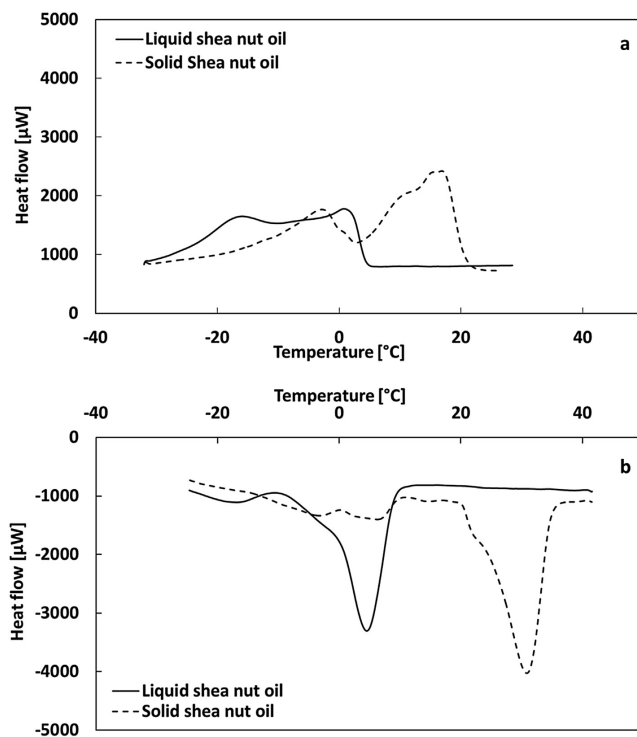

Fig. 4. Crystallization and melting of liquid shea nut oil and solid shea nut oil, a. Crystalization, b. Melting.

percentage of the initial amount added) showed that 39.3% ± 1.5% (n = 2) of dye in the case of liquid oil and 36.7% ± 0.9% in the case of solid oil was possible to recover (n = 2). Although this may be considered somewhat low, the method has been shown to have a high degree of reproducibility over the concentration range tested in previous work (Matos et al., 2013).

The encapsulation efficiency (EE%) was then calculated from the measured concentration of marker in the external phase ($C_{measured}$) by using the corresponding calibration curve and the equations below:

$$EE(\%) = \frac{C_{measured}}{C_{recoverable}} \times 100 \qquad (1)$$

Similarly the encapsulation stability (ES%) after exposure to storage over time or environmental stress can then be calculated by using equation (1) as well. The encapsulation properties (EE% and ES%) are presented in Table 5.

4. Results and discussion

4.1. Crystallization behaviour of the different oils

The results of thermal analysis of oil indicated that the onset (T_o) and peak (T_p) of crystallization for liquid shea nut oil 4.1 ± 0.1 °C and −15.9 ± 0.1 °C respectively (Fig. 4a, Table 3). These values for the solid shea nut oil were 20.5 ± 0.1 °C and 16.5 ± 1.1 °C. According to Fig. 4a, majority of crystallization of the liquid shea nut oil take place around and under 0 °C where crystallization of water takes place. On the contrary, majority of crystallization process of solid shea nut oil takes place at the temperatures higher than that of the water phase. Analysing the melting behaviour of the oils by DSC the showed peak (T_p) and endset (T_{end}) temperatures of 4.5 ± 0.2 °C and 9.0 ± 0.4 °C for the liquid and 31.0 ± 0.3 °C and 35.0 ± 0.5 °C for solid shea nut oil respectively (Fig. 4b, Table 3).

Table 3
Crystallization and melting temperatures of liquid shea nut oil and solid shea nut oil.

Sample	Melting		Crystallization	
	Peak [°C]	Endset [°C]	Onset [°C]	Peak [°C]
Liquid shea nut oil	4.5 ± 0.2	9.0 ± 0.4	4.1 ± 0.1	−15.9 ± 0.1
Solid shea nut oil	31.0 ± 0.3	35.0 ± 0.5	20.5 ± 0.1	16.5 ± 1.1

Values include Mean temperature ± Standard deviation.

4.2. Effect of heat treatment on structural properties of initial emulsions

4.2.1. Liquid shea nut oil

The overall microstructure of the non-heat treated and heat treated liquid shea nut oil can be observed in micrographs Fig. 5a, Fig. 5b. *In situ* heat treatment resulted in gelatinization of starch granules at oil-water interface (Fig. 5b). Particle size distribution of initial double emulsions formulated with liquid oil as the oil phase showed a major peak of 27.6 ± 2.3 µm, with some free starch indicated by the peak on the left of the main peak (sizes between 0.3 and 5 µm) and aggregates of droplets (sizes between 100 and 600 µm) as the one on the right side of the main peak (Fig. 6a, Table 4). When starch granule stabilized double emulsions are heated, partial gelatinization of starch granules occurs. Gelatinization causes both free starch and starch at the droplet interfaces to swell which can be observed in the particle size distribution results.
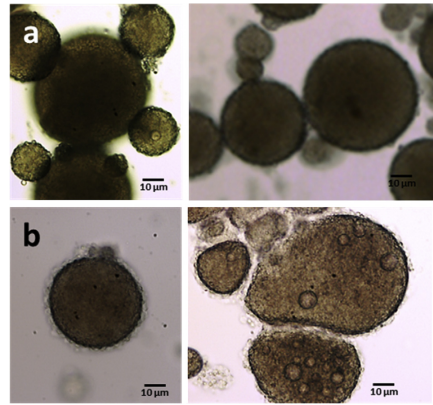
Fig. 5. Micrographs of a. non-heat treated (on the top) and b. heat treated starch granule stabilized double emulsions (on the bottom) with liquid oil as the oil phase.

The corresponding graph for heat treated emulsions represents a major peak of emulsion droplets with mode of 32.0 ± 2.0 µm, a left shoulder being interpreted as gelatinized clumps of free starch (sizes between 2 and 8 µm), and larger sizes between 100 and 2000 µm at the right shoulder of particle size distribution are aggregates of drops (Fig. 6a). Particle size distribution data together with the micrographs indicated that heat treated emulsions were slightly larger. This may be due to swelling of starch granules due to heat treatment and/or formation of networks of gelatinized starch where the emulsion droplets were entrapped within. This is reflected as a significant difference in the mean droplet size D[4,3] for non-heat treated (44.2 ± 4.9 µm) compared to heat treated samples (156 ± 8 µm).
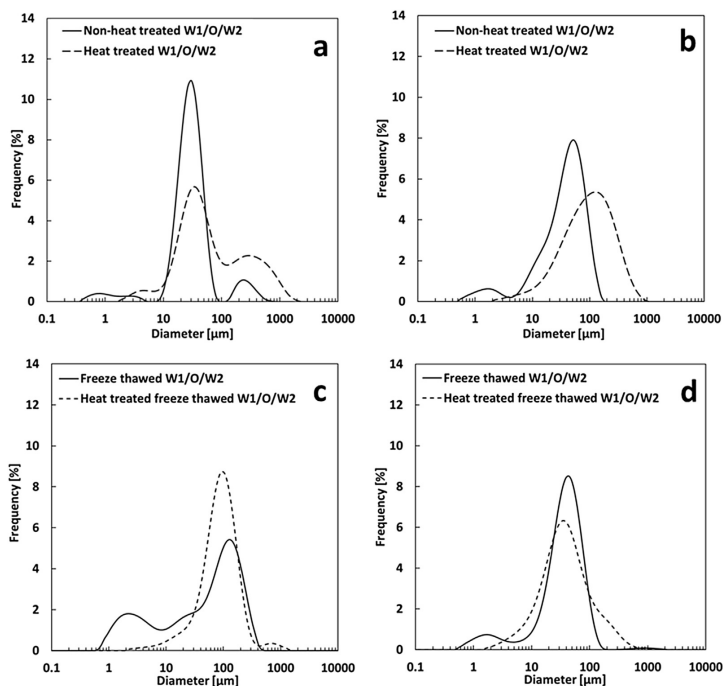
4.2.2. Solid shea nut oil

The initial emulsions with solid oil showed a bi-modal size distribution profile with a major peak at 48.2 ± 0.1 µm, and a minor peak with sizes between 0.5 and 4 µm representing free starch granules (Fig. 6b, Table 4). The difference between the droplet sizes of fresh emulsions between liquid and solid oil can be due to the difference in viscosity of the oil phase (49 mPa s and 59 mPa s for liquid and solid oils respectively) at the homogenization temperature of 40 °C. Viscosity of the oil phase is known to affect the droplet size developed at least in part in a high shear homogenizer. Heat treated samples showed a size distribution profile with larger sizes of gelatinized free starch and a major peak with 118 ± 3 µm in diameter with a larger span as a result of gelatinization of starch and emergence of gelatinized starch networks with entrapped emulsion drops (Fig. 6b, Table 4). The result agrees with previous results from liquid oil samples; however the effect of gelatinized layer and formation of clumps is more pronounced here.

4.3. Effect of freeze thaw cycling with and without heat treatment on structural properties

4.3.1. Liquid shea nut oil

When emulsions are frozen, larger particle size distribution of droplets has been observed with a major peak at 120 ± 1 µm (Fig. 6c, Table 4). As stated earlier in the introduction, it is likely that crystallization of the water phase (when the oil phase is still in

Fig. 6. Volume-weighted particle size distribution (D [4,3]) of particle stabilized double emulsion for a. initial non-heat treated and heated emulsions with liquid shea nut oil as the oil phase, b. initial non-heat treated and heat treated emulsions with solid shea nut oil as the oil phase, c. freeze-thawed and freeze-thawed heat treated emulsions with liquid shea nut oil as the oil phase, and d. freeze-thawed and freeze-thawed heat treated emulsions with solid shea nut oil.

Table 4
Different size parameters of W1/O/W2 emulsions with liquid and solid shea nut oil as oil phase including: freshly prepared initial W1/O/W2 emulsions and heated W1/O/W2 emulsions, frozen and thawed W1/O/W2 emulsions and heated and frozen and thawed W1/O/W2 emulsions, freeze-dried W1/O/W2 emulsions and heated and freeze-dried W1/O/W2 emulsions.

Sample	D [3,2] [μm]	D [4,3] [μm]	Span of D [4,3]	Mode of D [4,3] [μm]
Freshly prepared initial liquid $W_1/O/W_2$	11.3 ± 0.7	44.2 ± 4.9	1.6 ± 0.3	27.6 ± 2.3
Heat treated liquid $W_1/O/W_2$	27.4 ± 1.4	155.6 ± 8.1	10.0 ± 1.8	32.0 ± 2.0
Freeze thawed liquid $W_1/O/W_2$	7.3 ± 0.1	80.0 ± 2.1	3.2 ± 0.3	120.0 ± 0.7
Heat treated freeze thawed liquid $W_1/O/W_2$	48.6 ± 3.3	105.8 ± 8.0	1.8 ± 0.1	90.2 ± 6.5
Freshly prepared initial solid $W_1/O/W_2$	11.8 ± 0.0	41.8 ± 0.3	1.9 ± 0.1	48.2 ± 0.1
Heat treated solid $W_1/O/W_2$	42.7 ± 1.1	129 ± 7	2.9 ± 0.1	118 ± 3
Freeze thawed Solid $W_1/O/W_2$	11.2 ± 0.7	44.7 ± 7.5	1.9 ± 0.3	39.8 ± 0.1
Heat treated freeze thawed solid $W_1/O/W_2$	20.7 ± 0.3	59.2 ± 5.0	3.6 ± 0.2	33.0 ± 0.2
Reconstituted dried non-heated liquid $W_1/O/W_2$	Emulsion collapsed			
Reconstituted dried heat treated liquid $W_1/O/W_2$	104 ± 0.1	240 ± 5	2.1 ± 0.2	210 ± 11
Reconstituted dried non-heat treated solid $W_1/O/W_2$	10.6 ± 1.2	60.2 ± 19.4	2.7 ± 0.3	62.4 ± 3.2
Reconstituted dried heated solid $W_1/O/W_2$	47.1 ± 3.5	114 ± 16	2.5 ± 0.1	85.2 ± 11.1

Values include Mean of different size measurements ± Standard deviation.

liquid state according to the DSC results) forces the oil droplets into closer proximity of each other which promotes destabilization of emulsions. As water freezes, ice crystals expand forcing droplets together, potentially causing deformation of oil droplets and lateral displacement of the stabilizing particles, which eventually may lead to oil−oil contact and hence coalescence. Moreover, further development of crystals (both in the water phase and the oil phase) will cause disruption and detachment of stabilizing particles from

the surface of the oil droplets. These mechanisms have also been observed in studies by Thanasukarn et al. (2004). The particle size distribution profile in the freeze-thawed emulsions with liquid oil phase indicated a relatively higher amount of free starch compared to initial un-frozen samples as seen in the minor peak (sizes between 0.5 and 0.8 μm). This is interpreted as the detachment of starch, leading to lower surface coverage that may have resulted in coalescence. Micrographs in Fig. 7a confirm excess amounts of free
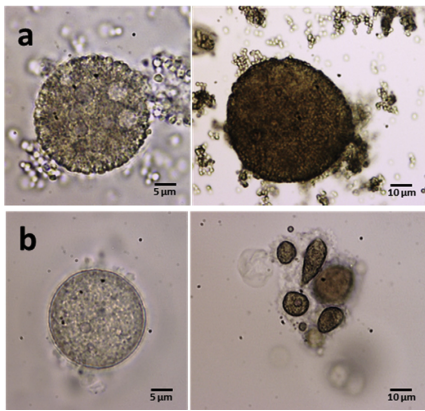
Fig. 7. Micrographs of a. non-heat treated (on the top) and b. heat treated starch granule stabilized double emulsions (on the bottom) with liquid oil as the oil phase after freeze thaw cycling.

starch when compared to Fig. 6c. This observation generally agrees with earlier observation of Marefati et al. (2013) where emulsion samples with liquid oil droplets were frozen and formation and shrinkage of fat crystals along with protrusion of fat crystals caused displacement of starch from oil-water interfaces. This displacement of starch can result in partial coalescence that will further results in destabilization of emulsions. As the emulsions are thawed both water and oil crystals undergo phase change. In the case of liquid oil, there is still a significant fraction of the oil that melts between 0 °C and 5 °C (Fig. 4a, Table 3). Penetration of oil crystals from one droplet to another droplet can cause the oil content of that droplet to flow out and make a linkage between two drops that may in turn result in complete coalescence upon thawing (Boode & Walstra, 1993; Cramp, Docking, Ghosh, & Coupland, 2004). This is reflected as larger droplet sizes in particle size distribution (Table 4).

A freeze-thaw cycling of partially gelatinized emulsion results in a size distribution with a major peak of 90.2 ± 6.5 μm and some gelatinized starch clumps (sizes between 300 and 1500 μm) as the right shoulder (Fig. 6c, Table 4). The level of free starch here is not as high as the non-heated samples after freeze-thaw cycling. However the larger sizes of free starch granules is a result of gelatinization and swelling of the free starch granules. The larger droplet sizes observed in particle size distributions may also be due to gelatinized starch layer and the formation of clumps where two or more neighbouring droplets with gelatinized starch adhere to one another (Fig. 7b).

4.3.2. Solid shea nut oil

The freeze-thaw cycling pre-treatments imposed changes in emulsions to a lower extent when solid oil was used. According to (Thanasukarn et al., 2004) when the emulsions are cooled down the temperatures where only the oil phase is crystalized (Fig 4a, Table 3) but the water phase is still in liquid state, partial coalescence can be observed which explains the slight shift in particle size. The particle size distribution graph in freeze-thawed samples showed a profile with a minor peak corresponding to, but slightly larger than that of the initial un-frozen emulsions having a droplet size peak of 39.8 ± 0.1 μm (Fig. 6d, Table 4). This result agrees well with the results reported earlier by Marefati et al. (2013) where freeze-thaw cycling has lower impact on solid shea nut oil emulsions.

The reason that solid oil does not follow the same freeze-thaw pattern as liquid oil can be attributed to the higher physical stability of the solid oil droplets during freezing step. According to observations by (Marefati et al., 2013) when the oil phase of the emulsion is crystalized before the water phase, susceptibility of the oil droplets to compression due to crystallization of water phase is attenuated. The combination of heating and freeze-thaw cycling treatments showed a particle size distribution with a larger span and a major peak of 33.0 ± 0.2 μm (Fig. 6d, Table 4). This result is attributed to the protective barrier properties of gelatinized layer formed by heating emulsions.

4.4. Effect of oil type and heat treatment on stability during freeze-drying

Upon freeze-drying, only some of the Pickering double emulsions resulted in oil filled powders. Due to the lower freezing stability of emulsions produced from liquid shea nut oil, only heat treated samples resulted in powders, whereas the non-heated samples collapsed during drying. However for solid shea nut oil in both cases of heated and non-heated samples powders were produced. Fig. 8 shows the dried double emulsions from solid shea nut oil. Non-heat treated samples produced a very fine powder while heated samples represented a more pellet looking dried system. Upon rehydration, samples exhibited some degree of droplet aggregation (Fig. 10), however non-heat treated samples with solid fat showed individually existing droplets and therefore lower size in particle size distribution measurements (62.4 ± 3.2 μm, Fig. 9b, Table 4) compared to heated samples (210 ± 11 μm for liquid oil and 85.2 ± 11.1 μm for solid oil, Fig. 9a and b, Table 4). Comparing the size distribution of original and dried rehydrated emulsions showed that there was an increase in overall size distribution that is due to aggregation, rather than coalescence of individual drops.

4.5. Encapsulation properties

The encapsulation properties are classified in two different categories: encapsulation efficiency (EE%) for initial emulsions measured immediately after emulsification, and encapsulation stability (ES%) for the sample after different treatments and process steps (heat treatment, freeze-thaw, freeze-drying and re-hydration). In general, a double emulsion is considered to have a good stability when the initial encapsulation efficiency is around 95% and when the encapsulation stability after a few weeks of still storage is around 70–80% (Dickinson, 2011). Encapsulation efficiency of the initial double emulsions was 96.6% ± 4.2% for liquid shea nut oil and 98.5% ± 0.2% for solid shea nut oil (Table 5). This results are in accordance with the results previously reported by Matos et al. (2013) in double
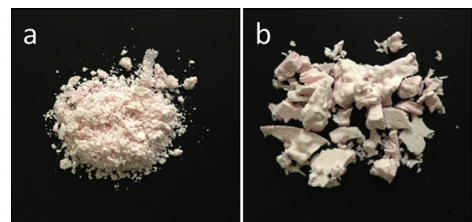

Fig. 8. Dried solid shea nut oil emulsion powders, a. non heated freeze-dried double emulsion left and b. heated freeze-dried double emulsion on the right.
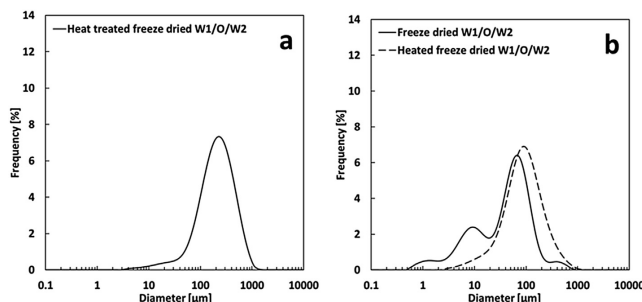
Fig. 9. Volume-weighted particle size distribution D[4,3] of dried particle stablized double emulsion a. with liquid shea nut oil as oil phase after rehydration and b. with solid shea nut oil as oil phase after rehydration.
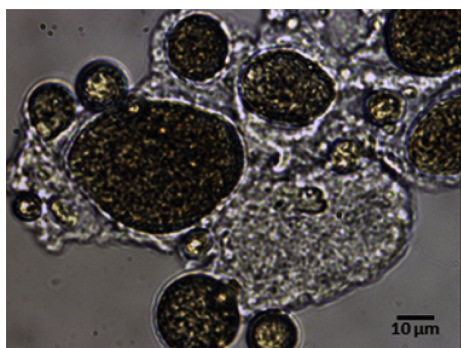

Fig. 10. Micrographs of dried heat treated starch granule stabilized double emulsions with liquid oil as the oil phase after rehydration.

emulsions using OSA modified starch as stabilizer for the external emulsions, and higher than the previously reported results by O'Regan and Mulvihill (2009) where sodium caseinate was used as stabilizer for the external emulsions. Moreover, the results showed that in all cases for all of the different pre-treated samples and even after drying procedure, with little change, encapsulation stability of the double emulsions for both cases of liquid and solid emulsions had high values (over 97.3%, Table 5) with the exception of non-heat treated freeze-dried emulsions in which case the emulsion collapsed upon water removal (as discussed under structural properties above).

Table 5
Encapsulation properties of starch granule stabilized W/O/W emulsions for liquid and solid oil.

Sample	EE % liquid	EE % solid
Freshly prepared initial $W_1/O/W_2$	96.6 ± 4.2	98.5 ± 0.2
Sample	EE % liquid	EE % solid
Heat-treated $W_1/O/W_2$	97.6 ± 2.0	99.4 ± 0.2
Freeze-thawed $W_1/O/W_2$	98.8 ± 0.1	98.5 ± 0.4
Non-heat treated Freeze-dried $W_1/O/W_2$	Emulsion collapsed	97.3 ± 0.6
Heat treated Freeze-dried $W_1/O/W_2$	97.5 ± 0.4	97.3 ± 0.2

Values are presented as Mean of EE % ± Standard deviation and Mean of ES % ± Standard deviation.

5. Conclusions

This study demonstrates the feasibility of production of starch granule stabilized double emulsions with high encapsulation efficiency using both liquid and solid oils as the oil phase. Moreover, this study showed that it is possible to develop oil containing powders from starch Pickering double emulsion with high encapsulation stability (over 97%) and high oil content ~70 wt%. Physical stability tests revealed that using modified starch granules may lead to stability of emulsions towards process stresses (freezing and freeze-drying in this case). The stability of solid oil based emulsions during freezing and freeze-drying was higher than that of the liquid oil formulations since liquid oil droplets are more susceptible to destabilization when the external aqueous phase crystalizes. Additionally, the stability of emulsions produced by liquid oil can be enhanced by formation of protective barrier made by partial gelatinization of starch. This system can be applied in versatile food and pharmaceutical formulations where encapsulation of an internal aqueous phase within O/W emulsions is desirable.

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Paper V

Storage and digestion stability of encapsulated curcumin in emulsions based on starch granule Pickering stabilization

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ARTICLE INFO

ABSTRACT

Stability and loss of encapsulated curcumin was investigated in starch granule stabilized Pickering emulsions. Heat treated and non-heat treated Pickering emulsions have been studied during storage, and during simulated oral, gastric and intestinal in vitro digestion. The amount of retained curcumin in Pickering emulsions was characterized based on spectrophotometric measurements as a function of time. Physical stability of emulsions was characterized using light microscope and light scattering particle size analyzer. Pickering emulsions showed good encapsulation efficiency (~ 80%) and good stability. Heat treated Pickering emulsions showed better encapsulation stability than non-heat treated Pickering emulsions during 24 h storage (78.2% vs. 38.3%), and during oral (95.3% vs. 69.6%) and intestinal (86.3% vs. 40.2%) simulated in vitro digestions. In the case of simulated gastric in vitro digestion, there was no statistically significant difference in the curcumin content between non-heat treated (86.2%) and heat treated (82.4%) emulsions (P > 0.05). Moreover, comparing the physical stability of emulsions during simulated in vitro intestinal digestion with and without bile salts, it was shown that samples with bile salts showed a larger extent of changes, and these changes were more gradual in heat treated samples. Overall, this study demonstrates that Pickering emulsions stabilized with quinoa starch granules have a potential for effective delivery of bioactive compounds such as curcumin.

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1. Introduction

There has been a growing interest in therapeutic properties of curcumin. Curcumin is a natural polyphenol present in the rhizomes of the turmeric plant (Anand, Kunnumakkara, Newman, & Aggarwal, 2007). Turmeric (Curcuma longa) is a popular spice in south and central Asian cuisines (Chattopadhyay, Biswas, Bandyopadhyay, & Banerjee, 2004). In addition, the rhizomes of turmeric have been used in Indian and Chinese traditional medicine for many centuries in treating a variety of disorders including biliary disorders, cough, diabetic ulcers, hepatic disorders, rheumatism, sinusitis, wounds (Ammon, Anazodo, Safayhi, Dhawan, & Srimal, 1992) and diseases associated with abdominal pain (Araújo & Leon, 2001). Curcumin, 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5 dione is the main curcuminoid present in the turmeric (Chattopadhyay et al., 2004; Noorafshan & Ashkani- Esfahani, 2013) which is responsible for its yellow color and was first isolated in 1815 (Vogel & Pelletier, 1815).

Curcumin has been suggested to impose wide range of biological and pharmacological activities including anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, antialzheimer's, as well as hypotensive and hypocholesteremic activities (Aggarwal, Surh, & Shishodia, 2007; Chattopadhyay et al., 2004; Hamaguchi, Ono, & Yamada, 2010; Maheshwari, Singh, Gaddipati, & Srimal, 2006; Noorafshan & Ashkani- Esfahani, 2013). Furthermore, studies on both animal models and humans have proven that curcumin is extremely safe even in very high doses (Anand et al., 2007). Therefore, there is a potential for development of a functional food for treatment or prevention of various diseases using curcumin as an active substance.

Curcumin shows low solubility in water at acidic and neutral pH (0.6 μg/mL), however it is soluble at alkaline pH and therefore the use of curcumin is somewhat limited (Kurien, Singh, Matsumoto, & Scofield, 2007; Tønnesen, Másson, & Loftsson, 2002). On the other hand, a study on solution stability of curcumin showed that the stability of curcumin at acidic pH is higher and decreases as the pH increases (Joshi, Ahmed, Suresh, & Kowti, 2010; Tønnesen & Karlsen,

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1985). At lower pH (3—7) the bis-keto form of heptadienone link dominates (Fig. 1a), and due to delocalization of unpaired electron on the adjacent oxygen and therefore presence of a highly activated carbon atoms in the resulting weak C-H bond, curcumin act as highly effective H-atom donor. In contrast, at higher pH, the enolate form of heptadienone link predominates (Fig. 1b), and curcumin acts as an electron donor (Jovanovic, Steenken, Boone, & Simic, 1999). The enolate form of curcumin is unstable and rapidly degrades to *trans*-6-(40-hydroxy-30-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane and vanillin (Lin, Pan, & Lin-Shiau, 2000). In addition, curcumin is a lipophilic molecule with rapid permeability through the cell membrane (Noorafshan and Ashkani- Esfahani, 2013). Over the past few decades, studies have demonstrated poor absorption and rapid metabolism that decrease the bioavailability of curcumin (Anand et al., 2007). Therefore there have been numerous approaches proposed for improving the bioavailability of curcumin. Some of these methods include addition of adjuvant to modulate the metabolism of curcumin, incorporation of curcumin in novel formulations through the formation of nanoparticles, liposomes, micelles, phospholipid complexes and emulsions, which result in longer circulation, better permeability and improved bioavailability (Anand et al., 2007; Tikekar, Pan, & Nitin, 2013).

Using emulsions as a delivery system and for encapsulation of bioactive compounds (especially those insoluble in water) is a common formulation approach to develop functional food materials in the food industry (McClements, Decker, Park, & Weiss, 2009; McClements, Decker, & Weiss, 2007). Emulsifiers are commonly used for stabilization of emulsions against creaming flocculation, and coalescence (Dickinson, 1992; Mun, Decker, & McClements, 2007). Emulsions, especially those stabilized with biopolymers and small molecular surfactants, have been extensively studied for encapsulation, oxidative stability and release of bioactive compounds (Li, Zheng, Xiao, & McClements, 2012; Malaki Nik, Wright, & Corredig, 2011; Mun, Decker, McClements, 2007; Singh, Ye, & Horne, 2009). Particle stabilized emulsions (Pickering emulsions) have demonstrated many beneficial properties including high stability and advantages in encapsulation, controlled and targeted release of active substances (Frelichowska, Bolzinger, & Chevalier, 2010). In addition, unlike other types of emulsifiers, particles form a rigid but porous shell structure with reduced mobility of the particles at the interfaces, and in most cases provide a thicker barrier (Aveyard, Binks, & Clint, 2003; Dai, Sharma, & Wu, 2005; Tambe & Sharma,

1993; Tarimala & Dai, 2004). Silica nanoparticles have been used in formulation of curcumin containing emulsions by Tikekar et al. (2013) who found a higher stability of curcumin in the Pickering system compared to distilled water. Moreover, they reported that 80% and 40% of curcumin was retained in the system after simulated gastric digestion and simulated intestinal digestion respectively. Although silica is one of the most commonly studied stabilizers of Pickering emulsions (Aveyard et al., 2003; Frelichowska, Bolzinger, & Chevalier, 2009; Simovic & Prestidge, 2004, 2007; Zou, Liu, Yang, Wei, & Wang, 2013) and silica nanoparticles are frequently used in various food and pharma formulations as stabilizers, processing aids and thickening agents, it is not a biodegradable or digestible material (Arditty, Schmitt, Giermanska-Kahn, & Leal-Calderon, 2004; Chaudhry, Castle, & Watkins, 2010; Frelichowska et al., 2010; Simovic & Prestidge, 2003).

There has been increasing interest in food-based particles for the stabilization of Pickering emulsions such as protein-based, lipid-based and carbohydrate-based particles. Carbohydrate-based particles studied include chitin nanocrystals, microcrystalline cellulose and starch (Berton-Carabin & Schroën, 2015). Recently, starch granules have been studied for use in Pickering emulsions (Li, Li, Sun, & Yang, 2013; Marku, Wahlgren, Rayner, Sjöö, & Timgren, 2012; Matos, Timgren, Sjöö, Dejmek, & Rayner, 2013; Rayner, Sjöö, Timgren, & Dejmek, 2012; Simsek, Ovando-Martinez, Marefati, Sjöö, & Rayner, 2015; Yusoff & Murray, 2011). Native starch granules are hydrophilic, and thus their partial dual wettability is enhanced by chemical modification using octenyl succinic anhydride (OSA) to increase their hydrophobic nature allowing better adsorption at the oil-water interface (Rayner et al., 2012). Chemically modified starch granules are biodegradable and accepted for use as food ingredient (E1450) and pharmaceutical excipient with <3% OSA (Timgren, Rayner, Sjöö, & Dejmek, 2011). Previously, starch granules from quinoa due to small sizes and unimodal size distribution were shown to have superior efficiency in stabilization of oil-in-water emulsions compared to starches from other botanical sources (Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013). In addition to emulsification properties of starch granules, due to the physicochemical properties of starch, it is possible to gelatinize starch granules at the oil-water interfaces by controlled heat treatment. The effect of starch gelatinization in improving barrier properties as well as optimization of functional utility and process stability of emulsions stabilized by starch granules has been studied previously (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013; Marefati, Sjöö, Timgren, Dejmek, & Rayner, 2015; Timgren et al., 2011).

Despite a broad understanding of the unique physicochemical properties of starch granule stabilized Pickering emulsions, there is limited characterization of stability and release of bioactive compounds encapsulated using this emulsification technology. However, some recent studies have evaluated the role of starch particles in improving oxidative stability of oil (Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012) or barrier properties of heat treated starch towards lipolysis (Sjöö, Emek, Hall, Rayner, & Wahlgren, 2015). Thus, this study aims to investigate the stability of both emulsions and encapsulated curcumin in starch granule stabilized Pickering emulsions during 24 h of storage, as well as during simulated oral, gastric and intestinal *in vitro* digestion. Since the bile salts play major role in lipid digestion, the intestinal digestion was performed with and without bile salts as control samples.

2. Materials and methods

2.1. Materials

Hydrophobized quinoa starch granules (0.6% OSA) with mode $D_{[4,3]}$ of 1.8 ± 0.1 μm (Fig. 2a, b and Table 1) obtained from white

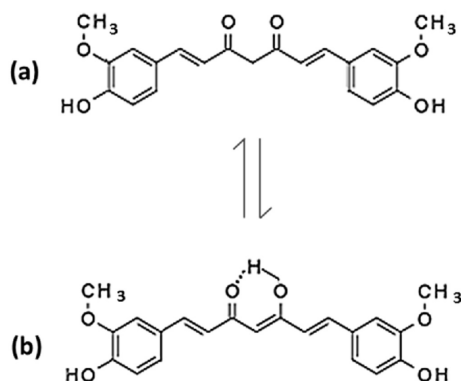**Fig. 1.** Tautomerism of curcumin in different pH ranges, **(a)** Bis-keto form under acidic and neutral conditions, **(b)** Enolate form under basic conditions.
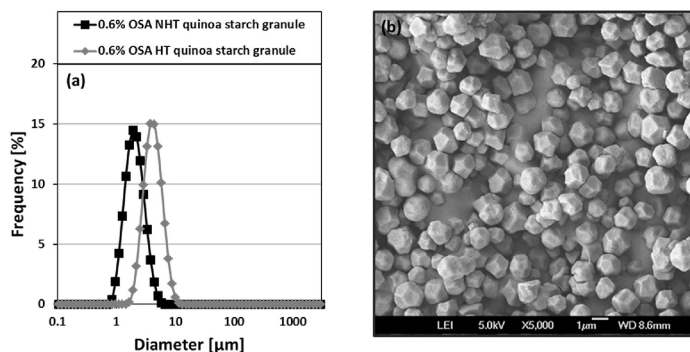
Fig. 2. (a) Particle size distribution ($D_{[4,3]}$) for non-heat treated (NHT) and heat treated (HT) quinoa starch granule, n = 6, **(b)** Scanning electron microscopy image of quinoa starch granule.

Table 1
Particle size parameters of starch granules and starch granule stabilized Pickering emulsions including: non-heat treated starch granule, heat treated starch granules, emulsion without curcumin, non-heat treated emulsions (NHT), heat treated emulsions (HT) and non-heat treated emulsions (NHT$_{t24}$) and heat treated emulsions (HT$_{t24}$) after 24 h.

Sample	$D_{[4,3]}$ [µm]	Span of $D_{[4,3]}$ [µm]	Mode of $D_{[4,3]}$ [µm]	$d_{(0.5)}$ [µm]
0.6% OSA NHT quinoa starch granules	2.0 ± 0.1	1.0 ± 0.1	1.8 ± 0.1	1.9 ± 0.1
0.6% OSA HT quinoa starch granules	4.1 ± 0.1	0.9 ± 0.1	3.8 ± 0.1	3.8 ± 0.1
Emulsions without curcumin	31.0 ± 3.0	1.4 ± 0.4	33.4 ± 1.0	30.4 ± 2.8
NHT	32.6 ± 0.6	0.9 ± 0.1	30.6 ± 0.3	30.7 ± 0.4
HT	26.2 ± 0.7	1.5 ± 0.1	28.0 ± 0.6	25.6 ± 0.4
NHT$_{t24}$	31.8 ± 1.2	0.9 ± 0.1	31.1 ± 0.7	30.8 ± 0.4
HT $_{t24}$	27.1 ± 0.7	1.5 ± 0.1	28.7 ± 0.7	26.4 ± 0.4

Values are mean ± standard deviation, n = 6.

Bolivian quinoa grains (Biofood-Biolivs AB, Sweden) according to a modified version of the method described previously (Rayner et al., 2012), were used as emulsion stabilizer in this study. The protein level of quinoa starch granules was found to be 0.22% ± 0.00 (n = 2) using a nitrogen/protein analyzer (Flash EA 1112 Series, Thermo Scientific, USA). The amylose level (%, w/w) determined using a amylose/amylopectin assay (Megazyme International, Ireland) which is a modified version of the method developed by Yun and Matheson (1990) for estimation of amylose content of starches after precipitation of amylopectin by concanavalin a and found to be 10.7% ± 1.1 (n = 6).

Phosphate buffer (5 mM, 0.2 M NaCl, pH 6.5) were used as continuous (aqueous) phase, and a medium chain triglyceride, Miglyol 812 (density 945 kg m⁻³, Cremer Oleo GmbH & Co, Germany) as the dispersed (oil) phase. Curcumin (Sigma-Aldrich, USA, Product No. C1386), with ≥65% purity according to manufacturer was used. Dialysis cassettes of molecular weight cut off 3500 Da (Thermo Scientific, USA), were used to characterize storage and digestion stability of curcumin. The molecular weight cut off of dialysis cassettes was chosen in such a way that it retained the emulsions and the digestive enzymes and allowed the transport of curcumin through the dialysis membrane.

Simulated salivary fluid (SSF) was prepared according to a previous study (Hur, Decker, & McClements, 2009) using α-amylase

from Aspergillus oryzae (Sigma Aldrich Product No. 86250, 1.5 U/mg), KCl (1.2 M), KSCN (0.2 M), NaH$_2$PO$_4$ (0.74 M), NaCl (1 M), NaHCO$_3$ (1 M), Urea (0.42 M), Uric acid (Sigma Aldrich Product No. U2625), mucin type I-S (Sigma Aldrich Product No. M3895), HCl (1 M) and NaOH (1 M). Simulated gastric fluid (SGF) was prepared by pepsin from porcine gastric mucosa (Sigma Aldrich Product No. P7000, >250 U/mg), NaCl, HCl (1 M), NaOH (1 M), NAHCO$_3$ (1 M). For simulated intestinal fluid (SIF), pancreatin from porcine pancreas (Sigma Aldrich Product No. P1500, activity at least equivalent to U.S. Pharmacopeia), bile extract from porcine (Sigma Aldrich Product No. B8631), KH$_2$PO$_4$ (0.05 M), NaOH (0.2 M), HCl (0.2 M) were used. Methanol was used to break the emulsions in order to measure the tracer. All chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Characterization of gelatinization properties of starch

The gelatinization properties of quinoa starch granules were analyzed using a differential scanning calorimeter (DSC, Seiko 6200, Seiko instruments Inc., Japan), calibrated with indium (M_p = 156.6 °C). Starch dispersions were prepared and weighed into coated aluminum pans (TA Instruments, USA) at a ratio of 1:10 with a scanning rate of 10 °C/min from 10 to 120 °C. The gelatinization transition enthalpy (ΔH, J/g dry matter), gelatinization onset temperature (°C), gelatinization peak temperature (°C) and gelatinization conclusion temperature (°C) were determined.

2.2.2. Curcumin-oil mix preparation

Initially the oil fraction was prepared. Curcumin was added to Miglyol 812 at the level of 1250 µg/mL and the combination was mixed at 22 000 rpm for 20 min with a rotor-stator high shear homogenizer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool. Thereafter, in order to separate the undissolved curcumin, the mix was centrifuged using an ultracentrifuge (Beckman LE-80K, USA) at 14 000 rpm for 10 min. The supernatant was collected and placed aside to be used in subsequent experiments. The concentration of curcumin in the mix was determined by addition of 0.49 mL of the oil to 6.51 mL of methanol to mimic the same concentrations as in the emulsion systems. Thereafter, 200 µL of the mix was once again mixed with 1.2 mL of methanol and absorbance was realized at 425 nm using UV–Vis spectrophotometry Cary 50 (Varian, Australia). The initial concentration of the curcumin in miglyol 812 was found to be 250 µg/mL.

This concentration was found to be the highest concentration that could be dissolved in miglyol 812 at room temperature.

Different concentrations of curcumin in methanol were prepared and a calibration curve was developed: *Absorbance* = 0.1844 × [*Curcumin* μg/mL] + 0.0076 (R = 1).

2.2.3. Pickering emulsion preparation

The emulsions were prepared in glass test tubes with a total volume of 7 mL and 7% v/v of oil fraction including curcumin. The continuous phase was prepared by dispersing OSA modified quinoa starch granules (214 mg/mL oil) in 6.57 g (corresponding to 93% v/v) of phosphate buffer solution. Emulsions were used for experiments immediately after emulsification. The emulsions were prepared by mixing the continuous phase and the oil fraction containing curcumin with a high-shear homogenizer with 6 mm dispersing tool at 22 000 rpm for 30 s. The whole emulsification process was performed at room temperature (~ 20 °C). These emulsions are referred as non-heat treated emulsions (NHT) throughout the text.

In previous work, it has been observed that heat treatment of starch granules stabilized emulsions creates a more impermeable layer and therefore, some of the emulsions were heat treated after emulsification. Emulsions in glass test tubes were placed in a water bath at 70 °C under moderate stirring at 500 rpm using an overhead mixer, Stir 20 (Inter Med, Denmark) to keep the droplets apart but without causing any additional homogenization. The heating process to 70 °C was monitored using a type K, 0.1 mm thermocouple which took around 3 min and the emulsions were kept at this temperature for an additional 1 min. The emulsions were kept stirred while being cooled to room temperature in air. These emulsions are referred as heat treated emulsions (HT) throughout the text.

2.2.4. Characterization of structural properties of the starch granules and the emulsions

2.2.4.1. Light microscopy.
The emulsions were characterized by light microscopy using a camera (DFK 41AF02, The Imaging Source, Germany) that was attached to a light microscope (Olympus BX50, Japan) and was connected to a computer. The emulsions were diluted 5 times with MilliQ water and then one drop was placed on a glass microscopic slide. In order to prevent deformation of droplets no cover glass was used. The microscopic images were taken using objective magnifications of 10× and 50×.

2.2.4.2. Scanning electron microscopy.
Starch granules and dried starch granule stabilized emulsions were characterized by scanning electron microscopy (SEM). The dried samples were coated with gold and examined under SEM (field emission SEM, JSM-6700F, JEOL, Japan) Operated at 5 kV with a working distance of 8 mm. Lower detection imaging mode (LEI) was used to give clear three-dimensional images of the sample surface. The LEI detector combine both signals secondary and back scattered electrons during operation.

2.2.4.3. Light scattering.
The particle size distributions of the starch granule stabilized emulsions were characterized with a laser diffraction particle size analyzer, Mastersizer 2000 (Malvern Instruments, UK). Each emulsion was added to the flow system (Hydro SM small volume wet dispersion unit) containing MilliQ-water and was then pumped through the optical chamber where it was measured. The refractive index of starch particles was set to 1.54 and the refractive index of the continuous phase was set to 1.33 which is the refractive index of the water and the obscuration was between 10 and 20%. For each emulsion sample added to the flow system three measurements were performed, and all emulsions were prepared in duplicates.

Some characteristics of the emulsions were then obtained including: overall particle size distributions, $D_{[4,3]}$, span of $D_{[4,3]}$, mode of $D_{[4,3]}$ and $d_{(0.5)}$. The $D_{[4,3]}$ refers to the volume weighted mean diameter of the droplets.

In the similar manner, the particle size distributions of non-heat treated starch granules and heat treated starch granules were characterized after performing the same preparation procedure in buffer but without the oil phase (and heat treatment if applicable). The particle size distribution of these starch granule dispersions were characterized as described above.

2.2.5. Characterization of retained marker in the emulsions

2.2.5.1. Measurement of retained curcumin in the system.
To be able to measure retained curcumin, methanol was used to break the emulsions. 200 μL of the emulsion sample was added to 1 mL of methanol and then centrifuged at 14 000 rpm for 10 min in order to remove the starch granules (Tikekar et al., 2013). Then 800 μL of the supernatant and 200 μL methanol were placed in a cuvette. The absorbance was measured using a spectrophotometer at 425 nm using methanol as blank. The wavelength was chosen according to a previous study (Taylor & McDowell, 1992) and checked with the scan performed on curcumin containing oil using the same procedure.

2.2.5.2. Curcumin stability in emulsions in excess of water.
In order to measure the release of curcumin from Pickering emulsions in
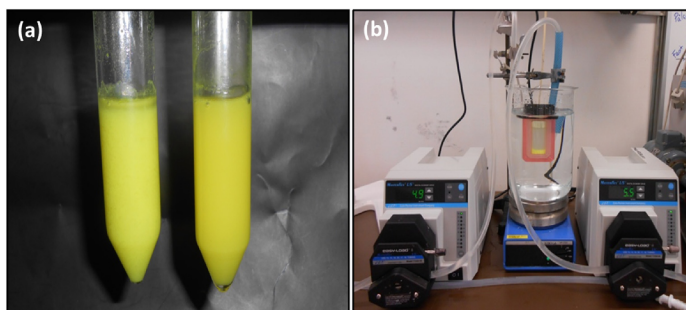
Fig. 3. **(a)** Non-heat treated emulsion (NHT, on the left) and heat treated emulsions (HT, on the right), both with encapsulated curcumin, **(b)** Experimental set up for release tests in excess water using dialysis cassette and 2 peristaltic pumps to maintain the water exchange.

water, dialysis cassettes have been used with excess of water, by adapting a previous method (Tikekar et al., 2013). 10 mL of the non-heat treated (NHT) or heat treated (HT) emulsions (Fig. 3a) were placed in a dialysis cassette (molecular weight cut off 3500 Da) then the dialysis cassette was placed in a 2.5 L beaker of distilled water at room temperature and under magnetic stirring. The dialysis cassette stayed in the water for 24 h and water was renewed at approximately 3 L/h thanks to two peristaltic pumps where one brought the water in and the other one took the water out of the beaker (Fig. 3b) to avoid the saturation of water from the released curcumin. After definite time intervals (1, 2, 3, 6 and 24 h) the cassette was taken out and the content was mixed. Thereafter, 200 μL of the emulsion was pipetted out of the dialysis cassette and the curcumin concentration was determined using the method described in Section 2.2.5.1.

2.2.6. In-vitro digestion

2.2.6.1. Simulated oral digestion. Simulated salivary fluid was prepared according to a previous method (Hur et al., 2009), by mixing 10 mL of KCl solution (1.2 M), 10 mL of KSCN solution (0.2 M), 10 mL of NaH_2PO_4 solution (0.74 M), 1.7 mL of NaCl solution (1 M), 20 mL of $NaHCO_3$ solution (1 M) and 8 mL of urea solution (0.42 M). Into this solution 290 mg α-amylase, 15 mg uric acid and 25 mg of mucin were added and the pH was adjusted to 6.8 before use. SSF was freshly made each day of experimentation and stored refrigerated until use. 4 mL of SSF and 4 mL of emulsion were transferred to a glass test tube and mixed. Then the test tube was placed into a water bath at 37 °C for 5 min. After 5 min the test tube was taken out of the water bath and the enzymatic reaction was stopped by adding 2 mL of 1 M HCl. Emulsions were then analyzed with the particle size analyzer and the amount of retained curcumin was determined by the methods described in Section 2.2.5.1. Both non-heat treated (NHT) and heat treated (HT) emulsions were investigated.

2.2.6.2. Simulated gastric digestion. The simulated gastric digestion method was adapted from a previous method (Tikekar et al., 2013) with slight changes based on the United States Pharmacopeia (United States Pharmacopeia Convention Inc., 2006). The simulated gastric fluid (SGF) was prepared by adding 2 g of NaCl in 7 mL of 1 M HCl. Then deionized water was added to have 1000 mL of solution in volumetric flask. The pH was then adjusted to 1.2 with 1 M HCl and 1 M NaOH (United States Pharmacopeia Convention Inc., 2006). Thereafter, 0.032 g pepsin was dissolved in 1 mL SGF and together with 9 mL emulsion were put in a dialysis cassette, mixed and then immersed in a 2.5 L beaker of SGF at 37 °C under magnetic stirring according to the method used previously (Tikekar et al., 2013). After definite time intervals (10 min, 30 min, 60 min, 90 min and 120 min), samples were taken out of the dialysis cassette and the enzymatic reaction was stopped by adjusting the pH to 6.5 with 1 M $NaHCO_3$. Then samples were analyzed with the particle size analyzer and the concentration of curcumin was determined according to the method described in Section 2.2.5.1.

2.2.6.3. Simulated intestinal digestion. The simulated intestinal fluid (SIF) was also adopted from a previous method (Tikekar et al., 2013) with some modification based on the United States Pharmacopeia (United States Pharmacopeia Convention Inc., 2006) by addition of 10 mg/mL of pancreatin mix into 0.05 M KH_2PO_4 at pH 6.5. Then 10 mL of emulsion were adjusted to pH 6.5 with 0.2 M HCl or 0.2 M NaOH. The emulsion was mixed with 10 mL SIF which contained 0.5 g bile extract. A set of control samples were also prepared without bile salts. Mixtures were then incubated at 37 °C. After definite time intervals (10 min, 30 min, 60 min, 90 min and 120 min) 200 μL of samples were pipetted out, the enzymatic

reaction was stopped by cooling down the samples on ice for 10 min and then the samples were analyzed with particle size analyzer and the concentration of curcumin was determined using the method described in Section 2.2.5.1.

3. Results and discussions

3.1. Characterization of gelatinization properties of starch

A starch gelatinization endotherm in presence of water as determined by DSC can be seen in Fig. 4. Gelatinization transition enthalpy was found to be 10.5 ± 1.4 J/g, while gelatinization onset temperature, gelatinization peak temperature and gelatinization conclusion temperature were 56.6 ± 0.5 °C, 63.7 ± 1.0 °C and 70.2 ± 1.4 °C respectively. Moreover, a previous study by Sjöö et al. (2015) showed that the gelatinization properties of quinoa starch granules did not change in the presence of oil.

3.2. Characterization of starch particles in dispersions

Characterization of non-heat treated and heat treated starch particles in dispersions showed the mode of $D_{[4,3]}$ changed significantly from 1.8 ± 0.1 μm in non-heat treated to 3.8 ± 0.1 after heat treatment (P < 0.05 using a student *t*-test, Fig. 2a and Table 1). This change is due to gelatinization of starch granules which accompanies absorption of water and swelling. The SEM micrograph of non-heat treated starch is also shown in Fig. 2b.

3.3. Characterization of fresh emulsions

3.3.1. Non-heat treated emulsions with or without curcumin
The structural characterization of initial non-heat treated emulsions (NHT) with and without curcumin was performed. In the absence of curcumin, the particle size distribution showed a mode of $D_{[4,3]}$ of 33.4 ± 1.0 μm (Table 1). The particle size distribution of emulsions with encapsulated curcumin represented a mode of $D_{[4,3]}$ of 30.6 ± 0.3 μm which indicated that addition of curcumin
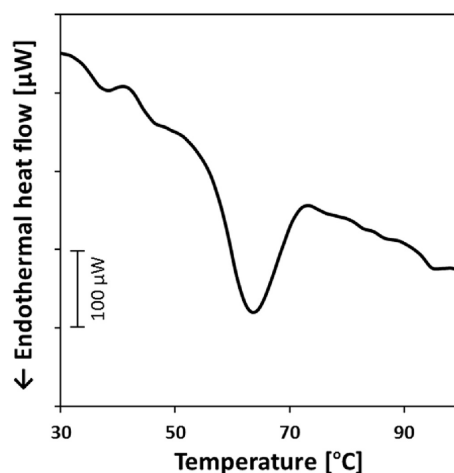
Fig. 4. DSC thermogram for gelatinization of quinoa starch in buffer, n = 6.
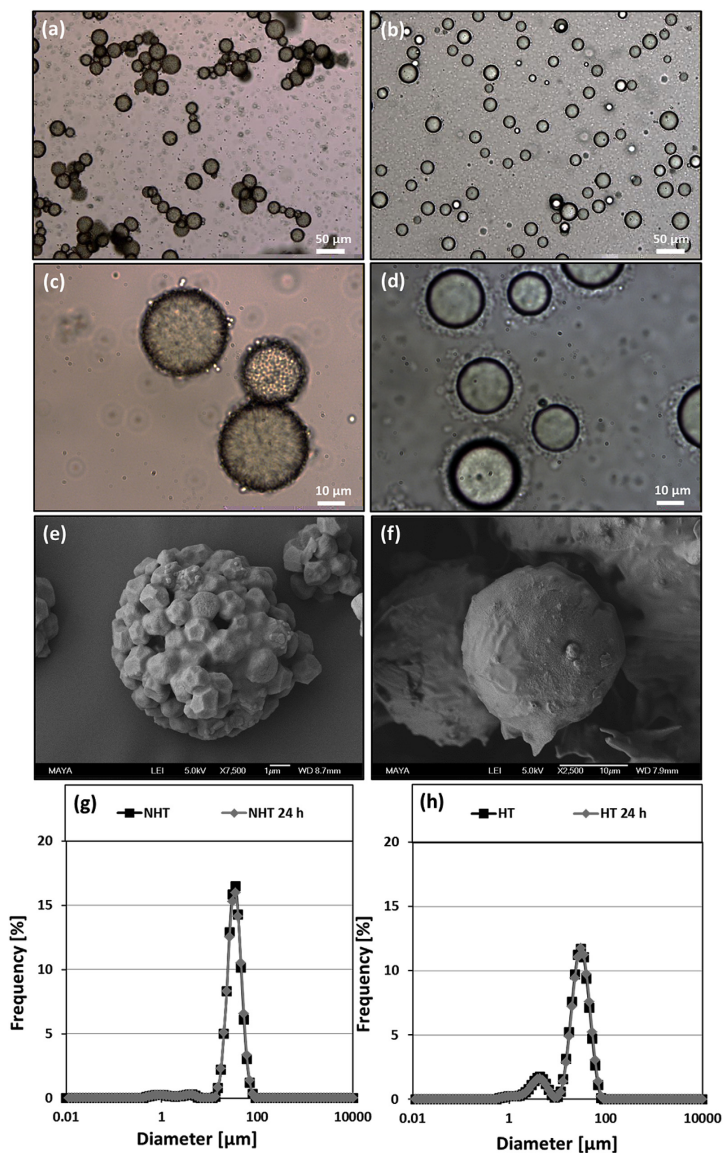
Fig. 5. **(a)** Light microscopy image of non-heat treated emulsion (NHT), **(b)** Light microscopy image of heat treated emulsion (HT), **(c)** Light microscopy image of non-heat treated emulsion (NHT), **(d)** Light microscopy image of heat treated emulsion (HT), **(e)** Scanning electron microscopy image of non-heat treated (NHT) dried starch granule stabilized emulsion, (f) Scanning electron microscopy image of heat treated (HT) dried starch granule stabilized emulsion, **(g)** Particle size distribution ($D_{[4,3]}$) for non-heat treated emulsion for fresh emulsions (NHT) and after 24 h (NHT_{24}), n = 6, **(h)** Particle size distribution for non-heat treated emulsion for fresh emulsions (HT) and after 24 h (HT_{24}), n = 6.

Fig. 6. Retained curcumin for non-heat treated (NHT) and heat treated (HT) emulsions during a 24 h period using dialysis cassettes, pH 5.7, n = 4, error bars represent standard deviation from the mean.
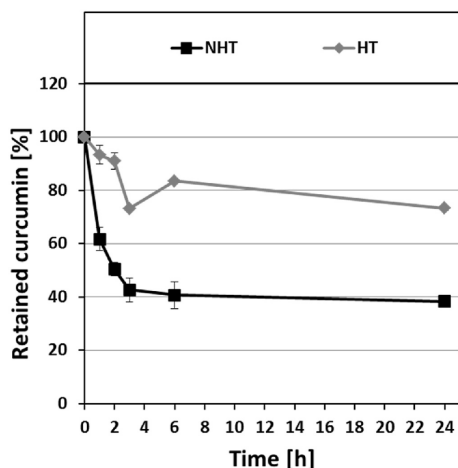
did not negatively affect emulsification, as emulsion droplets containing curcumin were slightly smaller (Table 1).

The encapsulation efficiency (EE%) of curcumin in these emulsions (NHT) was determined by measuring the retained curcumin in the system directly after emulsification as described in Section 2.2.5.1. The EE% was found to be 79.3% ± 4.3.

3.3.2. Effect of heat treatment on emulsions droplet size and encapsulation

Particle size characterization of heat treated starch granule stabilized emulsions (HT) created a particle size distribution with mode of $D_{[4,3]}$ of 28.0 ± 0.6 μm which was significantly smaller than the equivalent non-heat treated emulsions with major peak on

30.6 ± 0.3 μm (P < 0.05). This small decrease for heat treated emulsions (HT) is assumed to be due separation of some aggregated droplets by mixing during heating process, which can also result in release of free starch granules that are trapped in the aggregates (Fig. 5a, b, g, h and Table 1). This assumption was verified by comparing fresh, non-treated emulsions before and after mixing and the results showed that the change in mode of $D_{[4,3]}$ was statistically significant (P < 0.05). In addition, a minor peak (size of around 4 μm) appeared in the heat treated (HT) samples, representing free gelatinized and hence swollen starch which is similar to that in Fig. 2a. The particle size distribution of both non-heat treated (NHT$_{24h}$) and heat treated (HT$_{24h}$) remained unchanged compared to their corresponding initial samples after 24 h with mode of $D_{[4,3]}$ for NHT$_{24h}$ and HT$_{24h}$ were 31.1 ± 0.7 μm and 28.7 ± 0.7 μm respectively as it is shown in Fig. 5g, h and Table 1 (P > 0.05).

The amount of curcumin in each sample was determined directly after emulsification (EE%) and set as 100%. The encapsulation stability of the non-heat treated (NHT) and heat treated (HT) samples over a 24 h storage period using dialysis cassettes (Fig. 6) shows that heat treated (HT) emulsions retained more of encapsulated curcumin during storage. The initial detected amount of curcumin in non-heat treated (NHT) and heat treated (HT) declined to 38.3% ± 0.6 and 73.2% ± 1.6 after 24 h of storage with exposure to water respectively. The difference between non-heat treated (NHT) and heat treated (HT) samples is thought to be due to formation of a fused starch barrier as seen in SEM images of Fig. 5e and f which could improve encapsulation stability of the curcumin. Tikekar et al. (2013) proposed that increasing interfacial barrier thickness of Pickering emulsion systems by techniques such as layer-by-layer assembly of oppositely charged biopolymers or silica nanoparticles could also improve the stability of the encapsulated materials. Previous studies have shown that partial gelatinization and fusion of starch granules at the oil-water interface results in a lower rate of lipolysis (Sjöö et al., 2015; Timgren et al., 2011).

Comparing the results of starch granule stabilized Pickering emulsions with similar measurements in literature using silica nanoparticles (Tikekar et al., 2013) or Tween-80 (Lee, Lin, Chen, & Thomas, 2008) it was observed that approximately 40% and 50% of curcumin was retained within 24 h respectively. However, it should be noted that a direct comparison is perhaps difficult as different oils, particle sizes and somewhat different conditions
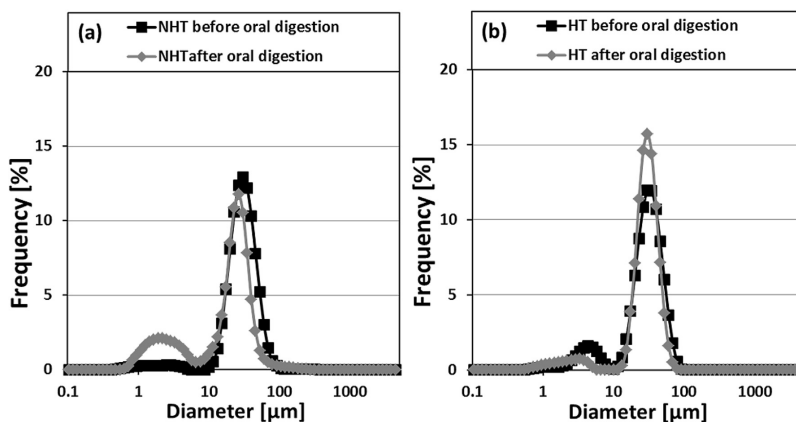

Fig. 7. Particle size distribution ($D_{[4,3]}$) for **(a)** Non-heat treated (NHT) and **(b)** Heat treated (HT) emulsions before and after simulated oral digestion (5 min), n = 6.

were used. According to a previous study (Tikekar et al., 2013) the high rate of release of curcumin from Pickering emulsions can be due to existence of a porous interfacial layer. Space between stabilizing particles leaves a large amount of oil in contact with the water and therefore facilitates release of encapsulated curcumin from the Pickering emulsions. This can also explain the difference between non-heat treated (NHT) and heat treated (HT) emulsions where the heat treatment creates a more cohesive layer of starch at the oil-water interface which increase the barrier effect. The increase in the relative barrier effect of heat treated starch granule stabilized emulsions has been reported previously (Marefati et al., 2015; Rayner et al., 2014).

3.4. Characterization of emulsions during In vitro digestion

3.4.1. In vitro oral digestion

Particle size distributions of the emulsions before and after oral digestion are shown in Fig. 7 and summarized in Table 2. In non-heat treated (NHT) samples a minor peak (around 2 μm) in addition to a decline in the mode of $D_{[4,3]}$ (from 29.5 ± 1.4 μm to

23.0 ± 1.8 μm, P < 0.05) was observed after oral digestion. These can be due to disaggregation of droplets as a result of mixing during oral digestion which in turn also results in release of free starch particles (Fig. 3a) that has been entrapped in the emulsion aggregates. In heat treated (HT) samples, there was a clear decline in the size of minor peak (sizes around 5 μm to around 3 μm) but the major peak was not significantly changed (from 30.2 ± 0.7 μm to 28.4 ± 1.8 μm, P > 0.05). The slightly higher initial size of heat treated (HT) samples is a result of swelling and partial gelatinization. The decline in particle sizes in size of the gelatinized free starch aggregates could be due to higher susceptibility of gelatinized starch to amylolytic hydrolysis (Holm, Lundquist, Björck, Eliasson, & Asp, 1988). However, the partially gelatinized starch on emulsion droplet surfaces could protect emulsions from destabilization even during longer exposure by continuation of oral digestion up to 60 min, which did not impose any drastic changes to particle size distribution (Table 2).

The curcumin content before and after oral digestion showed that heat treated samples (HT) retained a higher amount of curcumin than non-heat treated samples (NHT). The concentration of

Table 2
Particle size parameters for non-heat treated (NHT) and heat treated (HT) starch granule stabilized Pickering emulsions before and after simulated oral and gastric digestion.

Oral digestion					Gastric digestion			
Time [min]	$D_{[4,3]}$ [μm]	Span of $D_{[4,3]}$	Mode of $D_{[4,3]}$ [μm]	$d_{(0.5)}$ [μm]	$D_{[4,3]}$ [μm]	Span of $D_{[4,3]}$	Mode of $D_{[4,3]}$ [μm]	$d_{(0.5)}$ [μm]
			Non-heat treated (NHT)					
0	29.7 ± 0.4	1.2 ± 0.3	29.5 ± 1.4	28.3 ± 0.1	34.5 ± 2.1	0.9 ± 0.1	32.1 ± 1.4	32.4 ± 1.6
5	20.8 ± 0.8	1.9 ± 0.6	23.0 ± 1.8	19.6 ± 3.7	–	–	–	–
10	–	–	–	–	23.7 ± 0.8	1.4 ± 0.1	26.9 ± 0.4	25.3 ± 0.6
30	–	–	–	–	23.3 ± 1.1	1.4 ± 0.1	26.8 ± 0.5	25.0 ± 0.8
60	19.5 ± 1.7	1.8 ± 0.4	23.0 ± 2.3	18.7 ± 4.0	24.3 ± 0.3	1.3 ± 0.1	27.0 ± 0.5	25.0 ± 0.8
90	–	–	–	–	24.5 ± 0.3	1.3 ± 0.1	26.9 ± 0.4	25.6 ± 0.3
120	–	–	–	–	24.9 ± 0.4	1.3 ± 0.1	26.9 ± 0.4	25.8 ± 0.4
			Heat treated (HT)					
0	28.7 ± 0.8	1.5 ± 0.1	30.2 ± 0.7	28.0 ± 0.4	28.5 ± 0.4	1.3 ± 0.1	30.4 ± 0.3	28.8 ± 0.4
5	27.7 ± 2.0	0.9 ± 0.1	28.4 ± 1.8	27.4 ± 1.7	–	–	–	–
10	–	–	–	–	27.1 ± 0.4	1.4 ± 0.1	28.8 ± 0.4	27.0 ± 0.3
30	–	–	–	–	26.3 ± 0.4	1.5 ± 0.1	27.9 ± 0.4	26.0 ± 0.6
60	27.4 ± 1.5	0.9 ± 0.1	28.0 ± 1.2	27.1 ± 1.2	28.1 ± 0.3	1.3 ± 0.1	30.2 ± 0.2	28.5 ± 0.2
90	–	–	–	–	27.2 ± 0.8	1.4 ± 0.1	29.0 ± 1.0	27.1 ± 0.8
120	–	–	–	–	27.1 ± 0.8	1.5 ± 0.1	28.9 ± 0.8	26.8 ± 0.3

Values are mean ± standard deviation, n = 6.
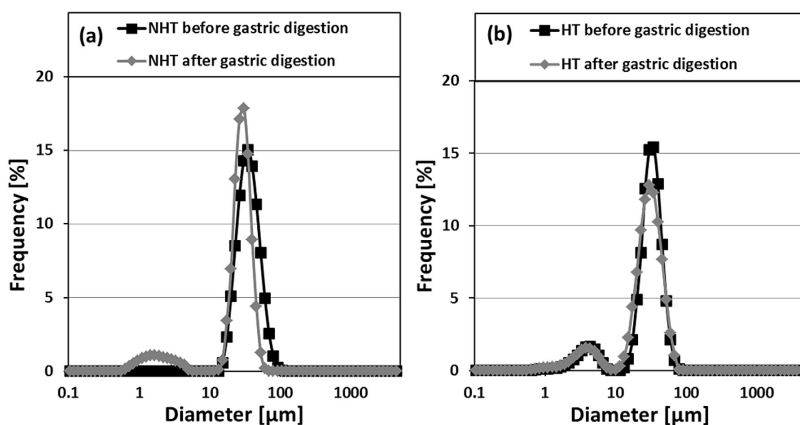
Fig. 8. Particle size distribution ($D_{[4,3]}$) for **(a)** Non-heat treated (NHT) and **(b)** Heat treated (HT) emulsions before and after simulated gastric digestion (2 h), n = 6.

curcumin for non-heat tread samples (NHT) dropped to 69.6% ± 9.0, while for heat treated samples (HT) the concentration remained 95.3% ± 5.5 of the initial amount. This observation, once again, illustrates the effectiveness of a partially gelatinized starch layer in encapsulation of curcumin.

3.4.2. In vitro gastric digestion

Comparison of the particle size distribution of non-heat treated (NHT) samples (Fig. 8) before and after gastric digestion showed appearance of a minor peak (around 2 μm) which corresponds to the free starch and a slight shift in the mode of $D_{[4,3]}$ to the smaller sizes (from 32.1 ± 1.4 μm to 26.9 ± 0.4, P < 0.05). These observations could as well be due to the mixing during the gastric digestion, which separates the aggregated droplets and release free starch

Fig. 9. Retained curcumin before, during and after simulated gastric digestion for non-heat treated (NHT) and heat treated (HT) emulsions, pH 1.2, n = 4, error bars represent standard deviation from the mean.

that is entrapped in these networks. In contrast, the heat treated samples did not show any statistically significant changes before and after gastric digestion (from 30.4 ± 0.3 μm to 28.9 ± 0.8 μm, P > 0.05). The extant of changes in particle size measurements during gastric digestion are shown in Table 2.

Gastric digestion had a slight effect on the retention of curcumin (Fig. 9). The amount of retained curcumin for non-heat treated (NHT) and heat treated (HT) samples after 120 min of gastric digestion was 86.2% ± 1.6 and 82.4% ± 7.0 respectively; however, the difference between samples were not statistically significant (P > 0.05). The higher initial and final encapsulation values comply with previous findings (Joshi et al., 2010) where they showed higher stability of curcumin in acidic pH which was expected. The results were in a close agreement with the retained amount of curcumin previously reported (Tikekar et al., 2013) for silica nanoparticle stabilized emulsions, which was approximately 78%.
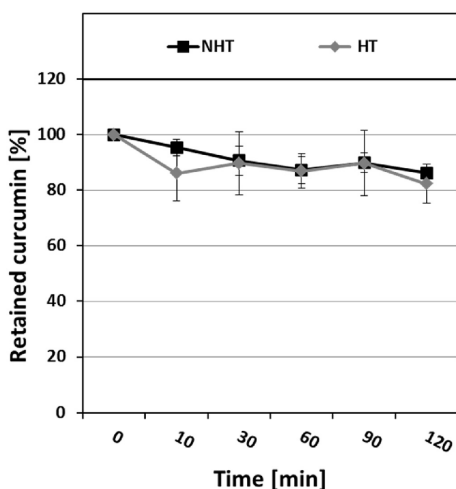
3.4.3. In vitro intestinal digestion

The results are summarized in Table 3. In order to separate the effect of bile salts from that of digestive enzymes, simulated intestinal digestion was performed with and without bile salts. For the digestion of emulsions without bile salts, some droplet destabilization was observed at 30 min for non-heated (NHT) sample (Fig. 10a), whereas it took 120 min for the heated (HT) sample (Fig. 10b).

In case of simulated intestinal digestion with bile salts (Fig. 10c and d) the destabilization was expected much faster, but the effect of heat treatment persisted as destabilization of the heated emulsion (HT) was only observed at 30 min.

The effectiveness of bile salts in displacing material from lipid surfaces, solubilizing lipids and assisting in lipolysis well established (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Thus the protective effect of partially gelatinized starch at the droplet interface at intestinal conditions is of particular interest.

Our interpretation is that in the non-heat treated emulsions (NHT), the starch granules block part of empty surface but the empty gaps at the oil-water interface that exist between starch granules allow the interaction of bile salts and lipolytic enzymes, leading to destabilization and coalescence and fast changes during intestinal digestion. In contrast, for heat treated samples (HT), the efficiency of intestinal digestion, especially the act of bile salts and formation of lipid-lipase interactions, may be delayed, which is in agreement with previous findings of Sjöö et al. (2015) and

Table 3
Particle size parameters for non-heat treated (NHT) and heat treated (HT) starch granule stabilized Pickering emulsions before and after simulated intestinal digestion, with and without bile salts.

	Intestinal digestion without bile salts				Intestinal digestion with bile salts			
Time [min]	$D_{[4,3]}$ [μm]	Span of $D_{[4,3]}$	Mode of $D_{[4,3]}$ [μm]	$d_{(0.5)}$ [μm]	$D_{[4,3]}$ [μm]	Span of $D_{[4,3]}$	Mode of $D_{[4,3]}$ [μm]	$d_{(0.5)}$ [μm]
				Non heat treated (NHT)				
0	49.9 ± 17.6	3.0 ± 2.9	25.4 ± 0.4	23.7 ± 0.8	53.0 ± 13.3	2.0 ± 0.3	28.2 ± 1.9	25.7 ± 1.9
5	–	–	–	–	–	–	–	–
10	49.2 ± 8.1	3.1 ± 1.5	24.6 ± 0.6	24.0 ± 0.6	82.9 ± 26.8	3.7 ± 2.3	58.3 ± 5.4	47.3 ± 3.1
30	60.4 ± 10.4	3.3 ± 0.7	25.2 ± 0.1	27.5 ± 0.9	86.5 ± 6.7	2.0 ± 0.2	73.5 ± 0.9	63.6 ± 2.8
60	83.7 ± 23.7	6.9 ± 3.5	25.3 ± 0.7	29.2 ± 3.0	81.1 ± 14.6	4.9 ± 3.4	55.4 ± 13.4	47.3 ± 9.8
90	62.5 ± 13.5	2.7 ± 0.3	51.5 ± 4.5	35.6 ± 3.5	108.2 ± 17.0	8.8 ± 1.8	37.7 ± 2.1	35.5 ± 2.1
120	68.8 ± 11.9	3.4 ± 0.5	52.6 ± 6.3	34.2 ± 3.7	60.9 ± 9.9	2.4 ± 0.4	60.9 ± 10.0	45.1 ± 10.0
				Heat treated (HT)				
0	51.3 ± 4.3	1.1 ± 0.1	26.2 ± 1.1	26.2 ± 1.1	63.1 ± 20.9	3.9 ± 3.0	27.8 ± 1.5	29.2 ± 1.5
5	–	–	–	–	–	–	–	–
10	73.3 ± 16.5	2.6 ± 2.2	25.6 ± 1.0	25.8 ± 1.0	45.1 ± 2.8	1.2 ± 0.06	28.0 ± 0.8	27.9 ± 0.7
30	78.1 ± 18.4	6.1 ± 3.9	25.4 ± 1.0	25.8 ± 1.0	70.7 ± 3.9	1.8 ± 0.2	50.0 ± 3.9	46.8 ± 2.7
60	66.9 ± 17.4	3.8 ± 2.9	24.5 ± 0.6	24.8 ± 0.5	67.2 ± 9.8	2.0 ± 0.3	53.0 ± 3.5	47.3 ± 2.6
90	69.2 ± 15.2	5.7 ± 2.5	23.5 ± 0.7	24.6 ± 1.2	90.8 ± 30.1	4.0 ± 2.3	59.5 ± 4.1	46.8 ± 4.0
120	75.6 ± 1.0	4.1 ± 1.5	24.5 ± 2.35	30.8 ± 4.6	73.0 ± 6.5	2.1 ± 0.2	71.9 ± 6.8	58.1 ± 5.6

Values are mean ± standard deviation, n = 6.

Timgren et al. (2011) where the lipolytic activity was shown to be delayed in heat treated emulsions based on starch Pickering stabilization. This could be interpreted as the effect of heat treatment in providing a fused barrier of partially gelatinized starch granules which makes them more difficult to displace from the oil-water interface, especially with regards to activity of bile. Even if bile salts can penetrate to the surface through imperfections in the fused starch layer, the layer will not be so easily detached as individual surface molecules and particles would. The fused starch layer would remain encircling the droplet and providing a mass transport barrier to digestion.

Despite partial starch gelatinization would increase the susceptibility of starch to amylase present in the intestine, one can hypothesize that the inner side of the granules towards the oil droplets may have a less easily digestible structure. However,

further research is needed to test this hypothesis and the exact mechanism for emulsion disruption and lipid hydrolysis during the intestinal digestion.

The encapsulation properties of the non-heat treaded (NHT) and heat treated (HT) emulsions for simulated intestinal digestion are shown in Fig. 11. These results showed that despite an overall decrease, significantly more curcumin is retained in heat treated emulsions (HT) with 86.3% ± 13.5 compared to non-heat treated emulsions (NHT) with 40.2% ± 3.4 after 2 h of simulated intestinal digestion.

The stability of curcumin at the simulated intestinal digestion conditions is remarkably high, the losses at 2 h were comparable to the losses at 3 h in the storage stability test (Fig. 6) and the protective effect of heat treatment similar.

The amount of retained curcumin in the non-heat treated starch

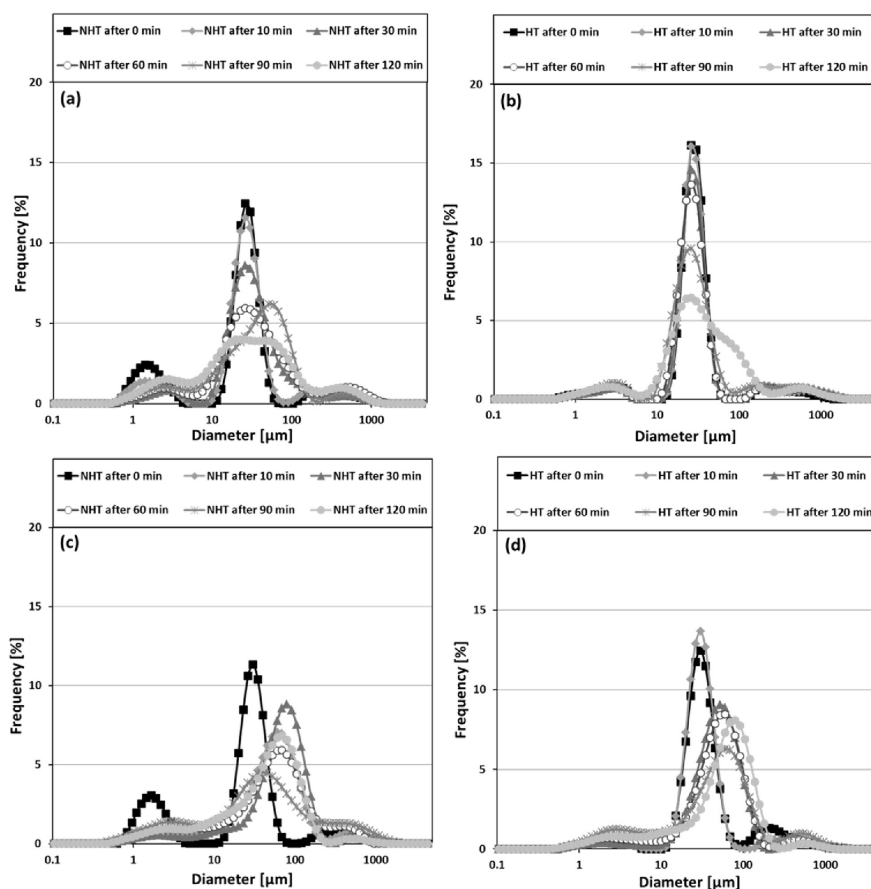**Fig. 10.** Particle size distribution (D[4,3]) for non-heat treated (NHT) and heat treated (HT) emulsions before and after simulated intestinal digestion (2 h), **(a)** and **(b)** without bile salts and **(c)** and **(d)** with bile salts, n = 6.
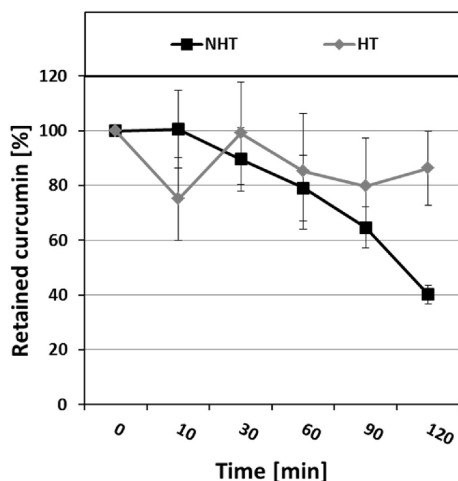
Fig. 11. Retained curcumin before, during and after simulated intestinal digestion for non-heat treated (NHT) and heat treated (HT) emulsions, pH 6.5, n = 4, error bars represent standard deviation from the mean.

granule stabilized emulsions (NHT) was in a close agreement with previously reported values (~ 40%) for silica nanoparticle stabilized Pickering emulsions (Tikekar et al., 2013), further supporting our observation that a fused starch layer is improving the retention of curcumin.

4. Conclusions

The results of this study demonstrated that starch granule stabilized Pickering emulsions can successfully encapsulate curcumin with high encapsulation efficiency (~ 80%) and retain curcumin during storage and simulated digestion in the upper gastrointestinal tract. The 24 h storage stability of encapsulated curcumin using dialysis cassettes showed a gradual release of curcumin with different rates for non-heat treated (NHT) and heat treated (HT) emulsions (with final content of 38.3% ± 0.6 and 73.2% ± 1.6 for non-heat treated, NHT, and heat treated, HT, samples respectively). It was demonstrated that starch based Pickering emulsion systems successfully preserved curcumin during simulated oral digestion (69.6% vs. 95.3% for non-heat treated and heat treated samples respectively) and simulated gastric digestion (86.2% vs. 82.4% for non-heat treated and heat treated samples respectively) and reaching in the intestine which is a desirable trait since most of nutrient absorption occurs in this stage. In the simulated intestinal digestion, larger degree of change was observed when bile salts were included. In the latter case, 40.2% and 86.3% of encapsulated curcumin was retained for non-heat treated (NHT) and heat treated (HT) emulsions, respectively. Thus, heat treated emulsions (HT) have shown to retain more of the encapsulated curcumin when exposed to environmental and physiological conditions. This is likely due to formation of fused barrier at the oil-water interface in the heat treated samples as a result of partial starch gelatinization. If this digestion was sequential, the amount of retained curcumin at the end of intestinal digestion for heat treated (HT) samples could be approximately 3 times that of non-heat treated (NHT) samples

(68% vs. 24%). Heat treated starch granule stabilized emulsions may be a useful encapsulation tool for delayed delivery of bioactive compounds to the larger intestine.

Acknowledgement

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Paper VI

In vitro intestinal lipolysis of emulsions based on starch granule Pickering stabilization

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Abstract

The effect of barrier properties on *in vitro* intestinal lipolysis of three different types of emulsions based on oil-in-water starch granule Pickering stabilization has been investigated including non-heat treated, heat treated (gelatinized) and heat treated and stored (retrograded) emulsions. The barrier properties of starch covered oil-water interfaces were characterized by the rate of lipid hydrolysis using a pH-stat. The results were compared with a sodium caseinate stabilized emulsion. The physical properties of the emulsions were characterized by a light scattering particle size analyzer and light microscopy. In all cases, Pickering emulsions showed lower extent of lipolysis compared to the protein stabilized emulsion. In addition, heat treated starch Pickering emulsions were more susceptible to lipolysis compared to freshly prepared emulsions with no heat treatment. This was thought to be due to the gelatinized starch barrier being more susceptible to amylase. The results of this study demonstrate the possibility of development of functional foods in the form of emulsions for controlled release and targeted delivery based on starch granule Pickering stabilization.

1. Introduction

There has been a growing interest in understanding the underlying mechanisms of emulsified lipid digestion in the pharmaceutical and food industries (McClements and Li 2010). Lipid based delivery systems are being developed in pharmaceutical industry to increase bioavailability of drugs or to control the release or target the delivery of drugs to specific locations in the human gastrointestinal tract (GI). In the food industry on the other hand, the emulsified lipids are used to protect, encapsulate and modulate the release of bioactive hydrophobic food components for increasing bioavailability. A number of such components is proposed by McClements and Li (2010) include: ω-3 fatty acids, butyrate, phytosterols, carotenoids, anti-oxidants and vitamin A and D. The knowledge of the stability and efficiency of these lipid based delivery systems can be useful for development of functional foods that maintain or improve health. Functional food can be developed to deliver bioactive components to specific locations of the GI tract where they function; such as anti-cancer components that could be released in the colon (McClements and Li 2010). Moreover, in order to prevent the negative health related side effects associated with obesity, energy intake can be reduced by slowing down the lipid digestion that leads to presence of nutrients in the distal part of the small intestine, which in turn results in so-called ileal brake mechanism and induces satiety through hormonal signals and eventually reduces food intake (Corstens, Berton-Carabin et al. 2017). However, the major part of dietary lipids are digested and absorbed in the proximal part of the small intestine and therefore do not reach ileum to induce this mechanism (N'Goma, Amara et al. 2012). Therefore, in order to stimulate ileal break, dietary lipids need to be protected against the digestive action of the GI tract. Dietary fat digestion (lipolysis) is determined by the physico-chemical composition of lipids and the interfacial properties of the emulsified lipids (Corstens, Berton-Carabin et al. 2017). Understanding the principal mechanism of emulsified lipid digestion may lead to fabrication of structured emulsions with controlled lipid digestibility to provide novel food materials and thereby promote satiety and control caloric intake and obesity (McClements and Li 2010).

There is a wide range of stabilizing agents for the emulsification of lipids. Surfactants and biopolymers are the main types of stabilizers used in the food the industry (McClements 2005). In addition, emulsions stabilized by solid particles (Pickering emulsions) demonstrate long-term stability even without surfactant additives (Aveyard, Binks et al. 2003). Higher stability of Pickering emulsions compared to surfactant stabilized emulsions make them more suitable for encapsulation and targeted delivery systems (Matos, Timgren et al. 2013). This stability can be attributed to the essentially irreversible adsorption of solid particles due to their large size, which results in a higher energy barrier compared to other stabilizing agents. Hydrophobically modified starch granules with octenyl succinic

anhydride (OSA) have been used to create Pickering emulsions and first reported by Timgren, Rayner et al. (2011), Yusoff and Murray (2011). Previous studies on starches of different botanical origin showed that relatively small and unimodal granules of quinoa starch have a higher stabilizing potential (Timgren, Rayner et al. 2013, Marefati, Wiege et al. 2017). Furthermore, Pickering emulsions stabilized by quinoa starch granules have shown to be extremely stable over an extended period of time, i.e. 2 years (Timgren, Rayner et al. 2013). Since the initial interfacial structure of an emulsion needs to be displaced by biological surfactant (bile salts), to allow adsorption of lipase-colipase complex and thereby facilitate lipolysis and release of fatty acids, it can be expected that highly stable and irreversibly adsorbed particles in Pickering emulsions could inhibit or control the displacement by the bile salts and therefore lipid digestion (Sarkar, Murray et al. 2016).

There have been previous attempts to reduce the rate of lipolysis such as layer-by-layer deposition, highly surface active surfactants or modification of the adsorbed layer at the interface with a mild heat treatment (Tzoumaki, Moschakis et al. 2013, Sarkar, Murray et al. 2016). In the same way, Pickering emulsions that are highly stable against severe environmental conditions, can potentially resist the lipid digestion process. In addition, a unique physico-chemical property of starch i.e. gelatinization can be utilized to create an impermeable barrier at the interface. Upon exposure to heat, starch granules gelatinize and form a cohesive barrier at the oil-water interface. Gelatinization is a transformation of crystalline structure of starch granules to amorphous state in presence of heat and water (Eliasson 2006). In this way, a fused or sealed barrier around the droplets are formed and thus, this characteristic of starch can improve physical (storage and process) and physiological stability of starch Pickering emulsions (Marefati, Rayner et al. 2013, Marefati, Sjöö et al. 2015, Sjöö, Emek et al. 2015, Marefati, Bertrand et al. 2017). In addition to contributing to the general structural stability of emulsions, partially gelatinized starch layer has been shown to reduce the rate of lipolysis by creating steric hindrance for the action of lipase (Timgren, Rayner et al. 2011, Sjöö, Emek et al. 2015). However, previous studies used lipase solely as the digestive enzyme, whereas, the intestinal digestion is a combination of enzymatic reactions governed by lipase, amylase and protease in the intestine. Furthermore, upon cooling and storage, the amorphous structure of gelatinized starch can transform into a more ordered crystalline state through a process called retrogradation, which improves firmness and rigidity of starch structure (Eliasson 2006). The newly formed crystalline structure is known to resist enzymatic hydrolysis and is therefore referred to as resistant starch (Sievert and Pomeranz 1989, Brown 1996). Gelatinization and retrogradation are common phenomena that happen during processing and storage in the food industry which may alter the properties of the food drastically.

In the humans, the digestion process of the dietary lipids initiates in the stomach and finalizes in the intestine by gastric lipase and pancreatic lipase respectively

(Brockman 2000, Mu and Høy 2004, Mun, Decker et al. 2006). In order for the pancreatic lipase to catalyze the lipid digestion, it needs to come into a close proximity of the lipid by adsorbing at the surface of the lipid droplets. This adsorption is proposed to be facilitated by the help of bile salts (Labourdenne, Brass et al. 1997, Brockman 2000, Mun, Decker et al. 2007). Bile salts not only help to enable the adsorption of co-lipase and lipase to the oil droplet interface, but also provide the means to solubilize the lipolysis products that have accumulated at the interface into micelles in order to facilitate their absorption (Wilde and Chu 2011). There have been numerous studies on digestion of starch (Chung, Lim et al. 2006, Shi and Gao 2011, Miao, Jiang et al. 2015, Dhital, Warren et al. 2017, Magallanes-Cruz, Flores-Silva et al. 2017) or emulsified lipids (Fave, Coste et al. 2004, Bauer, Jakob et al. 2005, McClements and Li 2010, Corstens, Berton-Carabin et al. 2017); however, the effects of starch granules as barrier properties on digestion of lipids have yet to be investigated.

The aim of this study is to further investigate the impact of gelatinization in heat treated (HT) and retrogradation in heat treated and stored (HT-stored) starch at the oil-water interface (compared to non-heat treated, NHT) on overall stability of the emulsions towards simulated *in vitro* intestinal lipolysis. The physical properties of emulsions were analyzed by laser diffraction particle size analyzer and light microscopy before the digestion process. The efficiency of barrier properties of emulsions was characterized by the rate of lipid hydrolysis in a pH-stat. In order to compare the interfacial barrier effect of Pickering formulation, sodium caseinate stabilized emulsions (Na-Cas), were used. Since the amount of the interfacial area between the lipids and the surrounding aqueous phase is important as lipolysis is an inherently interfacial process, in addition to the interfacial composition, a screening study was performed so that Na-Cas emulsions created similar surface area to the Pickering systems.

2. Materials and methods

2.1. Materials

Emulsions were prepared using starch granules isolated from quinoa grains and then hydrophobically modified by OSA (2.79%) using a previously described method (Marefati, Wiege et al. 2017). Phosphate buffer (5 mM, 0.2 M NaCl, pH 7) was used as continuous phase and Miglyol 812 (density 0.945 kg/m3, Caesar & Loretz GmbH, Germany) as dispersed phase. For protein stabilized emulsions, sodium salt of casein was purchased from Sigma Aldrich Co.

Pancreatin from porcine pancreas (8 × USP specification), sodium deoxycholate, sodium hydroxide 1 M, sodium hydroxide 0.1 M, trisaminomethane (Tris) and calcium chloride were purchased from Sigma Aldrich Co. Sodium Cholate was obtained from AppliChem, GmbH, Germany.

2.2. Methods

2.2.1. Characterization of starch

2.2.1.1. Characterization of gelatinization and retrogradation properties of starch

The gelatinization properties of starch granules were analyzed using a differential scanning calorimeter (DSC, Seiko 6200, Seiko instruments Inc., Japan), calibrated with indium (Mp = 156.6 °C). Starch dispersions were prepared by mixing with a rotor-stator high shear mixer (Ystral D-79828, Ballrechten-Dottingen, Germany) with 6 mm dispersing tool at 22 000 rpm for 30 s, and weighed into coated aluminum pans (TA Instruments, USA) at a ratio of 1:10 and gelatinization transition enthalpy (ΔH, J/g dry matter), gelatinization onset temperature (°C), gelatinization peak temperature (°C) and gelatinization conclusion temperature (°C) were determined. The scanning rate was 10 °C/min ranging from 10 to 120 °C.

2.2.2.2. Scanning electron microscopy of starch granules

Micrographs of starch granules were obtained by scanning electron microscopy (SEM). The dried samples were coated with gold and examined under SEM (field emission SEM, JSM-6700F, JEOL, Japan) operated at 5 kV with a working distance of 8 mm. Lower detection imaging mode (LEI) was used to give clear three-

dimensional images of the sample surface. The LEI detector combines both signals secondary and back scattered electrons during the operation.

2.2.2.3. Particle size analysis of starch granules

The particle size distribution of starch granules was determined using a laser diffraction particle size analyzer (Mastersizer 2000 Ver. 5.60, Malvern, Worcestershire UK). 140 mg of starch was dispersed in 7 mL of continuous phase phosphate buffer using a rotor-stator high shear homogenizer at 22 000 rpm for 30 s. The sample was added to the flow system containing Milli-Q water and was pumped through the optical chamber at a pump velocity of 2000 rpm. The refractive index (RI) was set to 1.54 for starch and the RI of the continuous phase was set to 1.33 (water) and the obscuration was between 10 and 20% (Bromley and Hopkinson 2002).

2.2.3. Preparation and characterization of emulsions

2.2.3.1. Preparation of emulsions

Oil-in-water (O/W) emulsions were produced in volume of 7 mL with 10% oil fraction and 200 mg_{starch}/mL_{oil}, and 90% phosphate buffer using the same rotor-stator high shear homogenizer, at 22 000 rpm for 30 s.

For heat treated samples (HT), emulsions were placed in a water bath at 70 ± 1 °C under moderate stirring (500 rpm) using an overhead stirrer, Eurostar (IKA lab elektronik, Germany) to keep the droplets apart while preventing additional homogenization. The heating procedure was monitored using type K (0.1 mm) thermocouple. When the temperature reached 70 °C, the emulsions were kept in the water bath for another 1 min before they were removed from the water bath. The emulsions were kept under stirring until they cooled down to the room temperature.

To induce retrogradation of the starch barrier, some of the heat treated emulsions were kept refrigerated at 6 °C for 4 weeks for further analysis (HT-stored). The protein stabilized emulsions (Na-Cas) were produced using 0.1% w/v sodium caseinate solution that was homogenized with high shear homogenizer at 13 000 rpm for 30 s. All samples were produced in triplicates.

2.2.3.2. Particle size measurement

The particle size distributions of the emulsions were characterized with a laser diffraction particle size analyzer, Mastersizer 2000 (Malvern Instruments, UK). Each emulsion was added to the flow system (Hydro SM small volume wet dispersion unit) containing Milli-Q water and was then pumped through the optical chamber where it was measured. The RI was set to 1.54 for starch particles and 1.46

for Na-Cas and the RI of the continuous phase was set to 1.33 which is the refractive index of the water and the obscuration was between 10 and 20%. For each emulsion sample added to the flow system, three measurements were performed, and all emulsions were prepared in duplicate.

In order to see the effect of addition of the bile salts and mixing during *in vitro* lipolysis on the stability of the emulsions, a set of control samples was made in the same way and mixed for 3 h in presence of bile salts and the size was measured using the particle size analyzer.

2.2.3.3. Microscopy

Micrographs were obtained with a light microscope (Olympus BX50, Japan) using a camera (DFK 41AF02, The Imaging Source, Germany) both were controlled by a computer. The emulsions were diluted 5 times with Milli-Q water and then one drop was placed on a glass microscopic slide. In order to prevent deformation of droplets, no cover glass was used. The microscopic images were taken using objective magnifications of 20× and 50×.

2.2.4. Lipolysis

The lipid digestion was monitored by measurement of the rate of lipid hydrolysis through the changes in pH from production of free fatty acids using a pH-stat, (Titrando 902, Metrohm, Switzerland), a device that continuously monitors the pH of a solution and maintains the pH constant by the addition of acid or alkali.

12 mL of the 25 mM Tris buffer together with 200 μL $CaCl_2$ 0.3 M and 2.5 mL of bile salt solution (80 mM sodium cholate and sodium deoxycholate bile salts 50:50 w/w) were transferred to a pH-stat vessel with thermo-stat jacket that kept the temperature at 37 °C. The concentration of $CaCl_2$ and bile salts were chosen in a way to achieve 0.3 mM and 10 mM in the final digestion mixture respectively. The mixture was set under a moderate mixing using a magnetic bar. Pancreatin was added to achieve 800 USP/mL in the final digestion mixture (Siqueira, Müllertz et al. 2017). Thereafter, the pH was carefully set to 7.0 using 1.0 M NaOH. Subsequently, 5 mL of emulsions were added and the process started. The amount of NaOH 0.1 M needed to maintain pH 7 over a 3 h period was used to calculate the concentration of free fatty acids (FFA) generated in the reaction vessel during digestion of the emulsified lipids and can be interpreted as availability of the oil for lipolysis (and therefore give a measure of comparison for barrier properties of samples). The percentage of free fatty acids released (FFA%) was calculated from the number of moles of NaOH 0.1 M required to neutralize the FFA released from triglycerols, considering 2 FFA can be produced per triglycerol, using the *Equation 1* below:

$$\text{FFA\%} = \left(\frac{V_{\text{NaOH}} \times M_{\text{NaOH}} \times M_{\text{W lipid}}}{2 \times W_{\text{lipid}}} \right) \qquad \text{\textit{Equation 1}}$$

where, V_{NaOH} is the volume (mL) of NaOH consumed, M_{NaOH} is the molarity of NaOH (0.1 M), M_{Wlipid}, is the average molecular weight of the Miglyol 812 (0.520 kg/M) (Ueck 2004) and W_{lipid} is the weight of lipid initially inserted to the reaction vessel (0.47 g) (Sarkar, Murray et al. 2016). All measurements were performed in triplicates.

3. Results and discussions

3.1. Characterization of gelatinization properties of starch

The starch gelatinization endotherm in presence of water as determined by DSC can be seen in Fig. 1. Gelatinization transition enthalpy was found to be 9.6 ± 0.5 J/g, while the gelatinization onset, peak and conclusion temperature were T_o: 56.1 ± 0.9 °C, T_p: 61.5 ± 0.9 °C, T_c: 67.4 ± 0.6 °C respectively which were in the range of the previous results of quinoa starch dispersions 50-74.9°C (Atwell, Patrick et al. 1983, Qian and Kuhn 1999, Li, Wang et al. 2016, Marefati, Bertrand et al. 2017). The results of gelatinization properties were used to choose the temperature of the heat treatment, i.e. 70 °C which is slightly above T_c of the gelatinization.
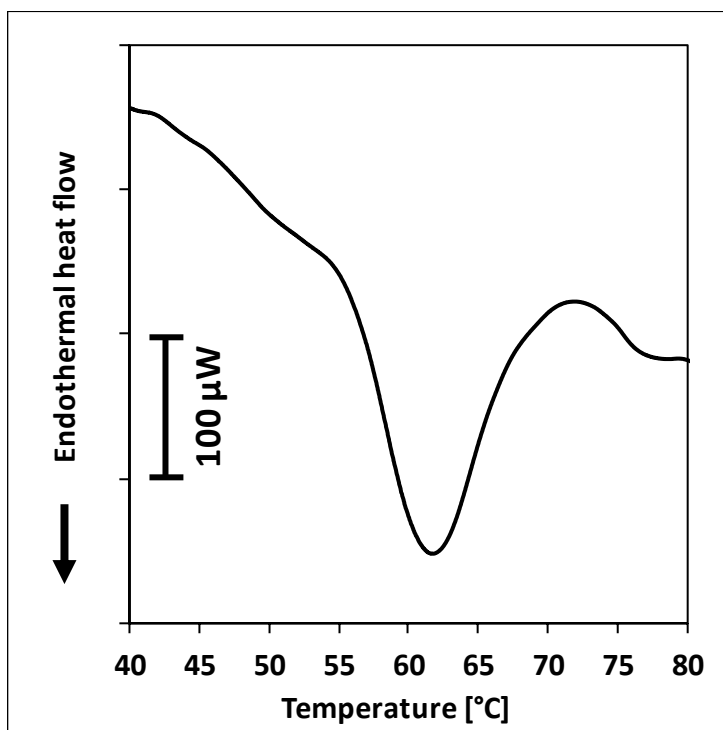
Fig. 1.
DSC thermogram of Gelatinization of starch in buffer, n=3.

3.2. Characterization of emulsions before digestion

Particle size distribution (PSD) of starch granules and emulsions are presented in Fig. 2 and Table 1. Starch granules were small and polygonal form having a mode of $D_{4,3}$ of 1.75 ± 0.09 µm and size range of 0.6-5 µm, which was attributed to partial aggregation caused by OSA modification as can be seen in the Fig. 2.

Non-heat treated emulsions (NHT) showed a bimodal particle sized distribution with a minor shoulder on the left of PSD for the free starch, and mode of $D_{4,3}$ of 35.4 ± 1.3 µm representing the droplets, and droplets aggregates as can be seen in the micrographs in Fig. 2. Heat treatment caused starch granule to gelatinize and swell which can be reflected in particle size distribution for HT and HT-stored samples with mode of $D_{4,3}$ of 38.9 ± 1.3 µm and 38.8 ± 0.4 µm respectively as well as the corresponding micrographs in Fig. 2. As mentioned earlier, the Na-Cas emulsions were formulated in a way to have similar sizes as of the Pickering emulsions with mode of $D_{4,3}$ of 37.0 ± 0.1 µm.

Table 1:
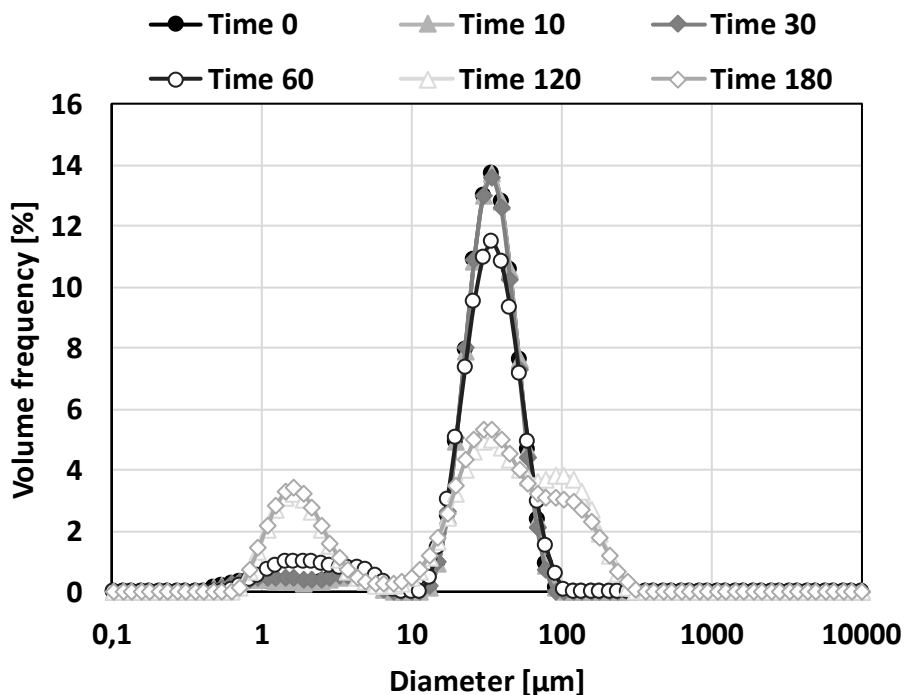Particle size distribution of OSA modified quinoa starch granules, NHT, HT, HT-stored, Na-Cas emulsions.

Sample	D [4, 3] [µm]	Span	Mode of D [4, 3] [µm]	D [3, 2] [µm]
Starch granules	1.80 ± 0.05	0.98 ± 0.11	1.75 ± 0.09	1.59 ± 0.01
NHT	39.8 ± 3.1	1.47 ± 0.28	35.4 ± 1.3	14.5 ± 0.7
HT	38.1 ± 0.7	1.31 ± 0.02	38.5 ± 0.1	18.2 ± 0.5
HT-stored	39.2 ± 0.6	1.20 ± 0.02	38.8 ± 0.4	19.8 ± 0.2
Na-Cas	36.1 ± 0.1	1.56 ± 0.01	37.0 ± 0.1	18.2 ± 0.1

Furthermore, the control samples showed that addition of bile or mixing in did not induce a destabilization effect on the droplet size distribution of the non-heated emulsions up to 1 h (Fig. 3). One could suppose that mixing or addition of biological surfactants would displace the starch granules at the interface, however, although there was a slight increase in the amount of free starch (left side of the main peak), there was no increase in the droplet sizes for the first hour. After 2 and 3 h some more starch granules were displaced which caused some coalescence as can be seen in Fig. 3. This result verified that neither addition of the bile salts, nor mixing caused destabilization of Pickering emulsions at least up to 1 h. In addition, previous study by Marefati, Bertrand et al. (2017) showed that the extent of changes induced by addition of bile salts was even lower for heat treated samples.
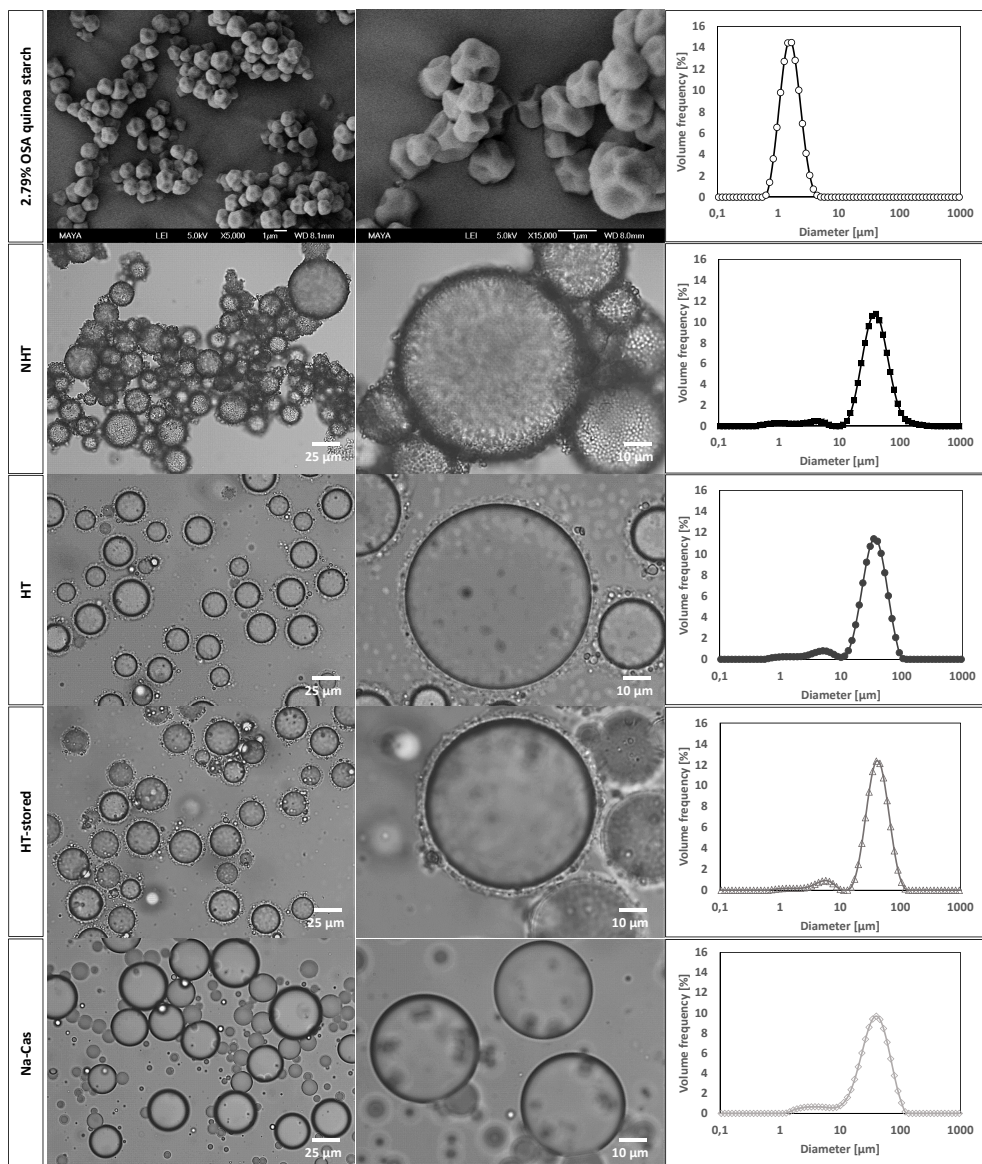
Fig. 2.
SEM micrographs of quinoa starch granules and particle size distribution ($D_{4,3}$) of starch granules (top row), light microscopy micrographs and particle size distribution of non-heat treated emulsions (2nd row), light microscopy micrographs and particle size distribution of heat treated emulsions (3rd row), light microscopy micrographs and particle size distribution of heat treated and stored emulsions, (4th row) and light microscopy micrographs and particle size distribution of protein stabilized emulsions (bottom row),n=6.

11

Fig. 3.
The effect of mixing and bile salts on the particle size distribution of Pickering emulsions stabilized by starch granules (NHT), n=6.

3.3. Lipolysis

The measurement of the rate of lipid hydrolysis was performed through monitoring the consumption of 0.1 M NaOH solution needed for neutralizing the drop in pH caused by production of FFA. The amounts of released FFA as a result of lipolysis for the different emulsions during the initial 20 minutes and 3 h are depicted in Fig. 4. The results showed that the initial rate was lower for all Pickering based emulsions compared to that of the Na-Cas emulsions from the very first minute (P<0.5). Fig. 4 also shows that the lipolysis rate was the same for the Pickering based emulsions during the first 5 minutes of the digestion which then increased for HT and HT-stored emulsions. However, the difference between production of FFA% for NHT and HT or HT-stored were not statistically significant (P>0.05) up until 15 minute but were significantly different afterwards (P<0.05). These results also indicated that the amount of FFA% produced by HT and HT-stored were

significantly different from those of Na-Cas (P<0.05). The extent of FFA production as a result of lipolysis during 180 min in Fig. 4-bottom was shown to be the lowest for NHT samples with 25.1% ± 1.2 followed by HT with 29.0% ± 0.8 and HT-stored with 28.9% ± 0.3 and lastly Na-Cas with 32.2% ± 0.2. Additionally, the amount of FFA% generated in these experiments did not exceed 32.2% (for Na-Cas emulsions). Despite the relatively low FFA release during the digestion, the results were in line with the previous works (McClements and Li 2010). The low amount of FFA released could be due to the large droplet sizes. According to McClements and Li (2010) the extent of FFA production from Miglyol 812 decreased from around 70% to around 45% during 20 min of *in vitro* lipolysis when the droplet size was increased from 178 nm to 758 nm. Similar observations about the effect of the size have been made in other works (Armand, Borel et al. 1992, Borel, Armand et al. 1994, Sarkar, Murray et al. 2016). In addition to the large droplet sizes of emulsions, accumulation of digestion products (FFA and mono glycerides) at the oil-water interface is proposed to inhibit sustained lipase activity (Wilde and Chu 2011). The saturation of the surface of the oil droplets due to accumulation of lipolysis products, can happen faster for Pickering emulsions due to larger droplet sizes and therefore, relatively smaller total surface area. McClements and Li (2010), Li, Hu et al. (2011) also showed that the concentration of the lipid present within the digestion cell has an inverse effect on the rate and the extent of lipid digestion and by increasing concentration of the oil from 1% to 2.5% the amount of FFA produced dropped over 10% and in this study all samples had same oil content at 10%.
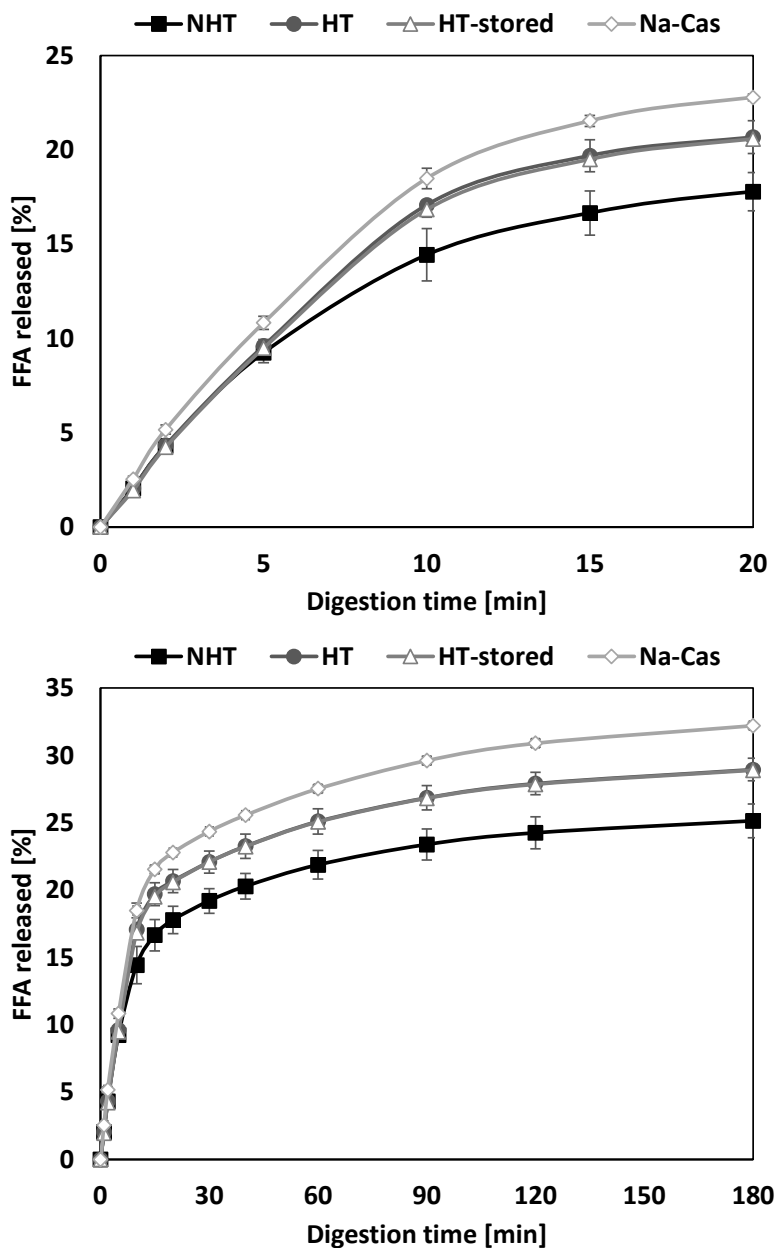
Fig. 4.
The amount of FFA% produced for the different emulsions during the initial 20 min *in vitro* intestinal lipolysis (top), the amount of FFA% produced for the different emulsions during the 3 h min *in vitro* intestinal lipolysis (bottom). Error bars represents standard deviation of the mean, n=3.

Since lipolysis is an interfacial phenomenon and in order to have a relevant comparison, the FFA released was normalized against the oil droplet surface area of the different emulsions droplets from *Equation 2a* by using *Equation 2b* and plotted in Fig. 5.

$$S = \frac{6 \varnothing V_{emulsion}}{d_{3,2}}$$ *Equation 2a*

$$\frac{FFA\%}{S} = \left(\frac{FFA\%}{\frac{6 \times \varnothing \times V_{emulsion}}{d_{3,2}}} \right)$$ *Equation 2b*

where S is the specific surface area (m^2), $\varnothing$ is the volume fraction of the oil phase (0.1%), $V_{emulsion}$ is the volume of the emulsions analyzed (5 mL). When normalized against the emulsion area, the initial lipolysis rate showed to be the highest for Na-Cas emulsions followed by HT-stored and then HT emulsions and NHT emulsions were found to have the lowest lipolysis rates (Fig. 5). This showed that NHT Pickering emulsions had a higher resistant towards lipolysis compared to HT, HT-stored and Na-Cas stabilized emulsions (P<0.05). This observation on non-heat treated samples was somewhat expected since the starch barrier in this case is still in its crystalline structure compared to HT and HT-stored (with lost crystallinity). Moreover, the HT emulsions were significantly more resistant to lipolysis compared to Na-Cas (from the very first minute) and HT-stored emulsions (from 5 minute) (P<0.05). However, except for the initial couple of minutes, and the end point of the digestion, there was no significant difference between HT-stored and Na-Cas emulsions (P>0.05). Gelatinization of starch in the heat treated samples causes starch granules to lose their crystalline structure and swell by absorbing water which in turn makes starch granules more accessible for the amylase enzyme present in the pancreatin Compared to non-heat treated samples. As a result, the erosion of the barrier by the action of amylase could result in a higher lipolytic activity. It was expected that HT-stored sample would regain a certain degree of crystalline structure through retrogradation during the storage at 6 °C that would resist amylolytic hydrolysis of the interfacial starch barrier slowing down the lipolysis in HT-stored samples. However, this cold storage did not provide a higher interfacial barrier towards lipolysis. Finally, protein stabilized emulsion stabilized by Na-Cas had the lowest resistance to lipolysis.

The lower rate of lipolysis in the NHT compare to HT and HT-stored does not agree with previous observation by Timgren, Rayner et al. (2011) where application of heat treatment on starch at the barrier reduced the activity of lipase up to 60% compared to non-heat treated samples. This difference can be explained by the different method of heat treatment and/or the composition of the digestive enzymes used. The heat treatment used to create the gelatinized starch barrier carried out by

Timgren, Rayner et al. (2011) differed in two ways compared to the present work. Firstly, the heating temperature (70 °C) was slightly lower than the conclusion temperature (T_c: 70.4 °C) of their quinoa starch, while the T_c of the quinoa starch used in the present work was slightly lower (T_c: 67.4 ± 0.7 °C) than the heating temperature (70 °C). Secondly, in contrast to this work where their emulsion was stirred during heat treatment, Timgren, Rayner et al. (2011) did not stir the emulsion during heat treatment but rather vortexed it for only 5 s after the emulsion had returned to room temperature. This could have resulted in a different microstructure with drop aggregates, this is also indicated in the larger D_{32}, specifically 33.5 µm at 225 mg/mL oil compared to 18.2 µm at 200 mg/mL oil in the present work. However, this difference cannot fully explain the differences the lower rate of lipolysis observed, since the lipase activity is reported in Timgren, Rayner et al. (2011) is scaled against the specific surface area which takes into account the available interface for lipolysis activity.

It is believed that the main reason for the difference in the rate and extent of lipolysis between the present work and the previous study by Timgren, Rayner et al. (2011) is the composition of the composition of the simulated digestive fluid. In the previous study lipase was used, whereas, in the present study pancreatin was used which is composed of lipase, amylase and protease. Therefore, the higher amount of lipolysis in the heat treated emulsions can be attributed to the gelatinized starch being more prone to activity of α-amylase present in the pancreatin, where the erosion of the gelatinized could allow better access of lipase to the oil as well as freeing droplets from starch-gel aggregates. This explanation can be corroborated by the results from Sjöö, Emek et al. (2015) where the addition of α-amylase before simple lipolysis with only lipase, reduced the observed barrier effect of the gelatinized starch layer. The results of the present study underline the importance of using a more representative composition of digestive enzymes.
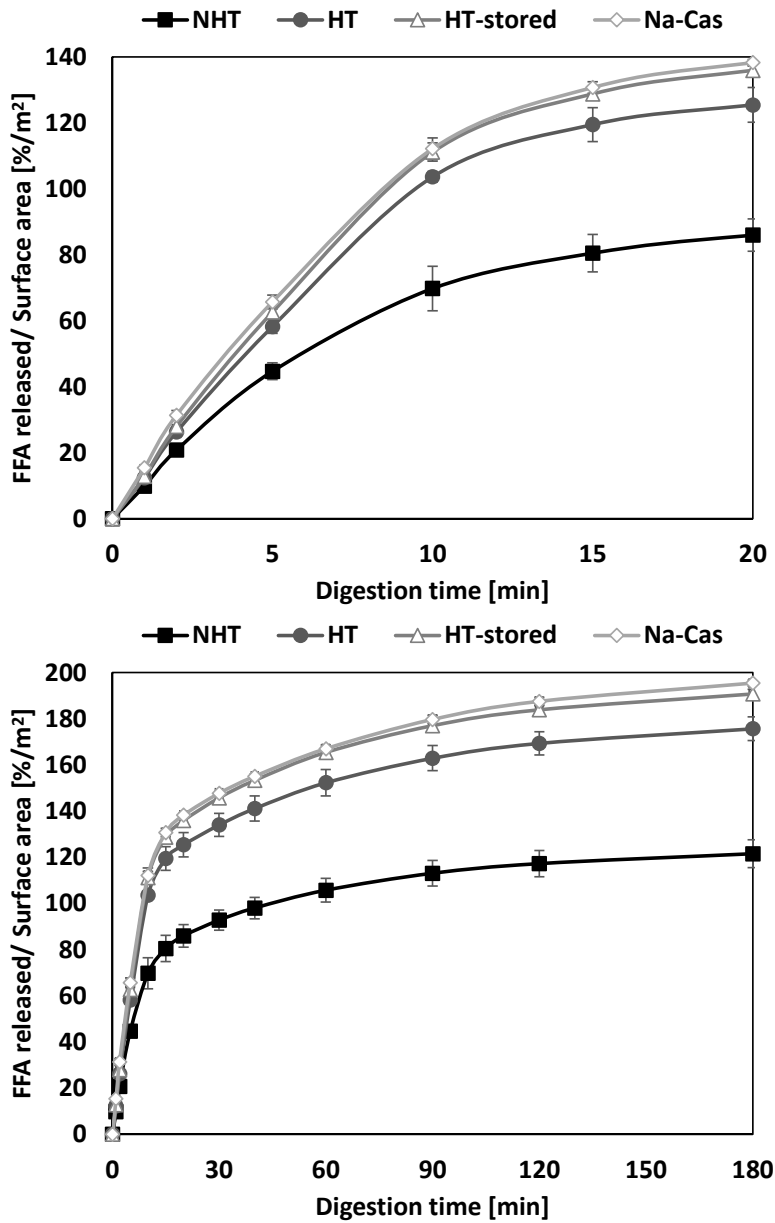
Fig. 5.
The amount of FFA% produced for the different emulsions during the initial 20 min *in vitro* intestinal lipolysis normalized against surface area (top), the amount of FFA% produced for the different emulsions during the 3 h *in vitro* intestinal lipolysis normalized against surface area (bottom). Error bars represents standard deviation of the mean, n=3.

4. Conclusions

The results of this study have shown that all Pickering emulsions stabilized by intact quinoa starch granules, irrespective of heat treatment or storage had a higher resistance to lipolysis compared to protein stabilized emulsion stabilized by Na-Cas. Furthermore, compared to heat treated and heat treated and stored samples, intact (non-heat treated emulsions) showed higher resistance towards the lipolysis. Moreover, the results of this study demonstrate the possibility of development of functional food in the form of emulsions based on starch granule Pickering stabilization where implementation of a simple thermal modification can affect the rate of digestion of starch at oil-water interface and therefore modulate the region in the intestine where the encapsulated compound is being delivered.

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Many food, pharmaceutical and cosmetic products are formulations based on emulsions. Traditional emulsions are stabilized by amphiphilic molecules including surfactants and biopolymers. It is possible to use solid particles with partial dual wettability to produce stable emulsions known as Pickering emulsions. Recently, starch granules have shown to have potential in producing Pickering emulsions. Starch granules meet the health and environmental requirements for ingredients and are also good candidates for stabilization of Pickering type emulsions due to their properties such as being natural, biodegradable and non-allergic as well as having neutral color, taste and odor. This thesis focuses on the development and application of starch Pickering emulsions for food, pharmaceutical and cosmetic applications.