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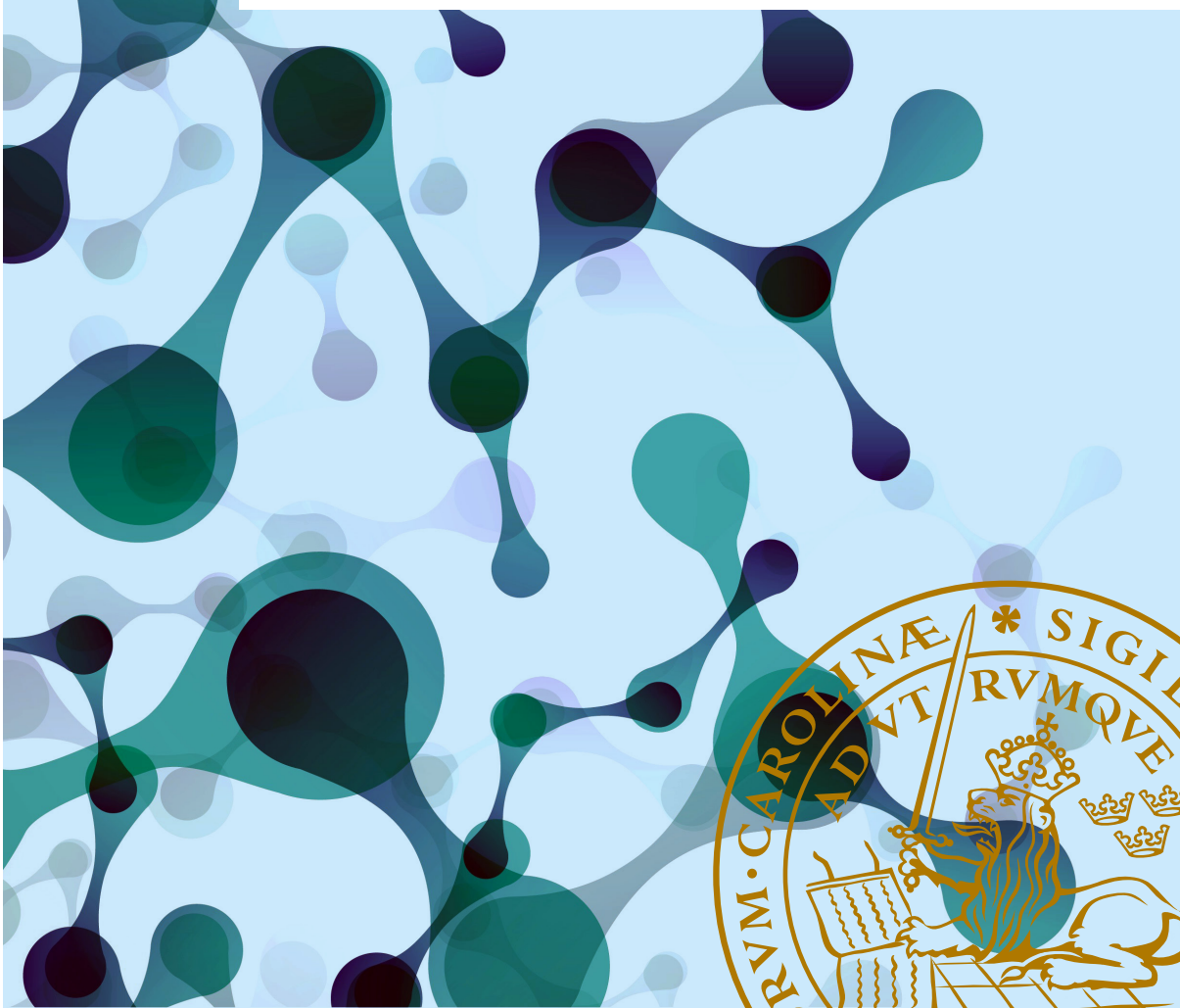

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Starch stabilized Pickering emulsions: Colloidal starch particles and their effects on emulsion properties

Particles can be used to stabilize multi-phase systems known as Pickering emulsions. The aim of this thesis was to investigate how starch particles affect emulsion properties. Starch granules were used individually as well as in binary mixtures. To obtain a wide variety of starch properties granules were selected based on botanic variation (quinoa, oat, waxy barley, waxy maize and potato). The properties of the starch particles were furthermore changed by size fractionation by sedimentation, acid hydrolysis, cold gelatinization, or dissolution-precipitation, which resulted in different particle sizes and shapes. Almost all samples were modified with octenyl succinic anhydride (OSA) to 1.7%-3%. The particle size was found to be important, since decreased particle sizes lead to decreasing emulsion drop size and increased stabilization against creaming. Decreased particle size up to 89% (potato), 64% (waxy maize) and 62% (waxy barley) by acid hydrolysis, and from 15μm to 120nm (waxy maize) by dissolution-precipitation, had a strong impact on decreasing emulsion drop size. The shape also influenced the affinity for the oil/water interface. Smooth rounded particles (waxy barley <10μm) seemed to provide smaller emulsion drops than smaller/similar sizes of polyhedral particles (quinoa and oat). Starch particles from ~120nm (nanoparticles) to <10μm (granules) were suitable for stabilization of emulsions, and the smaller the particle, the lesser weight of starch was needed for droplets stabilization. The best emulsifying capacity and stability was obtained with the nanoparticles (~120nm particle size) which gave emulsions that were stable for up to 1 year. Mixtures of starch granules in emulsion systems showed that when two types of starch granules are mixed, one is more likely to dominate on the interface but when the starch content is low both starches might adsorb. In the case of oat starch, it was seen that Pickering emulsion could be formed also with granules that had not been OSA-modified and that these adsorbed granules could be gelatinized in situ using $CaCl_2$. Cold gelatinization of oat resulted in an increase in particle size and at a minimum level of gelatinization these swelled granules increased the emulsion index for the emulsions they were stabilizing. The work in this thesis shows the potential and versatility of starch granules and its derivatives as emulsifiers.

Key words: starch granules, Pickering emulsions, starch, colloidal particles, sedimentation, acid hydrolysis, cold gelatinization, dissolution, non-solvent precipitation, nanoparticles

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To Luqman and Luthfia

The PhD babies, whom I carried in my womb during this 5years+ journey.
May this journey will inspires you in your future undertaking.

"There are better starters than me but I'm a strong finisher." —Usain Bolt
Strength and growth come only through continuous effort and struggle.
Never stop learning, never give up.

Abstract

Particles can be used to stabilize multi-phase systems known as Pickering emulsions. The aim of this thesis was to investigate how starch particles affect emulsion properties. Starch granules were used individually as well as in binary mixtures. To obtain a wide variety of starch properties granules were selected based on botanic variation (quinoa, oat, waxy barley, waxy maize and potato). The properties of the starch particles were furthermore changed by size fractionation by sedimentation, acid hydrolysis, cold gelatinization, or dissolution-precipitation, which resulted in different particle sizes and shapes. Almost all samples were modified with octenyl succinic anhydride (OSA) to 1.7%-3%. The particle size was found to be important, since decreased particle sizes lead to decreasing emulsion drop size and increased stabilization against creaming. Decreased particle size up to 89% (potato), 64% (waxy maize) and 62% (waxy barley) by acid hydrolysis, and from 15μm to 120nm (waxy maize) by dissolution-precipitation, had a strong impact on decreasing emulsion drop size. The shape also influenced the affinity for the oil/water interface. Smooth rounded particles (waxy barley <10μm) seemed to provide smaller emulsion drops than smaller/similar sizes of polyhedral particles (quinoa and oat). Starch particles from ~120nm (nanoparticles) to <10μm (granules) were suitable for stabilization of emulsions, and the smaller the particle, the lesser weight of starch was needed for droplets stabilization. The best emulsifying capacity and stability was obtained with the nanoparticles (~120nm particle size) which gave emulsions that were stable for up to 1 year. Mixtures of starch granules in emulsion systems showed that when two types of starch granules are mixed, one is more likely to dominate on the interface but when the starch content is low both starches might adsorb. In the case of oat starch, it was seen that Pickering emulsion could be formed also with granules that had not been OSA-modified and that these adsorbed granules could be gelatinized in situ using $CaCl_2$. Cold gelatinization of oat resulted in an increase in particle size and at a minimum level of gelatinization these swelled granules increased the emulsion index for the emulsions they were stabilizing. The work in this thesis shows the potential and versatility of starch granules and its derivatives as emulsifiers.

Popular Science Summary

Do you realize that starch is everywhere in our daily life? Starch is not just foods to satisfy our hungry belly. It is part of the cloths we wear, medicine we eat, cream lotion for our face, the paper that we write on, the glue to stick things, to thicken our delicious puddings or soups and it is even useful when preparing our salad dressing. Starch is the most common carbohydrate in human food intake. It is abundant, cheap, naturally existing and easily found among our staple foods. As mentioned, starch is not only served as foods, but has wide applications in other industries such as textile, pharmaceuticals, paper making, printing, adhesive, bioplastic and many more. The highlight of starch application in this thesis is in food processing where it can act as stabilizer or emulsifier in food products. Different types of starches such as quinoa, oat, barley, potato and maize have been studied to evaluate their potential as emulsifiers based on the important characteristics of size, shape and structure.

Investigating these starches is mainly to improve the quality of food products. The challenge in food industry today is how to produce foods that are not only nutritious, but also fresh looking, have longer shelf life and stay in good structure or stability from production until it reaches consumer's hand. Furthermore, and maybe most important of all, it should also be cheap. When we buy food products in the supermarket, the appearance of the products is what we perceive the most, as well as the expiry date. The appearance is based on freshness, good condition, structure and the stability. Stability is the condition when the ingredients do not get separated individually resulting in collapsed structure, which is very important especially for oil- and water based food products like mayonnaise, vinaigrette, salad dressing, butter, margarine but also for non-food products like body lotion and moisturizers. Oil and water is not mixed by nature except in milk that was created naturally stable with protein (casein) present around fat droplets that disperse in the liquid. As we do not favour our mayonnaise when it becomes oily on top layer or when our salad dressing gets separated into individual components of oil, water and herbs it is very important for oil and water to be mixed and stable for a certain period of time. That is why an emulsifier is needed.

Emulsifier holds both oil and water phase together and form a structure called emulsion. The most common emulsifying agent that we use in food is egg yolk, mainly in making homemade mayonnaise. Egg yolk contains lecithin, a substance called a surfactant that has two different characteristics: one part is loving oil (hydrophobic) and the other part is loving water (hydrophilic). Emulsifier is therefore actually a 'middle-man' between two different parties and is also called amphiphilic, it stays in between the interface of oil and water and bind both components. To mix all components well, energy from mixing, blending, or homogenization processes is often required to form emulsions. This emulsion is the building block of all oil-water based products. The capacity in stabilizing emulsions highly depends on certain emulsifiers. Most emulsifiers are amphiphilic due to dual characteristics.

Starch is neutral by nature, not favouring either oil or water. Modification is sometimes needed to make it more oil-loving either by heat treatment or patching the outer part with hydrophobic element. This will enable starch to sit in between the oil/water interface. To be able to emulsify certain products, the classical way is to use emulsifiers that contain both the 'loving oil- and loving water' parts. Depending on the amount of oil (O) and water (W), we can produce O/W emulsions when oil is dispersed in water as background liquid or vice versa. In this case, starch particles will sit around oil droplets and act as a barrier layer and prevent meetings with other oil droplets. Due to this, coalescence, a process where two oil droplets meet and combine can be avoided which in turn will inhibit separation of oil and water. This type of emulsion is also known as a Pickering emulsion named after S.U. Pickering, who is among the first scientists that found this phenomenon of solid particle stabilization in emulsions around 1907, although record mentioned Walter Ramsden as discoverer already in 1903.

Starch shows strong attachment between the O/W phases that is found to be irreversible due to high energy of detachment. The energy is defined as the amount of work that is required to take away starch particles from the interface that is subjected to the particle size. The bigger the particles, the higher the energy needed to remove the emulsifier. This is an indication of how stable an emulsion is and the higher energy needed for detachment the higher is the stability. However, this stabilization energy does not only dependent on the size, it is also subjected to the properties of the starch particles. Starches at a certain level, even with or without addition of hydrophobic elements, could position themselves in between both the oil and water phase due to the presence of protein on the surface, or due to favourable sizes and shapes. This study has revealed a great deal of information about the feasibility of starches. Quinoa with the size of 1-2 microns has been an interesting particle to explore due to their small and edgy shape that can maximize the packing at the interface of oil and water with higher volume and

create strong binding. Also, oat with the average size of 7-8 microns and a round edgy shape may stabilize emulsions even without hydrophobic treatment since it has a naturally occurring thin protein layer. Barley has bimodal sized particles with the size of less than 10- but up to 30 microns. The particles have round and smooth surfaces with a size less than 10 microns that have been found to sufficiently stabilize emulsions. However bigger barley particles seem to sediment by gravity and not be able to stay successfully at the interface. Maize particles on the other hand have slightly larger sizes than oat with rough surfaces and sharp edges and were observed to have less affinity to attach at the interface unlike most of the other starches investigated.

There are many interesting aspects to look at starches as they naturally exist in such uniqueness and variations. We can use it as it is after applying slightly hydrophobic elements and it is already good to go. Another step in current research work is to generate starch nanoparticles through a process of dissolving starch in high temperature and regenerate it by precipitation methods using alcohol. This will result in extremely small starch particles (less than 1 micron) that highly maximize packing in volume and bind effectively the interface of emulsions. Starch is not only a naturally existing ingredient but the application as emulsifier is regarded as novel and new innovation. By understanding starch characteristics, functions, mechanisms and its potential as stabilizer, the technology used will hopefully be implemented in all emulsion based products to benefit the use of starch as a natural ingredient in replacing synthetic and chemically processed stabilizers in the future.

Rumusan Kajian Saintifik

To my family with love so that you also can understand what I have done.

Adakah anda sedar bahawa unsur-unsur bersumberkan kanji wujud di mana-mana sahaja dipersekitaran kita? Realitinya, kanji bukan sekadar makanan untuk mengalas perut, malah ia terdapat pada fabrik kain, ubat-ubatan, losen penjagaan muka, alat-alat tulis seperti kertas dan gam, juga sebagai bahan penstabil dalam produk makanan khususnya. Kanji adalah sumber karbohidrat yang paling umum dalam sajian makanan manusia. Ia amat mudah untuk didapati, berasaskan bahan semulajadi, murah, dan mudah dijumpai di dalam makanan ruji kita. Seperti yang disebutkan, kanji bukan sahaja berfungsi sebagai makanan, tetapi mempunyai aplikasi yang luas dalam industri lain seperti tekstil, farmaseutikal, pembuatan kertas, percetakan, pelekat, bioplastik dan banyak lagi. Tujuan utama penyelidikan tesis ini adalah untuk meneroka potensi kanji dalam pemprosesan makanan di mana ia boleh bertindak sebagai penstabil atau pengemulsi dalam produk makanan. Pelbagai jenis kanji seperti quinoa, oat, barli, kentang dan jagung telah dinilai berpotensi sebagai pengemulsi berdasarkan ciri-ciri penting seperti saiz, bentuk dan struktur.

Penyelidikan berkaitan kanji ini tujuannya untuk meningkatkan kualiti produk makanan. Cabaran dalam industri makanan hari ini adalah bagaimana menghasilkan makanan yang bukan sahaja berkhasiat, tetapi juga kelihatan segar, mempunyai jangka hayat yang lebih lama, kekal strukturnya, dan stabil dari pengeluaran sehingga ia sampai ke tangan pengguna. Tambahan pula dari semua faktor yang disebutkan ianya juga harus dihasilkan pada kos yang rendah. Apabila kita membeli produk makanan di pasar raya, penampilan sesuatu produk adalah apa yang paling kita perhatikan, berserta tarikh luput. Penampilan ini berdasarkan kesegaran, keadaannya yang baik, struktur dan kestabilan. Kestabilan adalah keadaan di mana bahan kandungannya tidak terpisah secara berasingan mengakibatkan strukturnya rosak, terutamanya bagi produk makanan berasaskan minyak dan air seperti mayones, sos vinaigret, kuah salad, mentega, marjerin tetapi juga untuk produk bukan makanan seperti losen dan krim pelembap. Minyak dan air tidak boleh bersatu secara semulajadi kecuali dalam susu yang distabilkan dengan kewujudan protein (kasein). Pengguna tidak mahu membeli mayonis yang apabila disimpan pada tempoh tertentu, struktur minyak dan airnya terpisah atau sos (*dressing)* untuk salad yang terpisah menjadi beberapa komponen berbeza seperti minyak, air dan herba. Disebabkan itu, adalah sangat penting bagi minyak dan air untuk bersatu dan stabil untuk jangka masa tertentu. Itulah sebabnya pengemulsi diperlukan.

Bahan pengemulsi berfungsi untuk menggabungkan kedua-dua fasa minyak dan air bagi membentuk struktur yang di kenali sebagai emulsi. Ejen pengemulsi yang paling biasa kita gunakan dalam makanan adalah kuning telur yang boleh digunakan sebagai bahan pengemulsi bagi menghasilkan mayonis buatan sendiri. Kuning telur mengandungi lesitin, suatu bahan yang disebut surfaktan dengan dua ciri yang berbeza: satu bahagian yang menyukai minyak (hidrofobik) dan bahagian lain yang menyukai air (hidrofilik). Oleh itu, pengemulsi sebenarnya adalah penghubung antara dua komponen ini – yang juga dipanggil `amphifilik', di mana ia kekal di antara minyak dan air bagi mengikat kedua-dua komponen. Untuk menggabungkan semua komponen dengan baik, proses homogenisasi sering diperlukan untuk membentuk emulsi. Keupayaan menstabilkan emulsi adalah bergantung kepada bahan pengemulsi itu sendiri. Kebanyakan pengemulsi adalah amphipilik di sebabkan oleh dwi-cirinya di antara minyak dan air.

Kelebihan kanji adalah kerana bersifat neutral, tidak memihak kepada minyak atau air. Kadang kala pengubahsuaian di perlukan bagi menjadikannya lebih mudah bergabung dengan minyak. Sama ada melalui rawatan haba atau melekatkan bahagian luarnya dengan unsur hidrofobik (sukakan minyak). Ini akan membolehkan kanji berada di antara permukaan minyak/air. Bagi mengemulsikan produk tertentu, kaedah klasik adalah dengan menggunakan pengemulsi yang mengandungi kedua-dua bahagian 'menyukai minyak dan menyukai air'. Ini akan membolehkan kanji menjadi penghubung di antara permukaan minyak dan air.

Bergantung pada jumlah minyak dan air, kita boleh menghasilkan satu produk emulsi minyak-dalam-air apabila minyak tersebar di dalam air atau sebaliknya. Dalam kes ini, zarah kanji akan duduk di sekitar titisan minyak dan bertindak sebagai lapisan penghalang dan mengelakkan pertemuan dengan titisan minyak yang lain. Oleh kerana itu, proses penggabungan, satu proses di mana dua titisan minyak bertemu dan bergabung menjadi lebih besar dapat dielakkan. Emulsi jenis ini juga dikenali sebagai emulsi Pickering yang dinamakan bersempena seorang saintis yang menemui kaedah penstabilan zarah pepejal dalam emulsi sekitar 1907, S.U. Pickering. Untuk rekod, Walter Ramsden telah menemuinya lebih awal pada tahun 1903.

Kajian ini membuktikan bahawa kanji dapat mengikat dengan kuat di antara fasa minyak-air yang tidak dapat diceraikan kerana memerlukan tenaga yang tinggi untuk memisahkannya. Tenaga pemisah ini ditakrifkan sebagai jumlah tenaga yang diperlukan untuk memisahkan zarah kanji dari permukaan antara dua fasa yang tertakluk kepada saiz zarah. Semakin besar zarah, semakin tinggi tenaga yang diperlukan untuk mengeluarkan pengemulsi

Ini adalah petunjuk betapa stabil emulsi dan tenaga yang lebih tinggi diperlukan untuk memisahkannya dalam menggambarkan kuasa kestabilannya. Walau bagaimanapun,

tenaga penstabilan ini tidak hanya bergantung kepada saiz, ia juga tertakluk pada sifat-sifat zarah kanji. Kanji di peringkat tertentu, walaupun dengan atau tanpa tambahan unsur hidrofobik, dapat meletakkan dirinya di antara kedua-dua fasa minyak dan air kesan dari kehadiran protein di permukaan kanji, atau disebabkan saiz dan bentuk kecenderungannya.

Kajian ini telah mendedahkan banyak maklumat tentang potensi kanji. Quinoa dengan saiz 1-2 mikron telah menjadi zarah yang menarik untuk diterokai kerana bentuknya yang kecil dan sedikit berkotak dapat memaksimumkan penyelaputan pada titisan minyak di antara 2 fasa emulsi minyak/air dengan jumlah yang lebih tinggi dan mewujudkan pengikatan yang kuat. Oat juga yang bersaiz purata 7-8 mikron dan bentuk bulat yang rata boleh menstabilkan emulsi walaupun tanpa rawatan hidrofobik kerana ia mempunyai lapisan protein nipis yang semulajadi. Barli mempunyai zarah berukuran bimodal dengan saiz kurang daripada 10 hingga 30 mikron. Zarahnya mempunyai permukaan bulat dan halus dengan saiz yang kurang dari 10 mikron yang didapati berpotensi menstabilkan emulsi, manakala zarah barli yang lebih besar dibantu graviti untuk mudah termendap dan menjadikannya kurang efektif untuk mengikat dua fasa air/minyak. Zarah jagung pula, mempunyai saiz yang lebih besar daripada oat dengan permukaan kasar dan tepinya tajam dan diperhatikan kurang berpotensi untuk melekap di antara 2 fasa minyak/air.

Terdapat banyak aspek menarik yang boleh dilihat pada kanji kerana ia wujud secara semula jadi dengan keunikan dan variasi tertentu. Kita boleh menggunakannya selepas pengubahsuaian dengan sedikit unsur hidrofobik atau mengaplikasinya secara semulajadi kerana ciri-cirinya yang unik. Selain itu, terdapat juga kaedah semasa dengan penghasilan nanopartikel kanji melalui proses mencairkan kanji dalam suhu tinggi dan membentukkan semula dengan kaedah pemendakan (*precipitation*) menggunakan alkohol. Ini akan menghasilkan zarah kanji yang sangat kecil (kurang daripada 1 mikron) dalam memaksimumkan penyelaputan dalam jumlah yang tinggi serta dapat mengikat dengan berkesan pada permukaan emulsi. Kanji bukan sahaja merupakan bahan semulajadi yang sedia ada, tetapi aplikasinya sebagai pengemulsi dianggap sebagai novel dan suatu inovasi yang terbaru. Dengan memahami cirian, fungsi, mekanisma dan potensi kanji sebagai penstabil produk, di harap hasil kajian ini dapat digunapakai dalam semua produk berasaskan emulsi untuk memanfaatkan kanji sebagai bahan semulajadi dalam menggantikan penstabil sintetik dan berasakan kimia yang diproses pada masa akan datang.

List of Papers

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

Paper I Saari, H., Heravifar, K., Rayner, M., Wahlgren, M., & Sjöö, M. (2016). Preparation and characterization of starch particles for use in Pickering emulsions. *Cereal Chemistry*, *93*(2), 116-124.

Paper II Saari, H., Rayner, M., & Wahlgren, M. (2017). Study of mixed starch granules for used in Pickering emulsion. *Submitted to Carbohydrate Polymers.*

Paper III Saari, H., Johansson, D.B., Sjöö, M., Rayner, M., & Wahlgren, M. (2017). Pickering emulsions based on $CaCl_2$ gelatinized oat starch. *Submitted to Colloids and Polymer Science.*

Paper IV Saari, H., Fuentes, C., Sjöö, M., Rayner, M., & Wahlgren, M. (2017). Production of starch nanoparticles by dissolution and non-solvent precipitation for use in food-grade Pickering emulsions. *Carbohydrate Polymers, 157, 558-566.*

Paper V Saari, H., Rayner, M., Wahlgren, M., Sjöö, M., & M. Matos. (2017). A comparison of emulsion stability for different OSA-modified waxy maize emulsifiers: Granules, dissolved starch, and non-solvent precipitates. *Submitted to PLOS One.*

Paper VI Fuentes, C., Saari, H., Choi, J., Lee, S., Sjöö, M., Wahlgren, M., and Nilsson. L., (2017). Characterization of non-solvent precipitated starch using AF4 coupled with multiple detectors. *Manuscript.*

Related paper, not included in the thesis:

Paper VII Saari, H. Review paper: An insight on starch granules size, morphology, properties and functionalities. *Manuscript*

Author's contributions to the papers

Paper I	Author performed all practical work except acid hydrolysis (done by 2nd author) and wrote the first draft of the paper.
Paper II	Author participated fully in the design of the experiments, performed all experiments and wrote the paper.
Paper III	Author participated fully in the design of the experiments, performed the experiments with the exception of cold gelatinization in optimized conditions (done by 2nd author) and participated in writing the paper.
Paper IV	Author choose the experimental methods used. Designed the plan for the study. Performed the experimental work with exception of AFM and AF4. Wrote the first draft of the paper and refined it with co-authors.
Paper V	Author choose the experimental methods used. Designed the plan for the study. Performed the experimental work. Wrote the first draft of the paper and refined it with co-authors.
Paper VI	Author of this thesis suggested that this study should be done and prepared the starch samples. The first author of the paper did design the experimental characterisation strategy and performed the analytical work. The first draft of the paper was written by the 1st author but 2nd author collaborated on the refinement of the manuscript.

Contributions to conferences and workshops

Saari, H., Rayner, M., Wahlgren, M., & Sjöö, M. (2012). 'Starch granules: Potential stabilizing agents in food-grade Pickering emulsion' at *Nordic Starch Network 2012 Conference*, 22 November 2012, Copenhagen, Denmark. (Poster Presentation)

Saari, H., Heravifar, K., Rayner, M., Wahlgren, M., & Sjöö, M. (2013). 'Size reduction of starch granules for use in Pickering emulsion' at *AACC International Annual Meeting 2013*, 29 Sept- 2 Oct 2013, New Mexico, USA. (Poster Presentation)

Saari, H., Rayner, M., Wahlgren, M., & Sjöö, M. (2014)."Starch nanoparticles for use in Pickering emulsions", at *Realizing Reformulation conference 2014*, 22-24th October 2014, Lund, Sweden. (Poster presentation)

Saari, H., Rayner, M., Wahlgren, M., & Sjöö, M. (2014). "Preparation and characterization of nanoparticle for use in Pickering emulsion by dissolution and nano-precipitation" at *28th EFFoST International Conference*, 25-28th November 2014, Uppsala, Sweden. (Oral presentation)

Saari, H., Rayner, M., Sjöö, M Wahlgren, M., (2015). "The influence of size, shape and hydrophobicity of starch for Pickering emulsion stabilization" at *AACCI International Annual meeting*, 22-24 Oct 2015, Minneapolis, USA. (Oral presentation)
Best Student Paper Award 2015 in Engineering and Processing division (3rd)/ Student Travel Awards 2015.

Saari, H., Rayner, M., Sjöö, M Wahlgren, M., (2016). "Stability of waxy maize starch based emulsion; a comparison of granules, molecules and nanoparticles", *EU Starch Round Table 2016*, 17-18 Nov 2016, Lille, France. (Oral presentation)

Saari, H., Rayner, M., Sjöö, M Wahlgren, M., (2017). "Design of colloidal starch particles to stabilize Pickering emulsions and investigation of their effects on the emulsions", *31th EFFoST International Conference* 2017, 13-16 Nov 20176, Sitges, Spain. (Accepted for oral presentation)

Contents

Introduction

Emulsions are the basic microstructure of many common products in our daily life such as milk and margarine in food, cream and lotion in skincare, to mention some. Emulsion consists of two or more immiscible liquids: for example, oil dispersed in water or vice versa. The main challenges in emulsion stability are to prevent phase separation due to coalescence and Ostwald ripening. To facilitate stabilization, an emulsifier is needed. Emulsifiers are adsorbed at the interface, decrease the interfacial tension of two phases and increase the steric hindrance and/or electrostatic repulsion between the droplets, which increase the stability of the emulsion [1]. Two common food emulsifiers are proteins and low molecular/non-ionic surfactants such as lecithin. Nowadays, particle based emulsifiers are becoming popular due to their unique performance and strong adsorption at the oil/water interface which gives high stability to these emulsions. The most common particles are synthetic based such as silica and latex or food-based particles such as fat crystals [2].

Using starch as stabilizing particles has been increasingly explored especially for the purpose of food-based products, since it is a natural ingredient, abundant, inexpensive and it exists in different size, shape and composition [2-4]. In this thesis starch particles of specific sizes, morphology and properties are designed and used as colloidal particles to stabilize emulsions. The aim is to understand their functions in relation to the interface they are adsorbed to. This has included investigation of natural starch granules as well generation of new particles. These different particles have been characterised primarily concerning size and morphology and then been used to produce Pickering emulsions. The Pickering emulsions have been characterised concerning droplet size and stability towards creaming and coalescence. The use of starch nanocrystals as emulsion stabilizers is novel, and finds practical use not only in food and pharmaceutical applications, but could also be a "green chemical" for formulated products in general. Furthermore, the almost irreversible adsorption of colloidal particles creates very stable emulsions in contrast to surfactant molecules.

Thesis outline and objectives

The overall aim of this research is to design starch colloidal particles and investigate the effect of starch and its properties for stabilizing Pickering emulsions. The work has especially been focused on the size of the particles, but also partly on the morphology and other properties of the starch granules or starch particles. The specific research objectives are listed in Table 1 for each of the research topics.

Table 1 Specific research objectives

Overview of the specific research objective in Paper I to VI

Publications	Research Area	Starch	Research objectives
Paper I	Natural granules	Quinoa (very small)	To study intact starch granules from different botanic variation and class size and their potential in stabilizing emulsions
		Oat (small)	
		Waxy maize (medium)	
		Waxy barley (bimodal)	
		Potato (large)	
	Size reduction	Quinoa, waxy maize, waxy barley and potato	To reduce starch size particle and study its impact on emulsion stabilization capacity.
Paper II	Mixture of granules	Quinoa, oat, waxy maize	To study emulsions produced using mixed starch granules with different class size.
Paper III	Size reduction, properties and encapsulation	Oat	To study potential effects on emulsions of oat starch that has been subjected to cold gelatinization. To use cold gelatinization to encapsulate liquid oil.
Paper IV	Re-generate/ Break up and reform	Waxy maize	To generate starch nanoparticles using waxy maize that contain mostly amylopectin.
Paper V	Stability study	Waxy maize	To investigate the long term emulsion stability different starch structures (granuels, non-solvent precipitated starch and dissolved starch).
Paper VI	Study of starch conformations	Waxy maize	To characterize conformation and size of different types of non-solvent precipitated waxy maize starch using AF4.

The thesis is divided in the following parts:

- Characterization of starch particles chosen based on the natural size of the granules and fabrication of smaller starch particles with reduced size from micron to nano-size.

- Investigation of the impact of different type of starch particles, class, size and morphology, individually as well as mixed starch systems on the stability and droplet size of emulsions.

- Study of the long-term emulsion stability based on 3 different forms of starch; granules, dissolved starch molecules and precipitated starch particles as a representative of each different class size and properties.

Starch

Starch is an important food source for humans, animals, and of course for the plants themselves, providing energy for the germination of seeds. Starch that is produced by green plants can be found in fruits, seeds, steams, leaves, roots, tubers and pollen and are mainly used by the plants for energy storage [5, 6]. Starch grains collected from plants undergo milling processes to remove husk, fibre, protein and other impurities [6-8]. Isolation methods include dry or wet milling to obtain the starch granules [6, 7]. Starch content of the final product comprise up to 75% - 95% of starch dependent on the nature of the botanical source [5, 7].

Size, shape and morphology of starch

Starch granules are found in various sizes and shapes specific to their botanical origin [9]. Sources of extremely small starch granules, 0.3-2 μm, are quinoa, amaranth, cow cockle, and pig weed [5, 10-12]. Small starch granules, 2-10 μm, are found in oat, rice, and buckwheat [9, 10, 13, 14] while medium-size starches, 5-30 μm, include tapioca, barley, maize and sorghum [9, 14]. Large starch granules are found in tubers such as potato and canna, with sizes up to 100 μm [5, 9, 10, 15].

Figure 1 Starch granules with different structure, morphogy and size.
Credit photos from wiki common images: quinoa (blairingmedia), oat (yonygg), barley (ltyoppyawit) and maize (hiltomilanese).

Some examples of starch are shown in Figure 1 as representatives of starch variations. Some botanical starch sources, mainly cereals, are bimodal, like barley, sorghum, and wheat [5, 9-11, 14, 16-19]. Starches have unique morphology and are found in different sizes and shapes: smooth, rough, sharp and flat surfaces or round/spherical, sharp-edged, irregular, ellipsoidal and disc-shaped [5, 9, 10, 12-16]. Table 2 listed various types of starch classes based on the size of the granules; very small size to large starch granules and general information on the morphology or shape of starch granules are provided.

Tabel 2 List of starches

General information about starch with different size and shape.

Class	Starch Type	Dimension/Size	Shape
Very small	Quinoa	Submicron - 2µm [5] (0.7-2.2 µm) [13] 0.5-3 [10]	Smooth edges, irregular polygonal [1] Small & uniform size [1]
	Amaranth	Submicrons - 2 µm [1] 1-2 µm [5] 0.5-2 µm [7]	Irregular and polygonal-shaped granules [7]
	Cow cockle	Submicrons - 2 µm [1] 0.3-1.5 µm [5] 0.5-2 µm [7]	Irregular and polygonal [7] and round polygons edges but distinct Polygonal and has tendency to aggregates [8]
	Pig weed	Submicrons - 2 µm [1] 1.5-4 µm [5] 1-2 µm [7]	Irregular and polygonal-shaped granules [7]
	Taro	Submicrons - 2 µm [1] 2-3 µm [5]	Irregular and polygonal shape [7]
Small granule	Rice	4.5 µm [2] 3-8 µm [3,7] 2-10 µm [5]	Slightly larger than quinoa, irregular polygonal and sharp edges.Smallest in the cereal range [3]
	Waxy Rice	5.4µm [2] 3-8 µm [7]	Sharp edges, shows some compound or fused granules [7]
	Buckwheat	2-14 µm [5] 5-10 µm [7]	Polygonal [7]
	Oat	2-14 µm [5] 2-15 µm [7]	Seen in compound cluster. Outside cluster is round, interior granule is polygonal [3] Irregular shape and polygonal granules [7]
Medium	Tapioca	5-25 µm [7]	Smooth and irregular shape. [7]

Medium/ Bimodal	Sorghum	5 µm (small) & 10-30 µm (large) [7]	Polygonal, round edges with flat surfaces [3]
	Maize	Normal : 7.7 µm (small) and 13.2 µm (large) [2] / 5-20 µm [4,7] Waxy : 6.3 µm (small) and 13.9 µm (large) [2]/ 5-18 µm [7]	Normal: Irregular and sharp edges [6,7] Waxy: Irregular depression on surface of some granules and additional graininess and pin holes [1]
Bimodal	Wheat	2-3 µm (small) [1] 22-36 µm (large) [1] <2.8 µm (very small) [4] <9.9 µm (small) [4] >9.9 µm (large) [4] <10 & 10 -35 µm [5] 22-36 µm [7]	Bimodal [5]/trimodal distribution [4] Round (small granule) to disc shape (large) [3]
	Barley	Bimodal size distribution [1]-[8] 10-30µm (Large) and 1-5 µm [2] Small (<10 µm) and Large (>10 µm) [3] 2-10 µm (small) and 12-26 µm (large) [4] 2-5 µm (small) and 15-25 µm (large) [5] Average size: 16 µm [6] 2-3 µm (small) and 12-32 µm (large) [7] 2-3 µm (small) and 15-32 µm (large) [8]	Majority in oval and irregular shape. Most surfaces appear smooth (not entirely) [1] Oval to round shape [4] Smooth edges, oblate spheroids [6] Large disk-shaped granules [8] No holes or hilum observed and has uniform depression [1] Higher proportion of large granules [5]
Large granule	Potato	15-75 µm [1,7] 20-100 µm [6]	Smooth granules [7]
	Canna	Up to 100 µm [5] 30-100 µm [7]	Smooth, ellipsoidal and spherically shaped granules [5,7]

Starch components and structures

The physical properties of starch granules are complex depending on their botanical sources. Amylose/amylopectin ratio highly affects the physical properties of starch [20]. The two major components of starch are amylose and amylopectin. Normal starch consists of 15-33% amylose [5, 7, 21] and 70-82% amylopectin by weight (dry matter) of total starch [5, 21]. Waxy starches contain less than 1% of amylose whereas high amylose starches contain between 35-70% [7, 21]. Both amylose and amylopectin consist of polymer of α-D-glucose units. Amylose consists of long linear chains of (1,4)-D-glucose units with some branches in the large molecules. Amylopectin has branched chains consisting of around 5% of 1,6 bonds linkage with a backbone of linear chains of (1,4)- D-glucose backbone. The degree of branching is very important for the physical and biological properties of different starches [5, 6, 22, 23]. The size of the starch polymers can be described by referring to degree of polymerization (DP) which is in the range from 480-22400 glucose units depending on plant origin [5, 7]. The average molecular mass of amylose and amylopectin are in the order of 10^5 to 10^6 and 10^6 to 10^9 g/mole, respectively [6, 7, 21]. Other minor components in starch comprise of proteins (0.1-0.7%) that is found on starch surfaces and interior, lipids (up to 1.5%) making lipid-complexes with starch and phosphorus that is highest in potato starch [5, 21]. A representative of amylose and amylopectin structure is given in Figure 2.

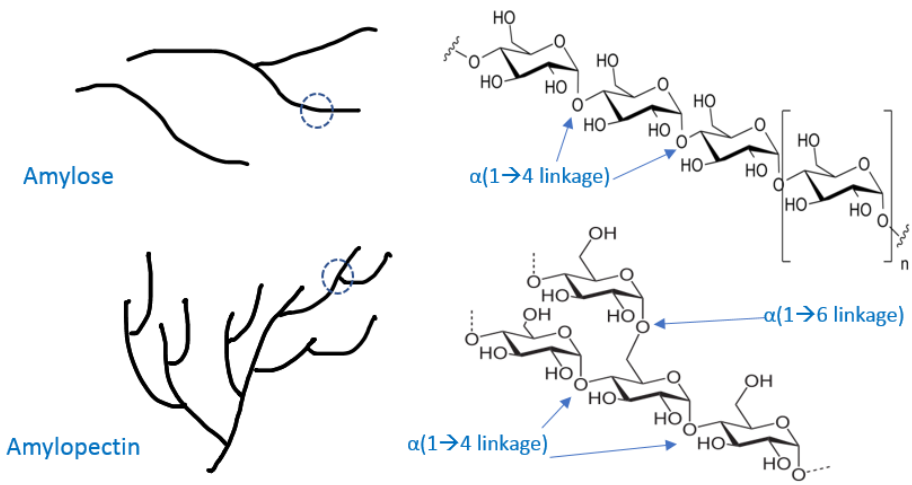

Figure 2 Chemical structure of amylose and amylopectin

Amylopectin has 3 unit chains as illustrated in Figure 3; the A-chain is the outer part and is the shortest, the inner B-chains and one C-chain that contains the only reducing sugar group in the molecule [5, 7]. Amylopectin is dominating in the crystalline parts of the granules and the crystallinity of the granule is mainly ascribed to double helices formed by amylopectin branches [20]. Starch contain around 15% and 45% of crystallite material and X-ray diffraction patterns reveals that there are two limiting polymorphs (A or B) and an intermediate form (C) [19, 20].

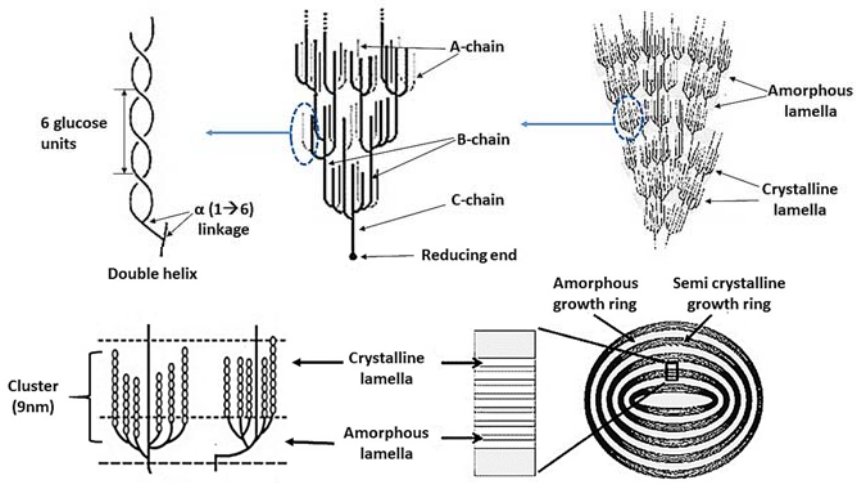

Figure 3 Lamella structure of starch granule
The representative diagram of crystalline layer separated from amorphous layer in growth ring, alternating layers of crystaline and amorphous containing chain of amylopectin and branch points respectively in both regions.

Crystallinity in the starch granule is related to the interlinking of outer chains of amylopectin that is forming double helixes in cluster structures [24, 25]. The 'cluster arrangement' of the amylopectin branches is characterized by alternating regions of ordered, tightly packed, parallel pattern of chains and less-ordered regions at the branch points. A unit of an amylopectin cluster comprises an amorphous region containing most of the tightly spaced branches (amorphous lamella) and a thin crystalline region containing the parallel glucans (crystalline lamella) [21]. Starch granules contain radial growth of amylopectin with 120 nm- 400 nm thickness and 16 clusters per growth ring [24].

Layers of alternating amorphous and semi-crystalline lamellas build up the granule structure in so called ‘growth ring’ [5, 7, 21, 24]. These growth rings develop from a periodicity in the biosynthesis. A periodicity of 9-11 nm is the repeat distances of crystalline and amorphous lamellae.

Dissolution of starch and its behaviour in solution

When starch is dissolved in water, it first gelatinizes and then the molecules leaks out from the gelatinized starch granules. In fact it is very difficult to fully dissolve the starch granule and thus special protocols needs to be applied to obtain a starch solution without residual ghosts of the granules [26, 27]. One way to obtain fully dissolved starch granules are pro-long heating at 140°C which results in starch being completely gelatinized and solubilized [28]. This process of dissolution was an important part of the method used in preparing nano-size particles followed by non-solvent precipitation as discussed in Paper IV.

In solution, amylose forms unstable random coil structure which with time can form double helices and recrystallize (see retrogradation below). In the presence of complexing agents such as alcohols, fatty acids, mono-glycerides etc. they can also form single helixes. Dissolved amylopectin on the other hand behave as a hyper- branched polymer.[29]

Gelatinization and **dissolution** occur primarily due to heating of starch in excess water. This leads to the breakage of the ordered structure of starch, where amorphous parts of the granule take up water and the granule swells. The double helixes start to uncoil, crystalline structure is lost and amylose is leached from the granule to the continuous phase. The whole process leads to increased viscosity and change morphological structure [7, 20, 30, 31]. In general, starch gelatinization occurs between 60-90°C [26]. During gelatinization starch granules swell in all direction, mostly following similar shape as the original granule and approximately double the size of the original form when heated in excess water [26, 32]. Amylopectin contributes to water adsorption, swelling and pasting of starch granules meanwhile amylose more likely hinder these processes [30, 32].

Gelatinization can also occur, at room temperature without heating, when the water phase contains high amount of salts such as $CaCl_2$ or LiCl. This is known as cold gelatinization. The mechanisms involved in salt-induced gelatinization are still not fully understood, but it has been reported that gelatinization of starch is affected by salts, and that the effect follows the Hofmeister series. The salting-in ions have been reported to reduce particle size, swelling and transparency, and lead to an increase in the gelatinization temperature, while salting-out ions, had the opposite effects [33, 34]. Cold gelatinization will be discussed further in Paper III.

Retrogradation happens due to re-arrangement of amylose-amylopectin during cooling and storage. This process is described as changes occurring in gelatinized starch from the amorphous phase to the more ordered crystalline phase due to thermodynamically

driven reorganization of gelatinized starch [20, 26, 35]. These changes includes re-association of double helixes that lead to partial recrystallization of the starch [36]. Rheological properties changes due to recrystallization and this is triggering firmness, rigidity, loss of water holding capacity and this increases during aging of the starch gels [26]. In solution, the recrystallization of starch leads to aggregation and precipitation of the starch molecules. Recrystallization occurs differently in amylose and amylopectin. Amylose has a high tendency to recrystallize and produce hard gels and films while amylopectin recrystallizes slower and create soft gels and films. The rate of crystallization for amylose gels reach a limit after 2 days while amylopectin gels will continue up to 30-40 days. Retrogradation has been shown to be responsible for both undesirable and desirable effects in starch-based products for example the process significantly contribute to staling of bread and other baked product. In the case of emulsion stability, starch recrystallization has been observed for starch in solution when longer term studies were conducted e.g. up to 1 year and as discussed in Paper V this might affect the stability of emulsions.

Non-solvent precipitation is the process of transforming starch molecules from a dissolved state in molecular form to precipitated particles after addition of a non-solvent that is miscible with the solvent that the starch is dissolved in [37]. This process of creating new particles is also known as nanoprecipitation as describe by Hornig *et al.* (2009) [37]. It has been shown that very small starch particles can be obtained with the non-solvent precipitation method [38]. Factors that control the particle size during precipitation include; the technique used, the initial concentration of the polymer (starch), the ratio of the polymer solution to the non-solvent solution, whether the polymer is added to the solvent or vice versa, and the type of solvent used [37-41]. Various kinds of precipitation techniques have been described for the production of nanoparticles, including dialysis and the dropping technique. The initial polymer concentration has been found to be crucial, and a narrow size distributions could be obtained from low polymer concentrations, in the range of 1-8 mg/mL. The size distribution may increase rapidly with increasing polymer concentration, and aggregation occurred at >20 mg/mL [38]. Low polymer concentrations are desirable to ensure that the molecules are in a dispersed state, and that they can be separated into nano-domains upon the addition of the non-solvent, as described by Hornig *et al.* (2009). Ratio of polymer solution and solvent has also been found to affect particle size and shape, and could lead to particles with different shapes, such as elongated fibers, spherical particles or a mixture of spherical particles and elongated fibres [39]. Finally, the order of adding polymer solution and non-solvent has been seen to affect the final particle size. [40].

Pickering emulsions

An emulsion is a system of two immiscible liquids where one phase is dispersed in the other. To make an emulsion, we need 3 basic ingredients; oil, water and emulsifier as represented in Figure 4 (left). A classical way to produce emulsions is by using emulsifiers such as proteins, surfactants and hydrocolloids [2]. Depending on conditions, volume and type of emulsifiers, we can obtain oil-in-water emulsion or water-in-oil emulsion.

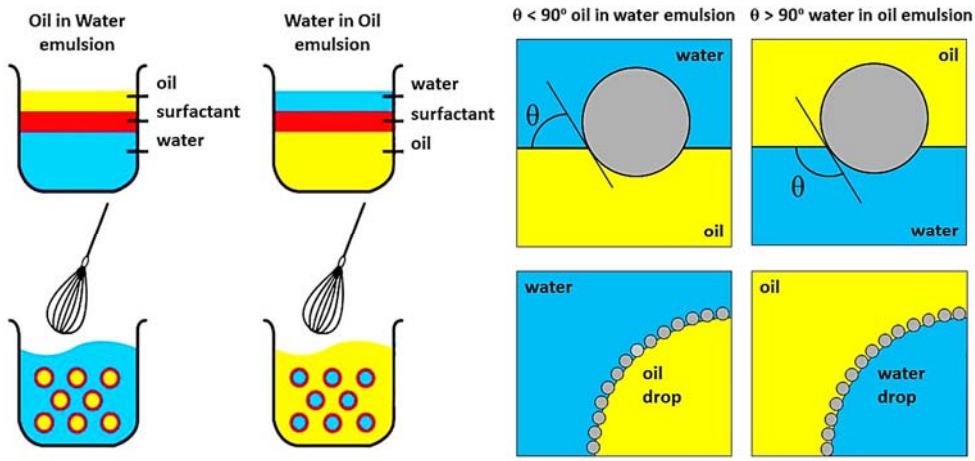

Figure 4. Classic emulsions (left) versus Pickering emulsions (right).
A classical way to make emulsions either for water or oil-based. A comparison to particle stabilized emulsions that showed the contact angle θ of particle that influence the position of particles at the interface.

Pickering emulsions are stabilized by solid particles, and thus it is the properties of the particles for example size and hydrophobicity that are critical for the formation and stabilization of the emulsion droplets. Particle stabilization of emulsions were first observed by Ramsden [42] and Pickering [43]. Commonly used particles for Pickering emulsions are synthetic particles such as silica and latex or food-based particles such as fat crystals [3]. Other particles of biological origin such as thylakoid membrane has also been used to stabilize emulsions [44-46]. However, starch granules have increasingly been used to stabilize emulsions mainly after chemical modification using octenyl succinic anhydride (OSA) [1, 13, 47-56]. In contrast to synthetic particles, starch is

mainly a natural ingredient which is accepted in food and also has nutritional value. Food based particles are preferred in the food and pharma-industries and particles like starch have been successfully used to stabilize emulsions [1, 3, 13, 48-58].

Principle of Pickering emulsions

The principal of Pickering emulsions is simply defined as particles being accumulated at the oil-water interface mainly acting as physical barriers that prevent the contact between droplets. Stable oil in water Pickering emulsions can be observed when particles have a size above 10 nm and are adsorbed to the oil-water interface with contact angle of $\theta < 90°$, as illustrated in Figure 4 (right) [59]. In this case the particles have the appropriate wettability and a tendency to adsorb irreversibly at the oil-water interface. The position of particle at the interface is described by contact angle. Since the desorption energy per particle is very high, there is a significant energy barrier to droplet shrinkage [47]. The energy of desorption, ΔG_d can be calculated according to equation 1 [3].

$$\Delta G_d = \pi r^2 \gamma_{ow}(1 - |cos\theta|)^2 \qquad \text{(eqn.1)}$$

Where γ_{ow} is the interfacial tension at the oil/water interface, r is the particle radius and θ is the contact angle. Therefore, when θ is not very close to 0° or 180° and ΔG_d per particle is extremely high, the particle can be irreversibly adsorbed at the interface and this would result in stability against coalescence and in some cases Oswald ripening [2] [3]. The desorption energy per particle can be as high as ~10^6-10^8 kT, in comparison to surfactant (~0.5-1kT) and protein (~1-20kT) when the size of the particle is in between 500nm to 30μm [1]. Figure 5, shows how the energy of detachment changes with size and contact angle. Pickering emulsions have also resulted in improved oxidative stability when silica and food-grade particles acted not only as excellent emulsifiers but also as physical barriers to inhibit lipid oxidation [60-62].

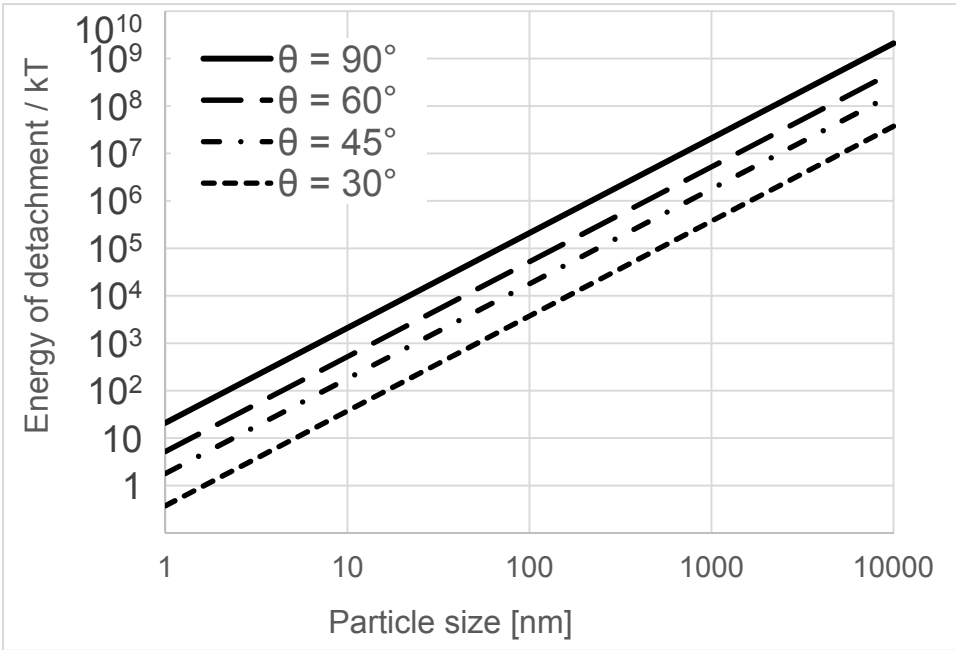

Figure 5 Energy of detachment
The effect of particle size, contact angle and the energy detachment in stabilizing emulsions.

Another instability problem in emulsions is creaming or sedimentation. This is reduced when emulsion droplets size or the density difference between the dispersed and continuous phase decreases. By reducing the particle size of starch more surface area of drops is covered per unit mass starch. Normally, leading to a reduction in the droplet size of the emulsions. To clarify the effect of starch particle size, the maximum surface coverage possible for constant starch concentration as a function of particle size can be calculated. The theoretical maximum coverage, Γ_M is estimated using the equation 2 [1]:

$$\Gamma_M = \rho_{sg} \frac{2}{3} d_{sg} \varphi . 10^6 \qquad \text{(eqn.2)}$$

Where ρ_{sg} is starch density (approximately 1500 kg. m-3), d_{sg} is the surface mean diameter of starch particles (d_{32}) and φ is the packing density (φ=0.907, theoretical maximum coverage with tight packing); i.e. the droplet surface area that is covered by the starch particles. The contact angle (θ) of 90◦ is assumed for all attached particles to the oil-water interface. It should be noted that the assumption that $d_{32} = d_{sg}$ is highly simplified, that in many cases φ is not close to 1, and that starch granules are not perfect spheres.

According to equation 2, more surface area can be covered per unit mass with smaller particles. This is one of the main reasons for reducing the starch granule size. The maximum surface coverage possible for each starch with a given particle size was calculated in order to examine the effect of the type of added starch and starch particle size, see Table 3.

Table 3. Maximum surface coverage

The calculation of theoretical and measured surface coverage for different starch particles.

STARCH	SIZE	Theoretical Γ_m (mg/m^2)	Theoretical,D_{32} (emulsion drops)	Measured D32/D43 (µm)
Waxy maize precipitates	~60-400nm 260nm (OSA)	56-375 243 (OSA)	1.68µm	4.62µm/9µm 1.66 (mode)
Quinoa	1-2µm	1033-1874	31-56µm	8µm/41µm
Rice	2-10µm	1874-9372	56-281µm	-
Maize	9-17µm	8435-15,933	253-478µm	14µm/47µm
Barley	2-30µm	1874-28,117	56-843µm	22µm/85µm
Potato	2-100µm	1874-103,000	56-3089µm	34µm/107µm

The theoretical surface coverage calculated in Table 3 shows for example for quinoa, oat and waxy maize given the value of roughly ~1000, ~8000 and ~15,000 mg/m^2 respectively based on their particle sizes which explains why smaller particles need smaller amount of starch to cover the surface compare to larger starch particles. This explains why quinoa starch granules even in small amounts is sufficient for stabilizing emulsion droplets compare to larger starch granules. However, it should be noted that adsorption of starch granules does not always give monolayer coverage. Instead there are cases when the starch form aggregates or is adsorbed to the oil/water interface in multilayers. In addition, it has been observed that full coverage of the droplets as calculated based on a closed packed monolayer is not a prerequisite for stable emulsion and emulsions with sub monolayer coverage has been found to be stable for long periods [58, 63]. Finally, one should be aware that all starch does not adsorb to the interface and as discussed in paper I and II, there can sometimes be considerable amounts of free starch in the emulsions.

The focus of this work was to characterize starch particles (either obtained from the botanical sources with naturally small granule size or with reduced granule sizes) and to investigate their potential as stabilizers of Pickering emulsions.

Formulation of emulsions

An emulsion is a thermodynamically unfavourable system where energy is needed to disperse oil in water. This emulsification process is the transfer of mechanical energy to generate the interfacial area of newly created emulsion droplets. High energy emulsification (as shown in Figure 6) involves; mechanical energy to mix the disperse phase with the continuous phase in the presence of emulsifier to create emulsion drops at the premixing stage of emulsions. Then, intense mechanical input is needed that deform and disrupt the emulsion to smaller and fine droplets that can go through fast or slow destabilization depending on the efficiency of the emulsifiers. This will further lead to either stabilization or coalescence of the emulsion droplets.

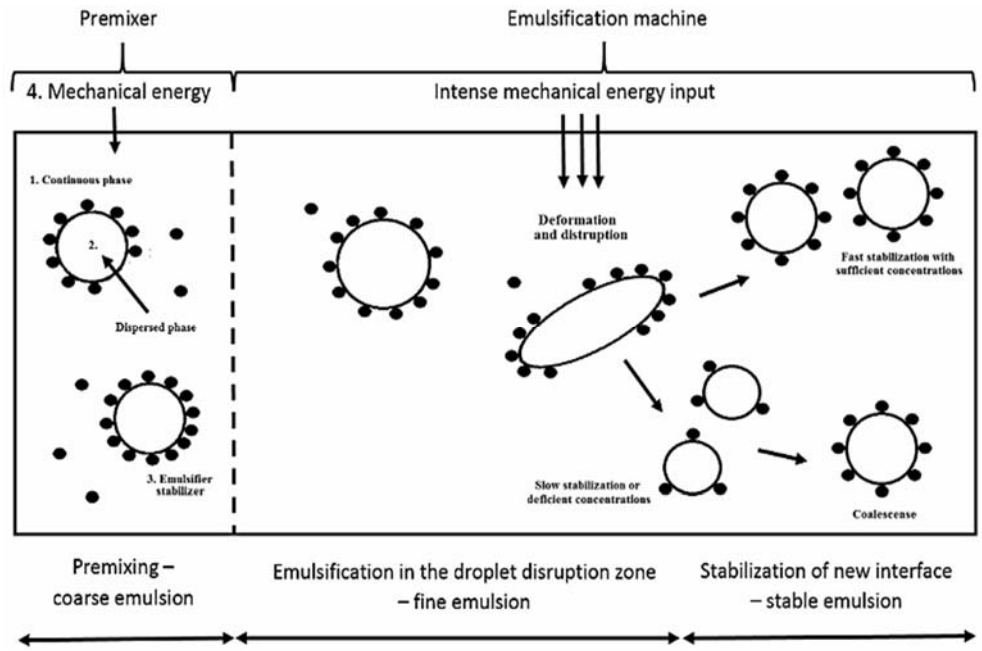

Figure 6 Emulsification process
High energy of emulsification process adapted from Rayner & Dejmek, 2015 [64]

Emulsification can be done by different types of equipment such as stirred tanks, colloid mills, high pressure homogenizer, ultrasonic emulsification equipment, micro fluidizations and high speed mixer [64]. Emulsification parameters were varied in Paper II where longer duration of mixing and shearing from 60-300 s were carried out to see the changes in droplets size. Meanwhile, in the rest of the papers, the emulsification time was fixed to 60 s to limit the parameters in the experimental studies.

Prolonged emulsification time has been seen to reduce drop size especially for the larger starch granules which needed more time to adsorb at the interface compared to the smaller starch granules (Paper II). One possible explanation for this difference in effect of emulsification time, is the difference in kinetics of transport of the granules to the droplet interface. The transfer of starch granules to the oil-water interface from the bulk continuous phase is governed by convective transport. This differs from the case of surfactants, protein molecules, and nanoparticles < 2 nm, where Brownian motion is the main transport mechanism overcoming adsorption barriers [4]. Convective transport during emulsification is subjected to the size and inertia of the emulsifier relative to the turbulent eddies created by the homogenization device used. This will govern the rate of adsorption of emulsifier to the emulsion droplets and thus the optimal emulsification time. Turbulence is characterized by the energy dissipation rate (power density) of eddies in the flow. In this study, a rotor-stator type homogenizer operating at 22000 RPM was used, generating turbulent flow that acts to break up the droplets and transports starch particles in order to stabilize the interface. This results in a power density of $\varepsilon = 6.6 \cdot 10^6$ and a minimum eddy size (Kolmogorov scale) of l_o =3.5 μm [53]. The rate of these collisions is determined by several factors including: the size and number concentration of the particles. This has been described for emulsions by Walstra (1993). In this work, it is obvious that at the same weight of emulsifier, the smaller the particle is the more frequent the collisions and thus the system becomes less sensitive to the time of emulsification compared to the larger ones.

Emulsion stability

Processing and composition of emulsions are designed with the aim to create emulsions with preferred stability. Emulsions are thermodynamically instable and the instability is caused by different mechanisms such as creaming/sedimentation, flocculation, coalescence and Ostwald ripening [65-67]. The most common instability mechanism observed in this study is creaming/sedimentation and coalescence.

Creaming/sedimentation is described as the separation of the oil droplets due to gravity, where the less dense phase will move upward and the denser phase goes downward for example oil droplets are separated upward in a water phase. Creaming/sedimentation may cause changes in space concentration that could lead to further instabilities such as phase separation and coalescence. Increasing the viscosity of the continuous phase will decrease the rate of creaming. The volume fraction of the dispersed phase will also influence creaming as it at high concentrations (above space fill) will restrict the movements of the droplets. Thus, at low concentration of the dispersed phase, there will with time, be a separation of the emulsion in to a concentrated emulsion layer and a liquid phase. This can be described by the emulsifying index (EI), equation 3. At low

volume fraction of the dispersed phase a cream layer will be formed on top of the emulsion as seen in Figure 7.

$$\boldsymbol{EI} = \frac{\boldsymbol{V\ emulsion}}{\boldsymbol{V\ total\ volume}} \boldsymbol{.100\%} \qquad \text{(eqn 3.)}$$

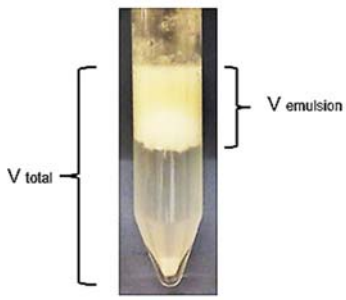

Figure 7 Emulsifying capacity of an emulsion, called the emulsion index (EI)
Image of an emulsion with volume fraction of 5% oil and 200mg/ml starch as emulsifier. The volume of the emulsion is indicating that the layer contained emulsion droplets stabilized by starch particles.

The emulsions investigated in this thesis (Paper I –V), have all a concentration of the disperse phase, ranging from 5-10% volume fractions of oil, which is below space fill. and thus, forms an emulsion layer on top or for heavy droplets sediment at the bottom of the container. However, full space filling of an emulsion was observed when the emulsion was stabilized by nano-size particles/dissolved molecule starch as presented in Paper IV and V.

Coalescence is the most common instability mechanism leading to increase in droplet size. The process is highly dependent on the film stability between the two droplets, collision, degree of flocculation of the system and concentration of dispersed phase. Factors that effects coalescence in traditional emulsions are, surface interaction, interfacial tension, interfacial viscosity, emulsifiers solubility, phase transition in the emulsifier layers and presence of solid particles In the case for Pickering emulsions, coalescence depend on the type of starch particles that stabilize the interface, for example granules or nano-size particles, size or surface morphology of these particles, and degree of surface coverage of the particles such as sub-monolayer coverage, close packed monolayer or aggregated multi-layers.

Coalescence has been one of the main factors of destabilization in our studies and it can be seen for most of the systems that there is an increase in droplet size over time indicated both by static and multiple light scattering as well as microscopy images. The stability of emulsions has been seen to vary between 24h up to 1 year depending on the starch particles used which is described in Paper I-V.

Characterization of emulsions and emulsion stability

Characterization of emulsion is of course an important step in evaluating the efficiency of starch as an emulsifier. Key parameters are the initial mean droplet size and droplet size distribution after emulsification as well as the change in these parameters during storage. The most common way to characterize emulsions is by measuring the size distribution with light scattering or microscopy.

The size distribution of emulsion is commonly calculated based on **volume frequency average diameter**, $\mathbf{d_{43}}$ or **surface average diameter,** $\mathbf{d_{32}}$ (known as Sauter mean diameter). These two means are functions based on the total number of particles in the emulsions with a given particle size according to equation. 4-5.

$$d_{32} = \frac{\sum_{i=1}^{n} d_i^3}{\sum_{i=1}^{n} d_i^2} \quad \text{(eqn.4)}$$

$$d_{43} = \frac{\sum_{i=1}^{n} d_i^4}{\sum_{i=1}^{n} d_i^3} \quad \text{(eqn.5)}$$

Where d_i is the diameter of the *i*th droplet and n is total number of drops measured.

In this study, mode and span are also used in characterizing size distribution of emulsion. **Mode** is defined as the peak of the frequency distribution. The highest peak representing the most common particle size seen in the distribution. This can be used when more than one population of particles is present in the distributions (for example oil droplets and granules) and thus using mean size of all particles is not anymore relevant. **Span** is used to define dispersion width that could be applied to all distributions as per equation 6:

$$Span = \frac{d_{90\%} - d_{10\%}}{d_{50\%}} \quad \text{(eqn.6)}$$

Another important parameter to highlight in relation to droplet size distribution is the surface area of the emulsion, S:

$$S = \frac{6\varphi}{d_{32}} \quad \text{(eqn.7)}$$

Where, φ is the volume fraction of the disperse phase (droplets), and d_{32} is the Sauter mean droplet diameter of the emulsion droplets. This calculation is important in producing emulsions as it relates to the energy to be put in the system in creating new

droplets and the amount of emulsifiers that is required to stabilize the emulsion which is further discussed in Paper II. Characterization of the size distribution of starch particles and emulsion droplets was mainly carried out using light scattering (i.e., Malvern Mastersizer) as well as microscope observations in Paper I-IV.

The stability study of emulsions was investigated in Paper V for short and longer term by Turbiscan Lab Expert (Formulation Co., France) using static multiple light scattering (MLS). The transmitted and backscattered light was monitored as a function of time and cell height. The backscattering (BS) measurement is directly dependent on the particle mean diameter and volume fraction. The main instability phenomenon observed in colloidal systems are: particle migration (bottom and top) and particle size increase (measured in the middle of the sample) [57]. The BS profile of the emulsions gives useful information of droplet size change, the formation of cream layer or sedimentation and makes it possible to calculate the velocity of these events [54].

Turbiscan stability index (TSI) was obtained using Turbisoft software (2.0.0.33) by computing it mathematically based on the scan to scan difference of the intensity of light over the height of the cell. Sums of the variations detected in the samples with respect to size/concentrations are calculated using the following equation:

$$TSI = \sum i \frac{\sum h \, |scan_i - scan_{i-1}|}{H} \qquad \text{(eqn. 8)}$$

where H represents the total height of the cell. The higher the TSI value is, the higher the instability of the emulsion will be.

Overview of the design of thesis work

This PhD project has been carried out using several different approaches and methodologies. A wide literature study was done at an early stage to determine the best strategies to achieve the research objectives. The initial start was to select starches from different botanic variation to obtain granules that naturally varied in size and morphology. Next, an experimental plan was designed to achieve the research objectives and to decide on the wanted starch properties to be investigated.

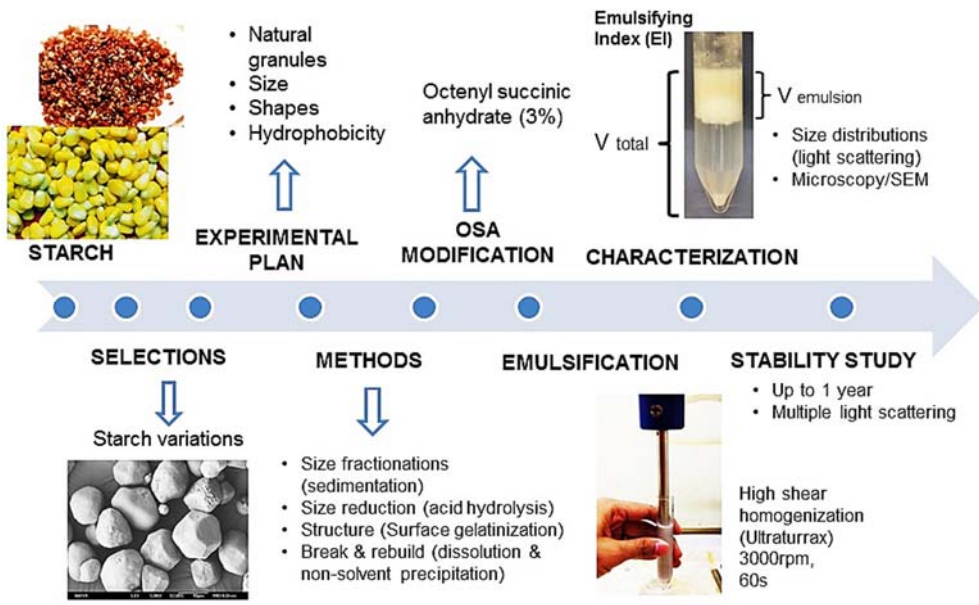

Figure 8 Overview of methods
Overview of methodology,approach, and the experimental design of this thesis.

The overview of this PhD project is presented briefly in Figure 8 above. A critical part in the start of the thesis work was the selection of suitable method for size reduction of the starch granules. The methods were; size fractionation by sedimentation, milling, acid hydrolysis, cold gelatinization, dissolution and non-solvent precipitation. All these

methods were studied and investigated as listed in Table 4. Normally the starches underwent different level of modifications before being used as emulsifiers. Emulsification and characterization of the obtained emulsions were also included in all studies in order to see the potential of starch as stabilizing agent. This section will first describe how the different starch materials was obtained and then the next section will discuss their effect on production of starch Pickering emulsions.

Approach to obtain starch particles for use as emulsifiers

Starch has been carefully studied and selected in this research based on the size, morphology and properties. Each project has different research objectives and selection of starch materials used is listed in Table 4.

Table 4 Selection of starch
List of selected starch used based on the research objectives.

Publications	Subject areas	Starch	Research topics & methods
Paper I	Size (very small to large granules)	Quinoa Oat Waxy maize Waxy barley Potato	Affect of size and morphology (1) Natural granules (2) Size fractionation (sedimentation) (3) Acid hydrolysis
Paper II	Mixed granules	Quinoa Oat Waxy maize	(4) Study of how mixed granules from different botanical sources affect the formation of emulsions.
Paper III	Shape/structure/ Properties	Oat	(5) Cold gelatinization
Paper IV	Size (small particles)	Waxy maize	(6) Studies on how to obtain starch particles by non-solvent precipitation and if these could be used for Pickering emulsion
Paper V	Stability of emulsions	Waxy maize granules, dissolved starch, non-solvent precipitates	(7) Stability study of emulsions made by the three different waxy maize starch emulsifiers
Paper VI	Starch conformations	Waxy maize acid hydrolyzed, precipitate, nanoprecipitates and OSA-precipitates	(8) Characterization of acid hydrolyzed starch granuels and starch non-solvent precipitated particles by AF4

In paper I, very small, small, medium and large starch granules were selected to study the influence of granule size on emulsion stabilization. The granules were used in original form and made more hydrophobic by reactions with octenyl succinic anhydride (OSA). Some starches were modified using acid hydrolysis to reduce the size of the particle and to see if this improved their emulsifying capacity.

In paper II, 3 types of starches ranging from very small, small and medium size were selected to study the effect of mixing different size classes of granules on emulsion formation and short-term stabilization.

In paper III, oat starch granules had shown interesting properties from previous projects and were therefore selected to be studied in detail regarding surface activity by investigating granules with and without OSA-modification. It was also investigated how cold gelatinization using $CaCl_2$ could be used both to affect emulsification properties of the starch granules and for encapsulation of oil droplets.

In paper IV-VI, waxy maize was used to produce non-solvent precipitate particles. The emulsification properties of these species were investigated and a thorough investigation of the structure of non-solvent precipitated starch was conducted.

Modification of Starch

To enhance the functionality of starch it is sometimes needed to be modify, for example to increase solubility, or as in the case in this thesis to make the granules more hydrophobic. Chemical modification involves, esterification, crosslinking, oxidation, cationization and grafting [68-70]. Physical modification includes processes such as annealing and pre-gelatinization and are often preferred as they are perceived as safe as it constitutes a chemical-free way to modify the starch. The advantages of modification of starch is that it provides desirable functional attributes that is controllable and predictable, gives value added properties, ensure product consistency, improve product aesthetic and extend shelf-life of products [68, 70, 71].

In this study, starch has undergone chemical modifications to increase its hydrophobicity. This was done using a chemical compound called octenyl succinic anhydrate (OSA) which modified starch through substitution with dicarboxylic acids and the method was first discovered by Caldwell and Wurzburg in 1953 [72]. The substitution of OSA is known to preferentially occur in and around the amorphous branch point of amylopectin at carbon 2,3, and OSA reacts with the glucose unit as seen in Figure 9 [73].

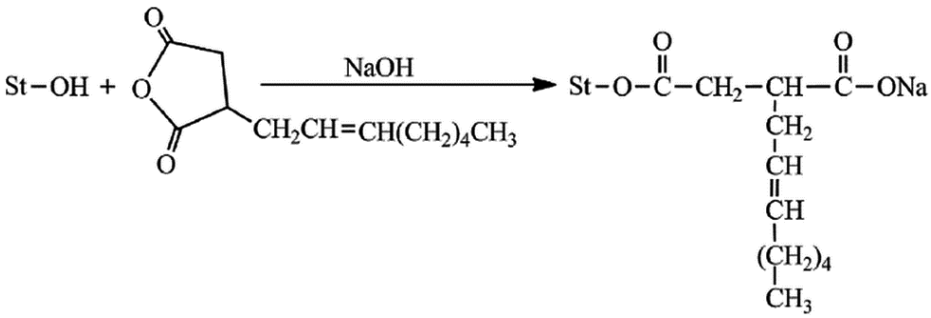

Figure 9 OSA starch
The representation of esterification reaction between starch and OSA [74]

Size fractionation

The objective of obtaining smaller sized starch particles is to facilitate better stabilizing effect in emulsions. Thus, several methods were tested to obtain and reduce particle size. A size fractionation method based on sedimentation was used to separate granules with different sizes. Sedimentation according to Stokes' Law and pipette method [75] were used to obtain small and large particle fractions of both starches. Duration of sedimentation was calculated based on the following equation;

$$t = \frac{18\mu h}{g(\rho s - \rho w)x^2} \qquad \text{(eqn.9)}$$

Where μ is the viscosity of the liquid media, i.e. water (kg/ms), g is the acceleration due to gravity (9.8 m/s), h is the sedimentation height (m), ρs is the density of starch, ρw is the density of water and x is the diameter of the particle (m).

Waxy barley has a bimodal size ranging from 2 -32 μm [10] and a fraction of small granules of <5 μm and large size of >10 μm [11, 17] and potato has a broad size ranging from 20-100nm [15]. For this reason, starch granules from waxy barley and potato

were chosen for separation into individual size fractions. It was observed that potato starch sediment faster than waxy barley maybe due to higher amounts of granules in the larger particle size range and that sedimentation does not fully follow Stokes' law at the concentrations used for the experiments. Potato starch showed almost complete sedimentation after 15.3 min compared to waxy barley where sediment occurred after 1 hour making it more difficult to harvest small size particle from the potato starch. This is probably the reason waxy barley has higher yield of small particle than potato starch. The sedimentation of barley granules at different times can be seen in Figure 10 and the cloudiness is an indication of the amount of particles available. Due to the low yield of small particles from potato starch, the study was only continued with starch from waxy barley.

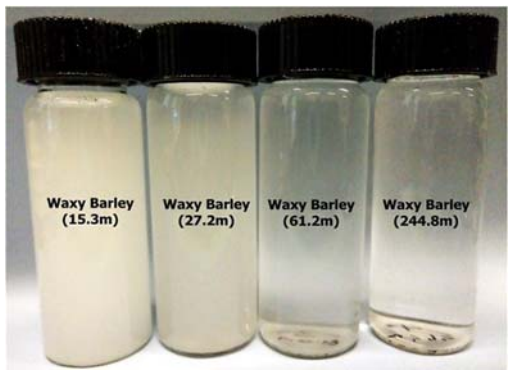

Figure 10. Sedimentation of waxy barley
Sedimentation of waxy barley at certain time (minutes) and at expected particle size.

Table 5 lists the sedimentation times needed to obtain fractions of the desired particle size, as calculated by stokes law as well as the average sized obtained experimentally when these sedimentation times where used.

Table 5 Sedimentation time
Calculation of sedimentation time to obtain certain diameter of the particle size and the experimenatly obtained avarge sizes.

Diameter range of particle (µm)	Theoretical Sedimentation time, t (min)	Theoretical Average size (µm)	Measure average particle size	
			D[3,2] (µm)	D [4,3] (µm)
≤20	15.3	16	9.2	15.2
≤15	27.2	14	8.3	13.5
≤10	61.2	9.5	7.9	9.17
≤5	245	6.2	-	-

The amount of sample obtained after 244.8 min of sedimentation was too small to be further analysed. Due to that sedimentation for 61.2 min were selected which gave <10μm sized granules. This method was successfully used to separate waxy barley with 2 groups of different sized particles, <10μm and >10μm. The method is simple however it was time consuming and resulted in low yield of granules.

Milling

Milling was considered as an option to achieve smaller particles. The objective was to reduce granule size by various types of milling methods as described in Table 6. However, the outcome was not as straight forward as expected. The first step in this study was to do a survey of the milling machine that might be purchased for the project. Inquiries were sent to at least 12 suppliers, only 3/4 responded that they could obtain the desired particle size (<0.1 μm) and 1 supplier offered free lab trial. Another issue was to select the right conditions to run trials, such as solvent for grinding and pre-treatment. Water and isopropyl alcohol were used as solvent for grinding. Pre-treatment were conducted with acid hydrolysis: 30% (w/w) starch in 4% HCL solution for 24h at constant temperature, 45°C.

Lab trial was conducted using Planetary Ball Mills from Fristch. However, the desired particle size was not achieved (0.1μm). 4 trials using this milling machine gave an unwanted outcome and instead of dry powder with reduced particle size was not obtained. In the case of waxy barley milled in a dry the samples become a sticky glue and the colour changed from bright white to clear grey and the particle size achieved was a d_{50}<20 μm. In the case of quinoa, a white paste was formed with a particle size of 1.74μm<d50<6μm and the starch became solid after 40 min of grinding. This was most likely due to effects on the starch by the heat that was produced by the process due to friction during dry grinding. Thus, grinding in solvent was carried out to solve overheating during the milling process. However even if no discoloration was shown using this method, the particle was still too large after 60 min of treatment. Thus, this method did not look promising and it was decided that the line of investigation should be discontinued. List of milling methods that was reviewed part of the literature review are given in Table 6.

Table 6. The list of milling equiments that was reviewed in the literature study.
The type of milling machine that were used reported in work by others in milling area.

Type of Milling/ Producer	Description	Starch	Performance (% Size reduction)	Sources
Planetary Ball Mill (NanDa, Nanjing, China)	QM-DK Low Temperature planetary ball mill and 4 ceramic milling cylinders.	Maize	Median diameter of granules after 3h 19.23 µm from 13.81 µm.	[76]
Centrifugal Ball Mill (Retsch S1000, Hann, Germany)	Mill with agate balls (10mm diameter, 8% w/v). Duration: 1,2,3h.	Cassava	Initial size: 3-30µm. Slight reduction after 3h milling: 8.6% < 6 µm. % reduction: 10%.	[77]
Hammer Mill (WEG, model FK71439, Brazil) **Food processor**	Paddy grains de-straw and dehusked. Milling rice starch after starch extraction from rice flour.	Rice grain to rice flour	Median diameter: 9.2±0.4 µm (11% amylose) and 8.4±0.1µm (21% amylose).	[78]
Cryomilling (Freezer/Mill 6850 SPEX)	Using Freezer/Mill 6850 SPEX with liquid nitrogen. Duration: 0,15, 30, 45, 60 min.	Rice flour to rice starch	Average size: 7-14 µm. (error in determining size reported).	
Cryogenic Milling (CM) (Dry & Wet) **Freezer/Mill 6850 SPEX (Metuchen, New Jersey, USA)**	Using Freezer/Mill 6850 SPEX in liquid nitrogen bath. Duration: 20 min.	Potato (PS) Normal Maize (MS) Gelose 50 (G50) & 80 (G80)	Size (median diameter in µm): PS; 35.2, PSCM; 35.9 (-2%). MS;16.8, MSCM; 13.7 (19%). G50; 17.5, G50CM; 14.7 (16%). G80; 16.0, G80CM; 13.3 (17%). Average reduction: 12.5%.	[79]
Vacuum Ball-Mill (QM-1SPO4, Instrument company of Nanjing University, China)	Capacity 50mL, diameter of balls 10mm and 6mm. Duration: 0h to 54h.	Cassava	Size: d_{50} value reduce from 24µm to 7.9µm after milling time 0h to 54h. % reduction: 67%.	[80]

*The chosen method in the study was highlighted in grey

Acid hydrolysis

Another established method to reduce size is the use of acid hydrolysis and there are several different methods described in the literature as listed in Table 7. It has been shown that acid treatment affects the amorphous layer of starch granules which are partially removed during the treatment leaving crystalline starch layer producing starch crystals with different shapes and sizes [81]. The results of acid hydrolysis can be affected by several factors such as the botanic origin of the starch granules, crystalline polymorphs, amylopectin content, morphology of the granules, and the conditions used during the acid hydrolysis [82]. Amylopectin rich starch (waxy starch) was claimed to be a good candidate for acid hydrolysis due to its high content of crystalline regions that can be converted to starch particles of different sizes [82].

In Paper I, potato, waxy maize, waxy barley and quinoa underwent acid hydrolysis treatment using a method, adapted from Angellier (2004). This includes, using 3.16M H_2SO_4 at 40°C and stirring at 100rpm for a time of 5 days [82]. Using this method, potato underwent the highest reduction of particle size giving a decrease of size by 89% followed by waxy maize and waxy barley that had a size reduction of 64% and 62%, respectively. However, quinoa doubled in size when subjected to acid hydrolysis and it was observed that the outcome of hydrolysation was aggregates and not individual starch particle. The size reduction has impact on the emulsion stabilization as will be discussed further in the section on starch stabilized emulsions.

Table 7. List of acid hydrolysis methods found in the literature.
The different outcomes of starch granules after the acid treatment at various conditions.

Starch	Description	Size/Shape/Info	Sources
Maize	-Starch pre-treated with enzymes for 2h; α-amylase, β-amylase and glucoamylase. - Hydrolysis using sulphuric acid (3.16M) of 1 L volume mixed with 146g native starches.	Shape: - α-amylase-hydrolyzed starch; least porous and smallest. Enzyme hydrolyzed granules from external layers and reduced size without creating much porosity. - β-amylase-hydrolyzed starch; intermediate size and porosity. - glucoamylase; biggest size and porosity. - attack the outer layers and create channels through the granules. Size : Initial size: 14.4 μm. α-amylase hydrolysis – 12.8μm (11%). β-amylase hydrolysis – 13.7μm (5%). glucoamylase hydrolysis – 14.1μm (2%). Hydrolysis after 2h enzyme pre-treatment for 45h produced nanoparticles size of ~100nm.	[83]
Waxy maize	-147g waxy maize starch mixed with 1 L diluted sulphuric acid (3M). -Suspension was kept under 400rpm mechanical stirring at 40°C using silicon oil bath. -Experiments were carried out for 1, 3 and 5 days. -Final suspensions were washed by successive centrifugation at 10000rpm in deionized water until neutral pH. -In between centrifugation, suspensions redispersed using Ultra turrax for 1 min at 14000rpm to ensure homogeneity. -The obtained suspensions were filtered on coarse filter tissue to remove granules's ghost. -Sodium azide was added to suspension before storage at 4°C to avoid microbial growth.	Shape: -Irregular shape, multimodal size with some sharp edges. Smaller individual granules look round and smooth. -Small nanocrystals aggregated into bigger particles after day 5. Size: -After 1 day; 25-70nm, average length of 50nm and width of 35nm.	[84]

Waxy maize starch	**-Acid hydrolysis of waxy maize starch granules for 5 days at 40°C in 3.16M aqueous sulphuric acids.** **-Obtained suspensions were washed with distilled water by successive centrifugations at 10000rpm and 10°C for 20 min until neutral pH.** **-Solvent exchange from distilled water to acetaone, then to tolune by successive centrifugation at 10000 rpm, 10°C for 45 min.** **- Homogenous dispersion of starch nanocrystal in tolune using Ultra Turrax for 3 min at 13500 rpm.**	**Shape & Size:** **- Platelet-like nanoparticles around 6-8nm thick, 20-40nm long, and 15-30nm wide.** **-Found to occur as aggregates and barrets.**	[85]
Waxy Maize	-Acid hydrolysis of starch nanocrystals with 3.16M sulphuric acid for 5 days at 40°C, 100rpm with starch concentration of 14.96 wt% and yield of 15.7wt %.	Shape: - Parallelepiped nanoplatelet - Observed in aggregates or in barrets of several platelets. Size: -Aggregates (1-5µm).	[82]
Waxy Maize	-36.725g of native waxy maize granules was mixed with 250mL of 3.16M H_2SO_4 for 5 days at 40°C at 100rpm stirring speed. -Suspension washed by successive centrifugation with distilled water until neutrality.	Shape: - Crystalline nanoplatlet. -tend to aggregate in aqueous medium but not sufficiently to induce sedimentation. - Surface ununiformed. Size: -Platlets 6-8nm thick, 40-6-nm long and 15-30nm wide. -Average size in aggregates form around 4.4µm.	[86]
Waxy Maize	-Starch nanocrystals were obtained by acid hydrolysis for 5 days at 40°C in a 3.16M aqueous H_2SO_4 solution while stirring constantly. -A homogeneous dispersion of starch. Nanocrystals was obtained by using an Ultra Turrax T25 homogenizer for 3 min at 13 500 rpm. -This dispersion was frozen immediately by immersing the container in liquid nitrogen. Subsequent lyophilization of the frozen dispersion resulted in dried nanocrystals.	Shape: -Aggregates with a large specific area due to a porous structure and a fractal-like base structure. -loose aggregates of nanocrystals in the dried state. Size: -not determined.	[87]

*The methods chosen in the study were highlighted in grey colour.

Chemical Gelatinization

Chemical gelatinization or as in some literature referred to as surface gelatinization is another possible method to reduce the size of starch granules. The initial idea behind this method is to reduce size by peeling the surface of the granule using chemical or salt like calcium chloride or lithium chloride. The outcome of this method is dependent on the type of starch granules studied as listed in Table 8.

The principle of this method is that in the presence of some substances, for example high amounts of $CaCl_2$, the surface of the granules will gelatinize at room temperature (cold gelatinization) and this gelatinized surface layer can then be removed mechanically by high shear stirring of the dispersion [88-91]. Some of the previous studies were conducted targeting cold gelatinization of potato and maize/corn and this generated starch granules with rough surfaces and reduced size [88, 90, 92].It was seen that small granules where more sensitive to this treatment than larger ones [91].

In paper III, the effect of chemical gelatinization with $CaCl_2$ on oat starch was studied but in this case, there was no surface gelatinization as have been seen in the literature for other types of starches. Instead oat granules swelled/expanded in size during the gelatinization time used in our experiments. The gelatinization time used where 0.5h, 1, 2, 3, 6, 12 and 24h. After each gelatinization time, the gelatinized starch was removed using shearing and washing by cold water. The remaining solid material were collected and the size of the obtained particles was measured. In order to remove dissolved starch and to obtain a smaller particle size, blending (by commercial blender) was more effective in removing the gelatinized starch compared to ultra turrax. The yield of remaining oat granules decreased drastically from 0.5h to 24h of gelatinization time. This as gelatinization and dissolution of starch increase over time and that the dissolved starch is removed during washing step. There were only small changes in size of the remaining starch particles even after a duration of gelatinization of 6, 12, and 24 h and in general the size of the cold gelatinized starch was larger than for the native granule.

Table 8. Methods of chemical gelatinization found in the literature.

The effect after the gelatinization varied where the granule size were reduced with certain shape and structure

Starch	Description	Size/Shape/Info	Sources
Potato	Chemical gelatinization using aqueous calcium chloride (4M) at 23ºC. -Initial size of starch of uniform size with diameter 30- 52 µm.	Shape: Undefined shape. Showed rough surfaces with reductions in the granular size. Size (estimation from SEM): Average initial size: 42µm. Average size reduction: 22 µm. % of size reduction after chemical gelatinization: 52.4%.	[90]
Maize **Large (>5µm)** **Small (<5µm)**	-Starches defatted by refluxing with an aqueous methanol solution (85% v/v) for 24h. -Chemical gelatinization for large and small granules using LiCl solution (13M) and stirred at 22-23ºC. -Large granules were gelatinized using $CaCl_2$ solution for comparison.	Shape: -periphery starch removed. -rough surface with lamella structure. -evenly surface gelatinized using 13M LiCl than 4 M $CaCl_2$. Size: -Surface gelatinized starch degree (%) is from 8 % up to 27%. -Size reduction is not determined.	[88]
Corn starch	-20g native corn starch was suspended in 150mL of 13M LiCl. - The gelatinization was stopped by adding 1200mL of chilled (4ºC) deionized water with quick mixing. - The mixture centrifuged at 3480g for 15 min, and supernatant discarded. -The surface-gelatinized starch washed twice with 1500mL of chilled deionized water.	Shape: -rough appearance was observed in all remaining granules, indicating the granule was peeled from the surface. -erosion occurred to large and small granules, confirming the uniformity of surface gelatinization. Size: -Surface removal is up to 40%. -Size reduction is not determined.	[92]
Potato	-Chemical gelatinization profile monitored by home-made temperature-programmed light intensity meter (LIM). - Starch suspension (5% in 5M $CaCl_2$) was put in microscope slide and heated at 1ºC/min.	Shape: - not mentioned. Size: At highest degree of gelatinization (81.3%), particle size from small and large granules are 14.1 and 15.7µm respectively. Initial sizes are 28 and 54µm respectively	[93]
Acetylated waxy Potato	2.0g (dry basis) starch suspended in 4mol/L $CaCl_2$ at 20ºC and low stirring. Degree of gelatinization monitored using polarized light microscope.	Degree of gelatinization: 26-54% depending of type of potato starch.	[89]
Rice	20g starch suspended in 150mL of 13M aqueous LiCl and stirred at room temperature.Treated sample blended at 22000rpm, and centrifuged. Re-suspend with chill deionised water and precipitated with ethanol.	Starch periphery may not be responsible for maintaining starch granule integrity.	[94]

*The methods chosen in the study were highlighted in grey colour.

Dissolution and non-solvent precipitation

Another approach used to produce smaller particles was to dissolve starch granuels and then induce precipitation of the starch to obtain particles. The chosen method was a combination of dissolution of starch by autoclave and non-solvent precipitation using ethanol as the non-solvents. Such methods are often referd to in the literature as nanoprecipitation. Listed in Table 9 are some of the previous works done using non-solvent precipitation.

Table 9. List of precipitation methods found in the literature.

Several methods of precipitation were studied to design starch nano-size particles. These have also been used to produce nano particles from other polymers.

Materials	Methods	Size/Shape	Reference
Polymer	Adding polymer solution drop wise under stirring into distilled water (P→W) or vice versa; (W→P) dropping water into polymer solution Method: 10mg polymer dissolved in 2.5ml acetone → add dropwise under stirring to 10ml distilled water/deuterated water or vice versa. Acetone evaporated at 40° C overnight.	Particle size (DLS): 5.8nm-100nm (P→W). 190nm -680nm (W→P) Shape: Spherical shaped.	[40]
Polymer	Method: 4mg/ml polymer solution underwent dropping method of dissolving polymer in acetone and drop wise into water. (P→W)	Particle size: 106-313nm. (P→W) Shape: Ideally globe shape and more uniform nanoparticles/more spherical depends on polymers. (P→W)	[37]
Starch acetate	Method: 100mg starch acetate was dissolved in 20ml acetone → distilled water was added dropwise to polymer solution (W→P) and vice versa (P→W). Stirred and evaporated.	Particle size: 249-720nm as concentration of polymer increase in acetone from 1-20mg/ml. Shape: Sphere shape and uniform.	[38]

Sago starch	**Method: 1 wt% of sago starch was dissolved in NaOh/Urea (NU) –[Dissolution]. 1mL of starch solution was added dropwise into abs ethanol (10,15,20ml) which stirred continuously 30 min more. The resulting mixture was centrifuged and supernatant removed. Pellet was rinsed 3x with abs ethanol to remove N/U. Dissolution→Starch→Ethanol**	**Particle size: Between 300-400nm With surfactant → limited the growth of starch particles. -Tween 80 – 150-200 nm, -CTAB – 250-300 nm Shape: Ratio starch solution: ethanol -1:10 (5 ml/ml) → fibrous shape. -1:15 (10 ml/ml) → mixture of spherical and elongated fiber. -1:20 (20 ml/ml) → spherical shape..**	[39]
Corn	Method: 8 g corn starch was added into 150 ml distilled water and heated 90 °C for 1 h [Gelatinization] by stirring. Etanol was added dropwise into the gelatinized starch solution. During cooling, another 150 ml ethanol was added. Centrifuged at 8000 rpm for 20 min and the pellet was washed with ethanol to remove water. After washing, drying at 50 °C to remove ethanol. Gelatinization→Ethanol→Starch	Particle Size: Between 50-300nm. Shape: Spherical shape.	[95]
Starch-ester	Method: 20 mg starch mixed ester was dissolved in tetrahydrofuran. Alkaline distilled water was added dropwise into polymer solution (W→P). Resulting nanoparticles was stirred until all tetrahydrofuran vaporized.	Particle Size: 270 nm. Shape: Spherical shape.	[41]
Starch-ester	Method: Dropwise addition of water into tetrahydrofuran solution of starch mixed ester (W→P) in different starch concentrations of 2,5,8,10 and 15 mg/ml in tetrahydrofuran.	Particle Size: 260nm – 1um. Shape: Spherical shape.	[96]
Acylated allylic starch	Method: 0.5g of acylated allylic starch (AAS) was dissolved in acetone and 10 ml distilled water was added dropwise into the polymer solution (W→P). Stirred until completely vaporized.	Particle Size: Ca. 300 nm. Shape: Spherical shape.	[97]

*The methods chosen in the study were highlighted in grey colour.

In paper VI, the method was adapted from previous studies as listed in Table 9. During the adaption of the method the following issues where investigated: method of dissolution of the waxy maize starch using either autoclavation at 140 °C for 20 min or DMSO/LiBr 0.05% (w/v), optimiztion of the starch concentrations, choosing the ratio of solvent, finding the right condition of mixing the solvents and evaluation of the effect of added surfactants. Figure 11 shows some of the experimental set-ups.

Figure 11 Dissolution by autoclavation and precipitation processes by dropping technique
The preparation of non-solvent precipitation steps to produce nano-size particles; (left) dissolution of starch using autoclave and (right) the precipitation method using ethanol by dropping technique.

During the development of the non-solvent precipitation method it was found that the dissolution step which was adapted from Perez-Rea *et al* (2015) using autoclavation of an aqueous solution at 140 °C for 20 minutes produced smaller particles than dissolving the starch in DMSO/LiBr [98]. The starting point for selection of precipitation conditions regarding starch concentration and ratio of starch:ethanol was the work done by Tan *et al* (2009) [38]. The optimum starch concentration resulting in the smallest particles was 8 mg/mL, and this concentration was used for studying all other parameters. As seen in Table 9 the starch concentration has been seen to affect the not only the size of the particles but also the shape. The method used in paper IV generated spherical starch particles.

This precipitation method was adapted from work by Hornig *et al* (2009) and Chin *et al* (2011) who initially used the dropping technique which was later optimized to direct mixing [37, 39]. In the optimization of the method, both these methods were compared for ethanol: starch solution ratios of 1:1 and 1:10. The direct mixing technique generated precipitates with smaller particles compared to the dropping and was less time consuming. If the ethanol was added to the starch suspension or the starch solution was added to the ethanol also affected the precipitation, and ethanol added directly to the dissolved starch was found to produce the smallest particles. The two ratios of starch to ethanol tested produced similar particle distribution and thus the 1:1 ratio was preferred as this reduce the consumption of ethanol.

Other parameters investigated were the addition of surfactants of SDS and Tween 80. The hypothesis was that addition of surfactants could reduce aggregation of the formed nanoparticles. However, this was not the case and in fact the addition of surfactants led to larger particles than when surfactant free solutions were used. Particles were produced ranging from 30μm to 170μm by addition of 4% SDS while particles with 25μm - 780μm were seen when 4% Tween 80 was used. Thus at least at concentrations of 8 mg/ml starch, solutions without addition of surfactant seemed to be better which is in contrast to the studies performed by Chin *et al* (2011) [39] who found that surfactants like CTAB and Tween 80 successfully limit the particle growth for production of their nanoparticles.

Atomic force microscopy (AFM) and Asymmetric field flow fractionation (AF4) were used to characterize the particles obtained (OSA and non-OSA modified samples). Using these methods, it was found that the main particle size was in the range 100-200 nm. However, larger particles and aggregates of non-solvent precipitates were also observed using methods such as light scattering, light microscopy and AFM. The average size distribution of the smaller particles in the precipitates was measured by AF4 and the result was a radius of gyration (rms radius) around 60 nm for the non-modified starch particles and 260 nm for OSA-modified nanoparticles which indicates that the particles are more strongly aggregated due to the OSA modification. This size is smaller than what has been seen for dissolved waxy maize molecules [99, 100] and further studies of the precipitate was carried out in Paper VI. AFM were used to analyse OSA modified particles, and the average ferret diameter (measure of an object size along a specific direction) of these particles were 182 nm with a span of 14-7000 nm showing large variations but close to the particle size of the non-modified starch as measured by AF4. This indicates that there is a large fraction of nano-precipitated starch that is in the range of 100 nm when not aggregated. Thus, the larger size observed for the OSA modified starch in AF4 is most likely due to aggregation. The size is also in the same range as the smallest particles detected by light scattering. Non-solvent precipitation in ethanol using the optimised method developed in this thesis work produced smaller particles compares to the acid hydrolysis method with particles less than 1μm and the particles were considerably smaller than the waxy maize starch granules d_{43}~13μm (granules) to ~200nm for non-solvent precipitated starch.

To summarize, the optimized process for non-solvent precipitation of starch successfully generated a yield of 70% of at each production run using the following process:

- Dissolution with autoclave 140°C, 20 min, +N_2 gas.
- 8mg/ml dissolved starch concentration.
- Direct adding to ethanol with 1:1 ratio (starch:ethanol) instead of the dropping method.
- No surfactant needed to limit growth of particle size.

This led to the production of particles with sizes between 10 nm and 1 μm, but larger aggregates were also formed, especially in the case of the OSA-modified precipitate. These non-solvent precipitate particles were then used to produce emulsions as discussed later in this thesis.

Investigation of structure and size of non-solvent precipitated starch using AF4

In Paper VI, the size and structure of different non-solvent precipitated waxy maize starch particles was investigated by Asymmetric field flow fractionation (AF4). The type of starch particles used were: non-solvent precipitated starch from acid-hydrolysed granules (acid hydrolysed sample), non-solvent precipitated starch from native granules (non-modified sample) with and without OSA modification. All the starch precipitates seem to be amorphous as no endotherms were seen in DSC.

By measuring recovery of the starch samples with different filters one can get some indirect information on how much of the sample is in the size range of the AF4 methods. The results in paper VI indicates that most of the particles are in the nano-size range seen by AF4. The AF4 profile for the acid hydrolysed starch sample as well as the size of OSA-modified sample was the same independent of the solvent used to dissolve the samples in. This indicates that the samples do not form particles that are stable for 1 hour in a water solution. The AF4 profile for the non-OSA-modified sample were different between the sample dissolved in DMSO and the sample dissolved in water. This indicates that a non-solvent precipitation induces a stable change in this material. Interesting to note is that the size of non-solvent precipitated waxy maize actually according to AF4 is slightly smaller in water solution than in DMSO which could indicate that the DMSO sample contains material from large particles that are dissolved in DMSO but not recovered in the samples dispersed in water. The results

are in line with observation in literature that also found precipitated particles that was smaller than the amylopectin molecules of the original material [101],

Based on AF4 theory, the molecular conformation of the samples disperse in water was determined. Acid hydrolysed sample had a conformation that was close to a **homogeneous sphere** (average $r_{rms}/r_h = 0.70$), whereas the OSA-modified sample is very close to the values seen for amylopectin [102]. Finally, the non-modified sample have a changed structure compared to what is expected for amylopectin (average $r_{rms}/r_h = 2.06$). This value indicate that the structure is more rod like. This cannot be due to a preferential precipitation of starch having a very different molecular structure compared to the waxy maize amylopectin as the OSA modified sample does not display this high average ratio and this material are produced from the non-modified material. Thus, there are strong indications that non-solvent precipitation leads to starch structures that are changed compared to the structure of the native amylopectin. In non OSA-modified starch these structures survives when the sample is dispersed in water. This is not the case for the OSA-modified starch sample at least not after 1 hour of dispersion. The reason could be that the OSA modified starch have been hydrolysed to some extent and that this increases the solubility of the material. The time between the dispersion of starch in water and emulsification is much shorter than 1 hour thus it cannot be ruled out that at least during emulsification the OSA-modified starch still contains some of the changed structure seen for the non OSA-modified material, and that this, as discussed in the starch stabilized emulsion section, can be beneficial for the emulsions produced from this material.

Starch stabilized emulsions

Native granules

Starch particles have been seen to successfully stabilize emulsions and the properties of the emulsions are strongly dependent on the size but also other properties of the used starch particles such as morphology. In Paper I, quinoa (1-2 μm), oat (7-8 μm), and waxy barley (bimodal, 5-30 μm) were studied and the emulsions produced from these granules were investigated to evaluate their potential for stabilizing emulsions. All granules were modified with octenyl succinic anhydride (OSA) to increase their hydrophobicity and the level of OSA substitution varied between 1.7 and 3.1%. Sedimentation of waxy barley made it possible to separate granules of different size: small (<10 μm) and large (> 10 μm). Smaller size starches (<10 μm) are favourable in stabilizing emulsions and provide smaller droplets. Larger size of starch (> 10 μm) has restricted ability to stabilize emulsions and cause aggregates that will sediment by gravity. The stability of emulsions observed after at least 24h of storage was seen to decrease in the order quinoa > waxy barley (small) > oat > waxy barley (bimodal) following the size of the starch granules. Granules that are either round or smoother contributed to the higher stabilizing capacity compared to similar size but sharp/edgy granules. The droplet size was also strongly influenced by starch concentration leading to a decrease in droplet size with increasing starch concentrations.

Mixed starch granules

After investigation of the effect of size of individual starch granules on emulsion properties, the next research topic was to study mixed starch granule emulsion systems. The objective in Paper II was to understand how mixtures of starch granules from different botanic sources affected starch Pickering emulsions and which types of granules that would dominate at the oil-water interface. Pickering emulsions were prepared using mixtures of starch granules based on 3 types of starches. Quinoa was mixed with either waxy maize or oat at ratios based on either at similar weight (400mg/mg oil for both starches, called in this thesis for *weight*), or constant surface area either based on a quinoa weight of 400 mg which is the same as in the weight

system (called in this thesis for *area*) or a total weight of starch of 800 mg/ml oil which is the same as the total mass of starch in the weight system (called *constant*). The effects of individual as well as mixed starch on the stability and drop size of emulsions were investigated as a function of concentration and emulsification time.

In mixed granule emulsions, there were a difference between the two systems investigated. In mixed systems quinoa and oat seems to work well together with the larger oat granules dominating at the surface when present in high concentrations. This is in difference to quinoa/waxy maize emulsion where quinoa is dominating at the interface. Waxy maize was observed to be a weak stabilizer, even at higher starch mass in the system compared to oat. Oat is an interesting granule in the mixed system it dominates at the surface even if the emulsion droplets obtained are larger than thus obtained by pure quinoa. As discussed previously it does in paper III it does not need to be OSA-modified to stabilize emulsions. It gives a rather non-compact creaming layer even when the droplets are large probably because oat granules seem to form an aggregated network of particles between the emulsion droplets.

The emulsion index (EI) increased as the starch amount in the system was increased. It was seen that the quinoa/waxy maize system have almost similar EI with quinoa/oat (both at weight and constant) probably due to the fact that they have similar total mass of starch in the system. This shows that the total starch concentration in the system has a strong affect on the EI, independent of the size of individual starch particle.

The emulsification time (60 – 300s) did influence the droplet size of the emulsions made when waxy maize and oat starches were used for stabilization but not for quinoa starch. Longer emulsification time decreased drop size in all mixed samples but more notably for quinoa/oat emulsions where longer homogenization times (5 min) decrease the mean droplet size by up to 80%. Quinoa/waxy maize emulsions did not change as much since the system was dominated by quinoa that gave small drops already at 60 s of emulsification.

Size reduction

Acid hydrolysis. In paper I, acid hydrolysed starches were studied where most of the starch has been significantly reduced in size after acid treatment (Figure 13). Potato, waxy maize, waxy barley reduced their size by 89%, 64% and 62% respectively compared to the size of the native granules. On the other hand, quinoa doubled in size since it formed aggregates instead of individual particles. After OSA modification, starches were used to stabilize emulsions from both intact-OSA (native) and hydrolysed-OSA starches. The comparison of intact and hydrolysed starches showed a significant decrease in the resulting emulsion droplet size. Quinoa had increased in size from 1.66 to 3.31 µm after acid treatment. However, the hydrolysed quinoa starch could still stabilize emulsions with a decreased drop size, from 76 to 33 µm which represents a reduction of 57%. Waxy barley showed the highest decrease in drop size of the emulsions (71%) from 106 to 30 µm followed by waxy maize starch with a reduction of 70% from 98 to 30 µm. The least reduction was shown for the potato stabilized emulsion with resulting size decrease from 103 to 89 µm corresponding to a 33% drop reduction. The emulsifying capacity was observed to be highest in the following order; quinoa > barley > potato > maize where size of the particles and morphology of starch influenced the stabilization performance. However, it did not fully follow the order of size after acid treatment, which were as follows; quinoa (3 µm) < maize (5 µm) < barley (7 µm) < potato (12 µm). Considering that the shape of the acid hydrolysed starches was very rough and uneven, it could be speculated that it is booth changes in size and morphology of the acid hydrolysed starch granules that promoted smaller droplets.

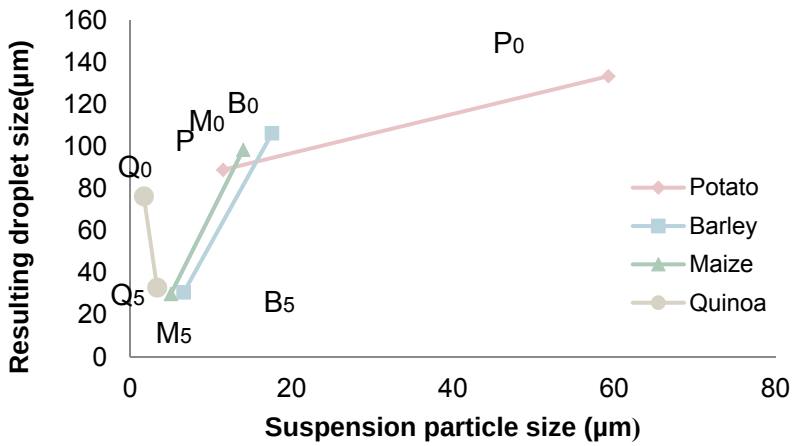

Figure 13 Emulsion droplets dependence on particle size for acid hydrolysed starch granules
The plot is showing the resulting emulsion droplet size of OSA modified starch particle emulsion produces with starch granules that have been acid hydrolysed for 0 and 5 days

Cold gelatinization. Another possible method to reduce particle size is by using the cold gelatinization process. The aim of the study presented in paper III was to investigate calcium chloride induced gelatinization of oat starch and how this affects the properties of oat starch based particle stabilized emulsion. As discussed previously cold gelatinization did not lead to a reduction of the size of the oat granules. Instead it did change the morphology of the starch. This was seen to affect the creaming layer of the starch based emulsions. All studied emulsions creamed after one day but the creaming layer differed due to the length of cold gelatinization of the starch, giving the least dense layer after 0.5 h of cold gelatinization.

Another interesting observation was that oat starch granules where found to stabilize emulsions both without and with OSA (octenyl succinic anhydride) modification of the granules. OSA modification of oat granules does not to any larger degree change the macroscopic properties of the emulsions compared to the corresponding non OSA modified oat starch, but the average size of the droplets was slightly smaller. This is in contradiction to most starch based Pickering emulsions that normally show very poor emulsification properties for native granules due to high hydrophilicity.

It was also investigated if emulsions formed from non-gelatinized granules could be cold gelatinized *in situ*. The results show that this was possible and that this leads to an encapsulation of oil. The gelatinized emulsions can be dialyzed and lyophilized without loss of oil. Upon reconstitution with water, the emulsion is reformed.

Non-solvent precipitation. In paper IV, the aim of the study was to investigate non-solvent precipitation of starch to produce nano-size particles that could be used in Pickering emulsions. It has been shown previously that modified starch particles could be successfully used to stabilize Pickering emulsions. Therefore, it was interesting to further investigate how to produce particles that were smaller than the granules traditionally used, in order to study how these, affect the size of the emulsion droplets.

It was shown that the non-solvent precipitation method produced starch structures that mainly was below ~200nm. However, larger aggregates of precipitates were also observed by light scattering and light micrographs. During emulsification with a high-shear homogenizer, these aggregates seemed to be partly de-aggregated into individual nanostructures, which resulted in a narrow droplet size distribution in the resulting Pickering emulsions.

The particles obtained subsequently influenced the emulsion droplet size in Pickering emulsions. Initially the emulsions had a size distribution from 0.5 to 45 μm ($d_{4,3}$, including free starch particles) with an average droplet size of 10±0.7μm and at day zero they had an emulsion index (EI) of 1 with as low amount of oil as 5%. An EI of 1 means that the emulsion is a space filling system with no observed creaming or

sedimentation. As comparison, OSA modified waxy maize granules at the same concentration as the non-solvent precipitated particles (200 mg starch/g oil) gave a droplet size of 76±10 μm and an EI of 0.25 for 5% oil. This showed that non-solvent precipitates can produced emulsions with much smaller droplet size than the granular waxy maize starch.

To understand the amount of starch needed to produce Pickering emulsions from non-solvent precipitated starch, a series of starch concentration from 200 mg/ml to 0.01 mg/ml were investigated. A starch concentration of 1mg/ml is able to create emulsion drops with 35 μm drops size whereas a starch concentration of 0.5mg/ml and below is not sufficient in covering oil droplets and oiling off. The highest concentration 200 mg/ml gave a droplet size around 10 μm which is thought to be around the limiting size for the emulsification equipment used.

The emulsions produced have been observed to be stable maintaining EI = 1 after 24hours and up to 1 week. It can thus be concluded that non-solvent-precipitation gave starch particles in the nano- to submicron range, and that these particles could be used to produce Pickering emulsions with smaller droplets sizes and higher emulsion index than Pickering emulsions based on starch granules.

Stability study

In Paper V, the stability of emulsions prepared by using octenyl succinic anhydride (OSA)-modified waxy maize in the form of granules, dissolved starch (molecules), and non-solvent precipitated starch (nanoparticles) as emulsion stabilizers were investigated. The aim of this study was to understand how starch in different forms affect the stability of the emulsions and this was studied using light microscopy, light scattering, and static multiple light scattering (Turbiscan). All three samples were prepared according to the previous studies in Paper I & IV. The results from light microscopy, and Turbiscan measurements indicated that coalescence occurred for all three types of emulsions. The coalescence was fast within days for the granule stabilized systems while it was considerably slower both for the dissolved starch and non-solvent precipitate-based emulsions. The latter showing the least degree of coalescence over time. Thus, it was concluded that differences in starch size and molecular structure influenced the resulting emulsion droplet size and stability, Table 10. The decrease in droplet size and the increase in stability towards coalescence and creaming were found to follow the order: granules (largest, least stable), dissolved starch, and non-solvent precipitated starch (smallest, most stable).

Table 10 Stability study

Stability data resulted from different starch structures in stabilizing emulsions.

Starch forms	Droplets size	Emulsion index (EI)	Turbiscan stability index (TSI)
Granules	45 - 74μm	0.47 → 0.28 (0h -24h)	31
Dissolved starch	19 - 86μm	0.87 → 0.41 (5-6 weeks)	21
Non-solvent precipitates	10 - 40μm	0.91 → 0.62 (up to 1 year)	16

Overview of results

The overview of the results in designing starch as colloidal particles and investigating starch and its potential in emulsion stabilization are given in Table 11.

Table 11 An overview of the results

Brief overview of the results from each paper listed for comparison and the further developement to achieve research goals.

Paper	Starch	Size/methods	Results
Paper I	Potato, waxy maize, waxy barley, oat, quinoa	OSA modified granules -Starch (1-100µm).	Particles (1-10 µm) are able to create emulsions. Bigger particles (>15 µm) → gravity & sedimentation .
	Waxy barley	Size fractionated granules -Starch (<10µm).	Particles (1-10 µm) are able to create emulsions – hydrophobicity and shape. EI: waxy barley (small)> Quinoa> oat>waxy barley.
	Potato, waxy maize, waxy barley, quinoa, oat	Size reduction of the particles. -up to 89% by acid hydrolysis.	Reduced droplet size and increased emulsifying capacity. EI: quinoa>waxy barley>waxy maize>potato
Paper II	Quinoa Oat Waxy maize	OSA modified granules in binerysystems	Oat dominates over quinoa at the surface. Quinoa dominates over waxy maize Waxy maize is a poor stabilizer.
Paper III	Oat	Cold gelatinization	Starch dissolved and formed a corona of barrier to stabilize emulsions. Non- OSA modified oat still stabilizes emulsions.
Paper IV	Waxy maize	Non-solvent precipitation (~60nm-1µm).	Reduced droplet size and increased emulsifying capacity.
Paper V	Waxy maize granules	OSA modifies granules -Starch (15µm)	Bigger particles (>15 µm) → gravity & sedimentation. Stability up to 24h.
	Waxy maize dissolved (molecules)	Dissolve starch molecules	Reduced droplet size and increased emulsifying capacity compared to granuels. Coalesence and creaming. Stability up to 5-6 weeks.
	Waxy maize Precipitates (nanoparticles)	Precipitate particles (~120nm -1µm)	Reduced droplet size and increased emulsifying capacity. Slow coalesence. The best emulsifying capacity is up to 1 year.
Paper VI	Waxy maize	Non-solvent precipitation	Non-solvent precipitation of waxy maize produces starch structures that are different from dissolved waxy maize amylopectin.

Application of Pickering emulsions

Starch is inexpensive and abundant which makes it a great choice as an ingredient in many application and products. Although they are mainly used in foods, such as in soups, sauces, gravies, bakery products, dairy, snacks, there are also application in non-food applications such as in pharmaceuticals, textiles, bioplastic, cosmetics and many more [70, 71]. Starch stabilized Pickering emulsions is a novel and new innovation where modification of starch particles show great potential for certain applications such as topical creams, encapsulation of oil, and stabilization of oxidation sensitive oils. The work done in this thesis shows several ways to improve starch based emulsions:

- Employing starch granule in natural size is one of the simplest way to obtain emulsions with different properties when it comes to droplet size and emulsion stability.

- Starch particles in nano-size range can function as good emulsifiers, fillers or coatings ingredients. Non-solvent precipitation produced nano-size material has potential for product that needs long term stability as they have been seen to improve emulsion stability.

- Mixing different type of starch granules maybe a good way to create product that need different ingredients functionalities.

- The cold gelatinization process may help in treating starch and other food ingredients that are heat sensitive but need stabilization properties in addition to the normal Pickering stabilization. Encapsulation of heat sensitive product can also be produce using this technique.

Conclusions

In this thesis, different methods to designing starch colloidal particles were developed in order to study the potential of starch particles as stabilizing agents for emulsions. The function of the starch particles as emulsifiers were seen to be dependent on their size, morphology and chemical and physical modifications applied to the granules. The following main conclusion could be drawn based on the work in the thesis;

- Investigation on starch granules size, morphology and properties of the starch granules shown that starch granules has potential as emulsifiers and small to medium size, round, slightly edgy, and smooth shaped granules such as for example quinoa, oat, and waxy barley (small fraction) show better performance as emulsifying agent than larger granules such as waxy maize and potato.

- The competition between different granules for the emulsion interface was investigated in binary systems. The conclusion is that size alone does not explain which granule will dominate at the interface. In the case of quinoa and oat, the larger oat granules dominate on the interface. However, for the waxy maize/quinoa system largest granules tested (waxy maize) the competing power was low and quinoa dominate at the interface. Future studies are needed to understand if this was due to a non-optimal size of the waxy maize or to other properties of waxy maize starch granules.

- Starch at reduced size has been an important factor in stabilizing emulsion as the smaller the particle, contributes to smaller emulsion droplets and this gives a higher emulsification index. New starch particles at reduced size from micron to nano-size has been successfully generated using several different methods such as size fractionation of native starch granules, acid hydrolysis, cold gelatinization, and dissolution followed by non-solvent precipitation.

- A method for producing non-solvent precipitated starch was developed and the material formed was characterized using a range of methods. It was seen using AF4 measurements that even if some of the particles produced by this method was not stable when re-dispersed in water for 1 hour, the structure of particles produced from unmodified waxy maize differed substantially from dissolved amylopectin.

- Increased emulsion stability was achieved for non-solvent precipitated starch compared to larger granules from the same botanical source obtaining reasonable good stability towards coalescence for up to a year for the non-solvent precipitated particles.

This study reveals another potential of starch besides functioning as our staple food to be useful as natural food ingredients for formulated products as well as the ability to use starch for emulsion stabilization in non-food products.

Comments and suggestions for the future

The works presented in this thesis mainly cover topics on the utilization of the starch with regards to its size and morphology. Only a few starches were selected or were able to be studied in this thesis, despite the facts that there are a number of botanic variations. Although experiments were always designed based on previous work found in the literature, the aim to create novel technologies and new innovations are the main key for research and development. Selection of the starch to be investigated is very crucial and if more research is done on uncommon starches, it will help to fill up gaps in the research field instead of studying the common starch that can already be found in the literature.

The main recommendations for studies that can be explored in the future are;

- A standard method could be set up to evaluate as many starch variations as possible, i.e., data collection of different varieties of starch standard analysis/methods/emulsions characterization and records in a starch database for future use within the group or to be compiled as review papers.

- Size had an important impact on stabilizing the emulsions. It can be seen that smaller particles contribute to high emulsifying capacity. Fabrication method for the nanoparticle can be improved and different kinds of starches can be explored beside waxy maize in order to produce novel starch nanoparticles. Extensive work has been done in finding the right method for production of starch nanoparticles by dissolution & precipitation and this method can also be used to produce bioplastic which was accidently produced during drying of the smallest fraction of the precipitates. It could be a very interesting research area to explore and study further.

- Morphology – shape, structure and morphology of the starch are unique and different at the surface as well as the internal structure. Unlike size, this thesis did not cover much on the shape/morphology and its effect on emulsion stability. More detailed studies could be done by a more systematic classifying starch shape, dividing the starch into categories based on shape as alternatives for studying the effect of shape based on individual starch material.

- Hydrophobicity – The OSA modification was applied to all starch in a similar standard method. However, because of the titration method the level of OSA varies between 1.7-3% depending on the morphology of the starch even though a fix amount of 3% of OSA is added based on the starch dry weight. A closer study could be done to investigate how and why OSA levels are not similar from one to another starch. Measurement by NMR to evaluate the levels of OSA by another method than titration could also be a possible alternative to validate the results. Other aspects to be studied is the distribution of OSA elements at the surface of the starch which could explain or give information on the wettability and how the stabilization occurs in certain ways for different starches.

- Contact angle is also an important parameter to be studied as it will explain how the starch positions at the interface. At the beginning of the research, we have tried to discover contact angles by confocal microscopy by taking the picture of the starch at the interface using 3D images. However, emulsions are large complex system and the attempt to measure relevant angles from microscopic images was not successful. Other methods of contact angle measurements or other methods which is related to the wettability of the starch particle itself can be investigated.

- Properties – A thorough study on a range of properties of different varieties of starch would also be an interesting area to explore. Starch occurs in various forms and there are more properties than studied in this thesis that can influence or determine how they work as emulsifiers. This includes knowledge of other components in starch such as protein, lipids, minerals, amylose/amylopectin as well as and contaminants, detailed surface structure, porosity of the starch granule to mention some important issues.

- The emulsification process effects the droplet size distribution. Different type of homogenizers used for the emulsification process were not studied which can be useful in order to understand how to generate the smallest droplet possible.

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"Being told you're appreciated is one of the simplest and most uplifting things you can hear."

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Paper I

Preparation and Characterization of Starch Particles for Use in Pickering Emulsions

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ABSTRACT

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Particle-stabilized emulsions, called Pickering emulsions, can be produced by using starch particles. In this work we studied how the properties of the starch particles affect the droplet size and creaming of such emulsions. In the study, various sizes of starch particles were generated by two different methods and used to stabilize Pickering emulsions. Sedimentation according to Stokes' law was used to separate small and large starch granules. Acid hydrolysis was another method used to obtain smaller particles. All samples were modified with octenyl succinic anhydride (OSA) to increase their hydrophobicity with a level of OSA substitution between 1.8 and 3.1%. The size of starch particles was the main factor influencing emulsion droplet sizes. Furthermore, the droplet size decreased as the starch concentration increased. Using small starch particles with sizes <10 μm produced stable emulsions with smaller droplet size compared with larger sizes of starch particles, >10 μm. When subjected to acid hydrolysis, smaller starch particles were generally obtained, which could subsequently create smaller emulsion droplets. The emulsion index increased for the acid-hydrolyzed starch owing to the size reduction of starch particles. The shape of the starch seemed to have a minor impact on the droplet size and the creaming of Pickering emulsions.

Emulsions are important structures in many everyday products, such as food, creams, and skincare lotions. Emulsifiers are adsorbed at the interface, decrease the interfacial tension of the two phases, and increase steric hindrance and electrostatic repulsion between droplets, thereby decreasing the droplet size and increasing the stability of the emulsion against creaming but also against coalescence. There is growing demand for natural nontoxic emulsifiers for stabilizing emulsions, which has led to an interest in new emulsion systems such as particle-stabilized emulsions. Particles have been successfully used to stabilize emulsions known as Pickering emulsions. These emulsions have shown excellent long-term stability against Ostwald ripening and coalescence. Pickering emulsions was first described by Pickering (1907) and Ramsden (1903) at the beginning of the 20th century. However, they have just recently gained a considerable interest when it comes to applications that use particles accepted for food and other life science applications (Rayner et al. 2014). The particles mainly act as a physical barrier that prevents contact between droplets, and the adsorption of particles is irreversible once they attach at the interface, which are the main reasons for the high stability seen for these emulsions (Tcholakova et al. 2008; Dickinson 2010; Rayner et al. 2014; Wahlgren et al. 2014).

Previous works have reported the use of synthetic particles in emulsion stabilization, such as silica, latex, and clay, or food-based particles such as fat crystals, globular proteins, and hydrocolloids (Dickinson 2009). Starches are being increasingly used to stabilize emulsions, mainly after chemical modification with octenyl succinic anhydride (OSA) (Timgren et al. 2011; Yusoff and Murray 2011). Unlike synthetic particles, starch is a natural ingredient with nutritional value, and as long as the OSA modification is below 3%, it is an accepted food ingredient (Rayner et al. 2012b).

It has been shown that starch particle size plays a key role in forming stable emulsions with small droplet size. Especially quinoa starch has been reported to successfully stabilize emulsions owing to its small size (Timgren et al. 2011; Rayner et al. 2012a, 2012b). The droplet size decreases with decreased particle size, provided that other properties such as wettability, shape, and surface remain the same (Timgren et al. 2013). It is the aim of this investigation to further study how starch particle character affects Pickering emulsions.

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Starches are found in various sizes and shapes specific to the botanical origin (Jane et al. 1994). Sources of extremely small starch granules (0.3–2 μm) are quinoa, amaranth, cow cockle, and pig weed (Hall and Sayre 1971; Pérez and Bertoft 2010). Small starch granules (2–10 μm) are found in oats, rice, and buckwheat, and medium-size starches (5–30 μm) include most starches such as tapioca, barley, maize, and sorghum (Hall and Sayre 1970; Jane et al. 1994). Large starch granules are mainly found in tubers such as potato and canna, with sizes up to 100 μm (French 1973; Lindeboom et al. 2004). Some starch sources, mainly cereals, are bimodal, such as barley, sorghum, and wheat (Hall and Sayre 1970; Jane et al. 1994); they have a large (A-type) and a small (B-type) granule fraction that can be separated through selective sedimentation. In addition to the size, starches have unique morphology and are found in different shapes: smooth, rough, sharp, and flat surfaces or round/spherical, sharp-edged, irregular, ellipsoidal, and disc-shaped (Jane et al. 1994; Lindeboom et al. 2004).

Starch size can also be reduced through several methods, such as chemical gelatinization (Jane and Shen 1993; Wang et al. 2007), starch dissolution (Stevenson et al. 2007; Syahariza et al. 2010), nanoprecipitation (Hornig et al. 2009; Chin et al. 2011; Tan et al. 2012), and acid hydrolysis (Putaux et al. 2003; Angellier et al. 2004). There is evidence in the literature that acid hydrolysis methods have been successfully used to reduce particle size. For example, Battista prepared starch crystals of sizes varying from nano to micron size by dispersing starch granules into HCl. However, this classic procedure involved a long process (40 days of treatment) and produced a low yield (0.5 w/w %) (Battista 1975). Angellier et al. (2004) optimized the method and produced starch crystals by H_2SO_4 treatment within five days. During acidic treatment, the amorphous region of starch is hydrolyzed and removed, whereas the crystalline part is converted to starch crystals of different sizes (Song et al. 2008). The result of acid hydrolysis can also be affected by factors such as the botanic origin, crystalline type, amylopectin content, morphology, and the conditions during acid hydrolysis (Angellier et al. 2004). Amylopectin-rich starch (waxy starch) has been claimed to be a more appropriate candidate for acid hydrolysis (Angellier et al. 2004), owing to its higher content of crystalline regions that can be converted to starch particles of different sizes.

The present study explored various types of starch particles for formulating starch particle-stabilized emulsions. Another focus has been on studying the effect of shape on emulsion stability. The samples for native starch were selected on the basis of differences in natural granule sizes: extremely small (quinoa), small (oats), and bimodal size (waxy barley). As a comparison, reduced-size starches were obtained from acidic treatment, and quinoa and waxy barley were compared with the more commonly studied waxy maize (medium size) and potato starches (large and nonwaxy).

MATERIALS AND METHODS

Materials. Starches used in this study were from potato, waxy maize, waxy barley, oats (all provided by Lyckeby-Culinar AB, Sweden), and quinoa (Northern Quinoa, Canada). Starch isolated from quinoa grains (Biofood, Sweden) was also used in this study. The oil used was medium-chain triglyceride oil (Miglyol 812, Sasol, Germany). All other chemicals were of analytical grade.

Sedimentation of Waxy Barley. Sedimentation was used to separate waxy barley starch granules into two fractions, small and large, according to Stokes' law and the pipette method (Dhital et al. 2010). Duration of sedimentation (t) was calculated according to the following equation:

$$t = \frac{18\mu h}{g(\rho_s - \rho_w)x^2} \quad (1)$$

where μ is the viscosity of the liquid media, that is, water (kg/m·s), g is the acceleration owing to gravity (9.8 m/s), h is the sedimentation height (m), ρ_s is the density of starch (1,500 kg/m^3), ρ_w is the density of water (998 kg/m^3), and x is the diameter of the particle (m). Waxy barley has a bimodal size ranging from 2 to 32 µm (Lindeboom et al. 2004), and the time used was calculated to separate small granules, <10 µm, from large granules, >10 µm. Starch (20 g) was dispersed in 1 L of distilled water and shaken for 30 s. The content was allowed to settle for 61.2 min. Particles larger than 10 µm sedimented, and the suspension above 20% of the sedimentation height was pipetted out and dried.

Acid Hydrolysis. The acid hydrolysis procedure was adapted from Angellier et al. (2004). Native starches (potato, waxy maize, waxy barley, and quinoa), each 36.725 g, were mixed with 250 mL of 3.16M H_2SO_4 solution. The suspensions were placed in a water bath (5B UC, Julabo, Germany) at 40°C and stirred at 100 rpm. After the targeted duration of hydrolysis (one, three, or five days for potato starch and five days for all other starches), the suspensions were collected and cleaned by using successive centrifugations with distilled water until neutral pH was achieved. The starch granules were then frozen at –20°C for 24 h before being freeze-dried at –50°C for 70 h (Hetosicc, Denmark). The yield of hydrolyzed starch (w/w %) was calculated as the ratio of the freeze-dried hydrolyzed particle and the initial dry weight of native starch.

OSA Modification. All starch granules were OSA modified to increase their hydrophobicity as described by Rayner et al. (2012a), and the OSA level was determined in duplicate for modified and control samples of 2.5 g. The amount of OSA used was based on the dry weight of starch as determined by an infrared balance (135°C, 5 min, duplicate). The starch was dispersed in 1.5–2 times the amount of distilled water based on starch dry weight, which was added while stirring. The pH was controlled at 7.4 and 7.8 and adjusted with citric acid, 1M HCL, or NaOH. Then 3–4% *n*-OSA (Ziyun Chemicals, China) was added in four portions with a 15 min delay between each portion. The automatic titration of 1M NaOH while stirring was set up to keep the pH close to 7.6. The titration was terminated when the pH was stable for at least 15 min. The suspension was then centrifuged at 3,000 × g for 10 min. The supernatant was discarded and the starch washed twice with water, once with citric acid (pH 4.5–5), and once more with water. The starch was frozen overnight and freeze-dried as described in the section on acid hydrolysis.

Emulsification. Oil–water emulsions containing medium-chain triglyceride oil as the dispersed phase and phosphate buffer (5 mM, pH 7, 0.2M NaCl) as the continuous phase were prepared by vortex mixing with starch (VM20, Chiltern Scientific Instrumentation, U.K.) for 10 s and then emulsified with a high-shear mixer (D-79282, Ystral, Germany) at 22,000 rpm for 30 s. Descriptions of the starches used for the emulsions are given in Table I.

Characterization of Starch Particles and Emulsions. The emulsifying capacities of the starches were determined from photographs (Fig. 1) that were taken one day after emulsification. The emulsion index (EI) (Rayner et al. 2012b) was calculated as follows:

$$EI = \frac{V_{\text{emuls}}}{V_{\text{total}}} \quad (2)$$

where V_{emuls} is the volume occupied by the emulsion, and V_{total} is the total volume of all the phases (oil phase, added starch, and continuous phase), as shown in Figure 1.

The size distribution of starch particles and emulsion drops was examined with a light-scattering particle size analyzer (Malvern Mastersizer 2000, Malvern, U.K.). A small amount of sample was injected into the flow system connected to the pump and optical chamber for measurement. The refractive index used was 1.54, which corresponds to starch (Bromley and Hopkinson 2002), whereas for the continuous phase it was set to 1.33, representing water. Obscuration was restricted between 10 and 20%. The data d_{43} and d_{32} refer to the sphere

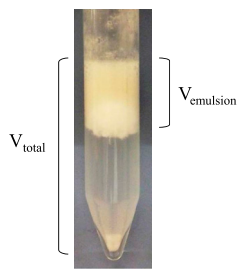

Fig. 1. Sample of emulsion photograph used for estimating emulsion index.

TABLE I
List of Starch Used for Analyses

Starch	Natural Sizes[a]	Acid Hydrolyzed[b]	Shape	References
Quinoa	X	X	Smooth edges and irregular polygons	Pérez and Bertoft (2010)
Oats	X	…	Irregular shape and polygonal granules	Jane et al. (1994)
Waxy barley (small, bimodal, large)	X[c]	X[d]	Oval to round shape, smooth edges, and oblate spheroids	Vasanthan and Bhatty (1996); Timgren et al. (2013)
Waxy maize	X	X	Irregular, deep depression, and graininess with pin holes; smooth edges	Hall and Sayre (1971)
Potato	X	X	Smooth, ellipsoidal, and spherical shaped granules	Hall and Sayre (1971); Jane et al. (1994); Pérez and Bertoft (2010)

[a] The intact starches were octenyl succinic anhydride (OSA) modified and used for emulsification with 5% oil and 200, 400, 800, and 1,200 mg of starch/mL of oil.
[b] The acid-hydrolyzed starches were treated with HCl, OSA modified, and used for emulsification with 7% oil and 214 mg of starch/mL of oil.
[c] Waxy barley starch was also separated into small (B-type) and large (A-type) fractions by sedimentation. The fractions were OSA modified and used for emulsification grouped as small, normal (bimodal), and large size fractions.
[d] Only normal waxy barley (bimodal) was used.

of equivalent volume and surface area of real-size droplets, respectively. All emulsions were analyzed 24 h after the emulsification process.

Light microscopy was used to analyze emulsion droplets and starch particles. Emulsions were observed under an optical microscope (BX50, Olympus, Japan) equipped with a live video camera. The samples were examined under Plan 2×, UMPlanFL 5× and 10×, and LMPplanFl 20× and 50× objectives (Olympus). Emulsions were diluted five times with buffer, and one drop was placed uncovered on the glass plate for microscopic observation.

A scanning electron microscope (SEM) was used to capture the morphology and size of the starch granules. The starch granule 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 (LEI) mode was used to give clear three-dimensional images of the sample surface. The LEI detector combined both signals from secondary and back-scattered electrons during operation.

RESULTS AND DISCUSSION

Starch Particle Characteristics. The starches selected for this study were chosen because of their unique sizes and shapes, as listed in Table I. To obtain even larger variations in the starch material, waxy barley starch also underwent sedimentation to separate A-type (large) from B-type (small) granules, because the native starch was bimodal. The yield obtained after separation was low (0.39%) for the small fraction (<10 μm) of waxy barley. The remainder was considered as the large fraction (>10 μm). The particle size of intact starches can be seen in Table II. The sizes of starch particles were found to be similar before and after OSA modification.

Selected starches (i.e., from quinoa, waxy barley, waxy maize, and potato) underwent acid hydrolysis treatment that generated particles of various sizes and shapes. The yield of starch and the particle size of the acid-hydrolyzed starches are presented in Table III. The results show that waxy maize starch had the highest yield (14.8%), which was comparable to the findings of Angellier et al. (2004), followed by waxy barley (10.0%), potato (9.1%), and quinoa (6.8%) starch.

Only potato starch particles were produced after one and three days of hydrolysis treatment, and as can be seen in the Table III, the yield decreased drastically with time. The increase of hydrolysis time caused a higher degree of acid degradation of starch and resulted, for all cases except quinoa starch, in mean particles of smaller sizes than the original starch (Table III). However, the particle size distribution of the hydrolyzed starches was considerably wider than the original starch, and in some cases aggregates with larger particle size than the native starch were formed. The potato starch showed a wide distribution in which the size varied with a range of 5–120 μm with a peak at 50 μm, 0.7–200 μm after one day of hydrolysis with a peak at 16 μm, 0.5–350 μm after three days of hydrolysis with a peak at 6 μm, and 0.4–40 μm after five days of hydrolysis with a peak at 5.6 μm. Five days of hydrolysis treatment reduced the particle size by up to 89% (potato), 64% (waxy maize), and 62% (waxy barley). This result corresponds with a previous study in which starches with high amylopectin content (waxy type) were prone to particle formation upon acid disruption owing to a higher content of crystalline regions that could be converted to starch particles of different sizes (Angellier et al. 2004). During acidic treatment, the amorphous region of starch is hydrolyzed and removed, whereas the crystalline part is converted to starch crystals of different sizes (Song et al. 2008). However, quinoa (as shown in Table III) did not follow the trend with reduced particle size after hydrolysis; instead, there was an increase in the measured particle size that probably could be attributed to aggregation. Figure 2 shows SEM pictures of quinoa starch and potato starch, illustrating the difference in behavior upon acid hydrolysis between the two starches. Potato starch goes from round well-defined granules to smaller disrupted structures, whereas the main particle size of quinoa starch seems to be intact but aggregated, probably owing to leached starch. The effect of hydrolysis on the starch structure is further illustrated in Figure 3, which shows light microscopy images of intact and acid-hydrolyzed starch particles. All intact starches show the characteristic birefringence under polarized light, whereas after acid hydrolysis birefringence was lost for all starches except for quinoa, for which the starch particles have been reduced in size and lost their well-defined rounded structure.

TABLE II
Particle Size of Intact Starches[a]

Starch	OSA Level (%)	d_{32}, Surface-Weighted Mean (μm)	d_{43}, Volume-Weighted Mean (μm)
Quinoa starch	…	1.40 ± 0.00	1.61 ± 0.00
Quinoa OSA starch	3.18	1.38 ± 0.01	1.52 ± 0.00
Oat starch	…	4.69 ± 0.02	8.76 ± 0.09
Oat OSA starch	2.51	5.32 ± 0.42	23.57 ± 6.72
Waxy barley (S) starch	…	6.16 ± 0.01	10.14 ± 0.04
Waxy barley OSA (S) starch	1.91	6.04 ± 5.17	9.63 ± 8.74
Waxy barley starch	…	9.43 ± 0.00	16.79 ± 0.01
Waxy barley OSA starch	2.79	10.21 ± 0.01	17.87 ± 0.02
Waxy barley (L) starch	…	10.42 ± 0.00	18.19 ± 0.00
Waxy barley OSA (L) starch	1.84	9.67 ± 0.02	17.63 ± 0.02

[a] OSA = octenyl succinic anhydride; S = small; and L = large.

TABLE III
Particle Size of Native and Acid-Hydrolyzed Starches[a]

Starch/Hydrolysis Duration (days)	OSA Level (%)	d_{32}, Surface-Weighted Mean (μm)	d_{43}, Volume-Weighted Mean (μm)	Yield (%)
Quinoa (0)	1.94	1.59 ± 0.01	1.74 ± 0.28	…
Quinoa (5)	1.93	1.59 ± 0.45	3.22 ± 0.46	6.8
Waxy barley (0)	2.02	10.7 ± 0.18	17.9 ± 0.40	…
Waxy barley (5)	1.98	5.02 ± 0.08	16.9 ± 0.81	10.0
Waxy maize (0)	1.98	7.83 ± 0.31	13.6 ± 5.37	…
Waxy maize (5)	1.81	3.78 ± 0.00	6.77 ± 0.00	14.8
Potato (0)	2.10	24.9 ± 1.29	53.5 ± 2.43	…
Potato (1)	1.97	8.33 ± 0.42	28.1 ± 3.02	20.1
Potato (3)	1.90	5.02 ± 0.92	16.9 ± 0.31	14.1
Potato (5)	1.86	3.87 ± 0.38	6.77 ± 0.42	9.1

[a] OSA = octenyl succinic anhydride.

In the case of quinoa, the light microscopy confirmed the presence of larger aggregates, as measured by light scattering (Table III).

Previous studies have shown that acid hydrolysis is an efficient method for reducing particle size, but our starch particles were generated primarily in micron size, ranging from 2 to 7 µm, compared with the smaller particle sizes seen in previous studies. Putaux et al. (2003) produced starch particles 5–7 nm thick, 20–40 nm long, and 15–30 nm wide by acid hydrolysis of waxy maize starch with HCl. Meanwhile, Song et al. (2008) used H_2SO_4 for hydrolyzing maize starch and prepared starch particles with a size of 50 nm. It is generally believed that the effect of acid hydrolysis may differ and is affected by factors such as the botanic

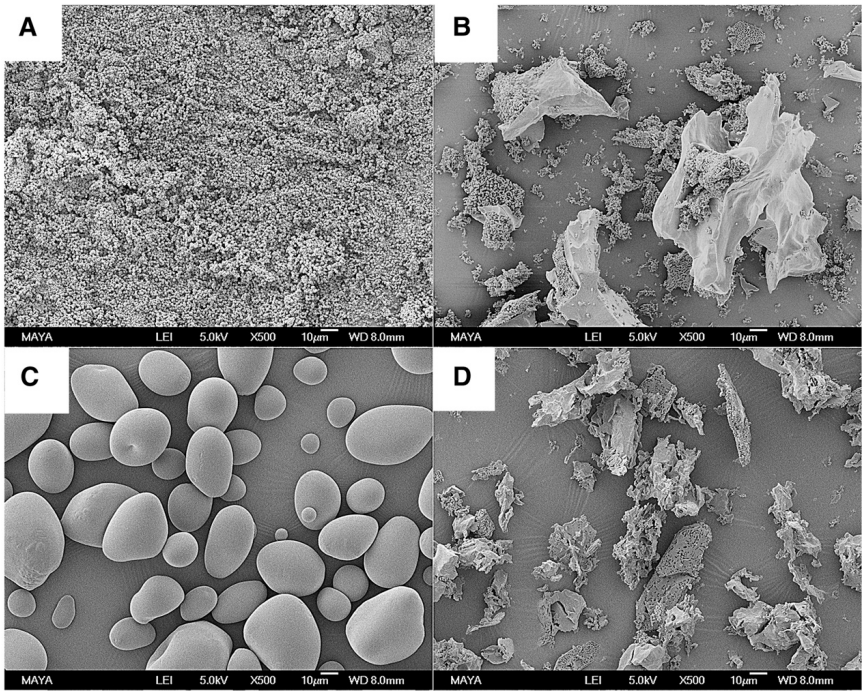

Fig. 2. Scanning electron micrograph of starches before and after acid hydrolysis treatment: **A,** intact quinoa starch granules; **B,** hydrolyzed quinoa starch after five days of acid treatment; **C,** the largest starch, nonhydrolyzed potato starch, for comparison; and **D,** hydrolyzed potato starch (five days). Scale bar: 10 µm.

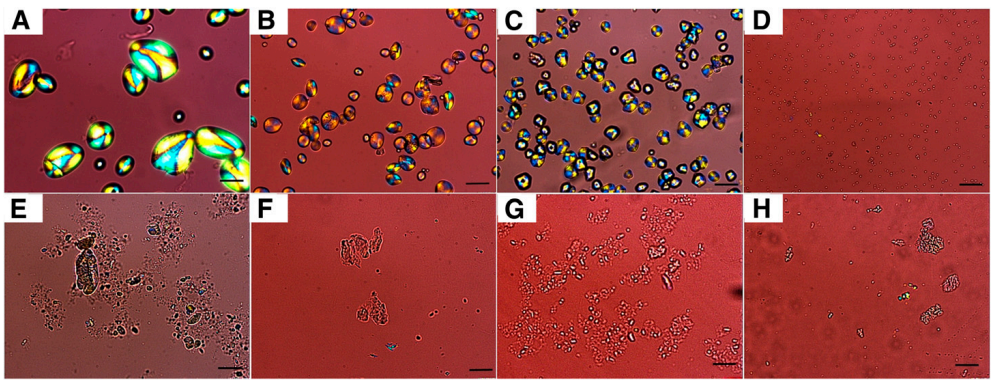

Fig. 3. Polarized light micrographs of starch particles before treatment (**A–D**) and after five days of acid hydrolysis treatment (**E–H**). Intact starches: **A,** potato; **B,** waxy barley; **C,** waxy maize; and **D,** quinoa starch. Hydrolyzed starches: **E,** potato; **F,** waxy barley; **G,** waxy maize; and **H,** quinoa starch. Scale bar: 20 µm.

origin of starch, crystalline type, amylose and amylopectin contents of the starch granule, granule morphology, and conditions of acid hydrolysis, mainly the acid type, acid concentration, temperature, and hydrolysis duration (Angellier et al. 2004). It is likely that if formed, nanocrystals were either not collected or not detected during the analysis. Furthermore, the conditions used in our study also led to aggregation of starch, not only quinoa starch, and that could partly explain the larger sizes and variations observed here.

Once all starch particles were obtained and collected, they were modified with OSA to a level between 1.81 and 3.18% before being used to stabilize Pickering emulsions (Tables II and III). Previous work showed that the OSA substitution degree within the range obtained here had a low to moderate effect on emulsion droplet size (Timgren et al. 2011; Rayner et al. 2012a).

Effect of Starch Particle Size and Shape on Pickering Emulsions. Emulsions were prepared by using both intact and hydrolyzed OSA-modified starch. All the studied starches were able to form Pickering emulsions, but the size of emulsion droplets and EI varied (Tables IV and V).

It has previously been shown that an increasing starch-to-oil ratio leads to decreasing oil droplet sizes until a limit is reached, which probably reflects the smallest droplets that can be obtained with the emulsion technique used (Rayner et al. 2012b). To understand if the starch properties affected this pattern, emulsions with nonhydrolyzed starches were prepared with different starch concentrations (200, 400, 800, and 1,200 mg/mL of oil) (Figs. 4 and 5 and Table IV).

Figure 4 shows photographs of the obtained emulsions. As can be seen, most of the samples contain an upper layer composed of creamed emulsion droplets and a bottom layer composed of sedimented free starch. However, owing to adsorption of starch at the oil–water interface, it cannot be excluded that some of the emulsion droplets might also sediment. In addition to the visual observation, the emulsifying capacity was determined by calculating EI. A thick, creamy emulsion layer was produced by quinoa, small waxy barley, and oat starches, which were observed to be stable after 24 h. In contrast, emulsions stabilized by bimodal and large waxy barley starch produced thick, creamy layers directly after emulsification; however, the layer became thin and the amount of free starch high after 24 h storage. For all these starches, EI increased, as expected, with the increased starch concentration, but the increase was minor for bimodal and large waxy barley samples (Table IV). The emulsifying capacity for intact OSA-modified starches showed the best performance (small droplets and high EI) for small waxy barley starch. This was followed by quinoa, oats, and other fractions of waxy barley at the highest starch concentration despite the order of particle size: quinoa < small waxy barley < oats < bimodal waxy barley < large waxy barley. The unique round shape and smoother surface of small waxy barley (small fraction) may explain the higher stabilizing capacity compared with granules that are polyhedral and sharp-edged (quinoa and oats), which were believed to have a better fit and maximize the packing at the interface of the oil and water

TABLE IV
Droplet Size of Emulsions Using Intact OSA-Modified Starch[a]

Samples	d_{32}, Surface-Weighted Mean (μm)	d_{43}, Volume-Weighted Mean (μm)	EI	Visual Observation
Quinoa, 200 mg/mL	13.39 ± 0.30	44.05 ± 1.88	0.40	O/W creaming
Quinoa, 400 mg/mL	9.10 ± 0.30	26.79 ± 0.93	0.48	
Quinoa, 800 mg/mL	6.52 ± 0.12	14.12 ± 0.34	0.56	
Quinoa, 1,200 mg/mL	4.69 ± 0.10	9.82 ± 0.20	0.64	
Oats, 200 mg/mL	17.77 ± 0.74	77.02 ± 4.95	0.46	O/W creaming
Oats, 400 mg/mL	14.45 ± 0.43	54.23 ± 1.91	0.41	
Oats, 800 mg/mL	11.06 ± 0.16	36.98 ± 0.53	0.52	
Oats, 1,200 mg/mL	8.52 ± 0.18	27.09 ± 0.84	0.62	
Waxy barley (S), 200 mg/mL	16.74 ± 0.75	53.35 ± 2.82	0.34	O/W creaming
Waxy barley (S), 400 mg/mL	11.71 ± 0.40	33.39 ± 1.76	0.49	
Waxy barley (S), 800 mg/mL	10.40 ± 0.22	38.90 ± 1.31	0.60	
Waxy barley (S), 1,200 mg/mL	9.35 ± 0.23	28.91 ± 1.60	0.76	
Waxy barley, 200 mg/mL	11.67 ± 0.09	31.82 ± 0.91	0.25	O/W some creaming
Waxy barley, 400 mg/mL	12.07 ± 0.12	37.65 ± 2.03	0.26	
Waxy barley, 800 mg/mL	11.56 ± 0.11	32.79 ± 1.43	0.38	
Waxy barley, 1,200 mg/mL	12.58 ± 0.08	36.28 ± 0.98	0.40	
Waxy barley (L), 200 mg/mL	12.56 ± 0.24	41.21 ± 1.69	0.30	O/W some creaming
Waxy barley (L), 400 mg/mL	12.55 ± 0.46	43.80 ± 3.34	0.33	
Waxy barley (L), 800 mg/mL	12.02 ± 0.40	41.87 ± 2.72	0.35	
Waxy barley (L), 1,200 mg/mL, $n = 2$	11.77 ± 0.03	40.06 ± 0.58	0.39	

[a] Standard deviation between replicates ($n = 3$); starch concentration, 200, 400, 800, and 1,200 mg of starch/mL of oil; 5% Miglyol oil-in-water emulsions. OSA = octenyl succinic anhydride; EI = emulsion index; and O/W = oil-in-water emulsion.

TABLE V
Droplet Size of Emulsions Using Hydrolyzed OSA-Modified Starches[a]

Starch/Hydrolysis Duration (days)	d_{32}, Surface-Weighted Mean (μm)	d_{43}, Volume-Weighted Mean (μm)	EI	Visual Observation
Quinoa (0)	7.86 ± 0.28	41.0 ± 3.65	0.48	O/W creaming
Quinoa (5)	4.33 ± 0.40	34.9 ± 3.39	0.64	
Waxy barley (0)	22.1 ± 1.07	85.1 ± 5.39	0.45	O/W creaming
Waxy barley (5)	7.24 ± 0.27	39.2 ± 3.60	0.55	
Waxy maize (0)	14.9 ± 2.78	47.0 ± 8.32	0.37	O/W creaming
Waxy maize (5)	7.10 ± 0.77	36.3 ± 7.66	0.46	
Potato (0)	34.3 ± 5.47	107 ± 8.48	0.43	O/W creaming
Potato (1)	13.1 ± 2.13	76.6 ± 4.50	…	…
Potato (3)	9.90 ± 0.85	55.9 ± 1.99	…	…
Potato (5)	8.06 ± 0.21	47.2 ± 0.70	0.53	O/W creaming

[a] Standard deviation between replicates ($n = 3$); starch concentration, 214 mg of starch/mL of oil; 7% Miglyol oil-in-water emulsions. OSA = octenyl succinic anhydride; EI = emulsion index; and O/W = oil-in-water emulsion.

phases. This result corresponds to the previous study on the effect of the smooth, round shape of starch that efficiently stabilizes Pickering emulsions (Timgren et al. 2011).

Figure 5 and Table IV show how the droplet size of Pickering emulsions varied with starch-to-oil ratio for the intact starches. As can be seen, the droplet size of emulsions produced from quinoa, small waxy barley, and oat starches decreased with an increasing starch-to-oil ratio. This was, however, not the case for bimodal and large waxy barley starch, which showed more or less the same average droplet size for all starch-to-oil ratios. This difference may be because the smaller-sized starches (<10 μm) seemed to be favorable for providing smaller droplets, as seen under microscope (Fig. 6) and in Table IV. It can further be seen that for starches with a reduction in droplet size with increasing starch-to-oil ratio, the EI also increases with the starch-to-oil ratio. These results indicate that the behavior of the waxy barley starch is dominated by the large particles. Further studies are needed to verify whether this is because this is the dominating fraction in number or because large particles are favored over small particles during the emulsion process.

Microscopic observations made it clear that quinoa, small waxy barley, and oat starch formed aggregates of emulsion droplets with free starch attached (Fig. 6). However, a substantial amount of free starch was present in the bulk of all samples. The amount of free starch was larger in the bimodal and large waxy barley samples. This result corresponds to previous works in which the particle size was found to highly influence the emulsifying effect (Timgren et al. 2011; Rayner et al. 2012a, 2012b). It is possible that larger-sized starch granules (>10 μm) have restricted ability to adhere to the oil–water interfaces, causing particles to sediment as seen in Figure 4. The emulsions made with smaller starch particles showed a high degree of flocculation at low starch-to-oil ratios, which decreased with increased starch-to-oil ratio. This decrease in flocculation could be indicative of higher close packing of starch at the interface as the starch-to-oil ratio increases. The higher coverage of oil droplets with starch in combination with the decrease in droplet size explained the lower amount of free starches in these samples.

The emulsion characteristic was greatly dependent on the particle sizes but also on the shape of the starch granules. Because oat starch and small waxy barley starch are similar in size, the results (Fig. 4 and Table IV) indicated that the more rounded and smooth shape of waxy barley starch is more favorable for stabilization than the edgy shape of oat starch. However, these samples also seemed to have more free starch in the emulsions (Fig. 4). A previous study has shown that small particles with smooth and round or edgy structure have shown better emulsion stability compared with large particles. The small size of smooth waxy barley and round-edged quinoa and oats explain their superior ability to position themselves at the interface, thereby stabilizing small emulsion droplets (Fig. 4). The small fraction of waxy barley showed some sedimentation but less than bimodal and large fractions (Fig. 4). Overall, the smaller particle size had a larger influence than the shape on emulsion stabilization.

As discussed previously, the hydrolysis of starch affected both the size and morphology of the particles. By comparing emulsions from both hydrolyzed and nonhydrolyzed samples it would be possible to further understand to what extent these parameters, size, and shape affect the emulsions. The effect of acid hydrolysis on the droplet size and EI of these emulsions is seen in Table V. All Pickering emulsions that were based on acid-hydrolyzed starch had smaller droplets and higher EI values than those based on intact starch. This effect on droplets and EI value was also true for quinoa starch, which, as previously discussed, had an increased particle size after hydrolysis that was probably owing to aggregation. The difference between hydrolyzed and nonhydrolyzed starch could indicate that other factors, such as changes in surface properties by removal of, for example, proteins adsorbed to

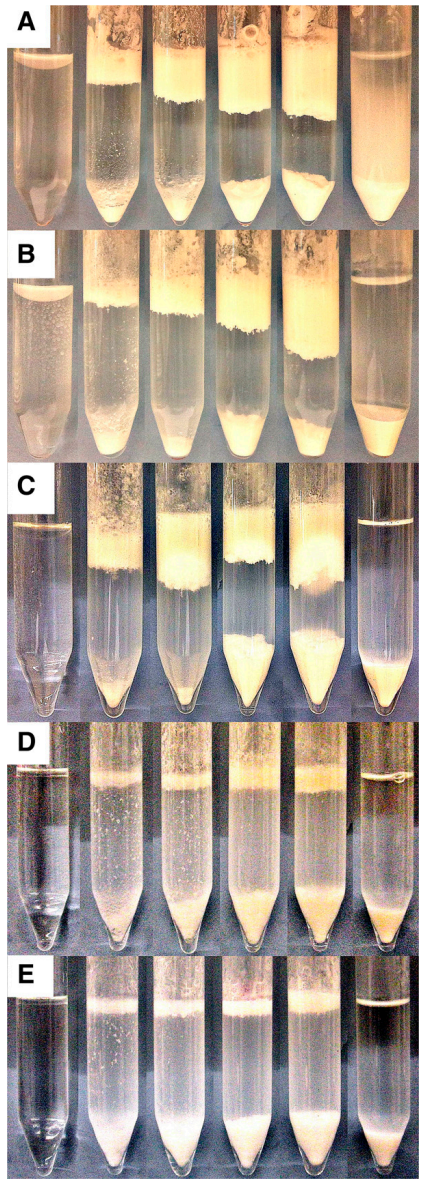

Fig. 4. Emulsion of 5% oil in buffer solution with different types of intact octenyl succinic anhydride modified starch at different starch concentrations. Starch samples: **A,** quinoa; **B,** oats; **C,** small fraction of waxy barley; **D,** bimodal waxy barley; and **E,** large fraction of waxy barley. Samples from left to right: oil with buffer, emulsions with 200, 400, 800, and 1,200 mg of starch/mL of oil, and samples containing starch with buffer only (i.e., no oil) as a comparison.

the starch or changes in particle morphology, influence the formation of Pickering emulsions. However, in general there seems to be a correlation between the particle size of the starch and the droplet size of the Pickering emulsions, especially at particle sizes above 10 µm (see Figure 7). The results seen here are in line with previous findings regarding nonhydrolyzed starches (Timgren et al. 2011). Considering that the shape of the acid-hydrolyzed starches was rough and uneven, it can be concluded that although shape and surface morphology might affect the results, the particle size is the main factor influencing the droplet size.

Effect of Starch Particles on the Surface Coverage of Emulsion Drops. To clarify the effect of starch particle size, the maximum surface coverage possible for a constant starch concentration can be calculated for different particle sizes. The theoretical maximum surface coverage, Γ_M (weight of starch/area) can be estimated with the following equation (Rayner et al. 2012b):

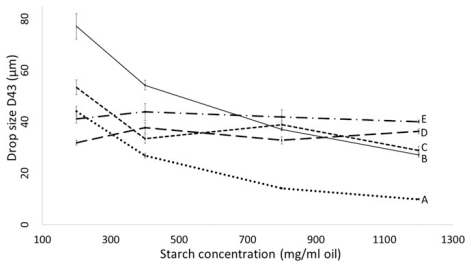

Fig. 5. The effect of starch-to-oil ratio on the droplet size (d_{43}) for emulsions with different intact octenyl succinic anhydride modified starch concentrations: **A,** quinoa; **B,** oats; **C,** small fraction of waxy barley; **D,** bimodal waxy barley; and **E,** large fraction of waxy barley.

$$\Gamma_M = \rho_{sg} \frac{2}{3} d_{sg} \varphi \quad (3)$$

where ρ_{sg} is starch density (approximately 1,500 kg/m³), d_{sg} is the surface mean diameter of starch particles (d_{32}), and φ is the packing density (φ = 0.907, theoretical maximum coverage with tight packing), that is, the droplet surface area that is covered by the starch particles. The contact angle (θ) of 90° is assumed for all particles attached to the oil–water interface. It should be noted that the assumption that $d_{32} = d_{sg}$ is highly simplified, that in many cases φ is not close to 1, and that starch granules are not perfect spheres (Rayner et al. 2012b). The theoretical surface area (S_t) estimated per unit volume of emulsion can be calculated as follows:

$$S_t = \frac{C_{sg}}{\Gamma_M} \quad (4)$$

where C_{sg} is the amount of added starch (weight/volume of oil) and Γ_M is the theoretical maximum coverage based on the specific size of the starch granule. According to equation 4, a larger surface area can be covered per unit mass with smaller particles. This is a main reason to reduce the starch granule size.

The specific surface area (S) per unit volume of emulsion is obtained as follows:

$$S = \frac{6}{d_{32}} \quad (5)$$

where d_{32} is the surface mean diameter of oil droplets.

The maximum surface coverage possible for each starch sample with a given particle size was calculated to examine the effect of the type of added starch and the starch particle size. The estimated surface area (x axis) was measured with equation 4, for which C_{sg} (mg/mL) was assumed as constant (200 mg/mL for intact starch and 214 mg of starch/mL of oil for hydrolyzed starch), and the measured droplet surface area (y axis) was calculated with equation 5. The

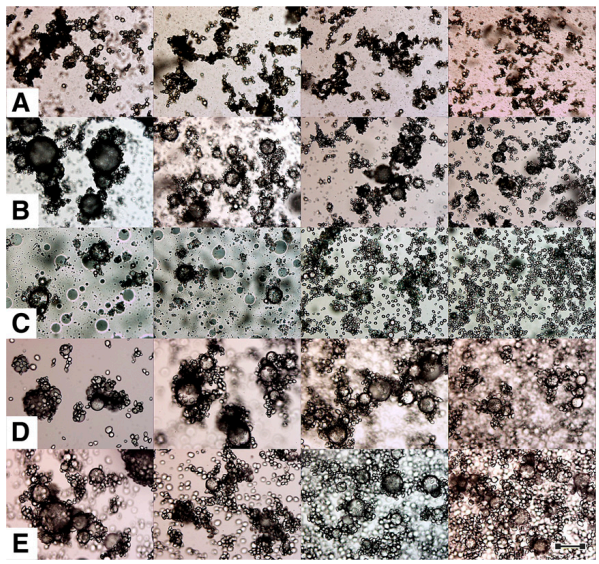

Fig. 6. Light microscopy images of emulsions stabilized by octenyl succinic anhydride modified intact starches: **A,** quinoa; **B,** oats; **C,** small fraction waxy barley; **D,** bimodal waxy barley; and **E,** large fraction waxy barley. Left to right: starch concentration of 200, 400, 800, and 1,200 mg/mL of oil, respectively. Scale bar: 100 µm.

results, seen in Figure 8, show that samples above the line had a larger drop size than predicted, whereas those below the line were smaller than predicted. Using an average d_{32} to estimate the droplet size when there is free starch in the system will lead to an underestimation of droplet size. However, this underestimation will in most cases only strengthen the trends seen. The reason for a surface area below the predicted is most likely that all starch was not adsorbed to the interface, thus leading to larger drops. This difference between theoretical and measured droplet size could either be because all starch did not adsorb to the drop interface or because the starch formed aggregates or adsorbed in multilayers. On the other hand, a larger surface area than predicted could indicate that the droplets were stabilized by a particle layer that is not close packed. Non-close-packed particle layers have also been observed previously for Pickering emulsions (Rayner et al. 2014; Wahlgren et al. 2014).

With the exception of quinoa, both intact OSA-modified starch and hydrolyzed starch provided droplets that were close to the predicted size or, in some cases, slightly larger than predicted. In the case of quinoa (for both the intact and hydrolyzed starch), a smaller area was generated than predicted. Because the quinoa starch had round, flat-edged particles and also the smallest size, these could adsorb on the drop surface with fewer spaces and gaps. Even though the acid treatment generated quinoa starch particles with larger size and more irregular shape, they were found to perform efficiently as a stabilizer, which was believed to be because of breakage into fine particles by strong forces during emulsification. One interesting observation is that when the particle size of the starch is taken into account, acid-hydrolyzed starches seem to have a higher tendency to create smaller droplets (larger surface area) than nonhydrolyzed starch. One reason for these smaller droplets could be that the structure formed by acid hydrolysis is less round and thus will not form a close packed layer on the surface.

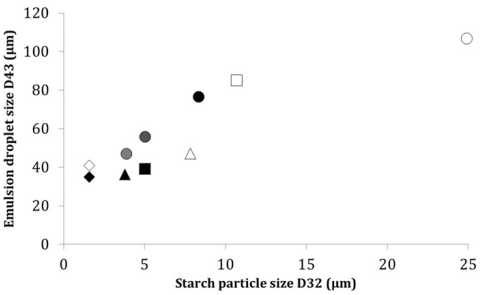

Fig. 7. Pickering emulsion droplet size as a function of starch particle size for potato (●), waxy barley (■), waxy maize (▲), and quinoa (♦). Open symbols are nonhydrolyzed starches.

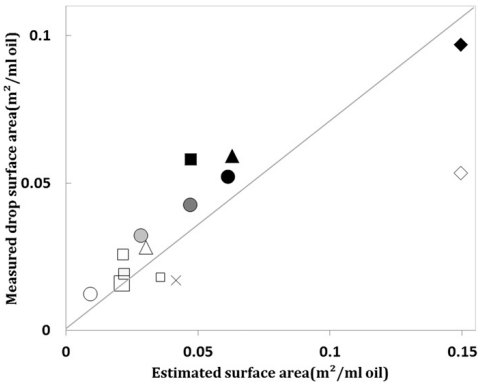

Fig. 8. Measured droplet surface area versus the estimated surface area for both intact starch and hydrolyzed octenyl succinic anhydride modified starch. Samples: potato (●), waxy barley (■), waxy maize (▲), quinoa (♦), and oats (×). Open symbols are nonhydrolyzed starches. For hydrolyzed potato starch, darkness indicates duration of hydrolysis: light gray (day 1), dark gray (day 3), and black (day 5). The difference in size of the waxy barley is indicated by the size of the marker from small to large.

CONCLUSIONS

This study showed that starches with sizes <10 µm produced stable Pickering emulsions with small droplet sizes. Microscopic images and data on drop sizes (d_{43}) confirmed that the emulsion droplet size decreased with increasing starch concentration, from 200 to 1,200 mg/mL of oil, for most starches except for waxy barley. The emulsifying capacity for intact OSA-modified starches based on EI was best for small waxy barley starch followed by quinoa, oats, and the other fractions of waxy barley starch, at the highest starch concentration. It was obvious that the larger barley starch had a lower emulsifying capacity than the small particles. The volumes of nonadsorbed starch were also larger than for the smaller starches.

Reduction of particle size by acid hydrolysis helped to reduce droplet size and increase the emulsifying capacity. Quinoa starch stabilized emulsions had the smallest droplet size, followed by waxy maize, waxy barley, and potato starch, but the difference between the droplet sizes of quinoa, waxy maize, and waxy barley starch emulsion was small. The EI increased as expected owing to the size reduction of starch particles; the overall emulsifying capacity of quinoa starch was definitely the best, followed by waxy barley, potato, and waxy maize starches.

ACKNOWLEDGMENTS

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Paper II

Mixed starch particles for use in Pickering emulsions

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KEYWORDS: Pickering emulsion, starch granules, adsorption from mixed systems, emulsification time

Abstract

The aim of this work was to investigate how mixtures of starch granules from different botanic sources affect starch-stabilized Pickering emulsions, and which type of granules that dominate at the interface of the emulsion droplets. Pickering emulsions were prepared by combining quinoa starch with waxy maize or oat starch. The effects of the individual starches as well as the mixtures on the stability and drop size of the emulsion were investigated as a function of concentration and emulsification time. The size of the starch particles influenced emulsion droplet sizes in emulsions of the individual starches. The droplet size was also strongly influenced by starch concentration, showing a decrease in droplet size with increasing starch concentration. The emulsification time (1–5 minutes) influenced emulsions containing waxy maize or oat starch, but not quinoa. In the quinoa/waxy maize system, quinoa dominated at the surface of the droplets, while in the quinoa/oat system, oat is more likely to dominate on the interface but when the starch content is low both might adsorb. Increasing the emulsification time led to a decrease in drop size in all samples, but more notably for quinoa/oat emulsions where the mean droplet size was decreased by up to 80% at the longest emulsification time (5 minutes). The emulsion index increased as the starch concentration in the system was increased.

Introduction

Pickering [1] and Ramsden [2] were the first to discover that emulsions could be stabilized by solid particles. These became known as Pickering emulsions. Synthetic particles such as silica and latex , are commonly used in Pickering emulsions, while fat crystals, globular proteins and hydrocolloids are common food-based particles [3]. Recently, starches have gained in popularity in Pickering emulsions. Normally, the starch is chemically modified using, for example, octenyl succinic anhydride (OSA) to increase its hydrophobicity [4, 5], and thus its tendency to adsorb to oil/water interfaces. The level of OSA for starch modification is restricted to 3%, for the starch to be an accepted food ingredient (E1450)[5].

The two main reasons for the high stability of Pickering emulsions towards coalescence are the irreversible adsorption of particles at the oil/water interface due to the high desorption energy, and that the solid particles act as a physical barrier between the droplets [6, 7]. Since the desorption energy in most Pickering emulsions per particle is very high, there is significant resistance to droplet shrinkage (Yusoff et al., 2010). It has been shown that a contact angle of the particle at the oil water interface of 90° gives the highest energy of detachment [8] and that large particles have higher energy of detachment than small. Slightly counter intuitive it has been shown that small starch particle seems to promote stability and small droplet size [9]. This could be due to emulsion conditions and packing at the surface. Especially quinoa starch has been reported to successfully stabilize emulsions and promote small emulsion droplets [10-12].

Very few investigations have been carried out on the stabilization of Pickering emulsions using combinations of different particles. One study has been carried out using a mixture of synthetic particles (silica and calcite). In this study, it was shown that while silica particles did adsorb from the mixture to the droplet interface, their high tendency to desorb due to high hydrophilicity (low contact angle) led to low emulsion stability. It was also seen that the larger the silica particles was the lower the stability became probably because the dominate over calcite at the interface but did not give long term stability [13]. The order of addition of the components can also have an importance as shown by Matos et al [14]. Who showed that nano-crystalline cellulose had to be added before starch to end up at the interface but in that case the two types of particles gave a synergetic affect promoting small stable emulsion droplets. Competition between surfactants and particles has also been studied to understand the effects of interfacial tension, interaction and contact angle. Conflicting results have been reported, in that the emulsifiers has been reported both to compete with the particles at the interface and worked together with the particles in stabilizing the emulsion [15, 16]. Finally, we have recently studied the competition between dissolved starch and starch granules from the same botanic source and found that a tendency for preferential adsorption of the dissolved molecules compared to the starch granules and that this led to less stable emulsions than the one obtained when only starch granules

where used [17]. To the best of our knowledge, no study has been reported on the effect of mixed starch granules from different botanic sources on Pickering emulsion properties.

Starch granules can have different shapes and sizes specific to their botanical origin [18]. In this study, we used starch granules from different botanic origin giving granules with a natural difference in particle: quinoa starch granules, regarded as extremely small, with sizes of about 0.3-2 μm, small size starch, oat with oat roughly 2-10 μm, and medium-sized waxy maize starch granules with sizes of 5-30 μm [18, 19]. In addition to their different sizes, they differ in shape and morphology. Quinoa has been reported to have an polyhedral shape [20]. Oat starch, on the other hand, has a round shape [18, 21]. Waxy maize has been reported to have deep depressions on the surface of some granules, smooth edges with graininess, and pin holes [9, 18, 21, 22].

Our previous study has been focusing on the effect of size and shape on emulsion stability when using granules from individual starches [23]. In the work presented in this paper we will study how mixed starch granules affects Pickering emulsions. The starches used were quinoa, oat and waxy maize. The effect on droplet size and the architecture of the adsorbed layer by emulsion time, amount of starch and ratio between the two starches in the mixture. The results were compared to the behaviour of emulsions from the individual starches.

Hypothesis

We hypothesize that today's knowledge cannot predict which component dominates at the interface of the oil droplets and that there are several plausible scenarios.

- Large granules dominate at the interface due to their higher energy of detachment or because they have higher energy of collision
- Small granules dominate as they diffuse more rapidly to the interface.
- There is a mixture of granules at the interface that depends on the ratio between the two kinds of granules used (either the ratio based on available surface area or the number of particles available).

Materials and methods

Materials

The starches used in this study were waxy maize and oat starch provided by Lyckeby-Culinar AB, Sweden, and quinoa starch supplied by Northern Quinoa Corp., Canada. The oil used was the medium-chain triglyceride (MCT) oil, Miglyol 812, with a density of 945 kg/m^3 (Sasol Germany), and the continuous phase used was 5 mM phosphate buffer containing 0.2 M NaCl, with a density of 1009.6 kg/m^3. The n-octenyl succinyl anhydride used to modify the starch granules was obtained from Ziyun Chemicals Co. Ltd, China. All other chemicals were of analytical grade.

Starch modification with OSA

All starch granules were OSA modified to increase their hydrophobicity, and the degree of OSA modification was determined using titration. The modification and determination methods used have been described previously [5].

Mix granules emulsification

Oil/water emulsions consisting of MCT oil as the dispersed phase and phosphate buffer (5 mM, pH 7, 0.2 M NaCl) as the continuous phase, were prepared by vortex mixing with starch (VM20, UK) for 10 s, and then emulsified using a high-shear mixer (Ystral D-79282, Germany) at 22 000 rpm, for 1 min, 3 and 5 min. The compositions of the emulsions are given in Table 1, and the total amount of oil used was 5 weight% on a total volume of 7 ml emulsion. The primary model system was based on a starch to oil ratio of 400 mg starch/g oil of each starch type in the system.

Table 1. Composition of the emulsions studied

Sample	Starch to oil ratio (mg/g oil)	Starch mass (g)		
		Quinoa	Oat	Waxy maize
Quinoa, 200 mg emulsion	200	0.07	-	-
Quinoa, 400 mg emulsion	400	0.14	-	-
Quinoa, 800 mg emulsion	800	0.28	-	-
Quinoa, 1200 mg emulsion	1200	0.42	-	-
Oat, 200 mg emulsion	200	-	0.07	-
Oat, 400 mg emulsion	400	-	0.14	-
Oat, 800 mg emulsion	800	-	0.28	-
Oat, 1200 mg emulsion	1200	-	0.42	-
Waxy maize, 200 mg emulsion	200	-	-	0.07
Waxy maize, 400 mg emulsion	400	-	-	0.14
Waxy maize, 800 mg emulsion	800	-	-	0.28
Waxy maize, 1600 mg emulsion	1600	-	-	0.56
Waxy maize, 3200 mg emulsion	3200	-	-	1.12
Quinoa/waxy maize (weight[1])	800	0.14	-	0.14
Quinoa/waxy maize (area[2])	2909	0.14	-	0.88
Quinoa/waxy maize (constant[3])	800	0.04	-	0.24
Quinoa/oat (weight[1])	800	0.14	0.14	-
Quinoa/oat (area[2])	1769	0.14	0.48	-
Quinoa/ oat (constant[3])	800	0.06	0.22	-

[1] Both starches have the same weight.

[2] Both starches have the same surface area with the same amount of Quinoa as in 1.

[3] Both starches have the same surface area with constant total weight as in 1.

Characterization of starch particles and emulsions

The emulsifying capacity of each starch or mixture of starches was determined from photographs taken one day after emulsification by measuring the emulsion index, EI, which is defined as follows [10]:

$$EI = \frac{V_{emuls}}{V_{total}} \qquad \text{(Eqn. 1)}$$

where V_{emuls} is, the volume occupied by the creamed or sedimented emulsion including sedimented free starch, and V_{total} is the total volume of all the phases (oil phase, added starch, and continuous phase).

The size distributions of the starch particles and emulsion drops were examined using static light-scattering (Malvern Mastersizer 2000, Malvern, UK). A small amount of sample was injected into the flow system connected to the pump and the optical measurement chamber. The values of refractive index used were 1.54 for starch [24] and 1.33 for the continuous phase which was assumed to have the same refractive index as water. Obscuration was restricted to between 10 and 20%. All droplet size measurements were performed on the same day as the emulsifications were made.

Light microscopy was used to analyse emulsion droplets. Emulsions were observed under an optical microscope (Olympus BX50, Japan) equipped with a live video camera. The samples were examined under Plan 2x, UMPlanFL 5x and 10x, and LMPlanFL 20x and 50x objectives (Olympus). Emulsions were diluted 5 times with buffer and one drop was placed uncovered on the glass slide for microscope observation.

A scanning electron microscope (SEM) was used to image the morphology and size of the starch granules. The starch granule samples were coated with gold and examined under SEM (FegSEM, JEOL JSM-6700F, Japan) operated at 5 kV with a working distance of 8 mm. Low secondary electron imaging (LEI) mode was used to give clear 3D images of the sample surface. The LEI detector combined the signals from both secondary and back-scattered electrons during operation.

Results and discussion

The starches used in this study, quinoa, oat and waxy maize, were chosen because of their different size classes. The starch granules were modified with OSA before being used to stabilize Pickering emulsions and the degree of OSA modification was 3.2%, 2.5% and 2.0% for quinoa, oat and waxy maize, respectively. We have previously worked with waxy maize with a degree of modification that was 3% and the droplet size after one day of storage was in the same size range as seen in this study. This is in-line with previous work has shown that variation of the degree of OSA modification in the range used here have a low to moderate effect on variation in emulsion droplet size

[5, 11, 12]. This could be because it is the surface modification not the total modification of the starch granule that is a key parameter for the emulsification. It is likely that as the surface is readily available for the OSA- reaction it will not vary to the same degree as the total degree of modification which will be affected by factors such as porosity and size of the granules.

Emulsions stabilised with single type of starch granules

The primary model system of 400 mg starch/ml oil

The three types of starch granules were used to produce emulsions with specific concentrations of 400 mg starch /ml oil which were emulsified for 1 min, Figure 1. The particle size distribution was measured using static light scattering. This distribution will show both the emulsion droplets and any free starch in the system and as can be seen in Figure 2 there can be some free starch observed.

Emulsions stabilized with starch often shows two peaks or a peak with a shoulder. In this case, the peak with the smallest size is normally related to starch. Quinoa starch granules with a granule size of 1-2 μm showed a size distribution with a peak related to the oil droplets around 25 μm. Oat starch granules, with an average size of about 7-8 μm showed a peak at 70 μm, while waxy maize granules with an average particle size of 15 μm showed a peak at 140 μm indicative of large oil droplets (Figure 1) but the smaller peak at 23 μm could also contain some small oil droplets as this deviates from the light scattering data for dispersed granules. Thus, the granule size seems to affect the droplet size, and in line with previous observations, larger granules give larger droplets which partially could be due to a lower amount of granules for the same weight [5, 23]. Quinoa, with the smallest particle size, leads to emulsions with the smallest drops, followed by oat and waxy maize. However, it cannot be ruled out that other factors, such as the shape of the starch granules, differences in surface properties including the difference in OSA-modification, or presence of other components in the starch used such as proteins and lipids, may influence stabilization. The SEM images of the starches used here (Figure 2) where in agreement with previous studies [18, 19, 22] and showed that quinoa and waxy maize granules had prominent edges, while oat granules were more round and smooth. We found in a previous study that small particles with smooth and round structure provide better emulsion stability than large particles [23]. This could explain the difference between oat and other starch granular based emulsions.

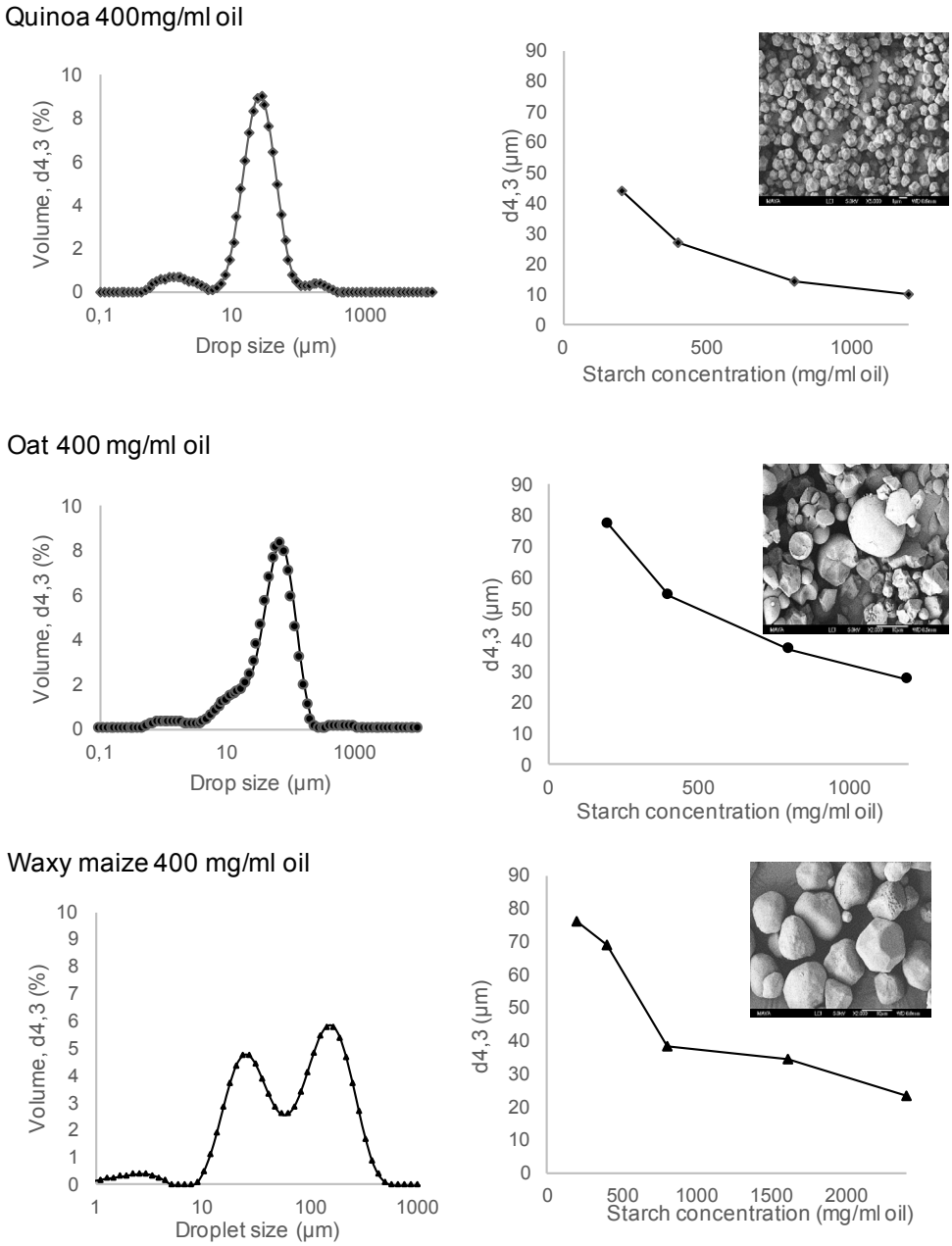

Figure 1. Left column: Size distributions of emulsion at 400 mg starch/ml oil ratio, Right column: Volume weighted mean emulsion droplet size as a function of starch concentration and SEM images of each type of starch granules investigated: (Upper row) quinoa, (middle row) oat and (lower row) waxy maize

For the primary systems, we also studied the effect of emulsification time since the transport of granules to the interface of the droplets could be a key component that affects the droplet size and also could be of importance for the outcome in a mixed system. As the emulsification time was increased, the droplet size did decrease to some extent and the effect of emulsification time varied between the three types of granules. The quinoa-stabilized emulsion showed only a minor change in droplet size, being reduced by only 3-5 μm when increasing the emulsification time from 1 to 2 min., and then remained constant at longer emulsifications times. When the droplet size decreases for waxy maize and oat starch, it is no longer possible to differentiate between the peak for the oil droplets and the free starch granules. Thus, we have chosen to look at the change in average $d_{4,3}$ values. The oat-stabilized emulsion exhibited a slight decrease in average $d_{4,3}$ value, from around 70 μm at one minute to 60 μm after 3 min., after which it remains unchanged. The waxy maize-stabilized emulsion showed a greater reduction, from 70 μm to 30 μm in average $d_{4,3}$ value and changes where noted both after 3 and 5 minutes of emulsification. These results show that extending the period of emulsification had a greater effect on emulsions with larger starch granules than with smaller granules and that this could partly be the reason for differences seen previous on the effect of granular size on droplet size [23]. One possible explanation to this difference in effect by emulsification time is the kinetics of transport of the granules to the droplet interface. The transfer of large particles such as starch granules to the oil-water interface from the bulk continuous phase is governed by convective transport. This is different from surfactants, protein molecules, and nanoparticles < 2nm, where Brownian motion is the main transport mechanism for overcoming adsorption barriers [7]. Thus, it is the convective transport during emulsification, i.e. their size and inertia relative to the turbulent eddies created by the homogenization device used that governs the rate of adsorption and thus the optimal emulsification time. The rate of these collisions is determined by several factors including: the size and number concentration of the particles. This has been described for emulsions by Walstra (1993). In our case, it is obvious that at the same weight of emulsifier the smaller the particle is the more frequent the collisions and thus the system becomes less sensitive to the time of emulsification.

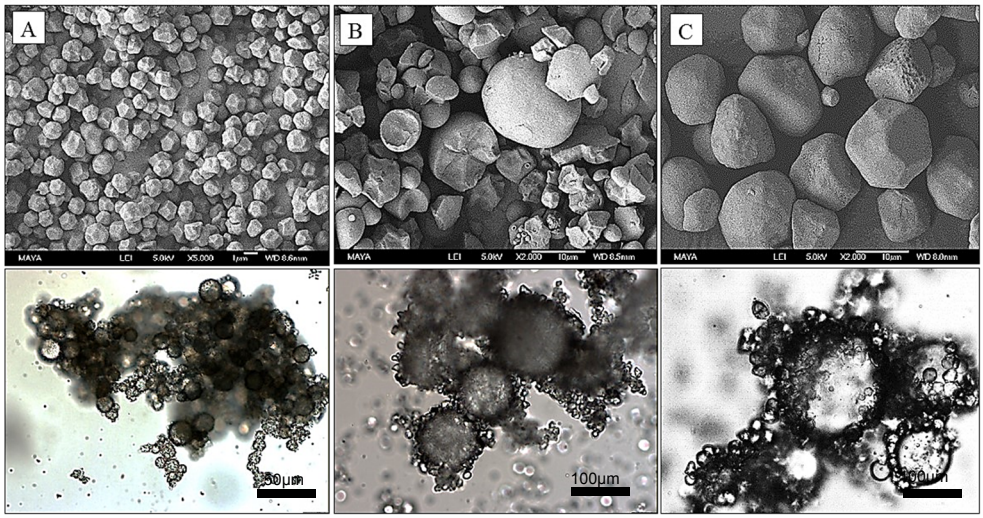

Figure 2. SEM images of (A) quinoa, (B) oat and (C) waxy maize starch granules (top row) and emulsions (bottom row).

Effect of starch concentration

To compare our mixed system with systems based on single starch type, we also need to understand how the concentration of starch granules affect the droplet size. Again, due to overlap between the size of starch granules and small emulsion droplets, the average particle size from the static light scattering does not represent the emulsion droplets alone. As discussed previously all system showed aggregates of emulsion droplets with some free starch attached (Figure 2) and a substantial amount of free starch was present in the continuous phase of all samples. The amount of free starch was higher in waxy maize emulsions. The emulsions made using smaller starch particles showed a high degree of flocculation at low starch/oil ratios, which decreased with increasing starch/oil ratio. This is in agreement with previous studies in which the particle size was found to highly influence the droplet size of the emulsions [10-12]. Thus, the change in average size will be affected both by the increase in free starch and a decrease in droplet size. The average $d_{4,3}$ size was seen to decrease for all three starch granules investigated as the concentration was increased from 200 to 2400 mg starch/ml oil, Figure 1 and Table 2. This is in agreement with our previous study, where the stabilizing effect of individual starches at different concentrations was investigated in Pickering emulsions [23].

Table 2. Description of starch based emulsions series

Starch emulsions	d4,3 (μm)	d3,2 (μm)	Mode (μm)		Span	Total amount of starch/ oil (mg/g)	Starch mass (g)		
			Peak 1	Peak 2			Quinoa	Oat	Waxy maize
Quinoa	28.1 ± 1.5	9.0 ± 0.2	26.3 ± 0.1	-	1.8	400	0.14	0	0
Oat	61.2 ± 7.4	17.2 ± 0.6	69.2 ± 0.1	-	1.9	400	0	0.14	0
Waxy maize	67.8 ± 6.4	16.6 ± 0.6	17.8 ± 1.1	109.8 ± 8.0	2.0	400	0	0	0.14
Quinoa	44.0 ± 1.9	13.4 ± 0.3	29.3 ± 0.3	-	2.6	200	0.07	0	0
Quinoa	26.8 ± 0.9	9.1 ± 0.3	21.9 ± 0.3	-	1.7	400	0.14	0	0
Quinoa	14.1 ± 0.3	6.5 ± 0.1	14.3 ± 0.3	-	1.6	800	0.28	0	0
Quinoa	9.8 ± 0.2	4.7 ± 0.1	10.5 ± 0.2	-	1.7	1200	0.42	0	0
Oat	77.0 ± 5.0	17.8 ± 0.7	100.7 ± 6.3	-	2.0	200	0	0.07	0
Oat	54.2 ± 1.9	14.4 ± 0.4	64.8 ± 1.2	-	2.1	400	0	0.14	0
Oat	37.0 ± 0.5	11.1 ± 0.2	34.6 ± 0.4	-	2.2	800	0	0.28	0
Oat	27.1 ± 0.8	8.5 ± 0.2	26.1 ± 0.6	-	2.5	1200	0	0.42	0
Waxy maize	75.9 ± 10.6	18.2 ± 3.3	20.0 ± 1.8	120.2 ± 0.1	3.2	200	0	0	0.07
Waxy maize	69.2 ± 7.6	16.3 ± 0.4	18.7 ± 1.8	112.5 ± 11	2.8	400	0	0	0.14
Waxy maize	38.2 ± 1.0	11.9 ± 0.2	17.4 ± 0	69.2 ± 0	3.4	800	0	0	0.28
Waxy maize	34.2 ± 6.7	10.4 ± 0.7	17.7 ± 0	85.3 ± 8.3	4.0	1600	0	0	0.56
Waxy maize	23.2 ± 0.4	8.8 ± 0.1	17.4 ± 0	98.0 ± 9.5	2.11	3200	0	0	1.12

Emulsions stabilized with mixtures of starch granules

Emulsions were prepared using OSA-modified starches denoted: weight, area and constant. *Weight* – combinations of different starch granules based on similar starch mass 400 mg/g oil of each granules. *Area* – combinations of different starch granules with similar surface area, and the same amount of quinoa starch as in the systems denoted *weight*. *Constant* –combinations of different starch granules with similar surface area, but constant total starch mass, as in the systems denoted *weight*.

Figure 3 and Table 3 show the droplet size distributions of Pickering emulsions for mixed granule emulsions, where it can be seen that most emulsions showed 2 major peaks (one for emulsion droplets and one with primarily free starch). Figure 4 shows pictures of the macroscopic emulsions and Figure 5 and 6 show microscopic observations on how the two types of granules position themselves differently at the interface.

Table 3. Description of combined starch emulsions

Starch sample	d4,3	d3,2	Mode 1	Mode 2	Span	Total amount of starch/ oil (g/g)	Starch amount (g)			Weight starch/ weight quinoa	Area starch/ area quinoa	Number starch/ number quinoa
							Quinoa	Oat	Waxy maize			
Quinoa/waxy maize (weight) 1 min	27.1 ± 1.4	8.2 ± 0.2	28.8 ± 6.8	-	1.98	800	0.14	0	0.14	1.00	0.16	0.00
Quinoa/waxy maize (weight) 3 min	18.6 ± 1.2	6.7 ± 0.4	17.5 ± 2.4	-	1.92							
Quinoa/waxy maize (weight) 5 min	18.2 ± 0.5	5.5 ± 0.1	15.1 ± 0	-	2.20							
Quinoa/waxy maize (area) 1 min	22.2 ± 3.8	8.0 ± 0.2	19.1 ± 1.5	-	2.09	2909	0.14	0	0.88	6.27	1.00	0.03
Quinoa/waxy maize (area) 3 min	16.5 ± 1.1	6.9 ± 0.0	15.1 ± 0	-	1.75							
Quinoa/waxy maize (area) 5 min	15.1 ± 0.1	4.3 ± 3.7	15.1 ± 0	-	1.74							
Quinoa/waxy maize (constant) 1 min	25.8 ± 4.5	8.6 ± 0.3	17.4 ± 0	94 ± 6.8	2.65	800	0.04	0	0.24	6.37	1.02	0.03
Quinoa/waxy maize (constant) 3 min	18.9 ± 0.9	4.9 ± 4.1	16.3 ± 1.6	-	1.72							
Quinoa/waxy maize (constant) 5 min	13.5 ± 0.1	5.4 ± 1.2	15.1 ± 0.0	-	1.65							
Quinoa/oat (weight) 1 min	36.3 ± 5.1	5.8 ± 0.1	39.6 ± 20	-	3.22	800	0.14	0.14	0	1.00	0.29	0.02
Quinoa/oat (weight) 3 min	17.7 ± 1.2	3.7 ± 0.1	15.1 ± 0	-	2.70							
Quinoa/oat (weight) 5 min	19.5 ± 1.0	3.1 ± 3.1	11.5 ± 0	-	4.32							
Quinoa/oat (area) 1 min	73.1 ± 5.7	5.9 ± 0.2	28.9 ± 16	-	12.9	1769	0.14	0.48	0	3.42	1.00	0.08
Quinoa/oat (area) 3 min	29.4 ± 2.5	5.7 ± 0.2	17.4 ± 0	-	3.58							
Quinoa/oat (area) 5 min	18.3 ± 0.1	4.3 ± 0.1	11.5 ± 0	-	3.75							
Quinoa/oat (constant) 1 min	53.4 ± 1.0	8.6 ± 0.2	49.7 ± 18	-	3.46	800	0.06	0.22	0	0	3.44	1.00
Quinoa/oat (constant) 3 min	37.7 ± 2.5	6.4± 0.1	17.4 ± 0	-	5.23							
Quinoa/oat (constant) 5 min	28.7 ± 1.1	5.4 ±0.2	11.5 ± 0	-	5.47							

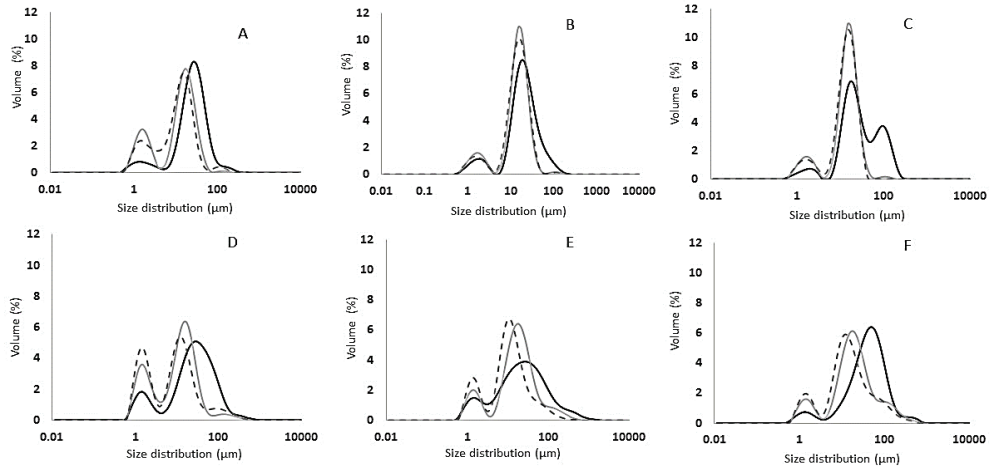

Figure 3. Size distributions from light scattering for emulsions produced with mixtures of starch granules from different botanic source. Upper panels Quinoa and waxy maize, lower panels Quinoa and Oat. Different times for emulsification 1 min. (full black line) 3 min. (full grey line) and 5 min (Dashed grey line). A and D mixture of the two granules based on 50/50 by weight, B and E mixture of the two granules based on 50/50 by area same weight of Quinoa as in A and B (400 mg/ml of Oil), C and F mixture of the two granules based on 50/50 by area same total weight of starch as in A and B (800 mg/ml of oil).

General observations of mixed particle systems

Figure 4 shows photographs of the emulsions obtained with mixed starch granules. All the mixed systems were able to form Pickering emulsions, but the size of the emulsion droplets and the EI varied (Table 3). For all the combinations of starch investigated in this study, it was observed that the emulsion was homogeneous immediately after emulsification and that there was no free oil on top of the emulsion. However, the emulsion did separate into a serum phase and an emulsion phase upon storage for 24 hours, Figure 4. In all cases, it was observed both a top layer and a sediment layer. The microscope pictures show that there are some small oil droplets with coverage of starch granules. As starch is heavier than water these might sediment according to previous observations by [10]. Thus, the sediment could contain both free starch and small heavy starch laden droplets. The EI increased as expected, with increasing amount of starch in the system. Thus, the *Area* based systems has the highest EI, 0.75 and 0.6, for quinoa/oat and quinoa/waxy maize respectively. Mixtures of quinoa/oat and quinoa/waxy maize based on the same *Weight* and *Constant* system had similar values of EI around 0.2-0.25. Indicating that the EI is mainly affected by the total amount of dispersed phase (granules and oil droplets together).

Quinoa/waxy maize emulsions

Weight – This quinoa/waxy maize emulsion showed droplets with a small, rather uniform size of about 27 μm, mostly dominated by quinoa. It can be seen in Figure 5A that quinoa starch covered the emulsion droplets, and the drop size was similar to the one for the reference systems containing only the same amount of quinoa. The waxy maize was observed mostly as free starch, not attached to the droplets. There was very little change in the drop size when emulsification time was increased from 1 min to 3, and then to 5 min, showing values around 18-19 μm and a slightly sharper particle size distribution (Figure 3 A and Table 3). The decrease is slightly more pronounced than for the pure quinoa system but much smaller than was seen for the pure waxy maize system, see section 4.1.1. The prolonged emulsification also showed an increase in the number of particles indicative of free quinoa starch. This could be due to a replacement of quinoa by waxy maize at the interface but the microscopy pictures do not substantiate such an interpretation. The other reason could be a brake-up of aggregated quinoa granules.

Area - In this emulsion system, the surface area of both starches was similar at a total weight of quinoa starch which is the same as for the mass based system (*weight*). Similar microscope observations were made as for the mass-based systems, where quinoa dominated at the interface on small uniform drops (Figure 5B). As the emulsification time increased, the size of the drops decreased from 22 to 15 μm (Table 3, Figure 3 B). The light scattering data indicates that this is primarily due to reduction of the larger droplets in the size distribution as can be seen by a less tailing in the sized distribution. The effect could both be due to that the prolongation of emulsification time decreases the droplet size but could also lead to a combination of more aggregated oil droplets and more free starch which would increase the volume % around the size of the starch. The microscope pictures indicate that both might occur but that there is an indication of smaller droplets.

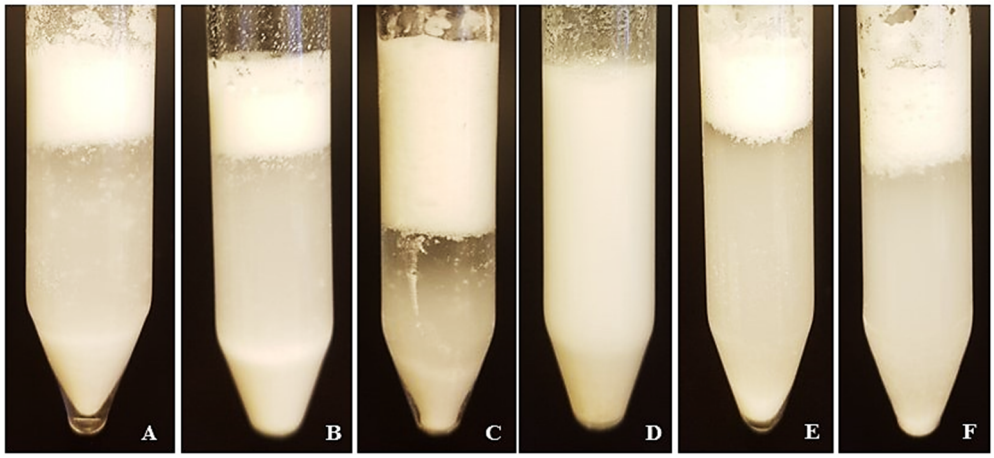

Figure 4. Photographs of the emulsions formed with the various combinations of starch granules. A (Quinoa and waxy maize) B (Quinoa and oat) the mixture of granules based on 50/50 by weight (400 mg/ml Oil of each starch), C (Quinoa and waxy maize) and D (Quinoa and oat) mixture of the two granules based on 50/50 by area same weight of Quinoa as in A and B), E (Quinoa and waxy maize) and F (Quinoa and oat) mixture of the two granules based on 50/50 by area same total weight of starch as in A and B (800 mg/ml total amount of starch per mg of oi

Constant - In this emulsion system, the surface area of both starches was the same at a total weight of starch that was the same as for the mass-based system. Thus, the weight of quinoa decreases substantially. However, the decrease in the amount of quinoa did not change that the fact that in waxy maize/quinoa based systems quinoa is dominating at the interface. The size of the drops was similar to that in the other quinoa/waxy maize systems around an average of 25 μm but the light scattering also showed a large peak probably related to larger emulsion droplets of a size around 94 μm. The large droplet peak is absent after longer emulsification times and the droplet size decreased after longer emulsification times to the same as for the other systems which was around 15 μm (Figure 3 C). Looking at the microscopy pictures in Figure 5C, there seems to be a shift from more waxy maize at the droplet interface at short times going towards larger dominance of quinoa after longer emulsification time. This is however not in line with the light scattering results where we see an increase of the peak around 1 μm indicative of free quinoa granules. As discussed previously this could be due to that the system is heavily aggregated as can be seen in the microscopy pictures and that prolonged emulsification changes the aggregate structure giving a higher amount of free individual starch granules.

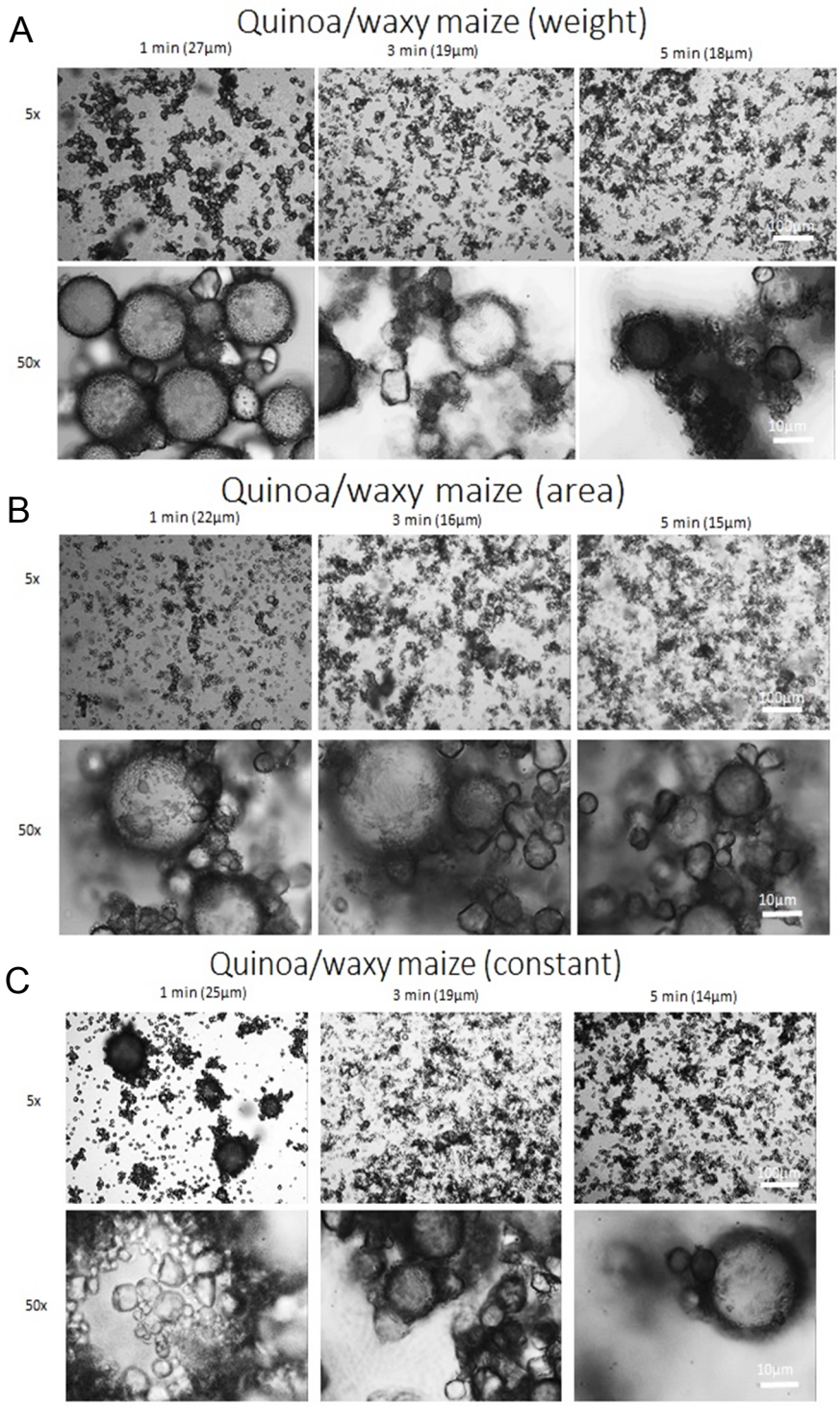

Figure 5. Microscope images of mixed quinoa/waxy maize starch emulsions, effect of ratio between the two starches, emulsification time and average droplets size.

It is obvious that although quinoa is substantially smaller than waxy maize starch. it still dominates at the interface. This could either be because the quinoa granules are more surface active than the waxy maize granules or that quinoa granules as discussed for the individual granules has a higher collision frequency with the oil droplets and that when they are adsorbed to the interface waxy maize cannot displace them. For the systems containing 400 mg of quinoa granules, the effect on the emulsion by emulsification time is as for the single system using quinoa rather small giving only minor changes in droplet size.

Quinoa/oat emulsions

Weight **-** The microscope images show that the droplets were covered with both types of starch granules. Furthermore, this system was highly aggregated with starch granules forming bridges between different oil droplets (Figure 6A). All of the system contains aggregated droplets but these aggregated droplets are more frequently seen in the emulsions containing quinoa/oat, especially for the shorter times of emulsification. The starch granules seem in this case to form a network that keeps the droplets together. With similar starch mass, there seems to be a mixture of quinoa and oat granules at the interface after 1 min of emulsification. When the emulsification time was extended from 1 min to 3 and 5 min, the drop size decreased by almost half, from an average of 36 to 18-20 µm, Figure 3D. The drop size in quinoa/oat emulsions is slightly larger than in quinoa/waxy maize emulsions, indicating that when oat starch adsorbs on the surface of the droplets, the size increases although there is enough quinoa to be able to produce smaller droplets. Thus, it is likely that oat is the dominant species at the interface.

Area – In this emulsion systems, the drops had larger sizes around 73 µm after 1 minute of emulsification. This size is actually larger than both droplets produced using only oat granules or only quinoa. At longer emulsification times, the size of the drops decreased by almost 80% to 18µm after 5min. The reason could be that large aggregates were formed in this emulsion at short emulsification time, as seen under the light microscope. Oat starch not only stabilized the droplets, but excess granules formed a network instead of remaining in the form of free starch (Figure 6B). The network might explain the high EI exhibited by this emulsion system, despite the larger droplet size. There where considerable amounts of clusters also at the longer emulsification times but the droplet size did decrease and maybe the clusters was easier broken during light scattering measurements Figure 3E. The microscopy pictures show a clear dominance of oat granules at the interface of the oil droplets.

Constant – In this emulsion system, which contains a lower total amount of starch by volume, the drops were fully covered with oat starch, Figure 6C. Thus, it's interesting to note the decrease of quinoa starch in the system had an effect even though the oat/quinoa ratio was the same. The average particle size was 53 μm, which decreased to around 29 μm after longer emulsification times, Figure 3F. It was seen that quinoa and oat work both adsorbed to the interface and that the size was in the range of the single system for oat.

Overall, the emulsions containing combinations of quinoa and oat starch showed results that are quite different from the waxy maize/quinoa system. From the light scattering data, we can see that, the amount of free quinoa starch granules is considerable larger than for the quinoa/waxy maize systems and it increases quite drastically with time indicating that oat granules are the species that would dominate at the interface and that it to some extent displaces quinoa at the interface as the emulsification time increases (Figure 6). This could be a case of ideal particle size and shape. However, it cannot be ruled out that other factors, such as the fact that oat granules are more hydrophobic than waxy maize due to higher degree of OSA modification as well as the fact that oat contains quite high amounts lipids ~1-2.5%. This could be the reason that oat dominates at the interface when mixed with quinoa in difference to waxy maize.

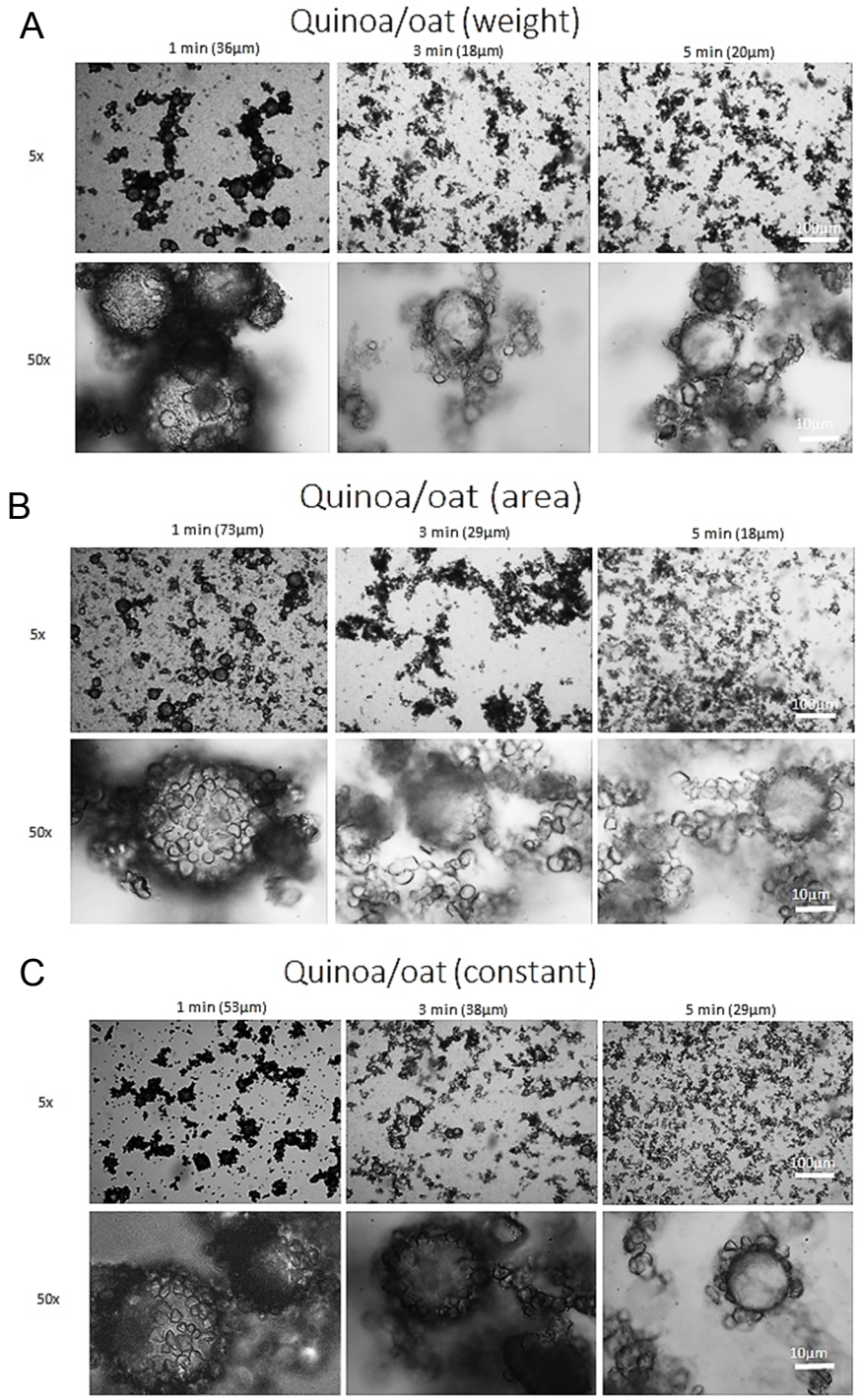

Figure 6. Microscope images of mixed quinoa/oat starch emulsions, effect of ratio between the two starches, emulsification time ad average droplets size.

Comparison between theoretical and measured particle sizes

The effect of the type of added starch and the starch particle size can be considered by calculating the theoretical droplet size from maximum surface coverage of starch at the oil droplet for each starch type. The theoretical maximum surface coverage, ΓM (weight starch/area) can be estimated using equation 2:

$$\Gamma_M = \rho_{sg} \frac{2}{3} d_{sg} \phi \qquad \text{(Eqn. 2)}$$

where ρ_{sg} is the density of starch (approximately 1500 kg m-3), d_{sg} is the surface mean diameter of the starch particles (in this case assumed to be d_{32}) and φ is the packing density (φ=0.907, theoretical maximum coverage with tight packing). A contact angle (θ) of 90° is assumed for all particles attached to the oil-water interface. It should be noted that the assumption that $d_{32} = d_{sg}$ is a simplification, as in many cases the starch granules are not perfectly closed packed and thus φ deviates from 0.907, and furthermore starch granules are not perfect spheres [10]. Despite these simplifications, the size of the emulsion droplet can be estimated for a given size and concentration of starch granules by performing a mass balance over the number of particles available to stabilize the emulsion droplets, and assuming, that there is no free starch in the limited coalescence regime, the theoretical diameter of emulsion droplets can be estimated by:

$$\frac{1}{D} = \frac{m_p}{\varphi\ 4\ d_{sg}\ \rho_{sg}\ V_{disp}} \qquad \text{(Eqn. 3).}$$

where D is the estimated emulsion droplet size, m_p is the mass of the starch granules added, and V_{disp} is the volume of the dispersed phase [25]. The sizes of the starch granules are given in Table 4.

Table 4. Characteristics of the starch particles

Starch	Size (μm)		Mode	Γ_m, surface coverage (mg/m²)	Protein content (%)
	$d_{4,3}$	$d_{3,2}$	μm		
Quinoa	1.56 ± 0.09	1.40 ± 0.03	1.45	1260	0.04
Oat	8.77 ± 0.11	4.69 ± 0.02	8.36	7893	0.28
Waxy maize	16.8 ± 5.67	8.00 ± 0.31	13.99	15120	0.15

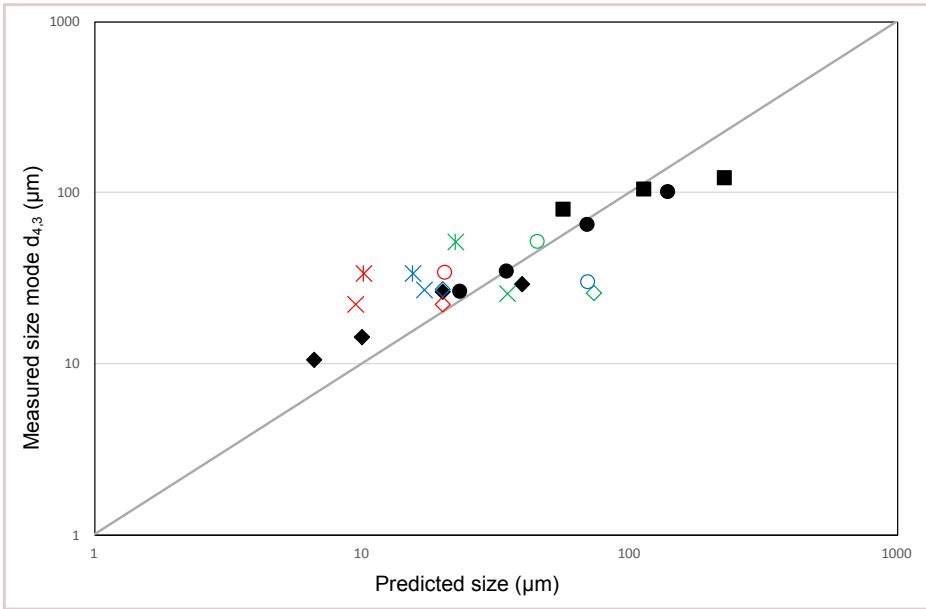

Figure 7. Comparison between measured average size of emulsion droplets and theoretically calculated one based on granular size. The line represents 100% agreement between the two. Filled symbols are individual starches; diamonds (quinoa), circle (oat) and square (waxy maize), mixed system comparison based on all starch in the system; crosses (quinoa /oat) and double crosses (quinoa/ waxy maize). Mixed systems based on the most abundant starch at the oil droplet interfaces non-filled; circles (quinoa /oat) and non-filled; squares (quinoa/ waxy maize); blue markers (weight), red markers (area) and green markers (constant).

In Figure 7, we compare the measured $d_{4,3}$ mode for the emulsion droplets with the theoretical $d_{3,2}$. The reason to use mode is that these better reflects the emulsion droplets and do to a lesser degree include the free granules in the system. The line defines the estimated droplet size while the markers show the measured values. There are several issues that could lead to larger drop size than predicted (points above the line) but primarily this is a reflection of that all starch is not adsorbed to the emulsion interface. Smaller droplets than predicted (below the line) could indicate that the droplets were stabilized by a particle layer that is not closely packed. This has been discussed previously [26, 27]. Not fully covered droplets could be especially important in the cases where the emulsion time has not been long enough to reach steady state but and also in cases where the amount of starch is low. As can be seen in the Figure 7, there is a reasonable good correlation between measured and predicted values. Quinoa especially at high amount of starch has a tendency to be larger than predicted indicating that there is substantial amount of free starch. Oat follows the line with the exception of low amount of starch where it is likely that one reaches a regime of not full surface coverage. Waxy maize seems to go from a regime with free starch to droplets that are below full surface coverage to smaller droplets than predicted. Microscopy shows that there is free starch in the system and it is likely that the large waxy maize granules cannot form a perfect close packing at the emulsion droplets. Thus, it is not unlikely that the final droplet size is a combination of both free starch and surface packing other than close packed and that the combined effect differs with amount of starch in the

system. For the mixed systems, the measured sizes are above the line in all cases except for quinoa/waxy maize (*constant*) indicating a high amount of free starch. To further investigate the system, we also plotted the predicted size of the droplets only using the most dominant species of the two granules in the mixed system as identified from the microscopy pictures. As can be seen in Figure 7, the adherence to the line was better with two exceptions quinoa/oat (*weight*) and quinoa/waxy maize (*constant)*. Indicative of these two out layers are that the amount of the starch for which the predicted value is based on is the lowest and thus one could speculate that at low amounts of the dominating starch, the less dominating one also adsorbs to the interface. There are some synergistic effects of the two starches that are not seen at higher concentration of the dominating starch.

Conclusions

The results of this study show that starch particle size has an influence on Pickering emulsions, where smaller starch granules seem to lead to smaller droplet size, especially in the case of quinoa and oat, compared to waxy maize starch.

In references to the hypothesis for this work we can conclude:

- That at high enough amount of starch granules, the large granules do not automatically dominate at the interface due to their higher energy of detachment or because they have higher energy of collision. This as quinoa and oat dominates over waxy maize at the interface.
- Small granules do not necessarily dominate at the interface as oat dominates over quinoa.

There might be a mixture of granules at the interface but this is not straight forward dependent on the ratio between the two kinds of granules used (either the ratio based on available surface area or the number of particles available)

Thus, what granule that dominates at the interface is more complex than this and could be a combination between an optimal size, shape and surface hydrophobicity.

The emulsifying capacity (expressed as the EI) was found to be increased in emulsion systems with mixed starches. It was found that the quinoa/waxy maize (*weight* and *constant* based) systems had similar EIs to quinoa/oat systems (*weight* and *constant*) due to the similar total weight of starch in the system. This shows that the total starch concentration in the system affects the EI.

Increasing the emulsification time from 1 to 5 min helped to reduce the droplet size in all samples. The droplet size in quinoa/waxy maize emulsions did not change as much since the system was dominated by quinoa, which led to small drops already from the beginning. A gradual effect of the emulsification time can be seen in quinoa/oat

samples, especially at higher concentration, where an 80% decrease in droplet size was seen after emulsification for 5 min. This may be due to the higher starch mass in the system, which needed more time to cover the surface droplets.

Acknowledgements

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Paper III

Pickering emulsions based on $CaCl_2$-gelatinized oat starch

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KEYWORDS: Pickering emulsion, cold gelatinization, oat starch, dry emulsions

Abstract

The aim of this study was to investigate calcium chloride-induced gelatinization of oat starch, and its effects on the properties of an oat starch-based Pickering emulsion. The effect of $CaCl_2$ gelatinization on oat starch granules was studied over 24 h. The results indicate that, in contrast to many other starches, the salt-induced gelatinization of oat granules is not primarily a surface phenomenon, as gelatinization and dissolution of the whole starch granule was observed. This could be because oat starch is more porous than most other cereal starches. Oat starch granules were found to stabilize emulsions both with and without modification of the granules using octenyl succinic anhydride (OSA). The hydrophobization of starch by OSA modification did not significantly change the macroscopic properties of the emulsions compared to the corresponding unmodified system, but the average size of the droplets was slightly smaller. This is in contrast to most starch-based Pickering emulsions, which normally show very poor emulsification properties when using native starch granules due to high hydrophilicity. All the emulsions studied had creamed after one day, but the creaming layer differed depending on the length of salt-induced gelatinization of the starch; the least dense layer being observed following 30 minutes of gelatinization. It was also investigated whether emulsions made from non-gelatinized oat starch could be gelatinized *in situ*. This was found to be possible and led to encapsulation of the oil. The gelatinized emulsions could be dialysed and lyophilized without loss of oil. Leading to dry emulsions that was reformed upon reconstitution with water.

Introduction

In this study, we investigated how salt-induced gelatinization (commonly called cold gelatinization) affected the properties of oat-starch-based Pickering emulsions. Pickering emulsions, as described by Pickering [1] and Ramsden [2], are emulsions that are stabilized by particles instead of traditional surface-active molecules such as surfactants, polymers or proteins [3-6]. In recent years, numerous articles have been published describing the use of starch granules in the formulation of Pickering emulsions [7-12]. One advantage of using starch as a stabilizer for Pickering emulsions is that the oil droplets can be encapsulated by heat-induced gelatinization of the starch, and that this can be followed by freeze-drying to produce dry emulsions of liquid oils [7, 13-15]. Dry emulsions are of interest in numerous applications within the pharmaceutical and food industries, for example, the controlled delivery of oil-soluble substances, the taste masking of oil, and the protection of oils from oxidation. However, one drawback is that heat-induced gelatinization may damage other heat-sensitive substances in the formulation.

We have previously studied the use of oat starch as a stabilizer of Pickering emulsions, and found that emulsions could be produced with droplet sizes varying from 20 to 80 μm using oat starch modified with octenyl succinic anhydride (OSA); the size depending on the amount of starch in the emulsion [10]. We also noted that, in contrast to several other starches, oat starch has a good ability to stabilize emulsions without OSA modification. We therefore used oat starch in the current study. Oat starch is characterized by a high lipid content (~1-2.5%), a low protein content (usually below 0.5%), particle sizes between 8 and 15 μm, a gelatinization temperature in the interval 60-70°C and an amylopectin content between 18 and 34% [16]. It has also been reported that oat starch granules contain pores about 10 nm in size, and that the pore volume and specific surface area of oat starch are higher than in most other cereal starches [17]. During swelling, oat starch granules initially swell along all three axes, but the swelling does result in the formation of flattened discs [18], also in contrast to other cereal starches. The structure of oat starch granules is also more sensitive to heat and acid than other starches, and starts to break down below 100°C, or due to mild acid hydrolysis [16].

In our previous study, we found that the properties of starch-based Pickering emulsions, such as droplet size and stability, are influenced by the size and, to some extent, by the morphology of the starch granules or starch particles used [10]. The present study was carried out in order to investigate how salt-induced gelatinization of oat starch granules affected Pickering emulsions. It has been shown that salts such as lithium chloride and calcium chloride can be used to gelatinize the surface of numerous cereal and non-cereal starches, changing both the size and morphology of the surface of the granules [19-22]. In these studies, it was seen that for several although not all starches studied, that salt induced gelatinization occurred at the surface of the granules. For potato and maize starch, salt-induced gelatinization led to granules with a rough surface and a reduction in the size of the granules [23-25]. This size reduction could be considerable, for example, the size of potato starch granules was reduced by up to 50% [23, 26]. It was also reported that small granules were more sensitive to this treatment than large ones [22]. Some starches, such as rice, waxy barley and maize starch, could not be separated into individual granules after salt gelatinization [22].

The mechanisms involved in salt-induced gelatinization are still not fully understood, but it has been reported that heat-induced gelatinization of starch is affected by salts, and that the effect follows the Hofmeister series. The salting-in ions have been reported to reduce particle size, swelling and transparency, and lead to an increase in the gelatinization temperature, while salting-out ions, had the opposite effects [27, 28]. To the best of our knowledge, salt-induced gelatinization of oat starch has not yet been studied.

We investigated whether salt-induced gelatinization could be used as a means of reducing the size of oat starch granules, as small granules have been found to produce stable emulsions with small droplets. We also wished to ascertain whether cold gelatinization could be used to produce starch-encapsulated oil droplets.

Material and methods

Materials

Oat starch was kindly provided by Lyckeby Starch (Kristianstad, Sweden). Medium-chain triglyceride oil, Miglyol 812, was obtained from Sasol Germany GmbH (Hamburg,Germany). OSA was obtained from Trigon Chemie GmbH (Schluechtern, Germany), and $CaCl_2$ from Sigma Aldrich (St. Louis, Missouri, United States).

Salt-induced gelatinization of starch

Starch granules (20 g) were mixed with 150 ml of a 3.3 M $CaCl_2$ solution. The mixture was stirred at room temperature for 0.5, 1, 2, 3, 6, 12 and 24 h, to achieve different levels of gelatinization. Gelatinization was stopped using 1200 ml chilled deionized water while mixing. The solution was then centrifuged at 3850 *g* for 15 min. The supernatant containing $CaCl_2$ was discarded, and the starch was washed twice with 1200 ml deionized water. To remove dissolved starch, the washed starch was diluted with 120 ml chilled deionized water transferred to a kitchen blender and blended for 4 min. The sample was then centrifuged at 3840 *g* for 20 minutes, and the supernatant was discarded. This washing procedure was repeated three to eight times until the supernatant was clear. The sample was then collected and freeze-dried at -50^{o}C for 4 days in a Hetosic freeze dryer.

OSA modification

Native and $CaCl_2$-treated oat starch granules were modified using OSA to increase their hydrophobicity. The degree of OSA modification was determined using titration, according to the method described by Rayner et al. [11]. The amount of OSA used for modification was 3 % of weight based on dry starch, and the degree of modification was about 2.5%. The OSA-modified starch was freeze-dried at described above.

Preparation of emulsions

Emulsions were made by mixing starch, oil and deionized water in a vortex mixer for 10 s or longer, and then emulsifying the suspension for a preset time using a high-shear mixer (Ystral GmbH, model D-79282 Ballrechten-Dottingen, Germany) at 22 000 rpm. In the initial experiments, the emulsion time was 60 s, and the proportions of starch, oil and water were 70 mg starch, 0.35 g oil and 6.65 g water. In later experiments the emulsion time was increased to 180 s to obtain as small droplets as possible. In these optimized experiments the proportions of starch, oil and water were 204 mg starch, 0.5 g oil and 5 g water.

In situ gelatinization of emulsions

In the initial experiments, an emulsion was produced as described above using native oat starch, and the emulsion layer was transferred to another tube after 1 day. Deionized water was added to give a final volume of 1.5 ml, and this was mixed with 1.5 ml of either 4 M or 6 M $CaCl_2$. Gelatinization was allowed to proceed for 0.5, 2, 3, 6, 12 or 24 h. Gelatinization was stopped by adding twice the sample volume of chilled deionized water.

In the optimized experiments, a 10% emulsion was produced and diluted with 6 M $CaCl_2$. The ratio between emulsion and $CaCl_2$ solution was 2:1 giving a final concentration of 4 M $CaCl_2$ and a 6.7% oil in water emulsion. Gelatinization over a shorter period, 10 to 120 minutes, was investigated. The system was stirred continuously during gelatinization and the temperature was controlled at 20°C using a water bath.

A larger sample of the emulsion (33 ml) was produced for freeze-drying. This was transferred to a mantled stirred glass vessel at 20°C to which 66 ml 6 M $CaCl_2$ was added. Gelatinization was continued for 10 minutes, after which it was stopped with 200 ml chilled deionized water. The sample was then dialysed for 2.5 days against distilled water, and then freeze-dried at -50^{o}C for 4 days.

Characterization of starch and emulsions

Starch granules and emulsions were observed with an optical microscope (Olympus BX50, Tokyo Japan) equipped with a video camera also providing still images. The sample was diluted 5 times and one drop of the diluted sample was placed uncovered on the glass slide for microscopic observation using Plan 2x, UMPlanFL 5x and 10x, and LMPplanFl 20x and 50x objectives (Olympus).

Dried gelatinized starch granules were also investigated using scanning electron microscopy (SEM). The starch granule samples were coated with gold and examined with a field emission gun microscope (JSM-6700F, JEOL, Tokyo Japan) operated at 5 kV, with a working distance of 8 mm. Low secondary electron imaging (LEI) mode was used to give clear 3D images of the sample surface. The LEI detector combined the signals from both secondary and back-scattered electrons.

The size distributions of the starch granules and the drops in the emulsions were measured using static light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK), with a refractive index of the dispersed phase of 1.54 (corresponding to the refractive index of starch [29]), and 1.33 (the refractive index of water) for the continuous phase. A small amount of sample was injected into the flow system with a pump circulating at 2000 rpm to give an obscuration of 10-20%.

The protein content of the oat starch was determined by measuring the nitrogen content using a FlashEA® 1112 N elemental analyzer (Thermo Fisher Scientific, Waltham, MA, USA). A sample of about 25-32 mg was combusted in a sealed furnace, and the nitrogen content was determined with thermal conductivity detection. Aspartic acid was used as standard and the nitrogen factor was set to 6.25 in the calculations. All samples were measured in triplicate.

Emulsions were photographed after 24 h to document the degree of creaming and the visual appearance of the creaming layer.

Results and discussions

Cold gelatinization of oat starch using calcium chloride

Several different conditions were tested for the cold gelatinization of starch. As can be seen from Table 1, none of these conditions reduced the particle size of the granules compared to native oat starch. The cold-gelatinized starch granules as measured by static light scattering were actually somewhat larger than the native oat starch, and the particle size distributions were broad (see Figure 1), indicating that the individual granules are swollen, and that these swollen granules have a high tendency to aggregate in solution. Aggregation is probably due to leakage of dissolved starch into solution.

Table 1. Effect of cold induced gelatinization with 4 M calcium chloride on oat starch granules and the droplet size of Pickering emulsions based on these starch particles

Duration of reaction (h)	Yield (%)	Starch granule size (d4,3) (µm)	Protein content in starch sample (%)[1]	Emulsion droplets Mode d4,3 (µm)
0 h	-	9.24±0.04	0.35±0.01	144±12
0.5 h	68	14.03±0.03	0.34±0.03	126±10
0.5 h (Ultra Turrax)	56	16.18±0.02	-	-
6 h	30	13.38±0.02	0.47±0.02	132±10
12 h	12	13.93±0.00	0.68±0.01	-
24 h	8.	15.58±0.15	1.16±0.01	115±9
OSA 0 h	-	12.96±1.57	0.28±0.01	77±5
OSA 0.5 h	-		-	62±7
OSA 1 h	-		-	96±20
OSA 24 h	-	-	-	113±10

[1]The mean and SD of three measurements.

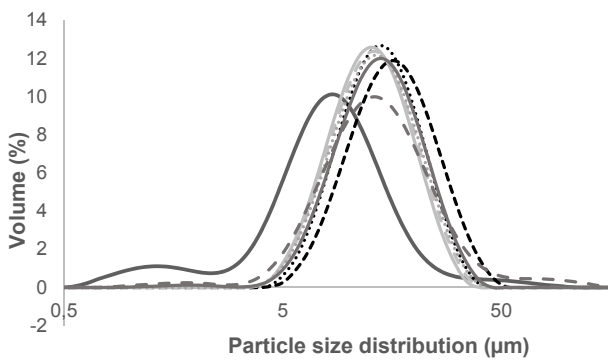

Figure 1. Granule size as measured by static light scattering for different samples of cold gelatinized starch after treatment with 4 M $CaCl_2$ at room temperature during the following times 0 h (black line), 0.5 h (black interrupted line) , 1 h (black dotted line), 2 h (light grey line), 3 h (light grey interrupted line) , 6 h (light grey dotted line), 12 h (dark grey line), 24 h (dark grey interrupted line).

It was also observed that after applying high-shear homogenization using an Ultra Turrax for 60 s, the average particle size decreased somewhat, probably due to a reduction in the aggregation of the starch, although all the aggregates were not broken up. The starch granule size increased

from 9 µm before cold gelatinization to 16 µm after 24 h salt-induced gelatinization*. However, the amount of starch granules decreased drastically, from 68% after 0.5 h cold gelatinization with 4 M $CaCl_2$, to only 8% after 24 h. Observations during the experiments showed that the starch was almost completely gelatinized after 24 h of salt-induced gelatinization, and subsequent treatment, including shearing in the kitchen blender and washing, removed the dissolved starch.

It can be seen from the light microscopy and SEM images in Figures 2 and 3 that the starch granules swelled and ruptured, rather than gelatinizing from the surface inwards. The structures seen in the SEM images indicate that the amount of dissolved dried starch increased with time. This shows that although the washing removes most of the dissolved starch, as evidenced by the decrease in yield, the samples still contain some dissolved starch molecules. None of the starch granules observed with SEM in the present study appeared to show structures of the type reported previously for surface-gelatinized starch [24, 25]. Rather, the present results are more in line with those reported by Koch and Jane [22] for starches such as waxy barley, waxy maize, and normal rice, who could not identify any individual granules after cold gelatinization with 4 M $CaCl_2$. One reason why oat starch granules swell and are not gelatinized at the surface might be their high porosity. A further indication that swelling occurs within the whole granule is similar to heat induced gelatinization where the starch granule swells in such a way that the result is a flattened discs (Figure 3). It is interesting to note that oat starch has many properties in common with rice starch [16], and similarities in the way in which these two starches react to cold gelatinization could, therefore, be expected.

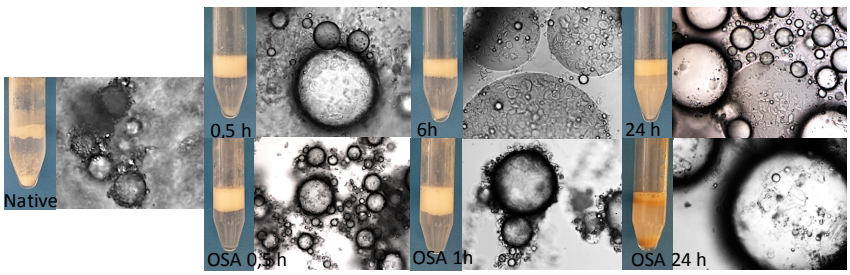

Figure 2. Light microscopy images of emulsions produced from oat starch gelatinized in 4 M $CaCl_2$. Native starch is shown on the left. Upper row: non-OSA-modified gelatinized starch after 0.5, 6 and 24 hours. Lower row: OSA-modified gelatinized starch after 0.5, 1 and 24 hours (200x magnification).

OSA modification was carried out on some of the gelatinized oat starch samples, and this probably led to further destruction of the granules, as the SEM images showed only an aggregated structure with no visible granules (Figure 3). OSA modification of the 24 h cold-gelatinized starch gave a product with a brownish colour, which could indicate some type of Maillard reaction between oat proteins and low-molecular-weight carbohydrates that could have been formed due to the presence of acid during OSA modification. However, some granules can be seen in the light microscope images of dried OSA-modified starch (Figure 2). It is likely that the sample preparation for SEM increased the dissolution of the starch. Based on the size distributions of the starch granules in the emulsion, the size of the gelatinized and OSA-modified starch particles was around 18 µm, which is slightly larger than the non-OSA modified gelatinized starch particles, which were about 13-16 µm.

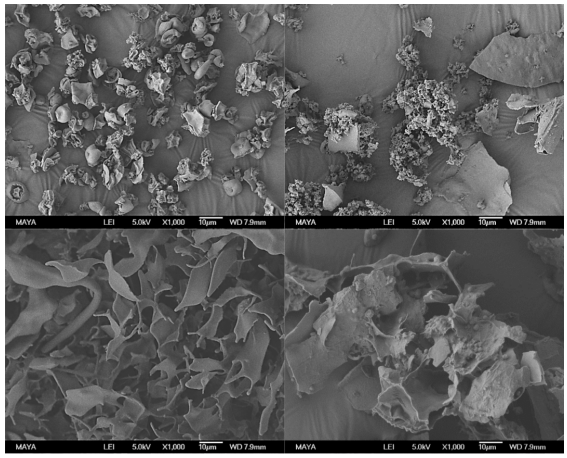

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Figure 3. Electron microscopy images of dried oat starch granules: after cold gelatinization for 0.5 hours (left column) and 24 hours (right column) with 4 mM $CaCl_2$. The upper row shows the results for non-OSA-modified gelatinized oat starch and the lower row OSA-modified gelatinized oat starch.

Cold gelatinization with $CaCl_2$ removes starch, but the treatment does not remove the proteins associated with the starch granules to the same extent, and the content of proteins in the starch sample thus increased (Table 1). This could affect the emulsions as proteins are surface active and, as discussed in the case of the OSA-modified starch, this will increase the risk of Maillard reactions.

Emulsions based on oat starch that have been cold gelatinized by $CaCl_2$

A selection of the cold-gelatinized oat starch samples was freeze-dried and then used to produce starch-based Pickering emulsions. Light microscopy images of these emulsions are also shown in Figure 2, and the sizes of the starch granules and droplets are presented in Table 1. All the starch granules were not adsorbed to the oil-water interface in the emulsion, and a considerable amount of free starch granules can be seen in the light microscope images in Figure 2. This free starch will be included in the particle size distribution determined by light scattering. The peak in the size distribution corresponding to starch may also contain some smaller emulsion droplets. However, the light microscope images indicate that most of the emulsion droplets are larger, and will be included with the larger particles in the light scattering measurements.

As can be seen in Figure 2, the surface of the emulsion droplets in Pickering emulsions made with native oat starch is covered with starch granules, and the light scattering measurements indicate that the droplet size is large, around 148 μm. The emulsion layer is rather compact, and light microscopy indicates that the droplets are aggregated. There is also quite a large amount of sediment, which is most likely free starch. When using cold-gelatinized starch in the emulsions, the microscope images show swollen, but more or less intact, granules at the surface of the oil droplets for the shorter periods of cold gelatinization, whereas only a small amount of very distorted particles is seen on the surface of the oil droplets after longer periods of gelatinization. This is especially apparent in the case of gelatinization for 24 h. The emulsion layer is less compact in the emulsions produced from cold-gelatinized starch than from native starch, and the droplet size decreases somewhat. The amount of sediment also decreases. The largest volume of the emulsion layer was seen for emulsions made with starch that was cold-gelatinized for 0.5 h. The thickness of the adsorbed layer of starch at the oil droplet interface deceases with time, as can be seen in Figure 4. The size of the droplets in the Pickering emulsions is slightly smaller in

those made with gelatinized oat starch, around 90-110 µm, than in those made with native starch. However, the difference in droplet size is not sufficient to explain the difference in the volume of the emulsion layer. This could instead be explained by a combination of smaller droplets and a less dense layer due to higher viscosity within the layer caused by the extraction of amylose and amylopectin from the damaged swollen granules. The increase in protein content could also lead to somewhat higher surface activity of the cold gelatinized starch which could partly explain the somewhat smaller droplet size in emulsions made from starch that has been cold gelatinized for 24 hours.

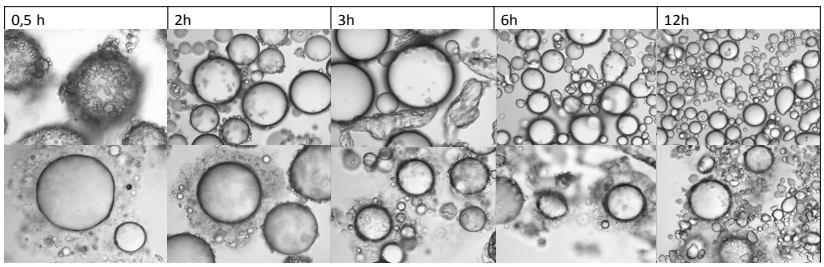

Figure 4. Light microscopy images of native oat starch emulsions after 0.5, 2, 3, 6 and 12 h cold gelatinization with 4 M $CaCl_2$ (upper row) and 6 M $CaCl_2$ (lower row).

OSA modification had no significant effect on the macroscopic properties of the emulsions, compared to the corresponding non-OSA-modified emulsions (Figure 2). However, the droplets are slightly smaller, and the material at the surface of the droplets is more like distorted flakes than swollen granules, as can be seen in the microscopy images in Figure 4, especially after 24 h of gelatinization. This is in line with the electron microscopy images in Figure 3, which showed aggregated material rather than swollen starch granules when using OSA-modified and gelatinized starch.

In situ cold gelatinization of Pickering emulsions produced from native oat starch

It has previously been shown that Pickering emulsions containing starch can be gelatinized by heating, and that the starch adsorbed on the oil droplets forms a cohesive layer that not only reduces the lipolysis of the oil in water emulsion, but is also dense enough to allow the emulsion to be freeze-dried to provide a dry emulsion [7, 14]. As cold gelatinization of oat starch showed similar behaviour to heat-induced gelatinization, we investigated whether this could offer an alternative way of producing dry emulsions.

Figure 4 shows light microscopy images of the emulsions after different gelatinization times with 4 M or 6 M $CaCl_2$ at room temperature. It can be seen in this figure that the lower $CaCl_2$ concentration does not cause gelatinization of the starch adsorbed on the emulsion droplets during the first half hour of the experiment, whereas the starch has started to gelatinize, forming a corona of leakage starch around the emulsion droplets, at the higher $CaCl_2$ concentration. As time progress, the starch at the droplet interface in the 4 M $CaCl_2$ system seems to be replaced by either free protein or adsorbed layers of dissolved starch, and the starch granules seem to swell and appear to aggregate, forming non-spherical structures. At the higher $CaCl_2$ concentration, a corona of gelatinized starch can be seen at all times, but as time increases it seems to dissolve slightly, and the sample appears to contain some retrograded starch or a residue of non-dissolved starch material. When measuring the size distribution of the emulsions using light scattering, the average particle size, which includes both the starch granules and the emulsion droplets, changes over time, showing an initial decrease in both systems, followed by an increase during the first 3 hours

(Figure 5). After 3 hours the size decreases again; more in the 4 M $CaCl_2$ system than in the 6 M $CaCl_2$ system. In the case of the 4 M $CaCl_2$ system, the increase up to 3 hours could be due to an increase in starch granule size, and the decrease seen thereafter could be due to the exchange of starch on the surface of the emulsion with other surface-active, low-molecular-weight substances. In the system with the higher concentration of $CaCl_2$, the swelling between 0.5 and 3 hours could be due to an increase in the size of adsorbed and non-adsorbed starch due to swelling during cold gelatinization, and the decrease afterwards could be due to the dissolution of the swollen starch.

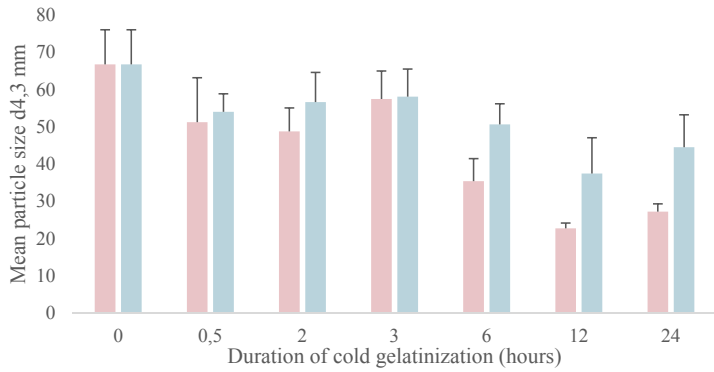

Figure 5. Changes in average droplet size over time during cold gelatinization of the oat starch solution, using $CaCl_2$ concentrations of 4 M (pink) and 6 M (green).

Optimization of gelatinization of oat starch

In order to gain better control over the gelatinization process, an attempt was made to optimize the system. It was found that the emulsification time should be increased to 180 s to achieve the smallest droplet size, of about 60 µm, in the non-OSA-modified system. In this part of the study, cold gelatinization was investigated for both OSA-modified and non-modified oat starch. Gelatinization was studied for 2 h (Figure 6) as the previous experiments had shown that the starch did not aggregate or become retrograded during this time. The emulsion was mixed with a high concentration of $CaCl_2$ (6 M) giving a final concentration of 4 M in the emulsion. This was observed to be sufficient to induce cold gelatinization, and it can be seen in Figure 6 that gelatinization occurred rapidly. The native oat starch granules were only partly gelatinized during the first 10 minutes, but a corona of gelatinized starch was seen around the oil droplet after only 20 minutes. There may be a slight decrease in the thickness of the corona, but it is obvious from Figure 6 that a considerable amount of starch is attached to the surface of the droplets even after 120 minutes. The addition of the $CaCl_2$ triggered an exothermic reaction leading to an increase in temperature to about 40-45°C. To ensure that this did not cause heat-induced gelatinization, the temperature was controlled during the emulsification. No decrease in the degree of gelatinization was seen when cooling the emulsion to 20°C.

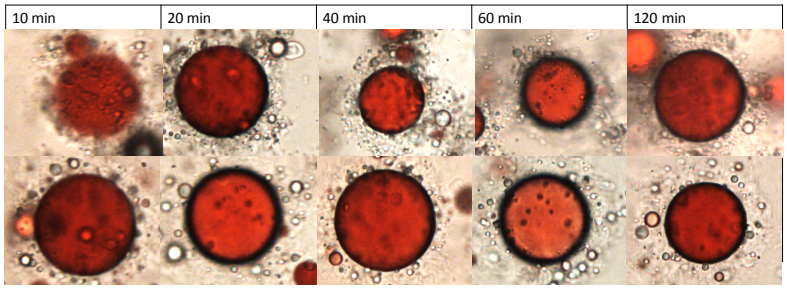

Figure 6. Light microscopy images of emulsions made using native oat starch (upper row) and OSA-modified oat starch (lower row) after 10, 20, 40, 60 and 120 min of cold gelatinization with 4 M $CaCl_2$. The oil is stained with Sudan red.

A slight difference was observed when using OSA-modified and non-modified oat starch granules for the in situ gelatinization. Firstly, OSA modification leads to slightly smaller droplets in the emulsion since these are more hydrophobic, and they will thus have a higher tendency to adsorb on the oil–water interface. Secondly, gelatinization occurred after only 10 minutes when using OSA-modified starch. OSA modification probably leads to partial acid hydrolysation of the oat starch as the process includes some acidic steps. A third difference was seen, in the swelling of the starch layer. OSA-modified starch appeared to exhibit a slightly denser layer of gelatinized starch than the non-modified starch (Figure 6). Finally, the non-modified oat starch adsorbed to the oil droplets swells, which is seen as an increase in particle size, from around 60 to 85 µm, while no such size increase was seen for the OSA-modified sample. It is possible that the higher hydrophobicity of the OSA-modified starch leads to higher cohesion of the swollen layer, leading to less uptake of water and thus a denser structure.

It was possible to stop gelatinization by diluting the sample and then removing the salt by dialysis. The dialysed sample was freeze-dried and this material contained the oil without leakage. The sample was not in powder form, but rather a network of gelatinized starch. This is probably due to the aggregation of free starch. However, the freeze-drying process was not optimized, and the sample became less aggregated by mixing the sample vigorously after gelatinization. Strategies to avoid aggregation during freeze-drying may include the addition of protective agents such as mannitol, optimization of the amount of starch used, etc. The freeze-dried emulsion was dissolved in water and investigated using light microscopy, and it was concluded that an emulsion was formed upon re-dispersion of the dried emulsions in water (Figure 7). As can be seen both emulsions based on in situ gelatinized OSA modified oat starch and non-modified starch can form dried emulsions that can be redispersed.

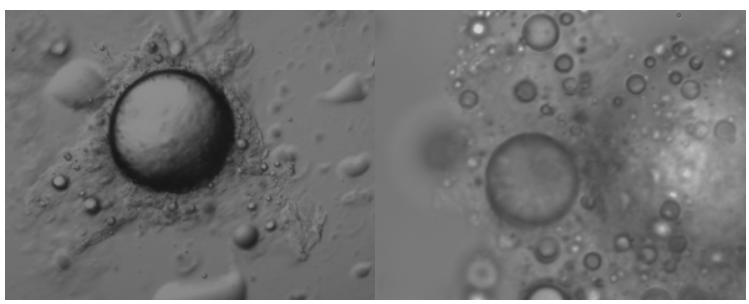

Figure 7. Light microscopy images of re-dispersed freeze-dried emulsions of native oat starch (left panel) and OSA-modified oat starch (right panel). The emulsion was produced using 400 mg starch/g oil and allowed to cold gelatinize for 10 minutes, after which it was dialysed and lyophilized.

Conclusions

Oat starch can be cold-gelatinized using 4 M $CaCl_2$, but, in contrast to other cereal starches such as maize and potato, gelatinization is not localized at the surface. Both OSA-modified and non-OSA-modified oat starch can form Pickering emulsions both prior to and after gelatinization. The droplet sizes obtained for non OSA modified starch granules were quite large, around 150 µm. The droplet size decreased slightly when cold-gelatinized oat starch was used, and the emulsion layer became less dense, especially in the case of starch granules that had been gelatinized for 0.5-1 hour. The volume of the emulsion layer measured after 24 hours storage decreased as the gelatinization time was further increased. OSA modification of oat starch led to a slight decrease in the droplet size in the resulting emulsion, but the macroscopic properties of the emulsions made with OSA-modified and non-OSA-modified oat starch granules were similar. It was shown that Pickering emulsions of native and OSA-modified oat starch could be gelatinized with 4 M $CaCl_2$ in situ, and that the layer of gelatinized starch formed could protect the emulsion during freeze-drying in such a way that a dry emulsion could be formed.

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Paper IV

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Production of starch nanoparticles by dissolution and non-solvent precipitation for use in food-grade Pickering emulsions

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ABSTRACT

The aim of this study was to investigate non-solvent precipitation of starch to produce nanoparticles that could be used in Pickering emulsions. The material used was waxy maize, modified with octenyl succinic anhydride. Different methods of non-solvent precipitation were investigated, and a method based on direct mixing of an 8% starch solution and ethanol (ratio 1:1) was found to produce the smallest particles. The particle size was measured using AFM and AF4, and was found to be in the range 100–200 nm. However, both larger particles and aggregates of nanoparticles were observed. The emulsion produced using the precipitated starch particles had a droplet size that between 0.5 and 45 μm, compared to emulsions produced from waxy maize granules, in which had a size of 10–100 μm. The drop in size contributed to increased stability against creaming. The amount of starch used for emulsion stabilization could also be substantially reduced.

1. Introduction

The aim of this study was to produce starch nanoparticles non-solvent precipitation. Starch nanoparticles could be used in several applications but in this paper we focus on the ability of these particles to stabilise Pickering emulsions. Pickering emulsions are emulsions that are stabilized by solid particles and are named after S.U. Pickering, who together with Walter Ramsden was the first to describe the phenomena (Pickering, 1907; Ramsden, 1903). The field of Pickering emulsions has recently been described in several reviews (Berton-Carabin & Schroën, 2015; Dickinson, 2016; Rayner et al., 2014; Wahlgren, Engblom, Sjöö, & Rayner, 2013).

It has previously been shown that modified starch particles can be successfully used to stabilize Pickering emulsions (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013; Marefati, Sjöö, Timgren, Dejmek, & Rayner, 2015; Saari, Heravifar, Rayner, Wahlgren, & Sjöö, 2016; Simsek, Ovando-Martinez, Marefati, Sj, & Rayner, 2015; Sjöö, Emek, Hall, Rayner, & Wahlgren, 2015; Villamonte, Jury, & de Lamballerie, 2016). Starch granules vary considerably in size and shape depending on their botanical origin (French, 1973; Hall & Sayre, 1970, 1971; Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994). This variation have been found to effect the stability and droplet size of Pickering emulsions (Saari et al., 2016; Timgren, Rayner, Sjöö, & Dejmek, 2011). Starch particles varying from nanometres to micrometres have been used to stabilize emulsions, for example waxy maize starch nanoparticles (Li, Sun, & Yang, 2012; Tan et al., 2012, 2014), and quinoa starch granules (Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013; Timgren, Rayner, Sjoo, & Dejmek, 2011). In a recent study we have shown that small particles obtained from hydrolysis of starch granules created stable emulsions with a smaller drop size, and the emulsifying capacity was also increased compared to intact granules from the same botanical source which had a larger particle size (Saari, Heravifar, Rayner, Wahlgren, & Sjöö, 2015).

We have previously shown that the hydrophobicity of starch particles must be increased in order to obtain stable Pickering emulsions (Timgren, Rayner, Dejmek, Marku, & Sjöö, 2013). This is usually achieved by chemical modification using octenyl succinic anhydride (OSA) (Sweedman, Tizzotti, Schafer, & Gilbert, 2013; Tan et al., 2014; Timgren, Rayner, Sjöö et al., 2011; Yusoff & Murray, 2011). A degree of chemical modification of less than 3% using OSA is acceptable in food ingredients and is also sufficient to obtain effective modified starch (Rayner, Timgren, Sjöö, & Dejmek, 2012; Wurzburg, 1995). Thus in this work we use OSA modification below 3% to obtain food-grade nanoparticles from starch.

The size of the particles used to stabilize emulsion droplets affects the amount of starch needed to cover the dispersed phase.

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The relationship between the amount of starch added and the droplet size can be described by Eq. (1):

$$d_{3,2} = \frac{(1-\phi)C}{\phi\Gamma} \text{where } \Gamma = \rho_{sg}\frac{2}{3}\varphi d_p \quad (1)$$

where $d_{3,2}$* is the surface mean diameter of the droplets, ϕ is the fraction of oil, C is the amount of emulsifier/stabilizing agent (in our case starch) added to the continuous phase (weight/volume of buffer), ρ_s is the density of the stabilizing agent (approximately 1500 kg/m^3 for starch), d_p is the average mean diameter of starch particles ($d_{4,3}$, and φ is the packing density (φ = 0.907, is theoretical maximum coverage with tight packing and has been used here)).

As can be seen from Eq. (1), a larger surface area can be covered per unit mass when using smaller particles. This is one reason why it is important to reduce the starch granule size as this could lead to smaller droplets of the dispersed phase.

Several methods can be used to obtain nanoparticles of starch, as recently reviewed by Kim, Park, and Lim (2015). In the present work we investigated non-solvent precipitation to obtain nano-sized starch. The first step in such a method is to completely dissolve the starch granules (dissolution). New smaller particles are then re-created from the dissolved starch molecules by non-solvent precipitation. Starch granules are insoluble in cold water due to their inter-chain hydrogen bonding. During the dissolution process, starch granules disintegrate completely and the intermolecular and intramolecular hydrogen bonds are disrupted, thereby destroying the crystalline structure of the granule (Gao, Luo, & Luo, 2012; Wang & Xie, 2010). The dissolution of starch makes it accessible for further processing, and the method should be carefully chosen as too harsh conditions may lead to changes in the molecular weight of the starch, while too mild conditions may not be sufficient for complete dissolution (Gidley et al., 2010). Several dissolution methods can be used; such as heating of starch in water either in the presence of nitrogen gas in an autoclave, or heating in a microwave oven, while other methods use other solvents such DMSO (dimethyl sulphoxide), ionic solvent or urea/KOH to dissolve the starch (Kim & Huber, 2006; Ortega-Ojeda, Larsson, & Eliasson, 2003; Perez-Rea, Bergenståhl, & Nilsson, 2015; Syahariza, Li, & Hasjim, 2010; Wilpiszewska & Spychaj, 2011). The DMSO method and the autoclave method have been shown not to alter the molecular size of the starch (Perez-Rea et al., 2015).

After dissolving the starch granules, new starch particles can be produced by non-solvent precipitation (also known as nano-precipitation as described by Hornig, Heinze, Becer, and Schubert (2009). Non-solvent precipitation involves the transition from the dissolved state to the solid state of a polymer after addition of a non-solvent that is miscible with a solvent (Hornig et al., 2009). The concentration of polymer (in this case starch) should be above overlap concentration for particles to form (Hornig et al., 2009). It has been shown that very small starch particles can be obtained with the non-solvent precipitation method (Qin, Liu, Jiang, Xiong, & Sun, 2016; Tan et al., 2009). Factors that control the particle size during non-solvent precipitation include; the technique used, the initial concentration of the polymer (starch), the ratio of the polymer solution to the non-solvent solution, whether the polymer is added to the solvent or vice versa, and the type of solvent used (Chin, Pang, & Tay, 2011; Hornig et al., 2009; Perevyazko, Vollrath, Hornig, Pavlov, & Schubert, 2010; Tan et al., 2009, 2012). Various kinds of non-solvent precipitation have been described for the production of nanoparticles, including dialysis and the dropping technique. The initial polymer concentration has been found to be crucial, and Tan et al. (2009) found that low polymer concentrations, in the range of 1–8 mg/mL, gave starch particles with narrow size distributions, which increased rapidly with increasing polymer concentration, and that aggregation occurred at >20 mg/mL. Low polymer concentrations will probably be required to ensure that the molecules are in a dispersed state, and that they can be separated into nano-domains upon the addition of the non-solvent, as described by Hornig et al. (2009). Ratio of polymer solution and solvent has also been found to affect particle size and shape, Chin et al. (2011) observed that such variations could lead to particles with different shapes, such as elongated fibres, spherical particles or a mixture of spherical particles and elongated fibres.

2. Hypothesis

One of the hypothesis investigated in this study is that starch nanoparticles can be used to produce Pickering emulsions with a smaller droplet size than emulsions produced from intact granules of the same botanic source, and thus reduce the creaming tendencies of such emulsions. We also investigated whether starch nanoparticles could be produced using non-solvent precipitation of starch with ethanol as the non-solvent. It was also hypothesized that the size of particles and the amount of submicron particles obtained using this method could be affected by changing the process parameters.

3. Materials and methods

3.1. Materials

The source of starch, waxy maize, was supplied by Lyckeby – Culinar AB, Sweden. The oil used was a medium-chain triglyceride oil (MCT), Miglyol 812 (Sasol, Germany). The *n*-octenyl succinate anhydride (OSA) was obtained from Trigon Chemie (Germany), DMSO 99.6% from VWR Prolabo (Belgium), and the ethanol was 99% European pharmacopeia quality (Sigma Aldrich, USA). Tween 80 and sodium dodecyl sulphate (SDS) were obtained from Sigma Aldrich, and all other chemicals were of PA quality.

3.2. Dissolution of the starch

The starch samples were dissolved either by autoclaving of a starch water solution or dissolution of starch in DMSO, as previous described by Perez-Rea et al. (2015).

3.2.1. Autoclaving

A sample of 4 g waxy maize starch was dispersed in 200 mL MilliQ water (Millipore Corp., Bedford, MA, USA) and heated in a high-pressure laboratory autoclave. This instrument was equipped with a magnetic stirrer and was connected to a temperature control unit (Withernm WRX 2000, Germany). Before heating, the system was flushed with nitrogen gas for 5 min to prevent oxidative degradation of the starch. The starch suspension was gradually heated from room temperature to 140 °C for 14 min, maintained at 140 °C for 20 min, and then cooled by immersing the autoclave cylinder in an ice bath. The starch solution (20 mg/mL) was diluted to concentrations of 1, 2, 4, 8 and 10 mg/mL before non-solvent precipitation.

3.2.2. DMSO method

Two grams of waxy maize starch (dry weight) was weighed into a glass flask and dispersed in 6 mL ethanol (80% ethanol in water v/v) with magnetic stirring for 3–5 min. Sixty mL of DMSO (including 0.5% w/v LiBr) was added. The sample was boiled for 1 h in a water bath with magnetic stirring (160 rpm) and then cooled to room temperature. The starch solution (20 mg/mL) was diluted to concentrations of 1, 2, 4, 8, 10 mg/mL before non-solvent precipitation.

3.3. Non-solvent precipitation of starch

Non-solvent precipitation was carried out using ethanol at different starch solution-to-ethanol ratios, 1:1 and 1:10, and the starch solutions obtained with the two techniques described above. The production technique was optimized to produce as small starch particles as possible. Non-solvent precipitation of nanoparticles was carried out by mixing ethanol and the starch solution directly, or by the slow addition of one to the other drop-wise. The effect of starch concentration on nanoparticle size was investigated. The effects of adding 4% surfactant (Tween 80 and SDS) based on starch concentration, on particle production were also studied. The size of the nanoparticles was determined directly after non-solvent precipitation using static light scattering and light microscopy (see Section 3.6). The solvent was removed by batch-wise centrifugation (2000 G, 10 min), and all the particles from the same production were collected together. For the last stage solutions containing very fine particles that could not be harvested by centrifugation was also added to the collection of precipitated starch and the collected solution was left in the fume hood overnight to allow the ethanol to evaporate. The nano-precipitates were then frozen at −20 °C overnight, after which they were freeze-dried in a Hetosicc freeze-dryer (Denmark), starting the process at −20 °C and increasing the temperature to 20 °C with 1 °C/min over a period of 4–5 days.

3.4. Modification of starch with OSA and determination of the degree of modification

A portion of the wet nanoparticles obtained by precipitation were directly modified by OSA to increase their hydrophobicity. Modification was performed using 3% OSA (based on the dry weight of the starch nanoparticles) and the degree of OSA substitution were analyzed. The methods are described by Saari et al. (2015).

3.5. Production of pickering emulsions

The emulsions were 5% oil in water emulsions using MCT oil as the dispersed phase, and phosphate buffer (5 mM, pH 7, 0.2 M NaCl) as continuous phase. Seventy mg of modified nanoparticles (equalling 200 mg starch per mL oil) was dispersed in a mixture of 0.35 g oil/6.65 g buffer, and mixed with a vortex mixer for 10 s. The suspensions were emulsified using a high-shear mixer (Ystral D-79282, Germany) at 22 000 rpm, for 60 s. The emulsions were observed directly and after 24 h. The emulsion index was measured after 24 h. The emulsion index EI is measured from a photograph of the emulsion and is the ratio of the emulsion phase divided by the whole volume of the sample. An emulsion index of 1 means that there is no visual segregation of the sample.

A 200 mg/mL starch suspension of was prepared and was diluted to 6, 2, 1, 0.5 and 0.01 mg/mL (in 6.65 mL buffer) and was used to produce series of emulsions containing 0.35 g oil (5%).

3.6. Characterization of nanoparticles and emulsions using optical methods

3.6.1. Static light scattering

The size distributions of the starch nanoparticles and emulsion drops were characterized using a light-scattering particle size analyser (Malvern Mastersizer 2000, Malvern instruments, UK). A small amount of sample (starch or emulsion) was injected into the flow system connected to a pump (2000 rpm) and the optical chamber for measurement. The refractive index of starch was set to 1.54 (Bromley & Hopkinson, 2002), and for the continuous phase, the refractive was set to that of water, 1.33. Obscuration was restricted to between 10 and 20% for emulsions. Due to the small amounts of sample available, the obscuration was reduced to less than 5% when characterizing nanoparticles, as these give a low amount of obstruction due to their size.

3.6.2. Light microscope

Emulsion droplets and starch nanoparticles were also analysed using an optical microscope (Olympus BX50, Japan) equipped with a live-video camera. The samples were examined under Plan 2×, UMPlanFL 5× and 10×, and LMPlanFl 20× and 50× objectives (Olympus). One drop of sample was diluted with 5 drops of buffer solution and, one drop of diluted sample was placed uncovered on a glass slide for microscopic observation.

3.6.3. Scanning electron microscope

The morphology and size of the starch granules and nanoparticles were investigated using scanning electron microscopy (SEM). The samples were coated with gold and inspected using a JSM-6700F scanning electron microscope, (JEOL inc, Japan) operated at 5 kV with a working distance of 8 mm. Lower detection imaging mode was used to give clear 3D images of the sample surface. The signals from secondary and back-scattered electrons were combined during operation.

3.6.4. Atomic force microscopy

Samples were prepared for atomic force microscopy (AFM) as follows. A $1 \times 1\ cm^2$ mica plate, freshly cleaved, was covered with a 20 μL drop of OSA-modified starch precipitate that had been suspended in water for 3 min and subsequently dried with nitrogen gas. A Ntegra atomic force microscope (NT-MDT, Russia), with Nova software, was used to analyse the surface of the samples. The specimen was scanned in semi-contact mode with a frequency of 1.01 Hz using an NSG01 cantilever (from NT-MDT) at a frequency of 150 kHz and a stiffness of 5.1 N/m.

The images obtained were analysed using Image J. The sizes of 660 particles were measured, from which the size distribution and average particle size were determined for each set of experimental conditions.

3.6.5. Asymmetric flow field-flow fractionation

The size of the non-solvent precipitated starch was analysed using asymmetric flow field-flow fractionation (AF4) based on a method used for starch previously described by Perez-Rea et al. (2015). After nanoprecipitation, the starch particles were dispersed in MilliQ water at a concentration of 1 mg/mL. A solution of bovine serum albumin (Sigma, A4378, St, Louis, MO, USA) with a concentration of 1 mg/mL in MilliQ water was prepared and used to determinate the channel thickness of the AF4 equipment and to validate the performance of the AF4 system. The AF4 channel was a short channel (Wyatt Technology, USA) with trapezoidal geometry (tip-to-tip length 17.4 cm and inlet and outlet widths of 2.17 and 0.37 cm, respectively) A regenerated cellulose membrane with a nominal thickness of 350 μm and a cut-off of 10 kDa was used. The composition of the carrier liquid was 10 mM $NaNO_3$ (AppliChem, A3125, Darmstadt, Germany) and 0.02% sodium azid (BDH, 10369, Poole, UK), dissolved in MilliQ water. Separation was performed using the following parameters: constant detector flow of 1 mL/min and injection into the channel at a flow rate of 0.2 mL/min for 4 min. After injection, a 3 min focusing/relaxation step was introduced prior to elution with a focus flow identical to the initial cross flow. Elution started at an initial cross-flow of 0.5 mL/min and was decreased exponentially with time to 0.07 mL/min, and then kept constant from 3 to 20 min. After elution the channel was flushed with buffer solution without any cross-flow for 3 min before the next analysis. Triplicate measurements were performed on each starch nanoparticle sample.

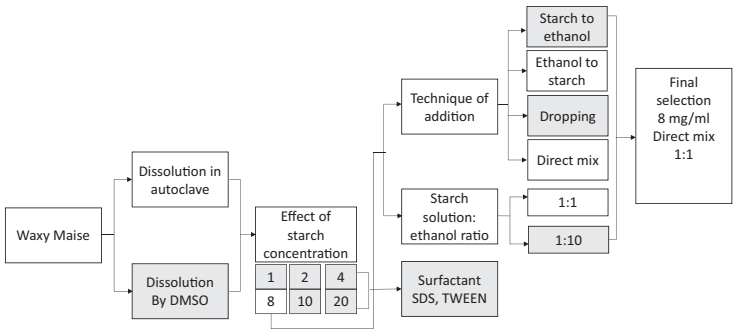

Fig. 1. The methods and experimental conditions used to determine the optimal method of producing non-solvent-precipitated starch nanoparticles. Grey shading indicates the method/conditions not chosen for further experiments.

4. Results and discussion

4.1. Method development for the production of starch nanoparticles

The methods employed were developed to generate particles with small sizes and low amounts of aggregated starch particles. The different steps in this method development are outlined in Fig. 1. The starting point for this development was based on two previous studies on dissolution (Perez-Rea et al., 2015) and non-solvent precipitation (Hornig et al., 2009). The parameters investigated were the method of dissolving the starch granules, the effect of starch concentration on particle size, the effect of the way in which the ethanol and starch solution were mixed in non-solvent precipitation, the effect of adding surfactants, and the effect of starch:ethanol ratio. Based on this screening process the final method and condition were decided upon, as described in Fig. 1.

4.1.1. Effect of method of starch dissolution

Two methods of dissolution were studied. These were adapted from Perez-Rea et al. (2015) who studied starch granule dissolution by autoclaving at 140 °C and dissolution in DMSO at 100 °C in order to form molecular dissolved starch without degrading the polymers. The method has been verified by Ortega-Ojeda et al. (2003) who showed that this was sufficient to completely dissolve the starch granules. Both methods resulted in dissolved starch in the molecular state, which then underwent non-solvent precipitation.

The results obtained for starch nanoparticle size using static light scattering are given in Table 1. It can be seen that dissolution using autoclaving leads to smaller starch particles after non-solvent precipitation than dissolution in DMSO. Using the autoclaving method for starch dissolution gave particles with an average size ($d_{3,2}$) of less than 18 μm, where almost 70% of the particles had sizes less than 1 μm. Particles precipitated from DMSO dissolution were as large as 200 μm, including some aggregates, and only 17% were small particles, <1 μm. One reason for this could be that the DMSO in the solution gives a solution that is a better solvent than the pure water/ethanol mixture, and it is thus a less effective medium in the transition from the molecular state to the solid state, leading to a slower precipitation which could allow for the formation of fewer larger particles compared to a fast precipitation that induces many nuclei for the precipitating starch.

4.1.2. Effect of starch concentration

Dissolved starch, produced both with autoclaving and dissolution in DMSO, were diluted and non-solvent precipitation was carried out at different starch concentrations: 1, 2, 4, 8, 10 and 20 mg/mL. Particles sizes are given in Table 1, and light microscopy images are shown in Fig. 2. It was found that the relation between starch concentration and precipitated particle size was not linear. The average particle size and the smallest particles observed show minima as the concentration is varied. For autoclaved solutions, smaller particles were observed at intermediate concentrations between 4 and 10 mg/mL starch, with 47%–66% of the particles being less than 1 μm, while larger particles were observed at lower and higher starch concentrations. For starch dissolved in DMSO, the precipitated particles were larger at low starch concentrations and smaller at higher concentrations around 10%, but the highest volume of particles below 1 μm was only 17%. It was found that starch solution obtained by autoclaving at an intermediate concentration (8 mg/mL) gave the best results, and produced nanoparticles with an average size of about 1 μm; the smallest particle detected was about 0.3 μm and 66% of the particles were less than 1 μm in size. It should be noted that precipitation was carried out at a starch-to-ethanol ratio of 1:10. Different starch concentrations from 1 to 20 mg/mL were investigated to establish whether there was any difference in particle size with varying starch concentration. At concentrations above 1 mg/mL, the precipitated particles seemed to form agglomerates, as can be seen in the light microscopy images in Fig. 2.

The results discussed above are in contrast to those reported by Tan et al. (2009) who found a linear relation between the increase in size of starch nanoparticles and increasing polymer concentration in the solvent. Rather than an increase in size, the light microscopy images indicate that more particles of a similar size are formed, and that they aggregate at higher starch concentrations (Fig. 2). Tan et al. (2009) suggested that lower starch concentrations of 1–8 mg/mL were preferable as the size distribution of precipitated particles was narrower, and widened rapidly at higher concentrations, leading to aggregation at starch concentrations >20 mg/mL. This is in line with our observations, of a wider particle size distribution and a higher average value of $d_{3,2}$ for a 20 mg/mL starch solution, which is indicative of aggregated particles. The reason for this could be, as pointed out by Hornig et al. (2009) that low concentrations are required to ensure that the polymer molecules are in the dispersed state, and able to separate into nano-domains following the addition of non-solvent.

Table 1
Particle size ($d_{3,2}$) of starch nanoparticles determined by static light scattering using different methods of starch dissolution and experimental conditions during non-solvent precipitation. All the experiments were conducted by adding starch to ethanol dropwise at a starch: ethanol ratio of 1:10 unless otherwise stated.

	Samples (Different starch concentration precipitate with ethanol)	n	d3,2 (μm)	%<1 μm	Smallest detected particle (μm)
Dissolution by Autoclave	1 mg/mL starch	9[a]	11.9 ± 7.2	0.0 ± 0.0	1.7 ± 0.1
	2 mg/mL starch	6[a]	8.2 ± 2.1	0.0 ± 0.0	2.2 ± 0.3
	4 mg/mL starch	3	3.8 ± 1.2	46.8 ± 33.1	0.4 ± 0.1
	8 mg/mL starch	3	1.2 ± 0.3	66.4 ± 4.2	0.3 ± 0.0
	10 mg/mL starch	6[a]	3.8 ± 2.2	52.4 ± 14.1	0.3 ± 0.0
	20 mg/mL starch	6[a]	17.6 ± 33.8	68.9 ± 34.6	0.7 ± 1.1
Dissolution by DMSO/LiBr	1 mg/mL starch	3	231.1 ± 276.2	0.0 ± 0.0	20.6 ± 27.1
	2 mg/mL starch	2[a]	48.2 ± 62.8	4.6 ± 8.0	1.6 ± 1.0
	4 mg/mL starch	3	6.2 ± 0.9	2.0 ± 0.6	0.7 ± 0.0
	8 mg/mL starch	3	4.6 ± 1.4	13.5 ± 2.0	0.7 ± 0.1
	10 mg/mL starch	3	5.1 ± 3.6	17.0 ± 15.6	0.5 ± 0.2
	20 mg/mL starch	2[a]	4.0 ±1.5	68.1 ± 1.0	0.3 ± 0.0
Surfactant (4% SDS)	1 mg/mL starch	3	103.8 ± 27.9	0.0 ± 0.0	40.1 ± 5.5
	2 mg/mL starch	3	66.5 ± 24.7	0.0 ± 0.0	10.7 ± 13.1
	4 mg/mL starch	3	143.0 ± 233.8	2.7 ± 2.7	12.1 ± 19.6
	8 mg/mL starch	3	170.7 ± 111.1	0.0 ± 0.0	34.9 ± 4.8
	10 mg/mL starch	3	38.7 ± 44.0	19.5 ± 25.9	10.4 ± 17.2
	20 mg/mL starch	3	26.7 ± 8.2	46.3 ± 14.4	0.4 ± 0.1
Surfactant (4% Tween 80)	1 mg/mL starch	1[a]	776.4 ± 0.0	0.0 ± 0.0	363.1 ± 0.0
	2 mg/mL starch	3	24.8 ± 21.0	2.2 ± 2.8	1.0 ± 0.4
	4 mg/mL starch	3	84.0 ± 120.3	13.5 ± 12.4	1.8 ± 2.2
	8 mg/mL starch	3	125.0 ± 3.1	0.0 ± 0.0	50.2 ± 3.9
	10 mg/mL starch	3	248.0 ± 147.5	0.0 ± 0.0	72.6 ± 5.9
	20 mg/mL starch	3	13.8 ± 2.5	63.1 ± 5.9	0.3 ± 0.0
Technique & ratio	8 mg/mL starch dropwise in ethanol (1:10)	3	1.2 ± 0.3	66.4 ± 4.2	0.3 ± 0.0
	8 mg/mL starch dropwise in ethanol (1:1)	3	2.4 ± 2.2	65.0 ± 22.0	0.3 ± 0.0
	8 mg/mL starch mixed with ethanol (1:1)	3	2.0 ± 1.4	71.5 ± 10.4	0.3 ± 0.0
	8 mg/mL starch concentration, ethanol mixed into starch (1:1)	3	0.9 ± 0.0	81.6 ± 0.8	0.2 ± 0.0

[a] The samples were prepared and normally measured at least in triplicate, or for some cases more especially at the initial steps. Some measurements were not obtained/not included due to technical problem of the instruments, insufficient samples to reach detection limit or detection of air bubble.

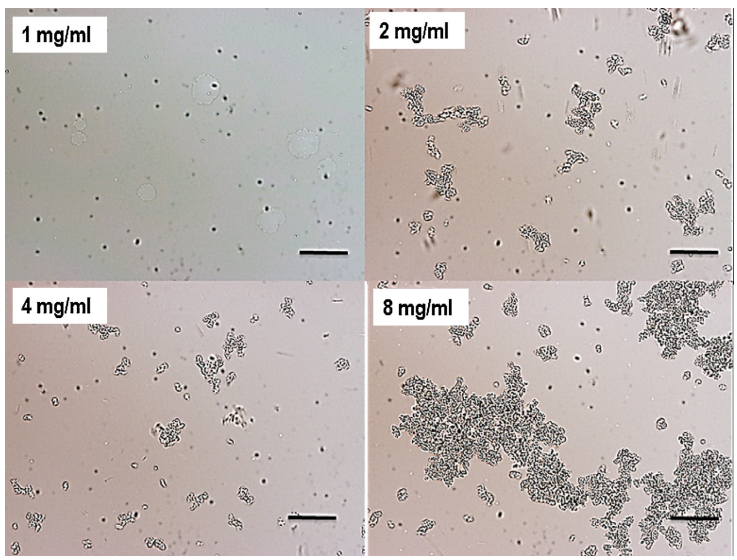

Fig. 2. Effect of starch concentration on particle size and aggregation (scale bar: 100 μm).

Table 2
Determination of size distribution using various optical techniques.

Structure	SEM	AFM	Light Scattering		AF4*
	Average Diameter	Ferret diameter	$d_{4,3}$ Volume weighted mean (μm)	$d_{3,2}$ Surface weighted mean (μm)	(rms radius)
Native starch granules from waxy maize	10–20 μm	–	Top mode: 13.98 16.8 ± 5.37 (native)[a] 14.7 ± 0.08 (OSA)[b]	Top mode: 2.0 8.00 ± 0.31(native) [a] 8.12 ± 0.07 (OSA) [b]	–
Nanoparticles	10 nm–400 nm	–	Top Mode: 0.83 Average: 9.87 ± 1.75	Top Mode: 0.63 Average: 0.92 + 0.1	~60 nm
OSA-modified nanoparticles	Aggregates formed	182 ± 0.44 nm	Top mode: ~4–5 24.4 ± 1.52 (shear) ~60–90 (non-shear)	Top mode: ~2.0 4.81 ± 0.05 (shear) ~7.5–7.8 (non-shear)	~260 nm

[a] Native starch means non-OSA modified.
[b] OSA modified native starch granule.

Thus a starch concentration of 8 mg/mL was the optimal choice for producing the highest fraction of submicron particles and the smallest detected particles. In these screening steps the data was based on static light scattering data and light microscope. For a more complete picture of the nano-precipitate characteristics, additional methods are needed.

4.1.3. Effect of adding surfactant to the starch solution

We examined the effect of two surfactants on the particle size (Table 1). In order to ascertain whether the addition of a surfactant would reduce aggregation of the nanoparticles. However, the addition of surfactants led to larger particles than when surfactant was not added. The size of the particles produced when adding 4% SDS ranged from 30 μm to 170 μm and when adding 4% Tween even larger particles were seen, 25 μm to 780 μm. Thus, at a starch concentration of 8 mg/mL, solutions without surfactant produced smaller particles than solutions containing surfactants. These results seem to be in contrast to those reported by Chin et al. (2011), who found that surfactants such as CTAB and Tween 80 limited particle growth when producing nanoparticles. The addition of surfactants will most likely lead to formation of starch inclusion complex (Eliasson & Wahlgren, 2004; Lundqvist, Eliasson, & Olofsson, 2002). The effect of these inclusion complex could be difficult to predict and as the effect in our case was non beneficial we did not further investigate this issue.

4.1.4. Effect of solvent: non-solvent ratio and how solvent and non-solvent are mixed

After determining the optimal starch concentration (8 mg/mL) and dissolution method (autoclaving) the precipitation conditions was studied. Hornig et al. (2009) proposed two non-solvent precipitation methods dialysis using a membrane and the dropping technique under stirring. The dropping technique was selected as a starting point for our work due to its simplicity. Starch was added drop-wise to the ethanol to a final starch:ethanol ratio of 1:10. However, this technique was time-consuming when large samples were used. Thus, the next step towards improving the production of starch nanoparticles was to reduce the amount of solvent required. Initially, 10 L of ethanol was used for every litre of starch solution. The possibility of reducing the amount of ethanol to 1 L (ration 1:1) was therefore investigated. Furthermore, both direct mixing and the dropping technique were investigated. As can be seen from Table 1, the particle size did not differ significantly between the two mixing techniques and the two ratios. However, after direct mixing, the fraction of small particles (<1 μm) was slightly higher (71%) than when using the dropping technique (65–66%). The smallest detected particle in both cases was about 300 nm. Thus, non-solvent precipitation can be achieved in a shorter time with a better quality of the particles using direct mixing at a starch:ethanol ratio of 1:1 than using the original method.

Finally, the effect of mixing of ethanol into the starch solution or mixing of starch into the ethanol solution were investigated. It was found that dropping at starch ratios of 1:10 and 1;1, and direct mixing of the starch solution into ethanol at a starch:ethanol ratio of 1:1 gave almost the same results, in terms of the amount and size of the particles produced. Interestingly, direct addition of ethanol solution into starch gave a considerably higher fraction of particles in the submicron range (81%) than the other mixing methods The smallest particle detected in static light scattering was 200 nm. From the above results it can be concluded that direct addition of ethanol into starch solution, at the ratio 1:1 was best for the production of nanoparticles. This method required the least amount of solvent, was most time-efficient and gave small particles. Thus, it was thus chosen for the production of starch nanoparticles on a larger scale.

To summarize, the production method found to be the best for producing nanoparticles was autoclaving at 140 °C of starch dispersed in water, a starch concentration of 8 mg/mL, using direct mixing by adding ethanol to starch, for precipitation at a starch:ethanol ratio of 1:1.

4.2. Characterization of particles

The starch particles produced using the optimal production method were then investigated using a range of optical characterization methods The results for the initial native waxy starch granules, precipitated starch particles and OSA-modified starch particles are presented in Table 2.

As can be seen, the characterization methods give quite different results. This is probably due to the fact that the different methods analyse different aspects of the samples. For examples the particle size distribution given by static light scattering will over emphasize large particles which leads to an overestimation of the average size of the sample while in the AF4 experiments on the other hand the largest particles have been removed thru a pre-filtration step. A further problem with static light scattering is that both the measured size and the size distribution is affected by the underlying models as well as parameters for these models, such as refractive index of the particles, that are used for transferring the measured light scattering to particle size and particle size distribution. The differences are substantially larger for the precipitated starch, especially for the OSA-modified precipitate, than for the original starch granules. This is probably because the nanoparticles are partially aggregated, in contrast to the original granules,

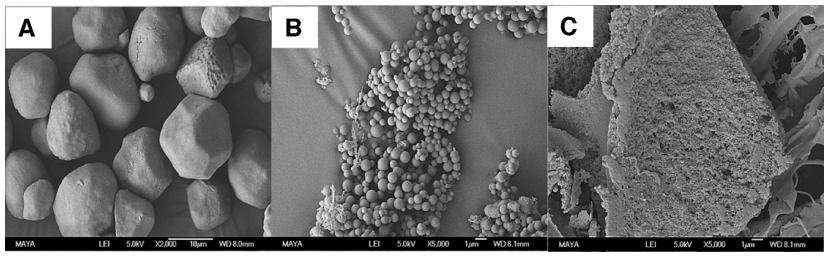

Fig. 3. Effect of non-solvent precipitation on size, shape and structure during the production of starch nanoparticles. (A) Native starch granules from waxy maize, (B) starch nanoparticles, and (C) Aggregation of OSA-modified starch nanoparticles.

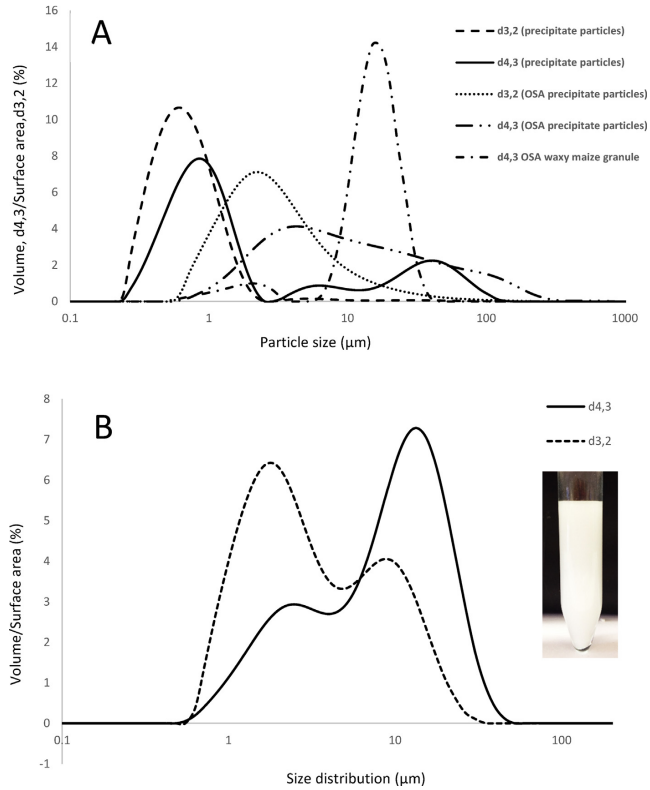

Fig. 4. (A) Particle size distribution for native starch granules, non-solvent precipitated nanoparticles and OSA-modified nanoparticles using the optimal production method. (B) Size distribution of an emulsion stabilized by nanoparticles is explained in *d3,2* (surface weighted mean, μm)) and *d4,3* (volume weighted mean, μm). Inserted is a photograph of the emulsion.

and their size is less optimal for the static light scattering technique used. But it is also an indication that the non-solvent precipitation gives a broad size distribution. The SEM images show that the native granule size is around 10–20 μm (Fig. 3A), which is in good agreement with the static light scattering measurements which showed a size distribution around 14 μm (top mode). It also shows that the particles formed are spherical. SEM of starch nanoparticles and OSA-modified precipitate (Fig. 3B & C) gave particles sizes varying from 10 nm to 400 nm, while values of $d_{3,4in}$ obtained by static light scattering were between 5 μm and ~0.25 μm (Fig. 4A). It can

be seen in Fig. 4A that aggregation was more pronounced for OSA-modified particles than for non-modified particles, as seen by the wider particle size distribution and secondary peaks. This was also substantiated by the SEM images Fig. 3C. The high degree of aggregation is further substantiated by the fact that the mean $d_{4,3}$ value (which is the value that best represents the fraction of large particles or aggregates) varied depending on whether the sample was sheared or not, the values being ~25 μm for sheared samples and 60–90 μm for unsheared ones.

AF4 was used to measure the average size distribution of the non-solvent precipitated starch. This method gave a radius of gyration (rms radius) of about 60 nm for the non-modified starch nanoparticles, while the corresponding value for the OSA-modified nanoparticles was about 240 nm (Table 2). This could indicate that the particles are more strongly aggregated as a result of OSA modification. This size measured for the non-solvent precipitate is smaller than that seen for dissolved waxy maize molecules and further studies of the precipitate are required to determine whether this precipitate is a mixture of particles and molecules. However, it is well known that particles can be denser than dissolved polymers, and thus have a smaller radius. The size is smaller than what is seen by light-scattering and it is possible that the larger aggregates observed in light-scattering was not measured in the AF4 experiments.

These OSA-modified particles were also analysed using AFM. A total of 660 particles from four AFM images were analysed, and the average Feret diameter of these particles was 182 nm (range of 14–7000 nm), again showing the large variations in the precipitate, but also having an average diameter that is close to the that of the non-modified starch particles, as measured by AF4. This indicates that there is a large fraction of nanoparticles with a size of about 100 nm when they are not aggregated. Thus, the higher value observed for the OSA-modified starch particles using AF4 most is probably due to aggregation. The size is also in the same range as the smallest particles detected by static light scattering.

4.3. Starch nanoparticle-stabilized emulsions

The reason for producing starch nanoparticles is for use in starch-stabilized Pickering emulsions. As can be seen in Fig. 4B, the particles produced using the method developed in this work can be stabilize emulsions. The initial emulsions produced with 200 mg starch/g oil have a size distribution from 0.5 to 45 μm ($d_{4,3}$) with an average droplet size of 10 ± 0.7 μm (triplicate measurements and had an emulsion index (EI, the volume of the emulsion phase compared to total volume)) on day zero of 1 with as little oil as 5% (Fig. 4B). An EI of 1 means that the emulsion is a space-filling system, with no creaming or sedimentation. This can be compared to previous results for an emulsion of OSA-modified starch granules from waxy maize which, at a slightly higher starch to oil ratio 214 mg starch/g oil gave a droplet size of 47 μm and a EI of 0.37 for 7% oil (Saari et al., 2015).

Thus, we have shown that the nanoparticles produced in this study can be used to form emulsions with a much smaller droplet size than is possible with starch granules from waxy maize. This is in line with studies that have shown that emulsion droplet size can be reduced by using smaller starch particles (Li et al., 2012; Saari et al., 2015; Timgren, Rayner, Sjöö et al., 2011). This is probably a result of that as the particle size decreases the available surface area per unit mass of starch increases. Thus, the same amount of starch can be used to cover a larger surface area of emulsion which is translated to smaller droplets There will, however, be a lower limit on the droplet size of the emulsion, where the addition of more starch particles will not decrease the droplet size. This limit is set by the equipment and conditions used for the production of emulsions. We have previously shown that, using the same set up in the present study, the minimum droplet size in an emulsion based on quinoa starch is about 10 μm, and is reached at about 1200 mg starch/mL oil in 5% oil emulsions (Saari et al., 2016). This will not be sufficient to obtain an EI of 1. Thus, the droplet size obtained at the present study using starch nanoparticles is in the range of the minimum droplet size already at the modest amount of 200 g starch/g oil.

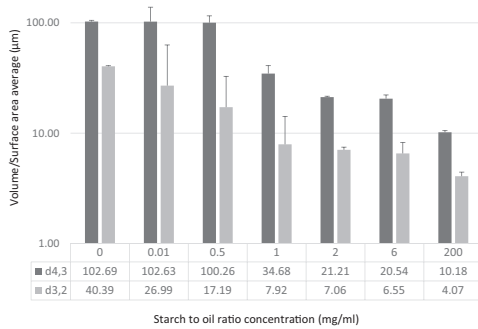

Fig. 5. The effect of starch concentration (based on oil fraction) on the mean droplet size of emulsions produced using 5% MCT oil in water.

Calculating surface coverage of starch of a 10 μm droplet by non-solvent precipitated starch using Eq. (1) (assuming a nanoparticle size of ~150 nm, which is in between the values given by AFM and AF4 measurements) gives that 140 mg starch would be required per m^2 for full surface coverage. This can be compared to the amount needed for starch granules from waxy maize (~14 μm) where 12,700 mg per m^2 would theoretically be needed to cover the same surface area. This shows how decreasing the particle size reduces the amount of starch needed by a factor of over 10^3.

As the droplet size seems to be in the region where the method of production limits the minimum droplet size limit, a series of dilution of starch (from 200 mg/mL to 6, 2, 1, 0.5 and 0.01 mg/mL) was used to determine the influence of particle size on the Pickering emulsion (Fig. 5). The result shows that the emulsions increase in drops size, from 10 to 100 μm at decrease starch concentrations until it reaches maximum drops size equivalent to what we have seen that free oil gives when it is pumped through the static light scattering equipment. This is around 100 μm. A starch concentration of 1 mg/mL is able to create emulsion drops with 35 μm drops size whereas a starch concentration of 0.5 mg/mL and below is not sufficient in covering oil droplet. The emulsions produced were stable, maintaining an EI of 1 for 24 h up to 1 week.

4. Conclusions

Precipitate of starch containing nanoparticles was successful produced after optimisation of the original method. The process identified here was dissolution of starch using autoclaving of an aqueous solution at 140 °C for 20 min, while flushing with nitrogen gas followed by ethanol precipitation. The optimum starch concentration was 8 mg/mL. Ethanol was added directly to the dissolved starch to achieve non-solvent precipitation, at starch:ethanol ratio of 1:1. This led to the production of nanoparticles with sizes between 10 nm and 1 μm, but larger aggregates were also formed, especially after hydrophobic treatment with OSA. The starch particles formed were spherical. Addition of surfactant was not able to limit the growth or aggregation of the particles. These nanoparticles were then used to produce emulsions with an emulsion droplet size

of about 10–30 μm already at the modest amount of 1 mg starch per mL oil.

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Paper V

A comparison of emulsion stability for different OSA-modified waxy maize emulsifiers: Granules, dissolved starch, and non-solvent precipitates

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Abstract

This work investigated the stability of emulsions prepared by using octenyl succinic anhydride (OSA)-modified waxy maize in the form of granules, dissolved starch, and non-solvent precipitated starch as emulsion stabilisers. The aim of this study was to investigate how starch in different forms affects emulsifying capacity using light microscopy, light scattering, and static multiple light scattering (Turbiscan). All starch samples were modified with 3% (w/w) OSA to increase the hydrophobicity. Dissolved starch was prepared by dissolving OSA- modified starch in water, in an autoclave at 140°C. Non-solvent precipitates where obtained by ethanol precipitation of dissolved waxy maize. The stability of the oil/water emulsions was different for the three forms of starch used. The granule-based emulsions were unstable, with only a small proportion of the granules attached on oil droplets, as seen under a light microscope. The emulsions where seen to cream after 2 hours. The dissolved starch and non-solvent precipitate-based emulsions were seen to be stable towards creaming for months, and they had a 100% emulsifying index (EI). The results from light microscopy, and Turbiscan measurements indicated that coalescence was occurring for all three types of emulsions. The coalescence was fast within days for the granule stabilised system, while it was considerably slower both for the dissolved starch and non-solvent precipitate-based emulsions. The latter showing the least degree of coalescence over time. Thus, it was concluded that differences in starch size and molecular structure influenced the resulting emulsion droplet size and stability. A decreased particle size contributes to a decreasing droplet size, thus increasing stabilisation against creaming. The stability towards coalescence was seen to be low for the large granules but best for the non-solvent precipitate starch indicating that there is a window of optimal particle size for stability. The best emulsifying properties was thus obtained with the non-solvent precipitates (~ 120 nm particle size) where the emulsions were reasonable stable also after one year of storage. This study revealed the potential of starch non-solvent precipitates for use as emulsifiers.

Introduction

The aim of this study was to compare the long-term emulsion stability of emulsions stabilised by octenyl succinic anhydride (OSA)-modified waxy maize starch in different physical forms, specifically intact granules (Pickering emulsions), dissolved starch (polymer stabilised emulsion), and non-solvent precipitated starch (stabilisation principle unknown). Emulsions are widely used formulations that consist of two immiscible liquids dispersed in one another in the presence of emulsifiers or stabilisers. Emulsions are thermodynamically unstable, and to improve the instability and production of emulsions, controlling the hydrodynamic conditions, decreasing the interfacial tension, and/or increasing steric hindrance or electrostatic repulsion are of importance [1, 2]. Surfactants and polymers including natural polymers such as polysaccharides and proteins are traditionally used to stabilise emulsions. Recently, particle-based emulsifiers have shown good performance, and are increasingly of interest in further research and technological applications [3-10]. This type of emulsion is known as a Pickering emulsion and was first reported by Pickering and Ramsden, and can be obtained using different types of particle such as silica, latex, and clay (synthetic/inorganic) [11, 12], as well as a range of bio-based particles [13, 14].

It has been concluded that solid particles create a physical barrier between droplets and that particles have a high desorption energy especially related to large particle size (10 nm–5 μm), and thus adsorb irreversibly to the interface to a higher degree than other emulsifiers such as surfactants [2, 15]. The Pickering emulsions have also shown better stability than other emulsions towards coalescence and Ostwald ripening [2]. However, due to their large size, they have slower kinetics of adsorption and the amount of particles (weight per volume of oil) needed for emulsification are larger than for traditional emulsifiers. These differences between particles and other stabilisers are strongly dependent on size, and it has been observed that nanoparticles around 1–5 nm display similar behaviour to globular protein because of comparable size and desorption energy [2]. One difference between polymers, proteins and particles should as we see it be in how they are affected by adsorption to the water/oil interface. The structure of a particle should be rather unchanged while polymers and proteins could have a considerable flexibility leading to changed structure when adsorbed to the interface compared to in solution. In this respect, we do not know if the nano precipitated starch used in this study is a Pickering emulsion or not, most likely the material consist of both dissolved molecules and some particles [16].

Starches have some general characteristics, such as possessing low toxicity, originating from a renewable recourse, and being tasteless, which make them interesting in a wide range of formulation applications such as in foods, beverages, cosmetics, pharmaceuticals, paints, and coatings [9, 17, 18]. Starch is in its natural state found as granules which are particles in the size range of 1- 100 μm. However, by extensive heating combined with mechanical treatment or by using solvents such as DMSO the granules can be dissolved and starch in its molecular form can be obtained. Both these physical forms of starch have been used as an emulsifier for oil in water emulsions. However, to our knowledge these forms have only been compared in one previous study [6] and in that case for quinoa starch and only for shorter times – 24 hours. Dissolved OSA-modified starch has been seen to be a good emulsifier [19-25]. The emulsifying capacity of OSA starches are dependent on a range of properties, such as molecular weight, where increased molecular weight improves stability but can increase droplet size; rigidity of the hydrophilic parts of the polymer chain, which increases steric stability; and higher amounts of amylopectin and a low hydrodynamic radius, which decrease droplet size [17]. During emulsification with OSA starches, there is a preferential adsorption of high molecular species on the emulsion droplets [23]. High shear rate emulsification has been shown to decrease the starches' molecular weight [20].

It has been seen that some emulsions stabilised using starch granules show long-term stability [15, 26, 27], for example, particle-stabilised emulsion from quinoa with 1–2 μm size was able to perform up to 2 years during storage [27]. The character of starch Pickering emulsions has been seen to be influenced by the size, morphology, and hydrophobicity of the starch [28, 29], as well as the starch concentration [2, 15]. In general, smaller starch granules produce more stable emulsions with a smaller droplet size [26, 27, 29].

In order to have a good adsorption to the oil/water interface, starch granules as well as dissolved starch have to be made more hydrophobic, and this has primarily been done by chemical modification with octenyl succinate anhydride [24]. The effect on the emulsion droplet size was found not to be linear with higher degree of OSA substitution, and the level of modification used in this study (3%) was found to be more than sufficient to stabilise emulsions and is also the limit for modification allowed within food applications [21].

Many research groups have been using waxy maize as a subject material, and it is widely studied, which makes this starch a popular source for comparison. Waxy maize has irregular and sharp edges, with graininess, pin-holes, and deep depressions on the surface of some granules [30, 31]. It has two populations of granules one in the size range between 1-7 μm and one in the range of 15–20 μm [30, 32]. In a previous study, waxy maize was used as a sole emulsifier and was able to stabilise the emulsion with a mean droplet size ($d_{4,3}$) of 47 μm and an emulsifying index (EI) of 0.37, which improved to 0.47 when the granule size was reduced to half the initial size after acid hydrolysis treatment [29].

Since small particle size has been seen to decrease droplet size with other parameters being constant, and at least in some cases to increase stability, it could be of interest to produce Pickering emulsions using starch particles in the nano size range. Nano-sized starch particles have been produced using non-solvent precipitation [33-36], and a recent study from our research group has revealed that non-solvent precipitation gave material in the sizes range between 10 nm -1 μm[16]. This non-solvent precipitate was seen to stabilise emulsions having an EI of 1. In the present study, we investigated the long-term stability of these non-solvent precipitated starches and compared them with dissolved OSA-modified waxy maize and OSA-modified granules, thus comparing how emulsion stability is affected by the configuration of the starch using starch with the same botanical origin and degree of hydrophobic modification.

Materials and methods

The native waxy maize starch used was supplied by Lyckeby Culinar, Sweden. The oil used was the medium-chain triglyceride (MCT) oil, Miglyol 812, density 945 kg/m^3 (Sasol Germany), and the water phase used was a 5 mM phosphate buffer with 0.2 M NaCl, density 1009.6 kg/m^3. The n-octenyl succinyl anhydride used was from Trigon Chemie, Germany.

Emulsions were made based on three different starch emulsifiers; Starch granules based emulsions (SGE); Dissolved starch based emulsions (DSE); Non-solvent precipitate starch based emulsions (NPSE). How these emulsifiers were prepared are described below. All emulsifiers were OSA-modified.

OSA-Modification

The amount of OSA to be used was based on the dry weight of starch determined by an IR-balance (135^{o}C, 5 min, duplicate). The starch was dispersed in 1.5 to 2 times amount of distilled water which was added during stirring. The pH was controlled at 7.4 and 7.8 and adjusted with citric acid, 1M HCL or NaOH. 3-4% n-octenyl succinyl anhydride (Ziyun Chemicals co. Ltd, China) was added in 4 portions with 15 min delay between each portion. The automatic titration of 1M NaoH while stirring was set up to control the pH near 7.6. The titration was terminated when the pH was stable for at least 15 min. The suspension was then centrifuged at 3000g for 10 min. The supernatant was discarded and the starch washed twice with water, once with citric acid (pH 4.5-5) and once more with water. The starch was frozen overnight and freeze dried.

Determination of OSA level was carried out in duplicate for modified and control samples. 2.5g of starch (dry weight) was wetted with few drops of ethanol before adding 25ml 0.1M hydrochloric acid and stirred for 30 minutes. The suspension was later centrifuged (3000g, 10 min) where the supernatant discarded. The pellet containing the starch was washed with 25ml ethanol once, and with distilled water twice before being dissolved in 150ml distilled water and heated in hot bath at 90^{o}C for 10 min. The solution was rapidly cooled to 25^{o}C using an ice bath, and the suspension was titrated while stirring with 0.1M NaOH until pH 8.3 was reached. The volume of NaOH used was noted for the calculation of % OSA below:

$$Degree\ of\ OSA\ (\%) = \frac{(Vsample - V\ control) \times M \times 210\ \times 100}{W}$$

Where *V* is volume of NaOH required in titration (ml), *M* is the molarity of NaOH (0.1M), *210* is the molecular weight (g/mol) of octenyl succinate anhydride group (OSA) and *W* is the dry weight of sample used.

Preparation of starch granules (SG)

Starch granules as obtained by the supplier Lyckeby Starch were modified by OSA to increase the hydrophobicity using 3% of OSA based on dry weight of starch following the method described above. After OSA modification, the wet samples were then put into a freezer overnight and freeze-dried (Hetosic, Denmark) starting at -20°C and reaching 20°C with 1°C/min over 4–5 days. The degree of OSA modification for starch granule was 3.1%

Preparation of dissolved starch solutions (DS)

The starting material for the dissolved starch was the OSA-modified starch granules prepared as described above. Thus, the OSA-modification was the same as above. OSA-modified starch granules were dissolved by adapting the dissolution methods reported by Perez-Rea et al. using an autoclave [38]. A sample of OSA-modified waxy maize starch (4 g dry weight) was dispersed in 200 ml milliQ water and heated with a high-pressure laboratory autoclave equipped with a magnetic stirrer and coupled with a temperature control unit (WRX 2000, Withernm, Germany). The heating was carried out for 20 min at 140°C. Before heating, the system was flushed with nitrogen gas for 5 min, to prevent oxidative degradation of the starch. The starch suspension was gradually heated from room temperature to 140ºC for almost 14 min, kept in 140°C for 20 min, and then cooled by immersing the autoclave cylinder in an ice bath. After the autoclave dissolution, the dissolved starch solution was diluted to 8 mg/mL from the initial concentration of 20 mg/ml before being used in emulsions.

Preparation of non-solvent precipitation starch (NPS)

Non-OSA modified starch was dissolved as described in the previous section. Non-solvent precipitation starch was carried out using ethanol by direct mixing of the ethanol into dissolved starch (8 mg/ml concentration) at a ratio of 1:1 (starch solution: ethanol). The precipitated starch was collected after centrifugation (2000 g, 10 min). The solvent was removed after centrifugation, and all precipitated particles were left in the fume hood overnight to evaporate the ethanol until no layer of solvent was seen on top of the precipitate samples. Detail procedure of preparing NPS was described in our previous work [16].The wet precipitates were then OSA modified with similar method in preparing OSA starch granule as described above and the OSA value was 2.03%.

Preparation of emulsions

The emulsions produced were 5% (w/w) of oil/water emulsions containing an MCT oil as the dispersed phase and a phosphate buffer (5 mM, pH 7, 0.2 M NaCl) as the continuous phase, with 200 mg of starch emulsifier per ml of oil. The emulsions were produced by mixing 70 mg of modified granule/non-solvent precipitate (in dry form) or dissolved starch (in solution) into the 0.35 g oil/6.65 g buffer (total of 7 g emulsion composition) and prepared by vortex mixing for 10 s. The suspensions were emulsified using a high-shear mixer (D-79282, Ystral, Germany) at 22,000 rpm, for 60 s. Emulsions were observed directly, after 24 hours, and weekly for up to six weeks and then also after a year for those emulsions that had not phase separated.

Characterisation of emulsions

Static light scattering

The particle size distribution of all emulsions was examined using static light scattering (Mastersizer 2000, Malvern Instruments, UK). A small quantity of sample was injected into the flow system connected to the pump circulating at 2,000 rpm. The refractive index (RI) used was 1.54, which corresponded to starch [39], while for the continuous phase, it was set to 1.33 which is the RI of water. Obscuration was restricted between 10% and 20% for the emulsions. Due to the low amount of the sample, the obscuration was reduced to less than 5% when measuring on emulsions based non-solvent precipitated/dissolved starch, since their size gives a low degree of obstruction. The data, $d_{4,3}$ and $d_{v,0.5}$, refer to the sphere of equivalent volume and the median size of the system based on $d_{4,3}$ distribution, respectively, of the particles or droplets. The mean $\bar{d}_{4,3}$ size of the systems based on the distribution was also determined. This includes all particles in the system. That is for emulsions both emulsion droplets and free starch particles. All samples were measured in triplicates and the average and standard deviations of the $\bar{d}_{4,3}$ mean was calculated. These averages were compared using two sided student t-test.

Light microscope

Emulsions were further observed with an optical microscope (BX50, Olympus, Japan) equipped with a video camera. The samples were examined under Plan 2x, UMPlanFL 5x and 10x, and LMPlanFL 20x and 50x objectives (Olympus). One drop of sample was diluted with five drops of buffer solution, and depending on the sample, one drop of the diluted sample was placed uncovered on the glass plate for microscopic observation.

Stability of emulsions studied by static multiple light scattering

A sample of 4 ml of starch-based emulsions was prepared to study stability during storage. The dispersion of OSA-modified starches (granule, dissolved starch, and non-solvent precipitate in buffer) and the MCT oil dispersion (oil in buffer) were also measured for reference and for comparison with the emulsions. The stability study of the emulsions was evaluated by Turbiscan Lab® Expert (Formulation Inc., France) using static multiple light scattering. The instrument operates by sending a light beam through a cylindrical glass cell containing 4 ml of the sample (12 mm height). The light source is an electroluminescent diode in the near infrared with a wavelength of 880 nm. Part of the incident light is then backscattered by the sample or transmitted through it, and received by two sensors with different locations: the sensor that receives the transmitted light is located 180º from the incident radiation, while the sensor that receives the backscattered light (BS) is located 45º from the incident light. The transmitted light and the backscattered light were monitored as a function of time and cell height at 30ºC. The transmission (TS) and backscattering (BS) data were measured every 40 µm in % relative to standards (suspension of monodisperse spheres and silicone oil) as a function of the sample height (in mm).

The backscattering measurement is directly dependent on the particles' mean diameter and on their volume fraction. The main instability phenomena observed in colloidal systems are: particle migration (i.e., local variation of the concentration of particles, which causes local variation of the transmission or backscattering level measured at the bottom and top of the sample); and particle size increase (i.e., global variation of the particle size, which causes global variation of the transmission or backscattering level measured in the middle of the sample) [6]. BS profiles build up a macroscopic fingerprint of the emulsions at a given time, providing useful information related to changes in droplet size and the appearance of a creaming layer or clarification front, and makes it possible to calculate the velocity with which these processes happen [4].

The Turbiscan stability index (TSI) was obtained using Turbisoft software (2.0.0.33) by computing mathematically based on the sum of scan-to-scan difference of the intensity of lights ($\sum h\,|scan_i - scan_{i-1}$) towards the cell height. The sum of the variations ($\sum i$) detected in the samples in respect of the size/concentration was calculated using the following equation:

$$TSI = \sum i \frac{\sum h\,|scan_i - scan_{i-1}|}{H}$$

with H as the total height of the cell. The higher the TSI, the higher was the instability of the emulsion.

Emulsifying index

The emulsifying index is the ratio between the volume of the emulsion layer and the volume of the whole sample. In the present study, the EI was calculated by measuring the height of the cell containing the emulsion layer (showing the backscattering peak) vs. the time from the Turbiscan profile. The emulsifying index was calculated as follows:

$$EI = \frac{Height\ of\ BS\ peak\ in\ the\ cell}{Total\ height\ of\ sample\ in\ the\ cell\ (12\ mm)}$$

Results and discussions

As described in detail in material and methods, three different emulsion systems were investigated based on the same waxy maize starch; OSA modified starch granules based emulsions (SGE); OSA modified dissolved starch based emulsions (DSE); and OSA modified non-solvent precipitate starch based emulsions (NPSE). SGE is a Pickering emulsion based on waxy maize granules that has a size 5-18 µm with average size around 15 µm [30] while DSE is a polymer stabilised emulsion that predominately contains amylopectin with a radius of gyration of around 250 nm [38]. The structure of the NPS is not yet fully understood but previous studies shows that it contains a broad range of different particles including some larger micro-particles; however, the bulk of the material is small, with an average radius of gyration of 260 nm as measured by AF4. In some cases, we have also studied the behaviour of MCT oil and dispersions or solutions of the starch emulsifier. This is done to understand how these components effect the measurements.

The macroscopic appearance of these emulsions at day 1 is seen in FIG 1. As can be seen SGE phase separates while the DSE and NPSE gives emulsions that are space filled. The phase separated SGE shows both creaming and sedimentation and this is because large oil droplets will cream while small droplets covered with starch granules and granules themselves will sediment. The degree of phase separation can be followed using multiple light scattering that also determined EI. There was a significant difference in the EI values, which showed the highest values for non-solvent precipitate (0.92), followed by dissolved starch (0.88), and the least for granule (0.47). As can be seen in Table 1 the EI decreases fastest for SGE and slowest for NPSE. In the case of SGE, there were also observed an oil layer on top of the emulsion layer already after 24 hours. This was not observed for the other emulsions. There are also according to student t-test a significant difference between the droplet sizes (Table 1) for the three emulsions and this difference can of course to some extent explain the difference in the macroscopic behaviour of the emulsions such as a higher tendency for the larger droplets of the SGE to cream.

The TSI-value (Table 1) is a measurement that includes all type of changes in a dispersion that changes the scattering profile. Thus, in our case it includes both creaming, sedimentation and coalescence. The higher the TSI the less stable is the emulsions. The TSI values is in line with the other results showing that NPSE are the most stable system and SGE is the most unstable one. As expected it also increases with time.

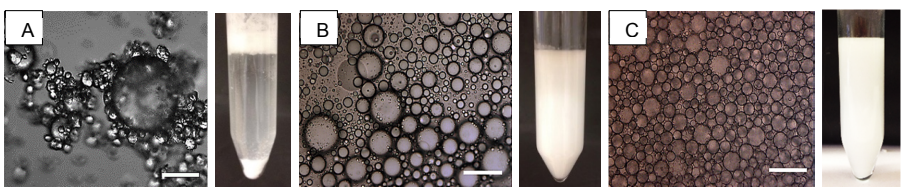

FIG 1. Macroscopic and microscope images 20x magnification, bar scale: 50 µm appearances of the emulsions at day 1 (A) SGE, (B) DSE, and (C) NPSE.

Table 1. Emulsion properties; size measured by static light scattering in terms of mean volume diameter $\overline{d_{4,3}}$ (n=3), median value of size distribution $d_{v,\,0.5}$ (n=3), emulsifying index (EI n=2), and Turbiscan stability index (TSI n=2). The values marked with * is according to student t-test significantly different from the previous time point with a 90% confidentiality.

Emulsions	Days	$\overline{d_{4,3}}$ (µm)	$d_{v,\,0.5}$(µm)	ΔBS (%)	EI	TSI
SGE	0	45.8 ± 7.9	26.0 ± 4.1	11.0	0.47±0.00	-
	1	74.0 ± 10*	56.4 ± 20.0	13.6	0.27±0.02	30.0±3.2
DSE	0	18.9 ± 5.3	15.2 ± 0.9	27.0	0.88±0.02	-
	1	20.2 ± 3.1	16.4 ± 1.0	45.4	0.85±0.04	7.5±7.0
	7	22.9 ± 6.0	17.2 ± 0.7	45.4	0.70±0.01*	14.2±2.5
	14	27.2 ± 6.0	17.7 ± 1.1	49.3	0.63±0.01*	16.2±0.6
	21	32.6 ± 4.2	18.3 ± 0.9	50.2	0.58±0.00*	17.8±0.8
	28	42.8 ± 7.0*	24.8 ± 6.1*	49.8	0.51±0.04*	18.8±1.5
	35	51.0 ± 4.0*	37.2 ± 4.0*	49.9	0.45±0.01*	19.5±1.6
	42	86.4 ± 0.6*	83.1 ± 1.0*	51.0	0.42±0.01*	19.8±1.3
	365	55.6 ± 10.2*	42.0 ± 19.0*	59.9	0.35±0.10	–
NPSE	0	10.6 ± 0.6	9.3 ± 0.7	39.4	0.92±0.01	-
	1	10.4 ± 0.8	9.0 ± 0.9	47.4	0.88±0.02*	5.7±7.0
	7	16.3 ± 4.2*	9.2 ± 1.0	49.6	0.76±0.01*	8.4±3.4
	14	19.1 ± 2.4	7.9 ± 1.5	53.0	0.75±0.01	12.5±0.6
	21	21.2 ± 5.0	7.3 ± 1.5	52.0	0.70±0.00*	14.9±0.4
	28	23.2 ± 9.6	6.7 ± 1.6	52.3	0.67±0.01*	15.8±0.4
	35	27.8 ± 8.0	5.7 ± 2.1	54.5	0.63±0.01*	16.5±0.6
	42	39.8 ± 10.3	8.3 ± 3.5	53.4	0.63±0.01	17.0±1.0
	365	48.9 ± 10.7	36.1 ± 16	44.6*	0.47±0.00*	–
MCT oil	0	123.8 ± 15.4	117.2 ± 14.5	–	–	–

Changes in droplet size over time as measured by static light scattering

In order to understand how the emulsions changed with time, the changes in size distribution as measured by static light scattering (FIG 2), including mean volume diameter $\overline{d_{4,3}}$ and median size $d_{v,\,0.5}$ (Table 1) was determined. The SGE system showed a significant increase in $\overline{d_{4,3}}$, from an initial value of 45 µm to 74 µm after the first 24 hours (Table 1), while initially there were no significant changes in the DSE or NPSE emulsions. However, as time progressed there were a gradual increase in measured $\overline{d_{4,3}}$ also for these systems. The main reason for changes in $\overline{d_{4,3}}$ is most likely coalescence of emulsion droplets. The solubility of MCT oil in water is quite low thus it is not likely that the main destabilising mechanism is Ostwald ripening. The DSE system shows significant change in $\overline{d_{4,3}}$ between day 21 and 28, but as can be seen the increase is gradual over the interior time span. The $\overline{d_{4,3}}$ for the NPSE also increases gradually but apart from day 7 the change between adjacent time points are not significant. However, over time it amounts to a significant change. Interesting to note is that $\overline{d_{4,3}}$ and $d_{v,\,0.5}$ is quite similar for SGE and DSE systems indicating that the particle size for these systems are close to normal distributed while for the NPSE these two values are not the same especially at later time points.

The change in particle size over time is shown in more detail in the cumulative graph distributions (FIG 2). As a comparison, the static light scattering of pure MCT oil as it is pumped through the particle size analyser is also shown in FIG 2. Due to the mechanical treatment during analyses, oil droplets will be formed also for free oil. The droplet size of MCT oil in buffer is around 90–140 µm, with average size of 123 ± 15 µm (Table 1). Thus, if the emulsions show values above 90 µm, this could be either large droplets—for example, obtained through coalescence, or aggregates of droplets—or free oil. For SGE, the measurements were terminated after only 24 hours as all parameters, indicated a relatively rapid coalescence of the system. Visual observations as well as the cumulative graph (FIG 2 A) indicates that there already at day 1 was some free oil in the emulsion. The instability of the system could have been due to low coverage of starch granules at the emulsion interface, which is in line with microscopy observations where large amounts of free starch were seen in the solution (FIG 1).

For DSE, the median droplet size was nearly stagnant (around 15-18 µm) until day 28, after which there was a significant increase to 85 µm. After 28 days, there was also, as seen in FIG 2B, an increase in the number of droplets above 90 µm. This is further increased after day 35, indicating that this probably is the stability limit for this emulsion. The one-year data again showed a high fraction of small particles; this change could however be due to starch particles formed through retrogradation, as discussed below. The change in the curves indicated a slow coalescence of the droplets, but visual observation revealed little free oil on top of the sample at the beginning of the study, but oiling-off occurred to some degree for the later time points. These findings indicate that even if the emulsions stabilised by dissolved starch is more stable than the granule-stabilised emulsions, coalescence is still occurring. The results are well in line with the results of Sweedman *et al* who showed that waxy maize give good long term stability to emulsion [17].

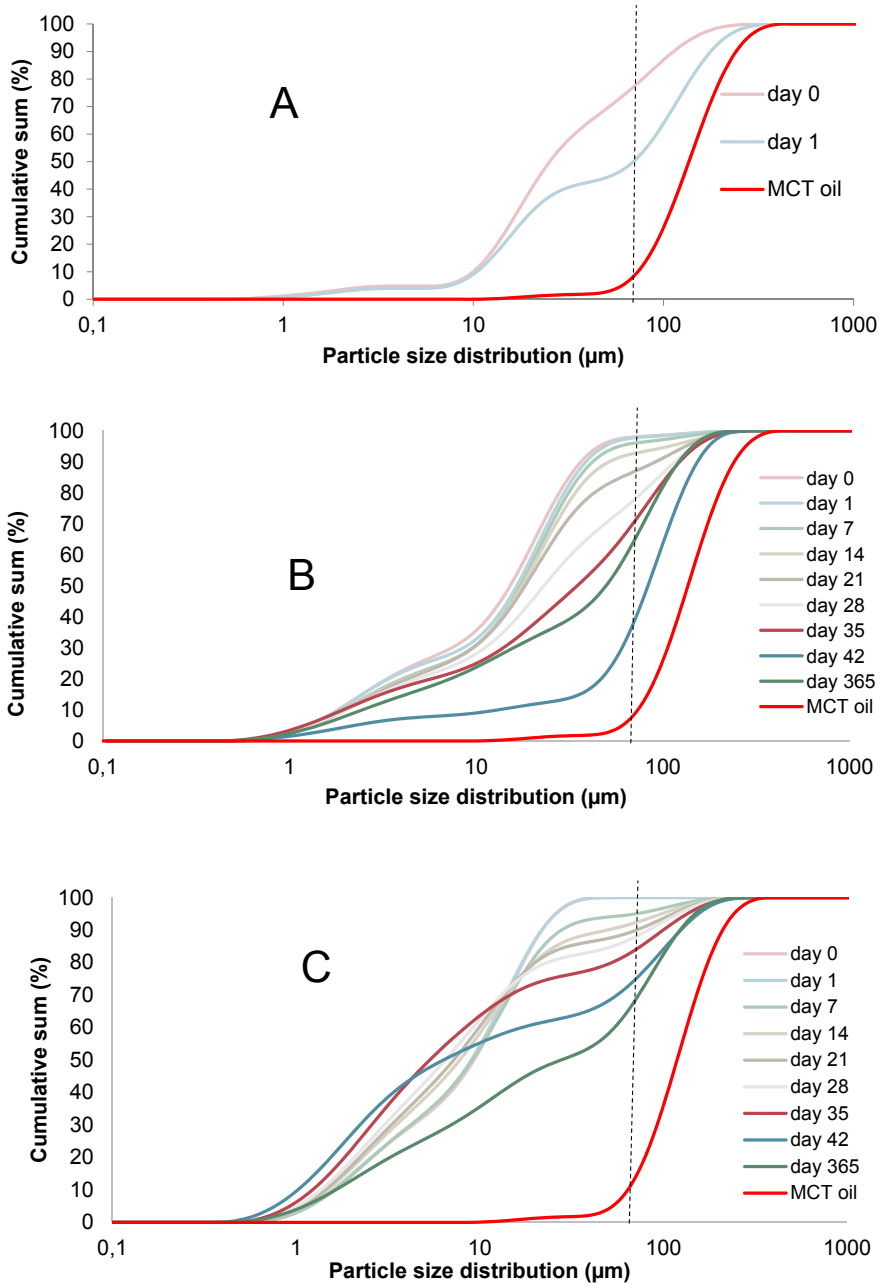

FIG 2. Size distribution of emulsions stabilised by (A) granules, SGE (day 0 to day 1), (B) dissolved starch, DSE (day 0 to day 365), and (C) precipitated starch, NPSE (day 0 to day 365).

For NPSE, the cumulative graph distributions (FIG 2C), show that emulsion droplets stabilised by non-solvent precipitated starch had a median droplet size of around 6–9 μm for 42 days. Thus, in difference to DSE these emulsions did not show any significant change in median droplet size for this time period. However, the number of droplets above 90 μm increased more rapidly for NPSE than for the DSE, and a gradual increase in this fraction of droplets was seen during the first 42 days, from 0% to around 40%. As can be seen from FIG 2C, the overall droplet size distribution is not shifting towards larger droplets instead it seems as if it is the fraction of droplets above 10 μm that is coalescing. Thus, there is a clear difference in the coalescence pattern between non-solvent precipitate starch and dissolved starch. As discussed previously non-solvent precipitated starch has a wide population of particles, from nanoparticles to micron-size particles. It is likely that this wide distribution also translates to a wide population of emulsion droplets stabilised by different entities of the precipitated starch. It can be speculated that some of these droplets are more stable than others towards coalescence and thus are the ones that make up the non-changing fraction of the emulsions, while droplets predominantly covered by other fractions of the non-solvent precipitate—for example, dissolved starch—are more susceptible to coalescence. Another explanation for the two populations seen could be that droplets that are larger have creamed to the top of the emulsions and the higher density of droplets in this region promoted coalescence. Finally, it was seen that even after 1 year of storage, this emulsion had a large fraction of small droplets.

Multiple light scatter measurements

The static multiple light scattering was measured by Turbiscan Lab® Expert and the changes in backscattering during the first two hours is shown in FIG 3, while the long term stability investigations is shown in FIG 4.

To better understand the results for the emulsions, multiple light scattering data were also obtained for samples containing only the starch emulsifiers (FIG 3B-3E) and for oil alone (FIG 3A). In the case of oil alone, the backscattering is low over the whole height of the cell but showing a small "bump" at the top indicative of the free oil. For the dispersed granules (FIG 3B) showed high initial values in backscattering (in the range of 2–15%) at the bottom of the cell (from 1–2 mm), indicating that sedimentation had already occurred from the beginning for the samples in this region.

When fresh dissolved OSA-modified starch was investigated (FIG 3C), backscattering decreased at the bottom of the cell (0–4 mm), indicating that clarification had occurred at the bottom. However, backscattering increased in the middle of the cell (4–8 mm), most likely owing to aggregation of the molecules. Initially the system did not change with time. The solution of dissolved starch was measured again after year of storage (FIG 3D). It had a similar profile to that of the fresh sample, but in this case, changes in backscattering were seen during the time of the measurement. The changes may well have been due to continuous aggregation of particles in the solution. The likely explanation for the formation of aggregates that are large enough to scatter light is that the dissolved starch molecules recrystallize in solution, a process referred to as retrogradation. During cooling and storage, both amylose and amylopectin crystalizes in solution [40, 41] but the rate of crystallization for amylose is fast within a couple of hours or days, in contrast amylopectin crystallization is slow occurring after a time span of as much as 30–40 days. Since waxy maize, which mostly contains an amylopectin fraction, the difference between the fresh sample and the stored sample could be attributed to long-term crystallization of the amylopectin fraction, which could then lead to long-term structural changes [40, 42, 43].

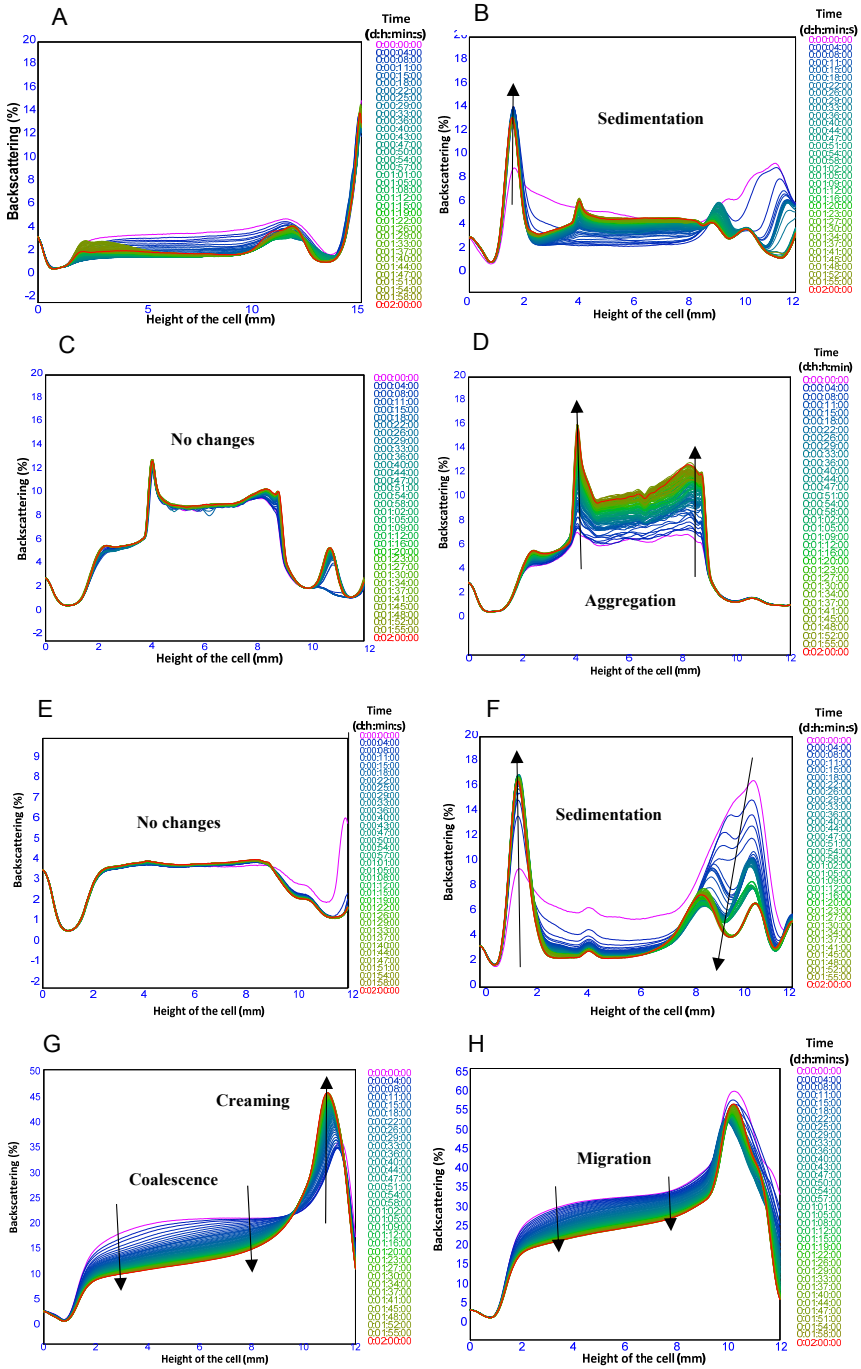

FIG 3. Turbiscan profiles for (A) MCT oil profile as reference, starches in buffer: (B) OSA –modified granules, (C) dissolved starch at day 0, (D) dissolved starched (DS) after 1 year, and (E) Non-solvent precipitated starch (NPS); and starch based emulsions samples:(F) SGE, (G) DSE, (H) NPSE.

The backscattering profiles of samples containing only non-solvent precipitated starch (NPS) showed that there were very small changes in the profile (FIG 3E). This indicates that the particles obtained with non-solvent precipitation are small enough to be subjected to Brownian motion and could thus stay suspended, not resulting in any clarification zones in the sample. Interesting to note is that they did not show any major changes over time that could be linked to crystallisation as was seen for the dissolved starch. Indicating that the non-solvent precipitation creates particles or starch molecules that differ in properties from the dissolved starch.

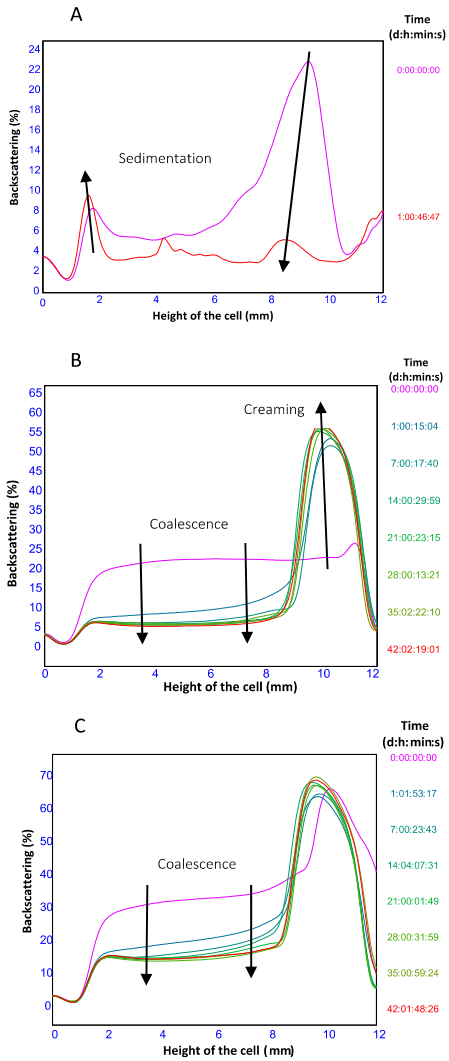

FIG 4. Turbiscan profiles for starch-stabilised emulsions: (A) SGE after 1 day, (B) DSE, and (C) NSPE from 0 to 6 weeks.

In terms of stability, SGE were very unstable, as seen in the backscattering profiles (FIG 3F and FIG 4A). At the top of the cell for SGE (FIG 3F), larger backscattering values (around 16%) were obtained at the beginning of the monitoring time resulting from the creaming of the emulsion droplets and in the bottom of the cell, high initial values in backscattering (in the range of 8–16%). In the latter case, the sedimentation may have resulted from the presence of free starch granules in the continuous phase. This process lasted until the end of the monitoring time, and even an increase in backscattering in the middle part of the cell (4–8 mm) was seen because of the presence of either starch or emulsion sediment. Similar behaviour had been obtained with starch Pickering emulsions in previous studies [6]. The cream layer on the other hand becomes thinner, with time and the appearance of a free oil layer, which is another indication of the low stability of these emulsions (FIG 4A).

The backscattering profiles obtained for the DSE (FIG 3G and FIG 4B) show large variations over time in the middle part of the cell as a result of a combination of both clarification and creaming processes. Creaming was noticed at the top resulting from droplet migration promoted by density differences between the oil droplets and the continuous phase, which explains the large changes in backscattering from 27% to 51% (Table 1).

The backscattering profiles of the non-solvent precipitate emulsion, NPSE (FIG 3H and FIG 4C) showed a similar trend as for DSE, with similar variations in the middle of the cell. These variations were promoted by a clarification process that took place at the bottom/middle of the cell accompanied by simultaneous creaming at the top. However, the change in the middle of the cell related to coalescence was slower than for DSE.

Based on the results above, a comparison of the three forms of starch can be made. The stability of the emulsion systems seems to increase in the order of SGE << DSE < NPSE. This was true for stability towards both creaming and coalescence. The SGE showed clear phase separation and release of oil after 24 hours, while the emulsions stabilised by dissolved starch (DSE) were stable in this respect for 6 weeks and the emulsions based on precipitated starch (NPSE) were stable for at least one year.

Since all the particles studied originated from the same starting material, the difference in the structure of the emulsifiers is most likely what gives the difference in emulsion stability. Especially, the difference in OSA-modification that is slightly higher for starch granules (3%) than for non-solvent precipitate starch (2%), it would be expected to promote smaller droplet sizes for the SGE systems compared to the NPSE, however it is not the case as seen from Table 1. Thus, the likely key properties that effect emulsion stability are size and morphology of the emulsifier. The most likely reason for the granules' low stability is that the particle size is not small enough to create small emulsion droplets, as indicated by the droplet size of the initial emulsion, and as seen in both the rapid creaming and the low stability towards coalescence. It is also obvious that the affinity of the starch granules for the oil/water interface is low, as can be seen in the high amount of free starch in the samples.

The dissolved starch (DS) and the non-solvent precipitate (NPS) are in the same size range, but obviously, the stability of the two emulsifiers differs from each other. Dissolved OSA waxy starch has been seen by other researchers to be a good emulsifier, which they have attributed to the adsorbed amylopectin inducing steric repulsion at the interface, as well as increased viscosity in the solution that to some extent hinders creaming [21, 22, 24, 44]. These previous observations are in line with our results, but as mentioned, we saw a loss in stability after 35 days, which we have attributed to slow retrogradation of the dissolved amylopectin. Retrogradation is a common phenomenon for dissolved starch [40, 42, 43] and is driven by the formation of starch double helixes that are further aggregated into ordered structures that lead to the formation of semi-crystalline arrays of these helixes [45]. Retrogradation can then in turn lead to the formation of

aggregates, and the reduction of free OSA starch can probably decrease the amount of starch available to stabilise the emulsion.

The lack of aggregates seen in the Turbiscan profile for the non-solvent precipitation (NPS) compared with the profile of the dissolved starch (DS) indicates that the NPS at least affects the tendency for any free amylopectin to later retrograde and form new aggregates in solution. This could be one of the reasons that non-solvent precipitate is stable for a longer time than dissolved starch. The fact that the NPSE has a smaller initial droplet size than DSE indicates either that NPS contains smaller particles than the dissolved starch and thus can cover a larger surface area, or that it has higher affinity to the surface. One further explanation could be that the particles do not have to be closely packed at the surface in order to stabilise the emulsion. It has previously been seen that for the Pickering type of emulsions, the stability can be obtained below full surface coverage [8]. However, for this to work, we probably need preferential adsorption of larger particles or aggregates of particles at the oil/water interface. The transport of molecules and particles to an interface can be either through diffusion or through convection; when convection dominates, it has been shown that the larger molecules are preferentially adsorbed to the interface [46, 47]. In the system used here, the larger particles could be in a size range of 1μm where they are transported through convection, and the presence of these larger particles could help to stabilise the emulsion. When large particles have been adsorbed to the interface, it is highly unlikely that they will desorb [2]. Thus, it is likely that the larger particles from the NPS will be preferentially adsorbed and the droplets that have enough of these particles will be more stable than, for example, drops only covered by dissolved amylopectin. This situation could explain the coalescence profile for the NPSE, since two fractions of droplets appear to exist: small droplets that are very stable over time and slightly larger droplets that appear to coalesce with time.

Conclusion

For emulsions using different types of waxy maize–based emulsifiers—granules, dissolved starch, and precipitated starch—it was found that the differences in the size and structure of the emulsifiers influenced the resulting emulsion droplet size and stability. The decrease in droplet size and the increase in stability towards coalescence and creaming were found to follow the order granules (largest, least stable), dissolved starch, and non-solvent precipitated starch (smallest most stable). If we take the formation of a substantial number of droplets that are larger than those seen in emulsions formed without emulsifiers as an indication of stability, the stability for emulsions using OSA starch-based emulsifiers was 2 days for granules, 35 days for dissolved starch, and more than 42 days and up to a year for starch non-solvent precipitates. Based on the droplet size, size distribution, microscopic images, and Turbiscan stability, the stabilisation of emulsion is clearly shown to be the best for starch non-solvent precipitates, followed by dissolved starch, and finally granules. Therefore, this study revealed the high potential of starch non-solvent precipitates as emulsifiers.

Acknowledgment

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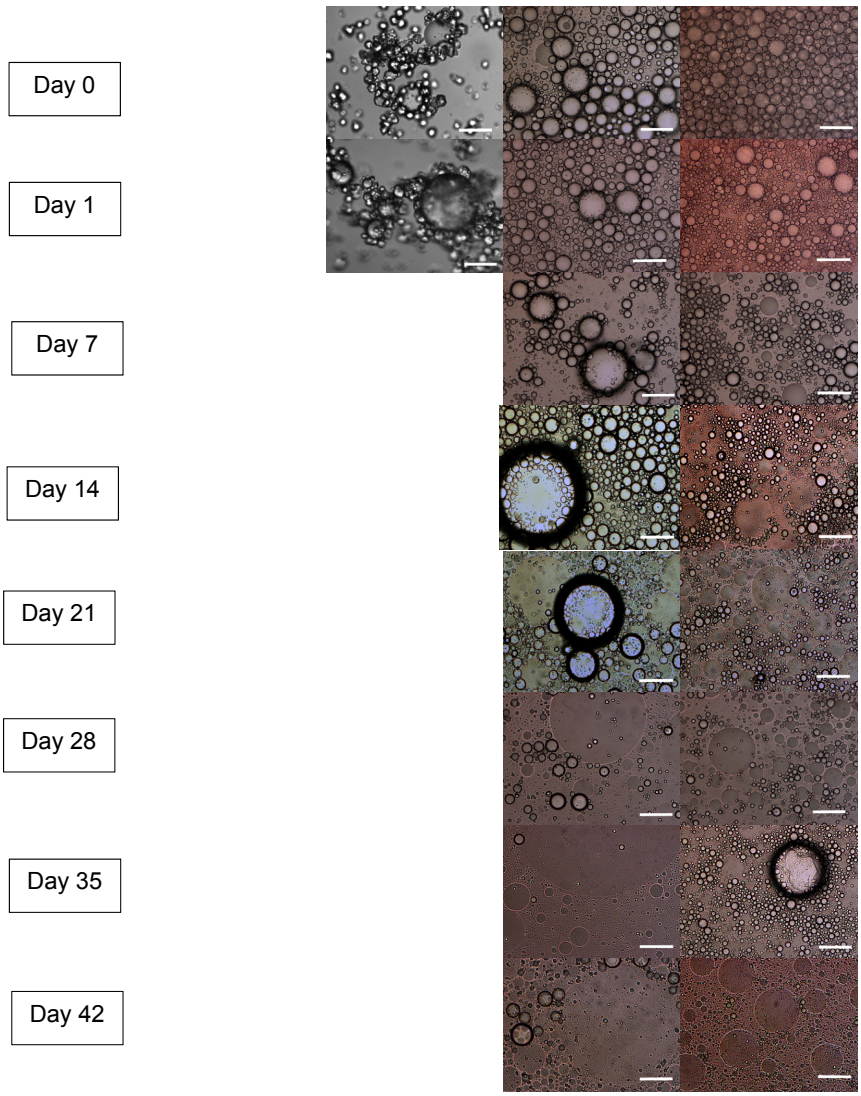
Supporting information

S1 FIG. Microscope images of emulsions.



Paper VI

Characterization of non-solvent precipitated starch using AF4 coupled with multiple detectors

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Abstract

The aim of this paper was to investigate size and molecular conformation of non-solvent precipitated starch. The non-solvent precipitated starch is a novel component for starch based emulsions. Previous studies have shown that the non-solvent precipitated starch gives particles in the nano-size range and one key question is if the starch structures obtained are different from dissolved amylopectin. Non-solvent precipitated starch was produced using ethanol precipitation of waxy maize starch granules. The granules were either unmodified (WMNSP) or acid hydrolysed (AHNSP). The non-solvent precipitated starch was also modified with 3% (w/w) OSA to increase the hydrophobicity (OSAWMNSP). The molar mass and conformation of the non-solvent precipitated starch was investigated using asymmetric flow field-flow fractionation (AF4). Prior to AF4 measurements, the non-solvent precipitated starches were re-dispersed in DMSO, aqueous solution at 100°C and aqueous solution at room temperature. This was in order to study possible changes in the size, molar mass, apparent density and conformation induced by different dissolution methods. The results show that molar mass, size and density depend on the type of starch samples studied with the molar mass decreasing in the order of WMNSP>OSAWMNSP>AHNSP. It was also showed that the acid hydrolyzed and OSA-modified samples where most likely dissolved after 1 hour of dissolution in water at room temperature while the non-acid hydrolyzed waxy maize sample still have a different AF4 profile compared to the same material dissolved in DMSO. The results show that we observe different conformation between the samples when they are re-solubilized in aqueous solution at room temperature.

Keywords: starch nanoparticles, size, molar mass, conformation

Introduction

Starch is the principal carbohydrate reserve in plants. It is a major source of energy in human diet, animal feed and has many applications in industry. It often contributes to characteristic properties of foods, and is added as a functional ingredient in many products. Non-solvent precipitation of dissolved starch has been claimed to generate starch nanoparticles. Such particles could have several interesting uses, one of these are as stabilizer of Pickering emulsions. In a previous study, we have investigated the stabilization of emulsions using octenyl succinic anhydride (OSA)-modified non-solvent precipitated waxy maize starch and compered this to OSA modified dissolved starch and granules [1]. It was seen that the non-solvent precipitated starch gave emulsions that were more stable towards coalescence than OSA-modified dissolved starch and granules. The question is to what extent especially the non-solvent precipitated starch and the dissolved starch differs. This is one of the main question for the study presented in this paper.

There is a large interest in producing nanostructures from starch as starch is a green renewable source of material and, as described in some recent reviews, there are several methods to obtain starch nanoparticles and the method used will affect the structure of the obtained particles for example if they are crystalline or amorphous [2, 3]. Nano starch can be prepared principally in three different ways: i) hydrolysis, either acidic or enzymatic, leads to nanocrystals, ii) from solution through a precipitation step which can give both amorphous and crystalline particles and iii) mechanical treatment such as microfluidizer leads to so called nano-colloids [4]. The method used in this study is non-solvent precipitation by mixing a starch water solution with a non-solvent in this case ethanol [1]. To obtain starch non-solvent precipitated starch the first step consists on dissolving the starch granules completely. During this process, the granules are disintegrating and inter and intramolecular hydrogen bonds are disrupted thereby destroying the crystalline structure of the granule and leaving only the amorphous region. Then new smaller particles are re-created by precipitation through the addition of a non-solvent such as ethanol. There has been extensive work done on methods to produce non-solvent precipitated starch or as also commonly describes as nanoprecipitation [5-13]. The size range reported for these starch nanoparticles are around 10-400 nm. The obtained structures reported both include crystalline and amorphous particles. The size range reported is interesting since the hydrodynamic radius of amylopectin from waxy maize starch is in the range of 140-175 nm depending on method for characterisation and dissolution media and method [14, 15].

AF4 has been used to determine the size, molar mass and conformation of the starch nanoparticles (NPs) during their preparation process [16-18] as well as to determine the molar mass and size of starch [14, 19, 20]. As mention above, most of the research work was focused on nano-starch characterization as final product in dried samples or during preparation; however, there is a lack of information about the behaviour of starch NPs when they are re-dispersed in an aqueous environment. Therefore, the present study has the purpose to investigate the effect on size, molar mass, apparent density (ρ_{app}) and conformation of different re–solubilized procedures for starch nanoparticles (NPs) using AF4 couplepd with multiple detectors.

Material and methods

Materials

Waxy maize (WM) starch was supplied by Lyckeby Culinar AB (Lyckeby Culinar AB, Malmö, Sweden), n-octenyl succinic anhydride (OSA) from Trigon Chemie (TRIGON ChemieGmbH, Hesse, Germany), dimethyl sulfoxide from VWR chemicals (DMSO ≥ 99.5%,VWR, Leuven, Belgium), ethanol from Sigma Aldrich (C2H5OH 99%, Sigma Aldrich, MO, USA), NaOH from Merk (Frankfurter, Germany), HCl from VWR chemicals (Leuven, Belgium), NaNO3 from AppliChem (A3125, Darmstadt, Germany) and NaN3 from BDH (10369, Poole, UK).

Production of precipitate starch nanoparticle

The experiments were performed on three different types of starch NPs shown in Table 1.

Table 1 Overview of samples

Samples	Sample ID
Acid Hydrolysed non-solvent Precipitate	**AHNSP**
Waxy Maize non-solvent precipitate	**WMNSP**
OSA Modified Waxy Maize non-solvent Precipitate	**OSAWMNSP**

The WMNSP sample was produced using an optimized method developed previously [1]. The method can be briefly summarized as follows: WM starch was dispersed in Milli-Q water, with a concentration of 8 mg/mL and autoclaving at 140 °C for 20 min in the presence of N2 in a laboratory autoclave (Roth Model II, 200ml, Karlsruhe, Germany) coupled with a temperature control unit. In order to precipitate the starch, ethanol was added in a ratio of 1:1 and mixed. The particles were obtained by batch-wise centrifugation at 2000 g for 10 min, and all batches were pooled. The collected particles were left in the fume hood overnight to allow the ethanol to evaporate completely. The precipitated starches were then frozen at -20 °C overnight, and freeze-dried in a Hetosicc freeze-dryer (Heto, Birkerød, Denmark), over a period of 4 - 5 days.

The AHNSP sample was prepared in similar as above with the addition of an acid pre-treatment. The procedure was performed as described elsewhere [21] with slight modifications. 50 g of WM starch was dispersed in 250 ml of HCl (1 M) with continuous stirring (200 rpm) at 40 °C for 120 minutes. The dispersion was neutralized to pH 5.5 with NaOH (1 M). The suspension was washed three times with Milli-Q water followed by centrifugation at 3500 rpm for 10 min. The precipitated solids were air-dried at room temperature for 3 days.

In order to produce an OSAWMNSP sample, a portion of the wet WMNSP obtained as described above, was modified with OSA. Modification was performed using 3% (w/w) OSA based on the dry weight of the starch WMNSP and the degree of OSA substitution was analysed. The procedure used for the modification was described previously [22]. The method can be briefly summarized as follows: WM starch was dispersed with continues stirring in 1.5 times of the distilled water (w/w) based on dry weight starch. The pH was keep between 7.4 - 7.8 and adjusted with HCl (1M), or NaOH (1M). Then OSA (Ziyun Chemicals, China) was added in four portions with a 15 minutes delay between each portion. The titration was terminated when the pH was stable for at least 15 min. The suspension was then, centrifuged at 3500 rpm for 10 min. The

supernatant was discarded and washed, once with citric acid (pH 4.5–5), and once more with distilled water. The starch was freeze-dried.

The degree of OSA substitution was carried out in duplicate as is described as follow: 2.5 g of OSAWMNSP and a control sample were weight and wetted with few drops of ethanol then 25 mL of HCl (1M) was added and stirred for 30 min. The suspension was centrifuged at 3500 rpm for 10 min. The supernatant was discarded and washed with 25 mL of ethanol once and twice with distilled water. The residue was dissolved in 150 mL distilled water, heated in a bath at 90 °C for 10 min, and then rapidly cooled to 25 °C in an ice bath. The suspension was titrated while stirring with NaOH (0.1M) until pH 8.2. The volume of NaOH was used for calculation of % OSA substitution using the follow equation.

$$Degree\ of\ OSA\ (\%) = \frac{(Vsample - Vcontrol)\cdot M\cdot 210}{w}\cdot 100 \qquad (1)$$

Where V is volume of NaOH, M is concentration of NaOH, 210 is the molar mass (g/mol) of octenyl succinate anhydride group (OSA) and w is the dry weight of sample. The level of OSA substitution was about 2.0 %.

Chemical analysis of starch nanoparticle samples

Moisture content was determined using a moisture analyzer (MAC 110/WH, Radwag, Radom, Poland). Total starch content was determined enzymatically using a commercial kit for total starch analysis (Megazyme amyloglucosidase/ α-amylase, Megazyme International, Bray, Ireland) subtracting the free glucose value.

Total protein content was determined by measuring nitrogen content using the elemental analyzer Flash EA 1112 N (Thermo Fisher Scientific, Waltham, MA, USA). The sample (25 - 32 mg) was combusted in a sealed furnace and the nitrogen content was determined with thermal conductivity detection. Aspartic acid was used as standard. Nitrogen to protein factor of 6.25 was used in the calculations. Each analysis was made in triplicate.

Thermal properties

The thermal properties were determined by using differential scanning calorimetry (DSC 6200, Seiko instruments Inc., Japan). The samples (20 mg) were weighed into a small tube, and MilliQ water (60 µL) was added in a 1:3 (w/w) ratio and left for 1 hour to equilibrate. Approx. 8 mg of these mixtures were weighed into an aluminium pan and lid (TA Instruments, Newcastle, Delaware, USA) using a micro balance (CAHN, C-30, Samotronic Microbalance, USA). The pan was hermetically sealed. Analyses were performed over a temperature range of 10 – 160 °C with a scanning rate of 10 °C/min with an empty sealed pan as reference. Thermo-gravimetric curves were recorded on the Exstar 6000 Thermal Analyses System equipped with the Muse Standard Analysis software, version 6.4 (SII Nano Technology Inc., Chiba, Japan).

Samples preparation for AF4

The samples were treated in three different ways i) dissolution at 100 °C in DMSO, ii) dissolution through boiling in aqueous media (carrier liquid) and iii) dispersion at room temperature in aqueous media (carrier liquid).

The samples in DMSO were prepared as described elsewhere [19] with modifications from [23]. When using aqueous media, samples (1 mg/mL) were heated at 100 °C (boiling) for 1 hour with continuous stirring. The aqueous sample solutions at room temperature were obtained by dispersing the sample (1 mg/mL) for 1 hour with continuous stirring. All samples, including the DMSO dissolved sample, were diluted to a final concentration of 0.25 g/mL using the aqueous media. The composition of aqueous solution was 10mM NaNO3 and 0.02 % NaN3 dissolved in Milli–Q water.

In addition, the filtration effect before injected the sample onto the AF4 system, was investigated using cellulose acetate filters (VWR Syringe Filter, 25 mm diameter, Leuven, Belgium) with different pore sizes: 1.2, 0.45, 0.2 and 0.1 µm. The radius, molar mass, apparent density and mass recovery, were investigated by injecting the unfiltered sample

AF4-MALS-dRI and online DLS

The samples were separated using an AF4 system (Eclipse 3+, Wyatt Technology, Dernbach, Germany) and characterized using online multiangle light scattering (MALS, Dawn Heleos II, Wyatt Technology) and differential refractive index (dRI) detection (Wyatt Technology). Both detectors operated at a wavelength of 658 nm.

An Agilent 1100 series isocratic pump (Agilent Technologies, Waldbronn, Germany) with an in-line vacuum degasser and an Agilent 1100 series auto-sampler delivered the carrier flow and handled sample injection onto the AF4 separation channel. Between the pump and the channel, a 100 nm pore size polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA) was placed to ensure that particle-free carrier entered the separation channel.

The AF4 channel was a short channel (Wyatt Technology, Dernbach, Germany) with trapezoidal geometry (tip-to-tip length of 17.4 cm and inlet and outlet widths of 2.17 and 0.37 cm, respectively) and with a nominal thickness of 350 µm. The ultra-filtration membrane forming the accumulation wall was made of regenerated cellulose (RC) with a cut-off of 10 kDa (Merck KGaA, Darmstadt, Germany).

Validation of the performance of the AF4 system and experimental determination of the channel thickness was carried out with bromophenol blue (BPB) and bovine serum albumin (BSA) (Sigma, A4378, St, Lois, MO, USA) (1 mg/mL, w/v) according to the procedure described in literature [24], using a MATLAB-based software (FFFHydRad 2.2) [25, 26]. The channel height was experimentally determined to 266 µm.

The separation method applied used a constant detector flow of 1 mL/min. Injection onto the channel was performed with a flow rate of 0.2 mL/min for 6 min. The sample volume injected onto the channel was between 5 - 80 µl for an injected sample mass of approximately 1.25 – 20 µg. The injected amount was optimized in order to ensure no overloading occurred i.e. retention time independence of the injected amount. After injection, a 4 minutes focusing/relaxation step prior to elution with a focus flow identical to the initial cross flow was performed. The cross-flow rate was programmed to decay exponentially using the following equation

$$Q_c(t) = Q_c(0)e^{\left(-\frac{ln2}{t_{1/2}}t\right)} \quad (2)$$

where $Q_c\ (t)$ is the crossflow rate as a function of time t after elution starts, $Q_c\ (0)$ is the initial crossflow rate and t1/2 is the half-life of the decay. For all samples, the elution started with an initial cross-flow of 2.5 mL/min and decreased exponentially over time to 0.13 mL/min, with t1/2

= 4 min, and then kept constant for 22 min. Finally, the channel was flushed without any cross-flow for 11 min before the next analysis.

Data was recorded using Astra software version 6.1.5.22 (Wyatt Technology). Molar mass (M) and root mean square radius (r_{rms}) were obtained using the Berry method [27, 28] by performing a 1st order fit to the data obtained from the scattering detectors 8 – 17 (angles 60.0° - 152.5°), second virial coefficient (A2) was neglected. The specific refractive index (dn/dc) of 0.146 was used for amylopectin in water [29]. The apparent density (ρ_{app}) were calculated from M and rrms [30], whereas the radius of hydration (r_h) was calculated using the MATLAB add-on FFFHydRad 2.2.

In order to run the samples with an online DLS detector, an Eclipse 2 separation system (Wyatt Technology) was used coupled with a DAWN EOS MALS detector (Wyatt technology) operating at the wavelength of 690 nm. The MALS detector 13 (i.e. 110.7°) was connected to the DLS apparatus (DynaPro NanoStar, Wyatt Technology) via a glass fibre. For this experiment, the channel height was determined to 258 μm.

Results and discussion

Chemical analysis of starch nanoparticle samples

The results from moisture content, total starch and total protein analyses are shown in Table 2. The moisture content in all samples were similar (11 - 13%). Total starch content ranges between 91 and 95%, with sample WMNSP having the highest value and WM the lowest. It is possible that, due to the addition of ethanol into the WM solution during the precipitation step, impurities were removed which could be reflected in slightly a higher starch content compared to the WM sample. In the case of AHNSP, the hydrolysis applied could affect the total starch content due to the formation of low molecular weight sugars and this could as discussed later also be the case for OSAWMNSP. Another reason for a lower starch content in OSAWMNSP sample is that OSA modification as the substituents could interfere somewhat with the enzymatic degradation to glucose. The protein content of starch NPs was similar (0.12 - 0.15%), but a bit higher than for WM. These results agree with the suggestion above that some impurities are removed during the precipitation step.

Table 2 Results obtained for moisture content, total starch, total protein

Sample	Moisture (%)	Total starch (%)	Total protein (%)
AHNSP	13.1 ± 0.7	93.7 ± 0.7	0.13 ± 0.00
WMNSP	12.9 ± 0.9	95.1 ± 0.6	0.12 ± 0.00
OSAWMNSP	12.1 ± 1.1	89.9 ± 0.7	0.15 ± 0.01
WM	11.1 ± 1.6	90.9 ± 1.4	0.07 ± 0.03

[a] Values reported on dry basis, ± standard error of the mean

Thermal transition properties

The results of the DSC analysis for WM sample were as follows: onset temperature (T_o) = 64.1 ± 1°C, peak temperature (T_p) = 70.0 ± 0.5°C, conclusion temperature (T_c) = 75.6 ± 1.4°C and

enthalpy (ΔH) 14.9 ± 2.0mJ/mg. Starch NPs did not show any transition in the endothermic curve. Accordingly, no crystallinity was detectable which means granule disruption is complete and that the non-solvent precipitation does not induce any major formation of starch crystals [31, 32]. Thus, the AHNSP, WMNSP and OSAWMNSP all predominantly include amorphous material.

Effect of filtration in the samples

The effect of the filtration and pore size of the filters on starch NPs prepared in aqueous dispersion at room temperature was investigated using AF4-MALS-dRI obtaining a weight-average molar mass (M_w), r_{rms} and the percentage of mass recovery (Fig 1). The obtained data are show in Table 3. From the results, it can be seen that all samples have a high recovery. However, the recovery does decrease with decreasing pore size of the filter and WMNSP is more affected than AHNSP and OSAWMNSP. The results indicate that the major fraction of the non-solvent precipitated starch are high molar mass species with $M_w = 3 \cdot 10^6 – 6.7 \cdot 10^7$ g/mol and z-average r_{rms} between 61-116 nm. However, there is a small fraction of the starch that is lost during filtration and this could be linked to particles with a larger particle size than the main fraction.

For AHNSP sample when filter 0.1 µm pore size was used, it caused a considerable decrease in recovery and a slight decrease in average M and r_{rms} for the first population, and a second population (i.e. elution times, approximately 37 – 45 min, Fig. 1) disappeared. M, r_{rms} and recovery for this second population were not possible to quantify due to an insufficient signal-to-noise ratio in the dRI detector. The second population, although present at very low concentration, was somewhat affected by the various filtrations as seen in the MALS signal in Fig 1.

For WMNSP sample, when filters of pore size 1.2 and 0.45 µm were used, M_w and recovery were lower than unfiltered sample. For smaller filter pore sizes, M_w is almost unaffected but recovery decreased, most likely due to sample loss in the filtration. For OSAWMNSP sample, when using filter 0.2 µm pore size, a drastic decreased in M_w, r_{rms} and recovery happened. For filter pore sizes, larger than 0.2 µm there was not a large difference in the results, whereas for smaller pore sizes, no signal was visible. This could be caused by sample loss in the filter due to a too small pore size or adsorption in the filter. The cut of is also in the size range of the molecules studied, see Table 3.

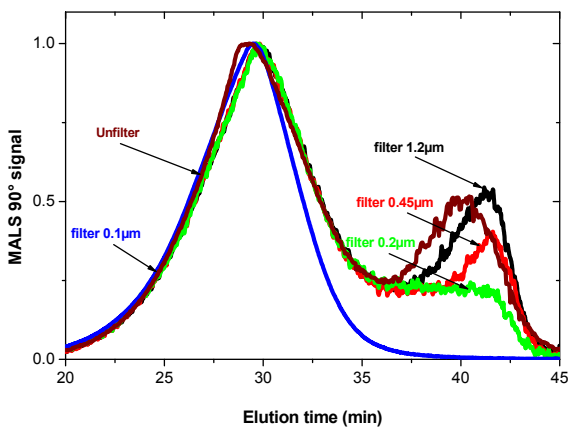

Figure 1: Elution profile for AHNSP sample dispersed in aqueous solution at room temperature using different porous size filters. MALS 90° signal (lines) vs Elution time (min).

Table 3 Average values obtained from AF4-MALS-dRI with different porous size filter for AHNSP, WMNSP and OSAWMNSP disperse in aqueous solution [a]

Filter porous size (µm)	AHNSP			WMNSP			OSAWMNSP		
	$M_w \cdot 10^7$ (g/mol)	r_{rms} (nm)	Recovery (%)[b]	$M_w \cdot 10^7$ (g/mol)	r_{rms} (nm)	Recovery (%)[b]	$M_w \cdot 10^7$ (g/mol)	r_{rms} (nm)	Recovery (%)[b]
Unfiltered	2.2	70	92	6.7	116	85	4.5	101	96
Filter 1.2	2.2	69	91	5.5	113	81	4.4	110	97
Filter 0.45	2.2	69	89	4.7	114	79	4.4	105	97
Filter 0.2	2.1	69	90	4.7	112	66	0.3	65	32
Filter 0.1	2.0	61	45	4.4	100	52	-	-	-

[a] M_w is the average molar mass, r_{rms} z-averages root-mean-square radius.

[b] The mass recovery was determined from the ratio of the mass eluted from the separation channel (integration of the dRI signal taking the complete peak) to the injected mass (based on the analysed starch content of the sample)

Effect of the sample preparation procedure in molar mass, size, and apparent density

AF4 fractograms of the samples after the 1 hour of dissolution/dispersion in the chosen solvent are shown in Fig 2A–C and the r_{rms} and molar mass is presented in Table 4. From the result, it showns that all samples have high M species with M_w from $3 \cdot 10^6$ g/mol to $1.2 \cdot 10^8$ g/mol, z-average r_{rms} between 38 - 148 nm and recoveries are equal or higher than 85%. It can be seen that the sample preparation methods do not to any larger extent change the fractograms for AHNSP and OSAWMNSP, but that the samples dispersed in water at room temperature for WMNSP is distinctly different from the ones that have gone through a more intense dissolution procedure. It is in our experience that the DMSO method will dissolve the starch samples and thus give difference in the molar mass and size of dissolved molecules [19]. Thus, difference from the DMSO samples indicates aggregation and maybe some particles formation. Thus 1 hour is enough to dissolve the AHNSP and OSAWMNSP in water solutions but not to fully dissolve the WMNSP. This does not rule out that at shorter times of dissolution which is often used in emulsification there are still particles present in the other samples. The acid hydrolysis had been conducted to see if this favored particle formation and especially if it favored crystalline formation as this has been seen to be the case for larger micro particles [33]. As can be seen this was not the case for the conditions used to produce these non-solvent precipitated particles.

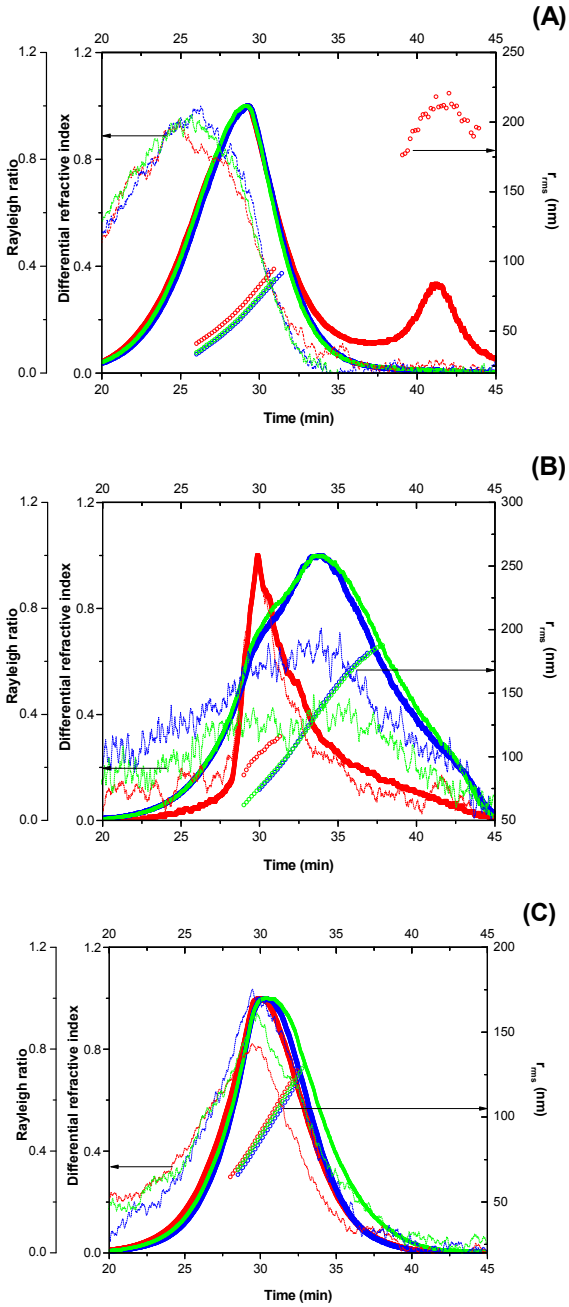

Figure 2: Fractograms from AF4 showing root-mean-squeare radius (r_{rms}), MALS-signal at 90¤ scattering angle, dRI-signal vs Elution time of AHNSP (A), WMNSP (B) and OSAWMNSP (C) samples considering different preparation method after the first hour. Samples illustrated in the plots; sample dispersed in aquous solution at room temperature (Red), dissolved in DMSO (Blue) and boiling in aquous solution (Green) respectively.

For AHNSP sample, after an hour of preparation shows a slight decreased in r_{rms} distribution as well as a second population in aqueous solution at room temperature (see Fig 2B). This second population at longer retention times (i.e. elution times, approximately 37 – 45 min) could corresponds to either a relatively dense starch nanoparticles or an aggregated structures. but the concentration in the population is very low. The aggregate formation was also observed in other studies [31, 34]. On the other hand, the recovery value in DMSO is higher (97%) which may be an indicator that the entire sample was dissolved. The acid hydrolysis has as expected decreases the molar mass and the size of the molecules.

For WMNSP sample, when was prepared in aqueous solution at room temperature, after 1 hour of preparation showed a considerable decreased in M and r_{rms} distribution (see Fig 2B, Table 4). In addition, the recovery varies between preparation procedures, being lower at room temperature (85%) and higher in DMSO (95%) having a loss of about 10%. Considering that the sample was completely dissolve in DMSO, it could be possible that some materials (possibly larger molecules or particles because the M is also lower), were immobilized at the accumulation wall and then eluted as a release peak when the crossflow is turn off. However, a release peak was not observed. The WMNSP still have the highest starch molar mass of the three investigated systems and the molar mass is in line with what have previously been reported for amylopectin from waxy maize [14, 15]. Thus, it is likely that the nanoprecipitation method does not to a larger extent changes the size range of molecules in the nanoprecipitate compared to the native starch. It can also be noted that the small particle size observed for precipitated waxy maize is in line with what has been seen by others [7]. The comparison with WMNSP dissolved with DMSO shows that the nanoprecipitate and dissolved molecules are in the same size range but still have quite different AF4 profiles.

In OSAWMNSP sample, the molar mass has been reduced compared to WMNSP (see Fig 2C and Table 4). In addition, the recovery value in boiling aqueous solution is slight low (92 %) than in the other preparation procedures (96 %). Those results are in contrast to the ones reported by Jiang, S *et al* [35] who found that OSA starch nanoparticles from Taro increased after modification with OSA as measured by DLS. This could be due to that OSA-modification of the WMNSP particles could have induced some hydrolyses of the starch as acid is used in the OSA-modification procedure. This have produced a dry non-solvent precipitated powder that are easier dissolved than the non-OSA modified WMNSP.

Table 4 Average values obtained from AF4-MALS-dRI with different methods of preparation for AHNSP, WMNSP and OSAWMNSP

Sample	$M_w \cdot 10^7$ (g/mol)	r_{rms} (nm)	ρ_{app} (kg/m^3)	Recovery (%)[b]
AHNSP sample DMSO	**1.8**	**60**	**21.6**	**97**
AHNSP sample boiling aqueous solution	**2.0**	**63**	**21.2**	**94**
AHNSP sample aqueous solution at room temp.	**2.2**	**70**	**16.2**	**92**
WMNSP sample DMSO	**9.4**	**148**	**7.9**	**95**
WNMSP sample boiling aqueous solution	**12.4**	**153**	**11.2**	**89**
WMNSP sample aqueous solution at room temp.	**6.7**	**116**	**8.3**	**85**
OSAWMNSP sample DMSO	**4.4**	**98**	**10.5**	**96**
OSAWMNSP sample boiling aqueous solution	**4.8**	**109**	**9.0**	**92**
OSAWMNSP sample aqueous solution at room temp.	**4.5**	**101**	**10.1**	**96**

Conformation using hydrodynamic radius obtained from online DLS and AF4 theory

To further understand the samples' conformational properties, samples of different starches dissolved at room temperature were investigated. A conformational parameter is given by the ratio between r_{rms} and r_h, where r_h used for the calculations was obtained in two different ways: online DLS and applying AF4 theory for calculating the values. The results are shown in Table 5. From the results, it can be seen that AHNSP sample (average $r_{rms}/r_h = 0.70$) appears to be close to a homogeneous spherical conformation ($r_{rms}/r_h = 0.77$). However, it should be noted that the value of 0.7 may also correspond to an object with microgel properties [24]. This is in line with the work of Juna *et al* who describes amylopectin as a compact, spherical structure [14] but a lower value compared to the ratio of around 1.2 measured by Roger et al [36] for corn amylopectin. It has been seen that acid hydrolysis have an impact on the structure of waxy maize amylopectin making the chain length distribution more narrow compared to native waxy maize with chain lengths with a degree of polymerization between 13-33 being the more dominating species [37]. This could of course also influence the conformation of the molecule.

The results for OSAWMNSP sample (average $r_{rms}/r_h = 1.43$) is a bit imprecise because when the comparison means test was done in the values obtained by online DLS and AF4 theory were significantly different. Nevertheless, the test with 0.01 significance level result in not significance difference from the statistical point of view. For that, it is important to consider when online DLS is used some challenges could be present. High concentration of the sample is typically required to obtain reliable scattering data. Furthermore, for large molecules, the diffusion is slow and the residence time in the measurement cell may be too low for adequate data collection and a stop – flow measurement could be necessary [38]. However, these deviations do not appear for the other samples where the agreement between the different methods is good. The conformation value for OSAWMNSP is in the range of starch [24] which could be mean that is more like hyper-branched polymer such as amylopectin with a typical values of 1.02 – 1.29 [36]. This indicates that the OSAWMNSP probably is dissolved into individual amylopectin molecules during the time used for dissolution.

For WMNSP sample (average $r_{rms}/r_h = 2.06$) which indicates that this structure differs seems that the conformation is substantially more elongated [39]. Thus, the structure is not the same as for dissolved amylopectin molecules even if the molecular size range is in the same range. This means that there is a structural change in non-solvent precipitated that still exist after 1 hour of dispersion in water at room temperature. This cannot be due to a preferential precipitation of starch having a very different molecular structure compared to the waxy maize amylopectin as the OSAWMNSP does not display this high average ratio and the OSAWMNSP are produced from WMNSP.

Table 5 Average values of r_{rms}, hydrodynamic radius and calculated values for the quotient r_{rms}/r_h obtained from online DLS, AF4 theory and batch DLS [a]

Sample	r_{rms} (nm)	online DLS		AF4 theory	
		r_h (nm)	r_{rms}/r_h [b]	r_h (nm)	r_{rms}/r_h [b]
AHNSP	57 ± 0.2	80± 5.7	0.73 ± 0.10	86 ± 4.7	0.67 ± 0.10
WMNSP	167 ± 6.1	79± 3.7	2.10 ± 0.30	82 ± 0.1	2.03 ± 0.10
OSAWMNSP	117 ± 1.5	73 ± 3.5	1.57 ± 0.10	88 ± 0.8	1.29 ± 0.10

[a] Values include standard error of the mean (±) based on three replicates

[b] According to comparison means test at 5% (critical value t_{ratio} = 2.78, P = 0.05), OSAWMNSP ($t_{OSAWMNSP}$ = 3.55) was significant difference, AHNSP (t_{AHNSP} = 0.81) and WMNSP (t_{WMNSP} = 0.94) were not significant difference Otherwise, OSAWMNSP was not significant difference at 1% (critical value t_{ratio} = 4.6, P = 0.01) based on triplicate analysis.

Conclusion

In this study, three different non-solvent precipitated waxy maize starches were studied and it was found that they differed in molar mass and behavior upon dissolution in different media. These changes depend on the treatment of starch used (acid hydrolyzed or not) as well as post non-solvent precipitation OSA modification. All the three non-solvent precipitated materials where amorphous. The acid hydrolyzed and OSA-modified samples where most likely dissolved after 1 hour of dissolution in water at room temperature while the non-acid hydrolyzed waxy maize sample still have a different AF4 profile compared to the same material dissolved in DMSO. The results show that we observe different conformations between the samples when they are re-solubilized in aqueous solution at room temperature.

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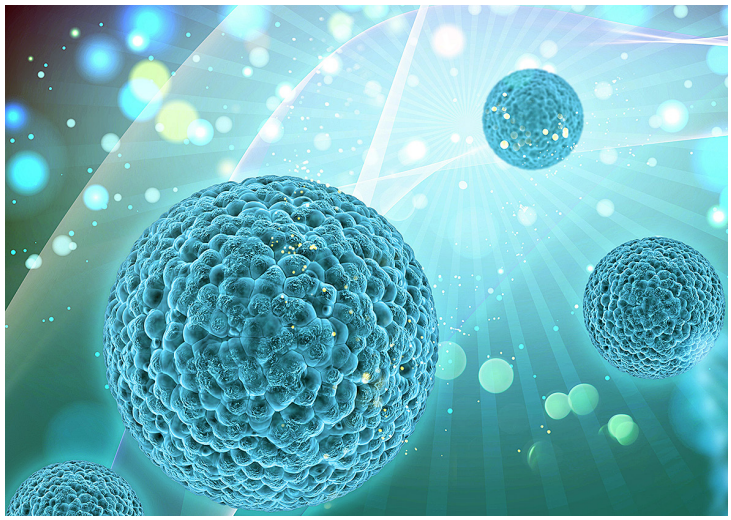

Hisfazilah Saari is a doctoral candidate at the Department of Food Technology, Engineering and Nutrition at Lund University. She was born and received her early education in Malaysia. In this thesis, she is investigating the functional properties of cereal starches for the application of food stabilizing products. Previously, she had 6 years working experiences in oil & fats industry in Malaysia and The Netherlands. In parallel, she had experience in protein chemistry and specializing in ingredients functionality during her MSc in Food Technology at Wageningen University, The Netherlands. From the diversity of food knowledge and combines with industrial experience, she hopes to contribute in research and development of more natural and highest quality of food products in the future.

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