Influence of starch botanical source on aqueous two-phase system phase behavior and starch microspheres preparation

By
Denis Oskolkov

Department of Food Technology
Faculty of Engineering, LTH, Lund University
SE-221 00 Lund, Sweden

LUND UNIVERSITY

RI.SE
Research Institutes of Sweden

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Abstract

Background:

Starch is a well-studied material, produced by plants in granules to store energy. Starch finds a lot of applications in day-to-day life, in the food and pharmaceutical industry. This project was focusing on the utilization of starch retrogradation and the ability to form aqueous two-phase systems to prepare microspheres from the starch of different botanical sources and characterize them.

Materials and Methods:

Three different starches were used: potato, corn, and barley, all waxy, meaning they have higher amylopectin compared to normal starches. Modification of raw native starch was needed, as the untreated starch could not be used for microsphere preparation. The behavior of an aqueous two-phase system comprising starch and polyethylene glycol was investigated, and acquired microspheres were characterized using microscopic evaluation, enzymatic degradation kinetics data and differential scanning calorimetry to assess the crystallinity of formed structures, aka microspheres.

Results and discussion:

Modification of the starches was generally successful; two out of three samples had the same profile as the reference and were assessed by doing a rheology investigation. Phase characterization followed the results achieved during rheology studies, providing information about the content of the phases and their distribution. Microscopic evaluation showed that microspheres received from different botanical sources of starch have different properties, such as porosity, surface and inner structure, shape and size. Enzymatic digestion and differential scanning calorimetry results have proven the same outcomes, as starch microspheres had different degradation kinetics and melting peaks.

Conclusion and future work:

Due to various constraints and time limitations, it was not possible to fulfil all the goals that were set during the planning stage of the project. Nevertheless, native waxy starches were successfully hydrolyzed, their phase behavior was studied, and their thermal properties have been investigated. Though, there is still a room for a vast amount of experiments: consequent phase behavior studies are required to further understand how microspheres are prepared, a potential study of rapidly dissolving potato starch microspheres based on the enzymatic degradation result, a study of microsphere preparation in presence of excipients and investigation of possible encapsulation of API in microspheres

Key words: starch microspheres, acid hydrolysis, DSC, microscopy, potato, barley, corn.

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Shuai Bai, a researcher, has shown me how the lyophilization equipment operates and how to input the appropriate method and how to manage this piece of equipment.

List of acronyms

ATPS – aqueous two-phase systems
DSC – differential scanning calorimetry
SEM – scanning electron microscopy
SNP – starch nanoparticles
PEG – polyethylene glycol
GI/GIT – gastrointestinal/gastrointestinal track
GOPOD - glucose oxidase/ peroxidase
WPSMS – waxy potato starch microspheres
WBSMS – waxy barley starch microspheres
WCSMS – waxy corn starch microspheres
API – active pharmaceutical substance

Popular Science Summary

Starch is a well-known and studied material that finds a lot of applications in both food and pharmaceutical production nowadays. Starch is a major polymeric carbohydrate in the human diet, and it consists of two types of polymeric molecules with the same monomer composition: threadlike amylose and branched amylopectin. Amylose and amylopectin have different properties so amylose is mostly found in amorphous regions and branched amylopectin composes crystalline regions in starch granules. If mixed with water and heated up, starch granules undergo gelatinization, which basically means that the granules swell as they take up water, and the amylase polymers leak into the solution. Subsequentially to starch gelatinization, after cooling down, starch polymers can recrystallize over time into semicrystalline structures. This feature can be utilized to produce starch microspheres in aqueous two-phase systems (ATPS) which potentially can be used in the drug formulation process. However, both starch gelatinization/retrogradation and starch/PEG ATPS phase behavior are complex and can be affected by several factors, like the type of starch, granule composition, the addition of salts or other substances to the system, and incubation parameters. Therefore, there is a need to get a further understanding of ATPS systems with starch and their control parameters to get consistent starch microspheres yield.

Contents

Aims

The aim of this master's thesis work was to study how different types of hydrolyzed starch from different plant sources could influence the preparation of starch microspheres. In addition to this, more detailed aims were:

- To hydrolyze native waxy starches from different botanical sources to get starch polymers with appropriate viscosity properties for microsphere production
- To investigate ATPS phase behavior and recrystallisation for the different starches
- To study the properties of starch that has recrystallized in these systems by means of DSC, light and SE microscopy, enzymatic degradation, and DSC

1. Introduction

1.1. Background and Motivation

Starch is a natural polymer produced and stored in granules by plants to save excessive energy. Due to the ability of some plants to produce it in high amounts, starch is a renewable material which can be yielded in high quantitates. It mainly consists of linear (1,4)-linked α-d-glucan amylose and highly (1,6) branched α-d-glucan amylopectin (Fig. 1**)**. [1] As both molecules are polysaccharides that can be digested by enzymes present in the human body, they are biodegradable and bioavailable for people. [1] These properties made it possible to utilize starch in the food industry and opens possibilities to use it in the pharmaceutical industry as well. Currently, in the industry, starch is employed as a binder, diluent, and disintegrant. [2] Due to the complexity of starch granules, its semi-crystalline nature and excessive length of polymer the application of starch as a drug delivery system is limited. Starch from different botanical sources has different properties and inclusions. [2] However, with specific pretreatment and/or isolation techniques, a range of micro and nanoparticles can be acquired. [3]

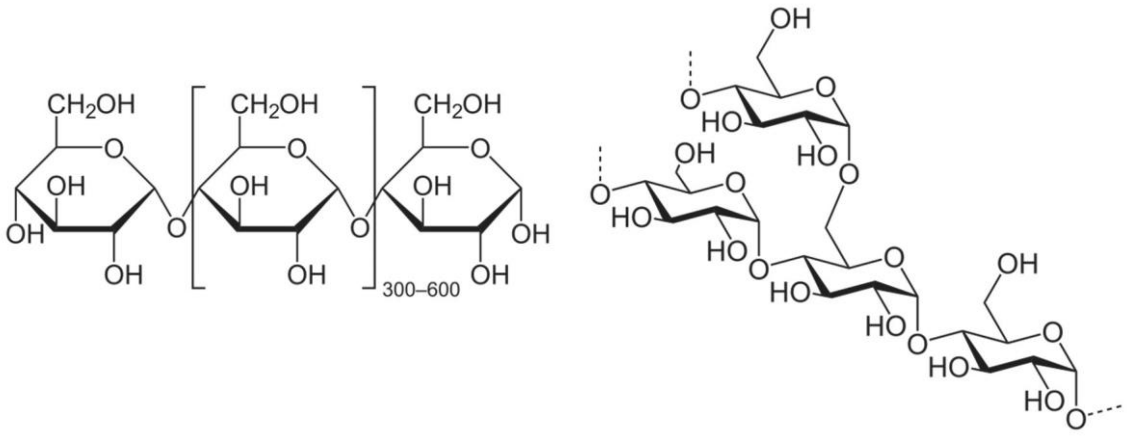

Fig. 1. Molecular structure of amylose (right) and amylopectin (left). From user NEUROtiker, Wikimedia Commons.

The starch microparticles (Fig. 2**)** [28**]** have been described in the literature as drug carrier molecules used for systemic and mucosal formulations, and their usage as a part of the nasal drug delivery system [4][28] and as a carrier molecule in the peroral and parenteral vaccine [5][6] has been described. In these studies, the starch particle itself has been produced by various techniques: either by polymerization of acryloylated starch in a water-in-oil emulsion [4] or by cross-linking soluble starch with epichlorohydrin [6]. Starch microspheres (Fig. 2**)** that have been investigated in this report are prepared from native waxy starch of different botanical sources: corn, barley, and potato, which have been hydrolyzed with hydrochloric acid and then incubated in an aqueous two-phase system (ATPS) with polyethylene glycol (PEG). In the literature, there is no evidence of studies, comparing different botanical sources of raw material prior to microsphere preparation. Studies involving starch microsphere preparation used cassava [7] and corn [8] starch to prepare microspheres, but there is no strong evidence of why this raw material has been chosen, despite the fact that it was successful in yielding microspheres in previous investigations.

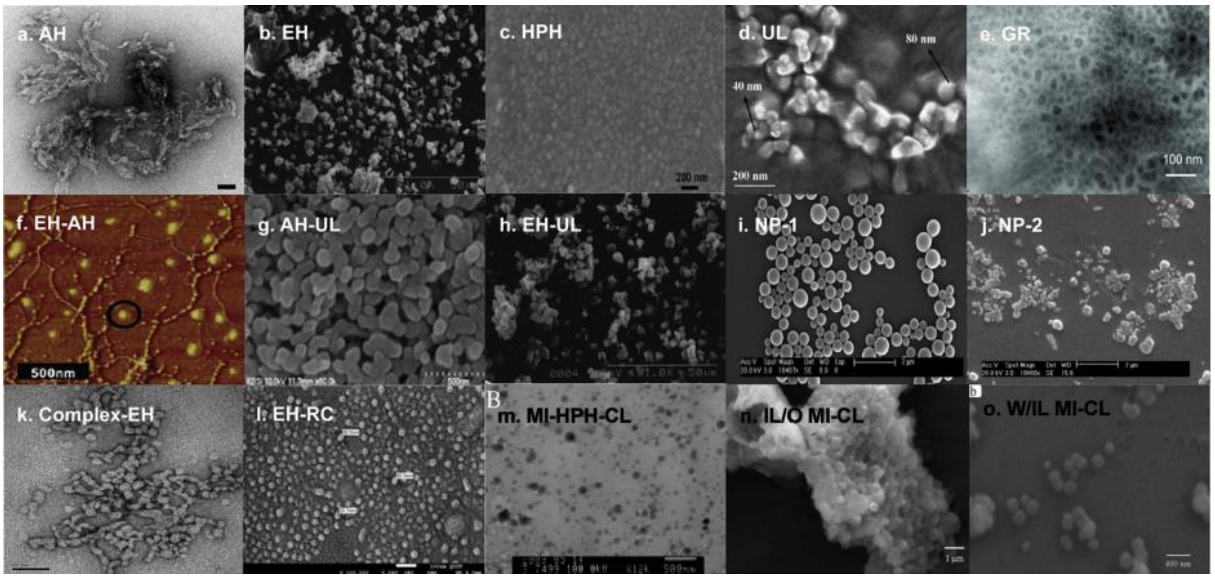

Fig. 2. Morphology of starch nanoparticles (SNPs) obtained by various preparation methods, from the article titled «Preparation, characterization, and utilization of starch nanoparticles. Colloids and Surfaces B: Biointerfaces» [28]

1.2. Starch properties

1.2.1. Granule structure

Starch naturally forms in starch organelles called granules (Fig. 3) [11]. Granules are tightly packed with a specific network and ratio of amylose and amylopectin. Plant species have their unique characteristic starch granules that differ in size, and shape, amylose to amylopectin ratio, crystalline to amorphous material ratio, starch supramolecular architecture, and amylose–lipid complexes, among other features. Combinations of these properties may result in differences in molecular weight, resistance to degradation, ability to form crystal structures upon retrogradation, etc. [9] In the scope of this project, only waxy starches of different botanical sources have been studied. Waxy means that granules of such starch have higher amylopectin to amylose ratio compared to nonwaxy alternatives. Amylose forms a single helix and is linear and is forming amorphous regions in the granules, whereas amylopectin is highly branched and forms a double helix, and is responsible for crystalline regions in the granules Fig**.** 3

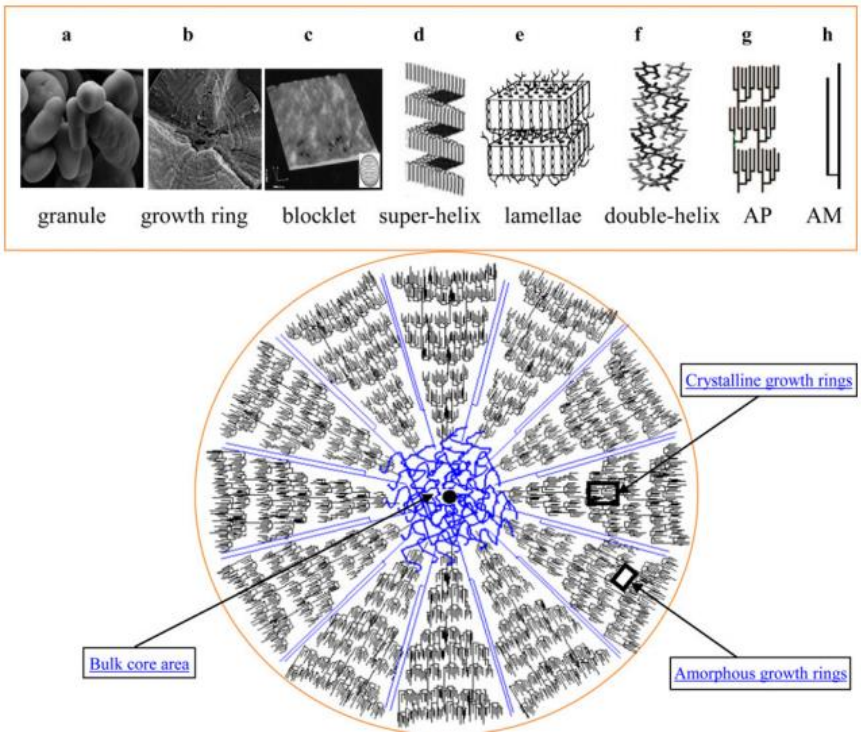

Fig. 3. Structure of the starch granule and its regions, from the article titled «Retrogradation: A Comprehensive Review. Comprehensive Reviews in Food Science and Food Safety» [11]

1.2.2. Gelatinization

Starch granules are generally composed of amorphous core and growth rings that alternate between amorphous and semicrystalline states. [10] In presence of water and heat, starch granules undergo a process of gelatinization, which is, basically, a disruption of the intramolecular bonds between starch molecules, allowing hydrogen bonding sites to attract more water, which acts as a plasticizer. As a result, the starch granule is irreversibly dissolved in water. [10] This process can be described in three steps: swelling of granules, melting of semicrystalline regions and amylose extraction with subsequent granule disruption. Due to heating, water is absorbed in the amorphous region of the granule, which causes swelling. Semicrystalline regions, because of heating, become diffuse and allow water to enter, where it attacks hydrogen bonding sites and dissolves amylose, which is linear and easier to attack. This reduces the number of crystalline regions and makes the granules more disordered, which leads to more water absorption. Eventually, amylose molecules are dissolved and extracted into the solvent, which leads to granule disruption. [10]

1.2.3. Retrogradation

When the solution is cooled down, the disordered high energy state of the formed starch polymer solution can gradually transform or condense into more rigid gel networks. Restructuration of starch gels happens, which alters the crystalline composition of starch making it less amorphous. Such a process is called retrogradation. [11] This process is crucial for microsphere preparation (Fig. 4**).**

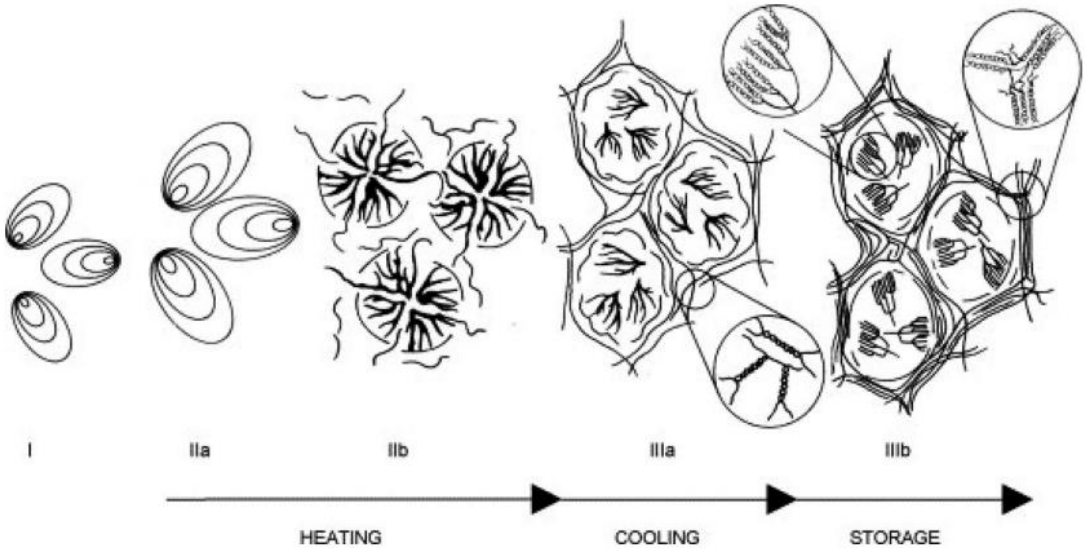

Fig. 4. Schematic representation of changes that occur in a starch–water mixture during heating, cooling, and storage, from the article titled «Retrogradation: A Comprehensive Review. Comprehensive Reviews in Food Science and Food Safety» [11]

Retrogradation, as a process following granule dissociation, depends on the amylopectin to amylose ratio. Amylose, released in the solution, recrystallizes faster than amylopectin, creating the network, in which amylopectin is trapped and slowly recrystallizes either. It is considered that the initial amylose rapid retrogradation determines the formation and the rigidity of the network [11]

For starches with higher amylopectin to amylose ratio, or waxy starches, retrogradation will not cause rigid network formation and the gel would be rather soft, containing aggregates instead. This results in such qualities as easier penetrability, slower retrogradation and higher adhesiveness.[11]

Due to differences in molecular weight of the polymers, granule composition, additional molecules on the surface of the granules and ratio of amylopectin to amylose, starches of different natural sources retrograde differently. Even the starches from the same plant can differ significantly between batches due to many reasons. This fact can affect the production of starch microspheres and alter microsphere properties, resulting in non-consistent results from batch to batch. [12] The addition of salts to the formulation can also affect the production profile which was highlighted in the literature. [13]

1.3. Starch modification

As was mentioned before, the natural starch does not have many applications, mainly due to not suitable properties of the raw material. Therefore, starches are usually modified either enzymatically, physically, or chemically. Modifications are usually applied to alter one or several properties of the starch, such as stability to stresses (heat, shear stress, degradation), gelatinization time, texture, viscosity, or thickening capabilities. By doing so, relative mechanisms associated with starch such as retrogradation, water holding capacity or syneresis can also be affected.[14]

One of the most straightforward and natural ways of modifying starch is acid hydrolysis or thin boiling at temperatures lower than gelatinization onset temperature. [15] It is natural because concentrated hydrochloric acid is also used by living organisms to treat food, in general, and starch, in particular, in their stomach. Acid hydrolysis can significantly alter the properties of starch without disrupting its granular morphology, which is achieved by reacting primarily with

amorphous regions of the granules, consisting primarily of amylose. This enhances the ratio of crystalline structures to the total dry weight of the starch by increasing the amylopectin to amylose ratio and overall double-helical structures amount formed by amylopectin in treated starch granules. Due to amylose hydrolysis, the viscosity of starch in water solution and the amylose network formation during gelatinization are also affected. [15]

1.4. Aqueous two-phase systems

Mixtures of two water-soluble substances that tend to separate in distinct phases in solution are called aqueous two-phase systems. Such systems, with appropriate agitation and stabilization procedures, can form water in water emulsions with a fine particle size of distributed phase. Such emulsions are typically referred to as water-in-water or W/W emulsions. [16]

1.4.1. Water-in-water emulsions

Water-in water emulsions are colloidal systems, where the aqueous solution of one hydrophilic macromolecule is highly dispersed in an aqueous solution of another hydrophilic molecule. Most important is that these macromolecules should be incompatible with each other thermodynamically, which drives rapid phase separation, which varies from seconds to hours. However, such emulsions are highly unstable, and the kinetic stability of such colloidal systems is almost uncontrollable as there is a lack of repulsion between particles, and they tend to coalesce. Surfactants commonly used for stabilization of conventional emulsions cannot be used as they do not absorb on the interface and continuous agitation is required to keep phases separated. [16]

1.4.2. Starch ATPS

Starch polymers or polysaccharides are capable of forming water in water emulsion when mixed with other polymers, e.g. polyethylene glycol (PEG)[8][16]. At certain concentrations, which are usually chosen by studying the phase behavior of such systems, starch-water microdroplets are dispersed in the PEG-water phase, forming emulsion upon vigorous agitation. If starch-water droplets would undergo retrogradation while being in this dispersed state, they can form rigid semi-crystalline starch structures of spherical form with size, defined on the process parameters, such as starch/PEG concentrations in the solution and speed of emulsification. To ensure microspheres formation, concentrations of initial starch-in-water and PEG-in-water solutions and final concentrations of polymers in solution are crucial as only a specific combination of starch/PEG initial concentrations will result in successful emulsification, yielding specific droplet size. Moreover, starch requires specific conditions to retrograde in ATPS, such as heat/cooling treatment and a specific amount of water, it is also dependent on the initial properties of the raw material. Both phases absorb a set amount of water when mixed and it should be taken into consideration as it can affect retrogradation. [8][13]

1.4.3. Phase characterization

Different polymer properties and concentrations can affect ATPS phase compositions and behavior, which in turn may affect starch retrogradation. We are characterizing the phase behavior in order to better understand starch/PEG ATPS preparation.

As phases in ATPS tend to separate, separation forces can be increased even further by centrifugation. Fully separated phases can be collected and analyzed to understand how they interact with water and with each other. To characterize the water content of the starch/PEG rich phases and how both phases interact with the solvent, pure phases can be dried, and the amount of dry mass can be quantified. And with the assumption that there is negligible mixing of the two polymers in the phases, initial phase behavior can be plotted. Based on information

received from phase diagrams and ATPS starch microsphere formation, microsphere preparation could be better understood.

1.5. Rheological investigation of hydrolyzed starch

As starch has complex rheological properties, that depend on concentration in water solution, temperature, and mechanisms such as gelatinization. [17] To ensure that modified by acid hydrolysis native waxy starches have appropriate properties, such as viscosity and rheological behavior for microsphere formation, rheological properties of modified starch were investigated after gelatinization have occurred. [18] A rheometer will be used.

A rheometer is a laboratory instrument that measures the flow of a viscous fluid in response to applied forces. Rheometers are divided into two categories. Rotational or shear rheometers control the applied shear stress or shear strain, whereas extensional rheometers apply extensional stress or strain. For this project, a dynamic shear rheometer was used for the evaluation of starch viscosity. It has a rotating cylindrical element and a cuvette of appropriate size and shape to fit the element. A sample is loaded in the cuvette and a rotational element is dipped in the sample. The forces and stress are applied by rotating the cylindrical element in the sample with a set rotational speed.

1.6. Light and electron microscopy

Researchers of all fields use light microscopy to magnify objects to a certain number of times their original size. In its most basic form, it consists of a transparent lens that magnifies the sample and a light source that illuminates it. Most light microscopes, on the other hand, are much more complicated, engineered with several lenses of precisely specified dimensions housed within the microscope's body and in components such as objectives and eyepieces, as well as with the ability to take real-time images and captions of the enlarged sample. Light microscopy will allow us to perform visual, real-time, direct characterization of the microspheres. [20]

A scanning electron microscope (SEM) is a type of electron microscope that uses a focused beam of electrons to scan the surface of a sample to obtain imagery. When electrons come in contact with atoms in a sample, they produce a variety of signals that carry information about the sample's surface topography and composition. A raster scan pattern is used to scan the electron beam, and the position of the beam is coupled with the intensity of the received signal to create an image. [21]

Modern SEM allows operation in a wide range of magnifications from about 3-10 times (that is, equivalent to the magnification of a strong hand lens) up to 1,000,000 times, which is about 500 times the magnification limit of the best optical microscopes.[21]

Today, the capabilities of scanning electron microscopy are used in almost all areas of science and industry, from biology to materials science. A number of different designs and types of SEMs manufactured and equipped with various types of detectors to obtain images of specific samples. In the scope of this project, it will help to characterize porosity, surface structure, and cleaved cross-sections of starch microspheres. [21]

1.7. Enzymatic digestion

Human digestion of food and medications is a highly complex system. In vitro technologies are employed in the pharmaceutical and food industries to imitate this process. Depending on the study's goal, the procedures can involve the entire GI tract (oral, gastric, small intestinal, colon digestion) or sections of it.

Starch is naturally digested in the body of many living species by an enzyme called amylase. Amylase is produced by saliva glands or by the pancreas. [19] The final formulation with starch microspheres could be intended as part of an acid-protected capsule formulation, where the microspheres are to be released in the lower intestine, where they would be exposed to the amylose secreted by the pancreas. Moreover, microspheres should dissolve prior to exiting GIT. This knowledge will be the basis to test how microspheres withstand enzymatic degradation and how fast they would be digested by the enzymes. However, other formulations may be intended for other ways of delivery, where the starch digestion could play a role in the release of active ingredients. Therefore, it was interesting to investigate the kinetic properties of starch microsphere digestion.

1.8. Freeze drying

Freeze during is a drying technique, which uses low temperatures and pressure to extract water by sublimation (primary drying) and drying residual water by increasing the temperature close to normal (secondary drying). Freeze drying will be utilized as the main method of drying on all stages due to the properties of starch to degrade at high temperatures. Starch tends to dissolve in water solutions at high temperatures and long exposure.

The samples must be thoroughly frozen when placed in the freeze drier for the lyophilization to work correctly. If there is liquid water with the sample, it will begin to boil as the pressure reduces, damaging the sample's structure.

Primary drying occurs when frozen water sublimates and secondary drying occurs when any moisture adsorbed to the substance is eliminated by desorption. To ensure that the water from the sample migrates to the condenser and vacuum pump, a pressure gradient from the sample to the condenser and vacuum pump must be established. The temperature of the condenser must be lower than the temperature of the lyophilized sample to catch any sublimated water. If not preserved in a dry environment (such as a desiccator), the lyophilized material will quickly absorb water from the air.

1.9. Differential Scanning Calorimetry

Differential scanning calorimetry is a thermoanalytical technique. The temperature of the crucibles in the chamber increases linearly as a function of time and the difference in the amount of heat required to raise the temperature of a sample and a reference was registered and plotted as a function of temperature.

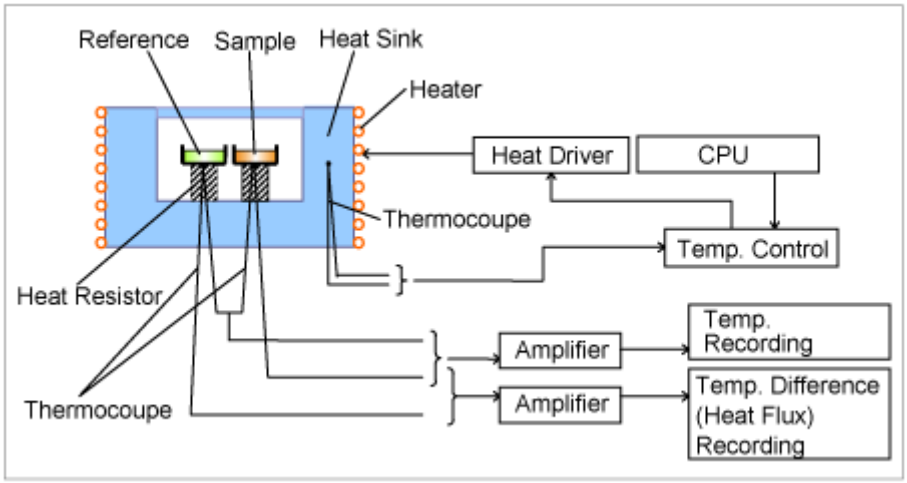

Fig. 5. Schematic representation of heat flux DSC differential scanning calorimeter, from internet source titled «Principle of Differential Scanning Calorimetry (DSC)», HITACHI [22]

The underlying premise behind this method is that, depending on whether the process under study is exothermic or endothermic, heat must be provided to the material during a physical transformation, such as phase transitions, to keep both sample and a reference at the same temperature. When a solid sample is melting, for example, it must transfer more heat to raise its temperature at the same pace as the reference. This is due to the sample absorbing heat when it transitions from solid to liquid in an endothermic phase transition. When the sample is subjected to exothermic processes (such as crystallization) [22], however, less heat is required to raise the sample's temperature compared to the reference because of ordered structure formation. Differential scanning calorimeters can quantify the amount of heat received or released during such transitions by monitoring the difference in heat flow between a sample and a reference. [22]

2. Materials and methods

2.1. Materials

Three starches of different biological origins were analyzed in the scope of this project: native waxy barley starch (BAP Sträkelse, Lyckeby-Culinar), native waxy potato starch (Emwaxy 100, Emsland Group) and native waxy corn starch (C*Gel 04201 01647541, Cargill). As a reference for starch modification and microsphere production, acid hydrolyzed waxy barley starch (PU 92-000 20182602, Lyckeby-Culinar) was used. For both microsphere preparation and phase characterization PEG 20 000 (ThermoFisher GmbH, Kandel) was used in ATPS preparation. As a source to heat treat starch to make it undergo gelatinization, a microwave at 600W was used.

For acid hydrolysis strong 5M HCl (CAS: 7647-01-0) was used. To neutralize the acid at the end of hydrolysis initially 5M NaOH (CAS: 1310-73-2) was used, and then an appropriate amount of Sodium Bicarbonate (CAS: 144-55-8) was added. As the main solvent MilliQ water was used.

For phase characterization as well as for microsphere preparation it was decided to use 0,9% sodium chloride as the main solvent, therefore NaCl (CAS: 7647-14-5) was used to prepare this solution.

For Amylose digestion, a commercially available Total Starch Assay Kit (AA/AMG) (Megazyme) was used. To prepare the buffer system for amylose digestion and perform the analysis, Sodium Phosphate Dibasic Heptahydrate (MW: 268,07 g/mol, CAS: 7782-85-6), Sodium Phosphate Monobasic Monohydrate (MW: 137,99 g/mol, CAS: 10049-21-5), Calcium Chloride (MW: 110,98 g/mol, CAS: 10043-52-4), Anhydrous or glacial Acetic Acid (MW 60,052 g/mol, CAS: 64-19-7), glucose oxidase/peroxidase (GOPOD) reagent (Total Starch Assay Kit (AA/AMG), Megazyme), heat-stable α-amylase (Total Starch Assay Kit (AA), Megazyme), amyloglucosidase (Total Starch Assay Kit (AMG), Megazyme), reference corn starch sample 86% of dry mass (Total Starch Assay Kit, Megazyme), standard glucose solution (Total Starch Assay Kit, Megazyme), porcine alpha-amylase Type VI-B (Sigma) and MilliQ water were used.

For DSC small hermetic aluminium DSC pans and compatible lids (TA instruments, 900793.901/900794.901) were used.

2.2. Methods

2.2.1. Starch modification

Initially, a specific glass chamber with mantle, gas diffusor system and a collector, and metal lid with propeller mixer installed in it were set up (as shown in Fig. 6) to create a closed system for starch hydrolysis. The method was based on the literature examples [15][23] and discussions in the department. The starch w/w dry mass to liquid concentration for this experiment was 20% w/w, the initial concentration of HCl was 1M and the incubation time was ranging from 1hr to 4 hours with samples of the same mass collected after each hour. After each sample collection, the acid was neutralized to stop the reaction, but later due to problems with the sample's chemical properties, neutralization was excluded, and four washing steps were proposed instead of two.

The trial setup was mounted and test-run with food-grade commercially available potato starch to assess the installation, operational and performance qualifications.

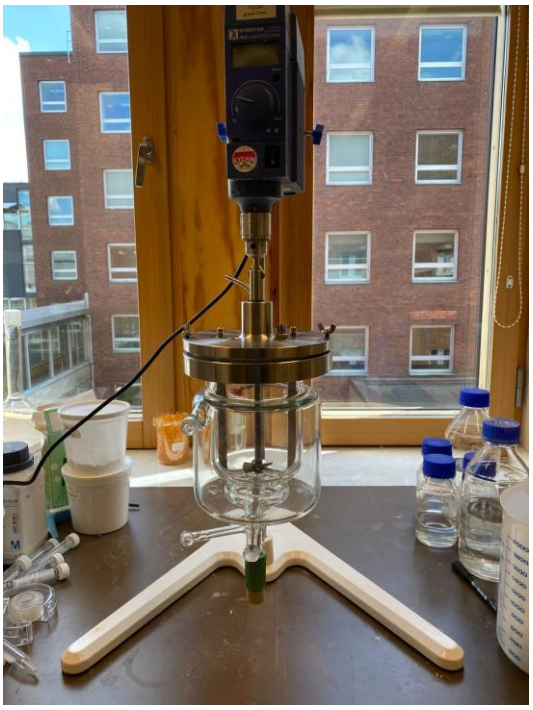

Fig. 6. Setup including torque stirrer attached to the lid with mixer and glass chamber used for acid hydrolysis of the native waxy starches.

After all preparation steps, a hydrolyzation run consisted of adding 20g of native waxy starch to the glass chamber, mixing 64g of MilliQ water and 16g of HCl and adding 1M HCl solution to the chamber to create 20% w/w starch dispersion (Small batch, SB). The lid with propeller mixer was installed on the top of the chamber and connected with a torque mixer. The torque mixer was set to output 500 rpm to ensure continuous mixing of the sample. The mantle was not connected to the water system and the sample was incubating at ambient temperature. 25 g of the total sample were collected every hour from the beginning (1,2,3,4-hour marks) which yielded around 4-5 g of modified starch each. After the sample was collected, it has undergone washing steps with subsequent neutralization. After studying how the sample behave, it was decided to increase the number of washing steps and remove neutralization. Washing was done by centrifugation of the sample at 2000g for 5 min at 25℃, removing the supernatant and exchanging it for fresh MilliQ water with 4 repetitions of the steps. After washing, the supernatant was decanted and the sample with an as low amount of liquid as possible was stored in the freezer at -20℃.

Subsequently, there were attempts to increase the batch size by increasing the amount of starch and 1M HCL respectively (2.5x) (Big batch, BB) and by collecting all the samples at the same time (3-hour mark e.g.).

After storage in the freezer, samples were distributed on the Petri dishes and freeze-dried, according to a program described in the lyophilization section.

2.2.2. Microwave treatment

The microwave was chosen as a controllable and reliable source of heat for starch gelatinization in water suspension. For rheology investigation, 20% starch in solvent w/w slurry was prepared, for other means, such as phase characterization and microsphere preparation, it was 30% w/w dispersion that was heat treated.

Set amount of starch was mixed with the solvent, either MilliQ water or 0,9% NaCl water solution, to create 20% or 30% w/w starch to solvent concentration in loosely capped plastic

beakers. The dispersion was vigorously mixed for 2 minutes until the starch appears dissolved by ocular inspection. The beaker with the loose cap was placed in the microwave with a 600W setting. The sample received microwave treatment in a pulsation manner for 5-6 seconds with a 5-second interval, when it was manually stirred. The samples were heated for a total of 20 seconds for 30% w/w starch dispersion and 40 seconds for 20% w/w starch dispersion to ensure boiling without leaking of the sample. The beaker was carefully removed from the microwave oven and stirred manually by rotating. Afterwards, the cap was closed tightly, and the beaker was left at ambient temperature to cool for 15 minutes.

2.2.3. Rheology assessment

For rheological studies, modular DS rheometer Kinexus Pro was used. OC14 C0088 AL lower geometry and CD 14 standard bob upper geometry were installed and calibrated. Calibration of the parameters with Ellis model fit was based on the standard sample study. The standard sample was prepared from acid hydrolyzed starch PU 92-000, which has previously yielded microspheres. All samples for rheology investigations were starch-in-water with 20% dispersions after microwave treatment. Samples were loaded into the equipment accordingly to the instructions to measure the shear stress and shear rate depending on the applied force and calculate viscoelastic properties.

2.2.4. Lyophilization

Samples, containing starch cannot be dried at temperatures higher than the starch gelatinization temperature, which is on average around 50-60℃. To remove water efficiently and quickly from the starch, freeze-drying was used. Two different freeze-drying programs were used in this project. One was implemented to dry starch after acid hydrolysis and during phase characterization, and another one to dry microspheres. The main difference between the techniques is the initial temperature of freezing, degree of vacuum or pressure in the chamber and time of drying. Both programs with steps and parameters are represented in Table 1

Table 1. Lyophilisation programmes' steps with parameters

Program 1 (for the raw mat.)				Program 2 (for microspheres)			
Step	Temp	Press	Time	Step	Temp	Press	Time
1. Precooling	-48℃	-	-	1. Precooling	20℃	-	-
2. Freezing	-48℃		2hr	2. Freezing	-45℃		2hr
3. Main drying	-48℃		1,5hr	3.Freezing	-45℃		2,25hr
4. Main drying	-20℃		0,5hr	3.Main drying	-10℃		1hr
5. Main drying	-20℃		12hr	4. Main drying	-10℃		16hr
6. Secondary drying	20℃		8hr	6. Secondary drying	20℃		2hr
7. Secondary drying	20℃		17hr	7. Secondary drying	20℃		10hr
8. Secondary drying	-40℃		1hr	-	-℃	-	-
9. Secondary drying	-40℃		24hr	-	-℃	-	-
Total time	66hr			Total time	33,25hr		

In the case of starch material, the received starch cake was broken down to powder (if it is not powder already) and stored in a glass or plastic bottle with the lid at +4°C.

2.2.5. Phase characterization

Preparations for phase characterization consisted of 3 solutions: NaCl 0,9 % (w/w) in MilliQ, PEG stock 45 % (w/w) made with NaCl 0,9% in MilliQ and 30% w/w starch stock in NaCl 0,9% in MilliQ, gelatinized using microwave by the method described in section 2.2.2. As PEG took considerable time to dissolve, after mixing ingredients, a magnetic stirrer was added to the beaker and the solution was slowly mixed by inducing 300 rpm rotation overnight and heated to approx. 40°C, covered in aluminium foil as PEG can be oxidized when exposed to light. Then it was stored at +4°C.

Based on pre-studies of microsphere formation, three total system concentrations were mixed for phase characterization. The concentrations are presented in Table 2. The total system mass of each preparation was 12 g mixed directly in the centrifuge tubes.

The techniques have been established using the waxy potato starch with three replicates and then reproduced with only two replicates for waxy corn and waxy barley starches.

Table 2. Chosen concentrations for phase characterisation preparations

12g total w.	Starch (%)	PEG (%)	Water with 0,9 % NaCl
10/30/60 prep.	10	30	60
8/24/68 prep	8	24	68
6/18/76 prep	6	18	76

At this stage, careful observation of the weight of stocks added to the preparation is crucial as it will directly affect results and will reflect on the phase diagram.

All stock solutions were added to the 15 mL centrifuge tube, according to the preparation specification from Table 2. Starch stock 30% was freshly prepared and heat-treated according to 2.2.2. All stock solutions were added to the tubes when they had reached ambient temperature. When all components were introduced, tubes were incubated for 1 hour in a vertical test tube rotator at 20 rpm at ambient temperature After incubation, tubes were centrifuged at 4000G for 15 minutes. After centrifugation, phase volume was recorded and photographed for subsequent analysis. 5ml of PEG phase is collected to investigate the density of the PEG rich phase. After that, the pure PEG rich phase and pure starch-rich phases were separated and collected, and their weight was noted. Samples were prepared for lyophilization and freeze-dried using program 1, from Table 1.

2.2.6. Microsphere preparation

This procedure follows procedure 2.2.5 above, with the difference in the preparation specification. Specification for this mix is 4% starch and 27% PEG with 69% of 0.9% of NaCl in water. Two different preparations of ATPS were mixed, 8 g total mass in 15 ml centrifuge tube and 30 g in 50 ml centrifuge tubes.

Another deviation from the phase characterization is that directly after adding all the stocks to the tube, it was vigorously emulsified by using a disperser at the lowest setting of speed (T 10 basic ULTRA-TURRAX® by IKA Dispersers) for 30 seconds, then 5 inversions for 15 seconds and then 30 seconds of disperser treatment again. After successful emulsification sample

changed color, became nontransparent and homogenized. After that ATPS was ready to be incubated for 24 hours on the rotating table at 20 rpm, ambient temperature.

After 24 hours, microspheres have retrograded and can be collected via centrifugation. 0,9 % NaCl MilliQ water was added to the tubes to reduce PEG concentration and make the continuous phase less vicious. After that, centrifuge tubes containing microspheres are centrifuged at 2000g for 5 minutes. The supernatant is carefully discarded and fresh MilliQ water is added, and microspheres are redispersed in it to wash any residual PEG. The abovementioned washing steps should be repeated 3 times.

After washing, microspheres are redispersed in a small amount of 0,9 % NaCl MilliQ water: 2ml for 8 g preparation and 10 ml for 30 g preparation. Some of the freshly prepared microspheres are taken as sample material for other investigations. Most of the material is distributed in lyophilization vials (not more than 2 ml of suspension each) and freeze-dried using program 2 from Table 1

2.2.7. Enzymatic digestion

This procedure was established to study the stability of starch microspheres in the presence of amylase. It was adapted from a protocol used in the literature and based on the total starch assay kit K-TSTA-100A AOAC METHOD 996.11, MEGAZYME. Stock solutions of: Sodium acetate buffer (100mM, pH 5.0) with 5mM CaCl, Amylase Phosphate buffer (100mM, pH 6.9) with 5mM CaCl were prepared. Porcine Amylase with the calculated activity of 2.7 U/mg has been used. The amylase activity had been previously determined, where one unit of amylase was the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmole of p-nitrophenol per minute at 25 °C. Stock solution based on Amylase Phosphate buffer with total amylase activity of 0.02 U/ml has been used for analysis. 100 mg of starch microspheres have been incubated in 10 ml of amylase stock solution for 15, 30, 60, 120 and 240 minutes at 37℃ in the oven on a rocking table. At the corresponding time points, 2ml samples were collected, centrifuged at 13000 rpm for 5 minutes in the Eppendorf MiniSpin centrifuge, and the supernatant was harvested to check for total starch concentration in the solvent. Furthermore, the supernatant was divided equally and added to two 15 ml tubes that represent sample and blank measurements, the weight of the added solution was noted. Sodium acetate buffer was added to the tubes to increase the final volume up to 10 ml.

Then, to sample and control tubes 0.1 ml of thermostable α-amylase from the total starch kit was added, and 0,1 ml of sodium acetate buffer was added to the blank tubes. Tubes were incubated at 95℃ for 15 minutes with continuous vortexing. After incubation, 0,1 ml of amyloglucosidase was added to the sample and control tubes, and 0,1 of sodium acetate buffer was added to the blank tubes. Afterwards, tubes were put in the incubation at 50℃ overnight.

After the incubation, tubes were brought to room temperature for the temperature of the solution in the tubes to equilibrate, 2 ml of the solution from the tube were transferred to new tubes and diluted according to the instructions from the kit depending on the expected total starch concentration. 0,1 ml aliquots were transferred from the tubes to 3 ml of the GOPOD buffer from the kit. The standard sample was prepared by following instructions in the kit by adding 0,1 ml of glucose solution from the kit to 3 ml GOPOD buffer. After the addition of all components, cuvettes have been incubated for 20 minutes at 50℃ to develop color. The absorbance of the GOPOD+sample in the cuvette was measured on Cary 50 Bio UV-VIS Spectrophotometer. To ensure that the color stopped developing the absorbance of the solution in the cuvettes have been tested twice at 20 minutes interval. The absorbance then was recalculated to the percentage of the starch microspheres degraded with amylase over time and plotted.

2.2.8. Light microscopy

Using light microscopy (BX50, Olympus, Japan), behavior of ATPS during the incubation, the overall structure, rigidity, and degradation of microspheres over time were assessed visually. During all tests, approximately 0.05 ml samples were collected and studied using a 5x linear magnification objective. Starch microspheres were assessed continuously during ATPS incubation to check the rigidity of the formed structures, after washing the PEG off and after freeze-drying in both dry and rehydrated in MilliQ water states, as well as during enzymatic degradation.

2.2.9. SEM microscopy

The samples were sent to RISE, Stockholm for SEM analysis, where microsphere cross-sections and morphology were characterised. The dried formulations were prepared by placing samples on circular stubs with double-sided adhesive carbon tape. A scalpel was used to carefully chop some of the microspheres on the stubs. The stubs were placed in a Quanta 250 FEG ESEM scanning electron microscope (FEI, Czech Republic). Photographs were taken in a low vacuum with a Large Field Detector (LFD), using a 5kV beam under 60 Pa pressure. The images were taken at magnifications of 50 ×, 500 ×, and 5000 ×.

2.2.10. Differential scanning calorimetry (DSC)

DSC was utilized to assess the crystallinity of the microspheres. Two types of samples have been analyzed: fresh microspheres that have finished 24 hr. incubation and were washed of PEG and resuspended in MilliQ water freeze-dried microspheres. Seiko DSC6200 / EXSTAR 6000 DSC Differential Scanning Calorimeter has been used for this experiment.

For freshly prepared microspheres, approximately 10 μg of microspheres together with washing buffer have been transferred to an alodined pan with a total volume of 15μl. The pans were sealed and put into the DSC chamber together with an empty pan as a reference. Analysis was performed from 5℃ to 95℃ with a 10℃ per minute increase. After the analysis pans were punctured and put in the oven for drying to assess the dry mass of the sample.

For dried microspheres, depending on the required concentration for the analysis, either 15 or 30% w/w, approximately 1,5 or 3 μg of dry microspheres have been transferred to the DSC pan and resuspended with an appropriate amount of MilliQ water to reach the required concentration. Afterwards, pans were sealed and analyzed in the equipment with the same temperature regime and parameters.

3. Results

3.1. Starch modification and rheology investigation

Based on the Ellis model fit, a special shear ramp has been programmed, and applied forces have ranged from 0.1 to 200 Pa (with exponential scale). To establish a reference sample, 3 preparations of acid hydrolyzed starch which was proven to yield microspheres were analyzed as shown in Fig. 7. Afterwards, several acid hydrolysis incubations have been done on all three starches of the different botanical sources to match the reference result. Acquired parameters are recalculated into average viscosity using equipment software.

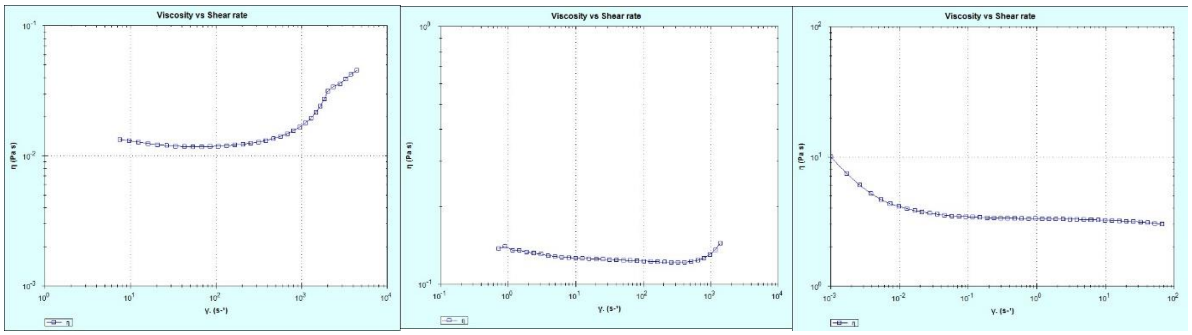

Fig. 7. Viscosity vs Shear rate graphs of three reference sample preparations, 10%, 20%, 30% (from left to right))w/w in MilliQ water after microwave treatment, Acid Hydrolyzed Barley Starch PU 92-000

An observation was made, that raw barley and corn starch after being incubated as a small batch have comparable parameters and viscosity profiles between each other, as shown in Fig. 8**,** and compared to the reference (Fig. 7, middle image). In addition to the same rheological behavior, measured viscosity between these three samples is also comparable (Table 3). The fact that the viscosity of starch regresses rapidly after the first hour of incubation and slowly afterwards was also noted.

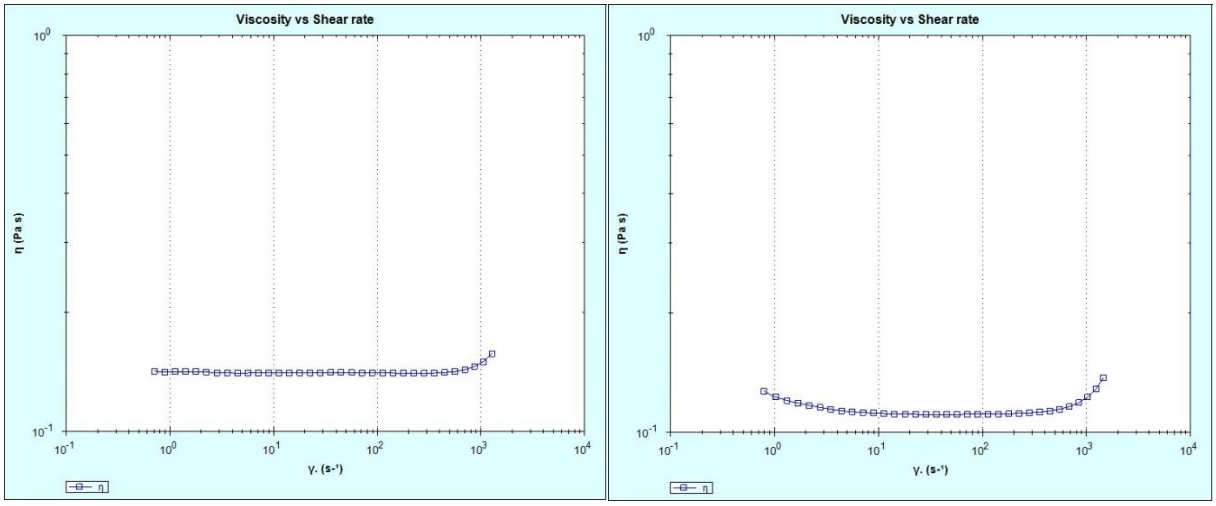

Fig. 8. Viscosity vs Shear rate graphs of two sample preparations, 20% w/w in MilliQ water after microwave treatment of native waxy barley starch (Left) and native waxy corn starch (Right) both incubated for 3 hours in 1M HCl small batch

However, the behavior of native waxy potato starch was different. After 3 hours of incubation in the same environment, it still showed relatively high viscosity(5-6 times of the reference) with different rheological profiles (Fig. 9 left). Therefore, an overnight 1M HCl incubation was performed, but it also yielded noncomparable to the reference results of too low viscosity compared to the reference (7 times of the reference) and altered rheological profile as shown in Fig. 9 right.

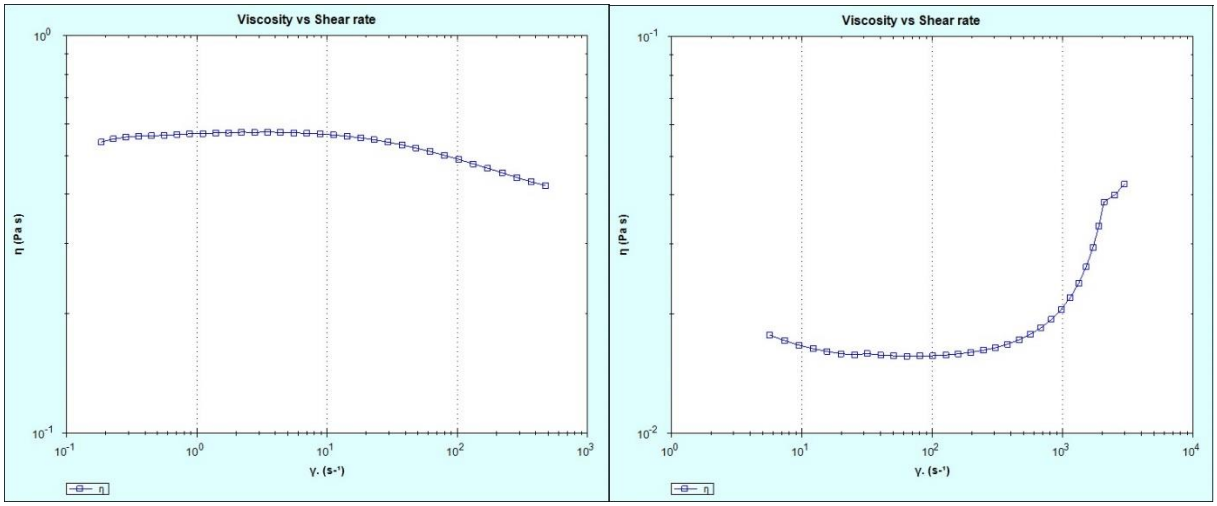

Fig. 9. Viscosity vs Shear rate graphs of two sample preparations, 20% w/w in MilliQ water after microwave treatment of native waxy Potato starch incubated for 3 hours (Right) and 16 hours (Left) in 1M HCl small batch

As the technique was established for small-batch, it was suggested to increase the batch size. Such action yielded strange results, both for corn and barley starches. The rheological profile of the corn sample is presented in Fig. 10. It is highly altered, due to significantly lower (more than 10x decrease) viscosity compared to the small batch size it was hard to yield the rheological profile. Similar results were achieved for barley starch and presented in Table 3.

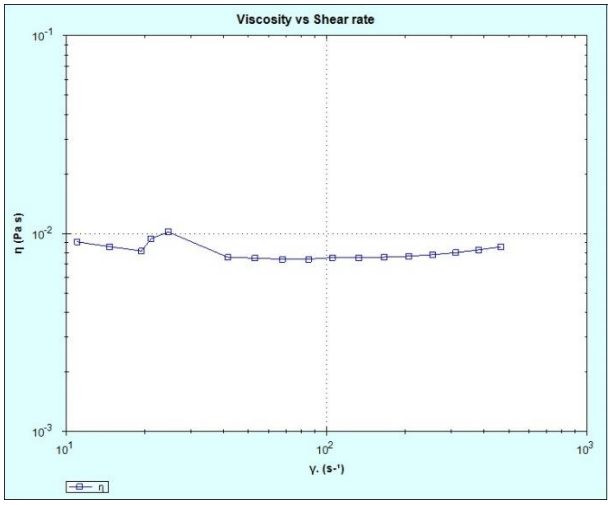

Fig. 10. Viscosity vs Shear rate graph, 20% w/w in MilliQ water after microwave treatment of native waxy corn starch incubated for 3 hours in 1M HCl big batch

There were also attempts to reduce the acid concentration for big batch preparation, but the viscosity only increased two times compared to the previous big batch run. The calculated viscosity of all analyzed samples is combined and shown in Table 3.

Table 3. The viscosity of the acid hydrolysed starches. Highlighted samples were used for further investigations

Sample	Time of incubation, hours	Viscosity, Pa/s
BARLEY ST. reference 10 %	-	0,0117
BARLEY ST. reference 20 % triplicate	-	0,1376
BARLEY ST. reference 20 %	-	3,3512
BARLEY ST. SmallB 1M HCl	1	0,1406
BARLEY ST. SmallB 1M HCl	2	0,1335
BARLEY ST. SmallB 1M HCl	3	0,1213
BARLEY ST. SmallB 1M HCl	4	0,1087
CORN ST. SmallB 1M HCl	1	0,1308
CORN ST. SmallB 1M HCl	2	0,1216
CORN ST. SmallB 1M HCl	3	0,1185
CORN ST. SmallB 1M HCl	4	0,1098
POTATO ST. SmallB 1M HCl	2	0,5689
POTATO ST. SmallB 1M HCl	2,5	0,6838
POTATO ST. SmallB 1M HCl	2,75	0,5974
POTATO ST. SmallB 1M HCl - outlier	3	0,0466
BARLEY ST. BigB 1M HCl	3	0,0112
BARLEY ST. BigB 0,5M HCl	3	0,0193
CORN ST. BigB 1M HCl	3	0,0075
POTATO ST.BigB 1M HCl	16	0,0157

3.2. Phase characterization

For further investigations, 4 small batches of hydrolyzed corn starch with different times of incubation were combined into one batch, 4 small batches of hydrolyzed barley starch with

different times of incubation combined into one batch and an overnight big batch of potato starch were used (highlighted in Table 3).

After lyophilization of separated phases, they were collected and carefully weighted, the results were compiled in the form of phase diagrams depicted in Fig. 11, Fig. 12 and Fig. 13. The purple dotted line on the diagrams depicts the dilution line, which was followed when the solutions for this test were prepared. Green, red, and blue lines represent three preparations for each type of starch material, 10/30/60, 8/24/68 and 6/18/76 % starch/PEG/water respectively. Highlighted by the orange ellipse area is the area of particular interest for this project, based on previous microsphere preparation screenings Yellow dot shows the phase concentrations, chosen to make a final microsphere preparation.

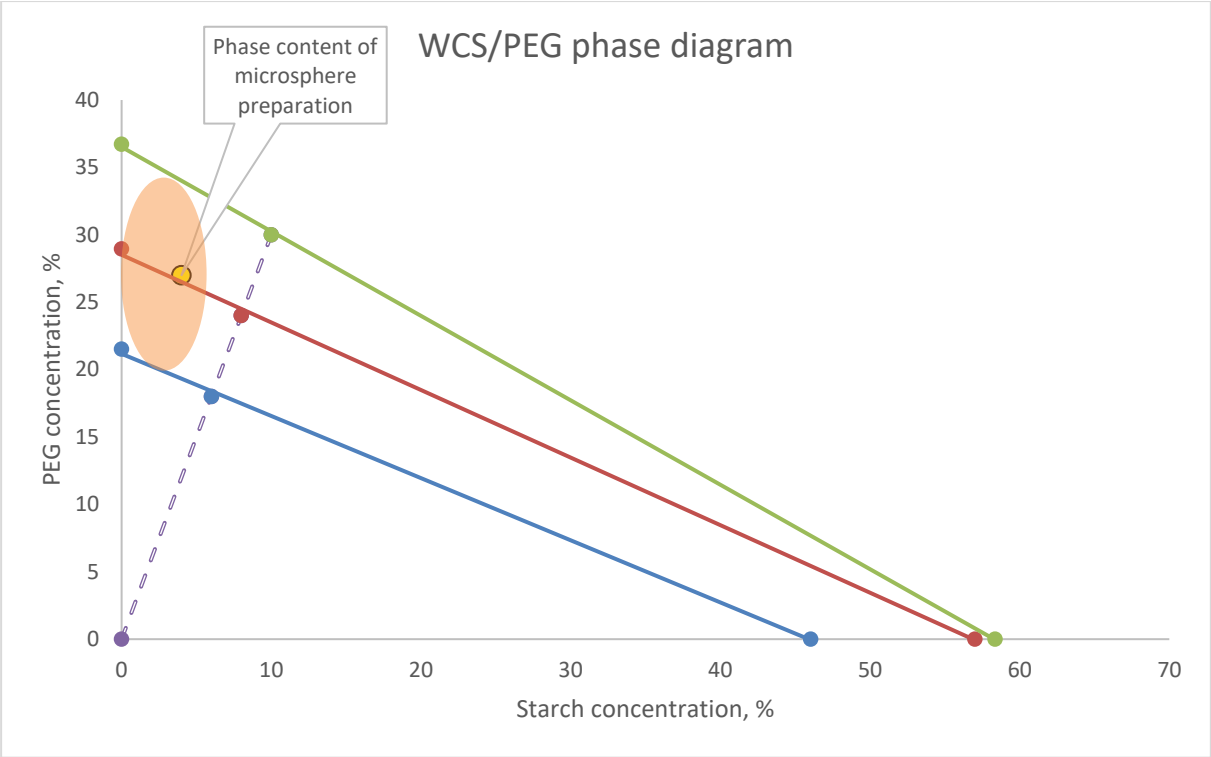

Fig. 11. Waxy corn starch, and PEG 20000 ATPS phase diagram

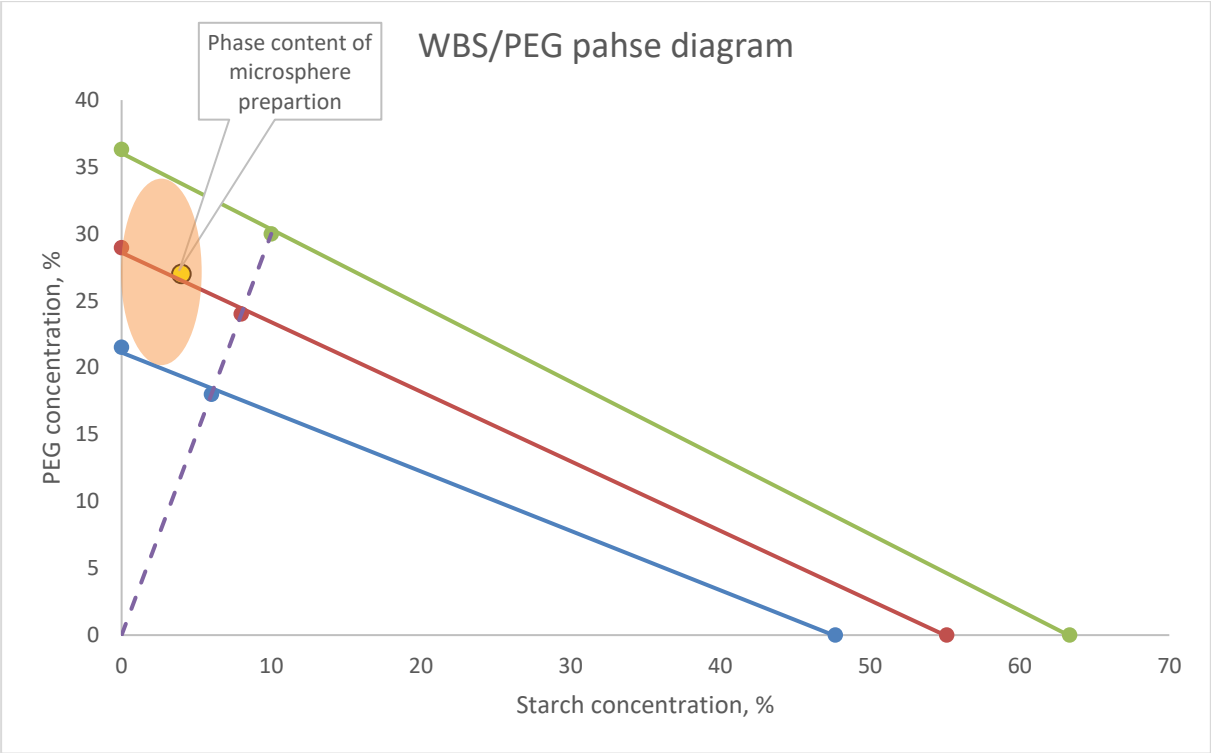

Fig. 12. Waxy barley starch and PEG 20000 ATPS phase diagram

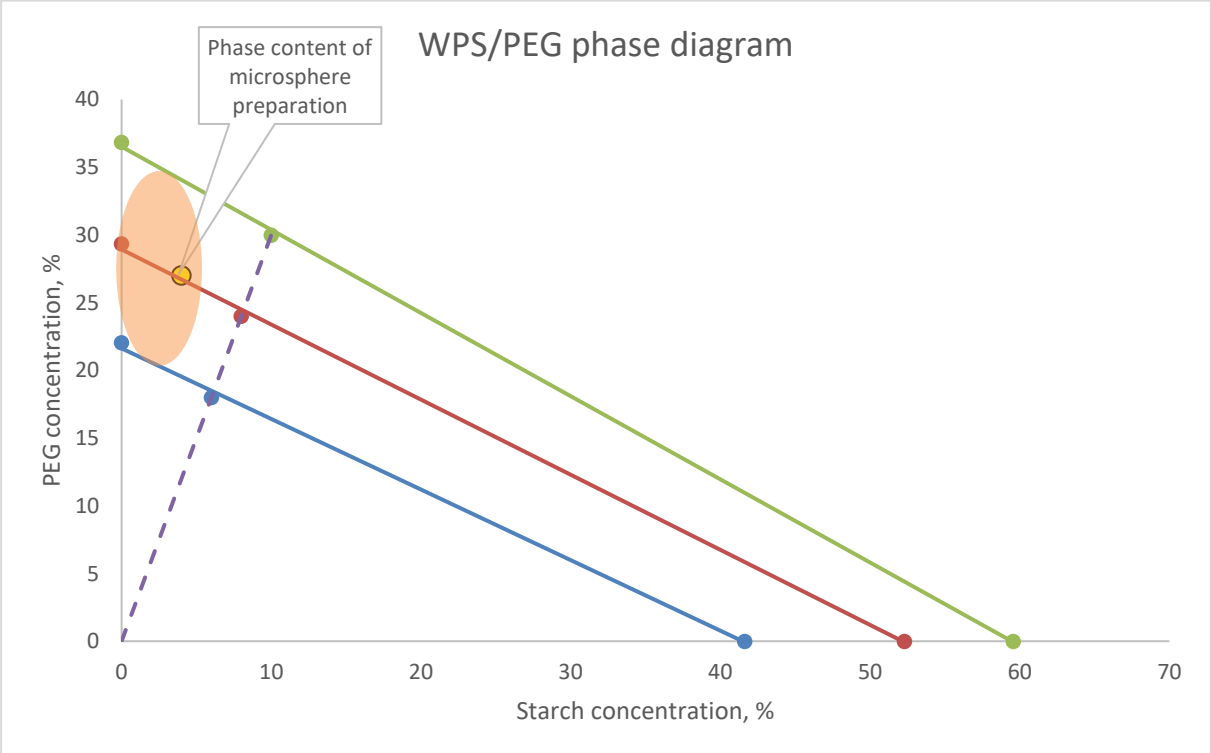

Fig. 13. Waxy potato starch and PEG 20000 ATPS phase diagram

Quantitative data from the experiment is compiled in Table 4. With grey highlighted the outlier value, which fails to behave in the predicted way. To prove that it is an outlier, further studies are needed. Even though the data for the water content of each phase is presented, the % of total water of the system that each phase has in grams is not presented. As can be seen in Figures 11-

13, the phase diagram position where microspheres were manufactured in this study is situated on the 8/24/68 % tie line. The water content of the starch-rich phases for this tie line (as presented in table 4) is highest for samples containing potato starch, with values for corn and barley. The water content for PEG rich phase is comparably the same for all the preparations.

Table 4. Phase content of waxy starches of different botanical origin/PEG 20000 ATPS. Highlighted value is an outlier.

Raw material and preparation concentrations	Water content of starch phase, %	Water content of PEG phase, %	Tie line coefficient
Corn/PEG/0,9 NaCl MilliQ water 6/18/76 %	53,97	78,48	-0,46
Corn/PEG/0,9 NaCl MilliQ water 8/24/68 %	42,98	71,06	-0,50
Corn/PEG/0,9 NaCl MilliQ water 10/30/60 %	41,64	63,27	-0,62
Barley/PEG/0,9 NaCl MilliQ water 6/18/76 %	52,32	78,48	-0,44
Barley/PEG/0,9 NaCl MilliQ water 8/24/68 %	44,88	71,64	-0,52
Barley/PEG/0,9 NaCl MilliQ water 10/30/60 %	36,66	63,69	-0,57
Potato/PEG/0,9 NaCl MilliQ water 6/18/76 %	58,36	77,95	-0,52
Potato/PEG/0,9 NaCl MilliQ water 8/24/68 %	47,68	70,65	-0,55
Potato/PEG/0,9 NaCl MilliQ water 10/30/60 %	40,42	63,16	-0,61

3.3. Microsphere preparation

The microsphere preparation was assessed by light microscopy and SEM

3.3.1. Light microscopy

Microscopic images of microspheres at various stages of production were taken to assess the process. In the initial stage, the starch phase was highly dispersed in the PEG phase as shown in Fig. 14.

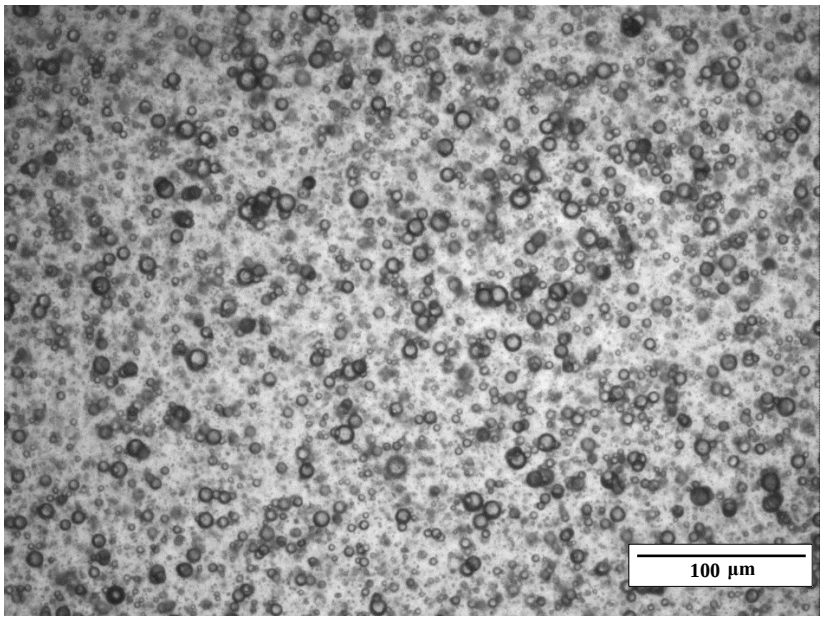

Fig. 14. In the initial stage, acid hydrolyzed waxy barley starch in PEG w/w emulsion. Same visuals were received for corn and potato starch.

After 4 hours of incubation, microspheres were assessed again and the microscopic images of those microspheres are shown on the left side of Fig. 15, Fig. 16 and Fig. 17. Potato starch microspheres have already formed and were rigid. Corn starch microspheres have formed and were quite rigid not to the same extent as potato ones. Barley's starch microspheres were not rigid at this point. The rigidity of microspheres was assessed by applying cover glass and pressing it. Rigid microspheres received very distinctive cracks, whereas soft microspheres deformed without cracking or were coalescing into shapeless bigger vesicles.

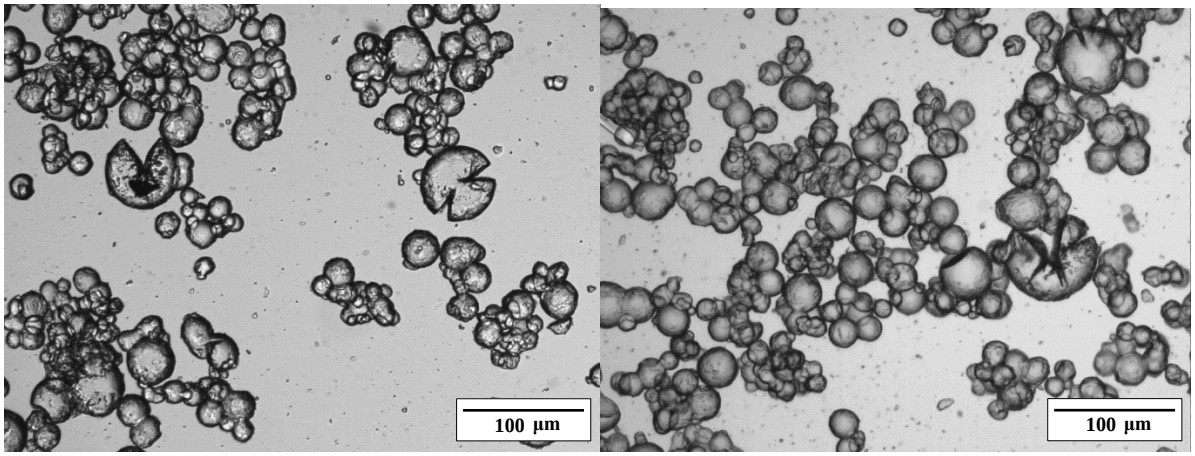

Fig. 15. Potato starch at microspheres during incubation at 4 hours and 24 hours timepoints

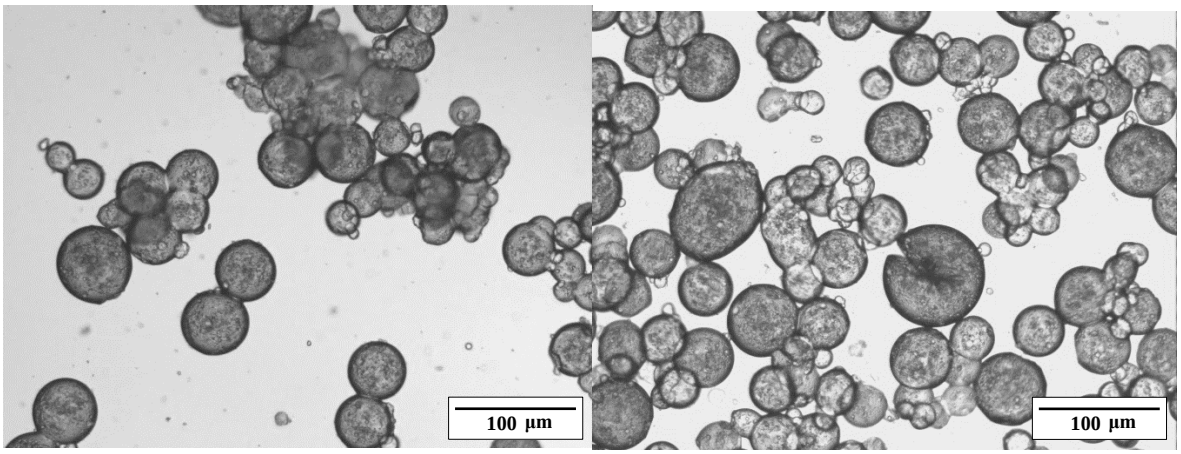

Fig. 16. Corn starch at microspheres during incubation at 4 hours and 24 hours timepoints

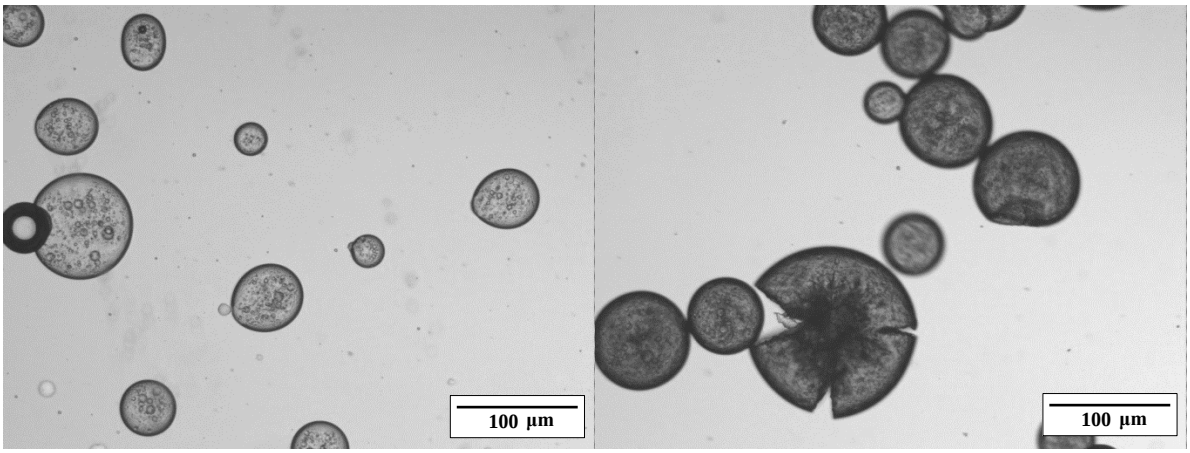

Fig. 17. Barley starch microspheres during incubation at 4 hours and 24 hours timepoints

After 24 hours of incubation, all systems have formed microspheres. These were washed and inspected again and the microscopic images of the microspheres at a 24-hour timepoint are shown on the right side of Fig. 15, Fig. 16 and Fig. 17. At this stage, all microspheres of all starches were rigid and well-formed, as they all received distinctive cracks when the pressure was applied. It can be seen that most of them have spherical forms but sometimes there are cavities on the surface that reduce the total volume of the microsphere. There is a striking difference in the average size of microspheres of the different botanical sources. The largest microspheres were obtained from barley starch, followed by corn starch and the smallest microspheres were received from the potato starch.

After washing, microspheres were harvested and freeze-dried. After freeze-drying, there were two types of structures in the vial: free-floating starch microspheres (Fig. 18, Fig. 19, Fig. 20 – left) and cotton-like structures with microspheres entrapped in it (Fig. 21, Fig. 22, Fig. 23 – left).

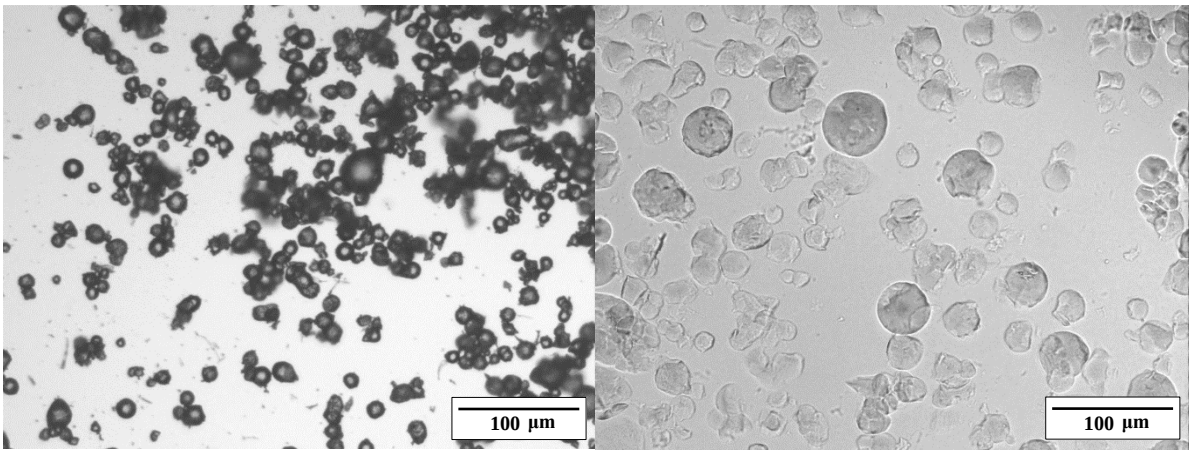

Fig. 18. Potato starch microspheres in dry (left) and rehydrated (right) states

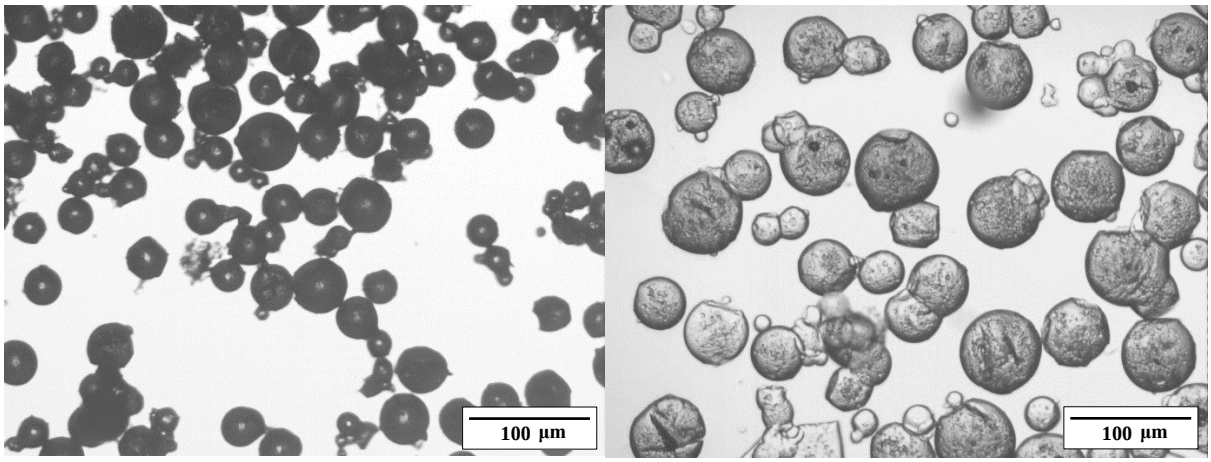

Fig. 19. Corn starch microspheres in dry (left) and rehydrated (right) states

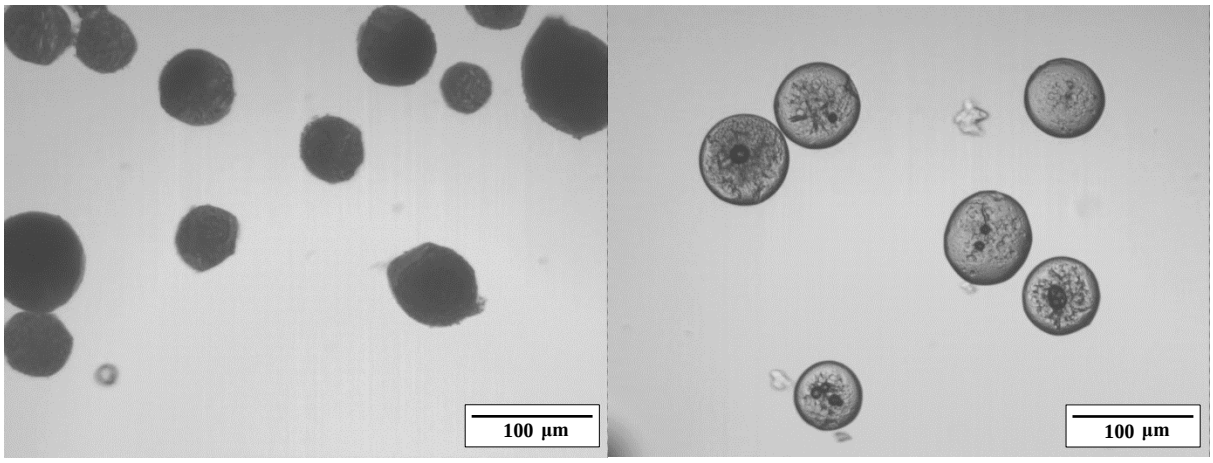

Fig. 20. Barley starch microspheres in dry (left) and rehydrated (right) states

All three types of microspheres made from the starch of different botanical sources represented both microspheres and network-like structures. Then a small number of microspheres and cotton-like network were also exposed to MilliQ water and examined in the rehydrated state, results of this experiment are shown in Fig. 18, Fig. 19, Fig. 20 (Right) for microspheres and in Fig. 21, Fig. 22, Fig. 23 (Right) for the cotton-like network system. Rehydration was achieved by applying a droplet of room temperature water to the microscopy slide It is interesting, that

the network seems to dissolve in relatively cold water almost completely as it can be seen from the figures below.

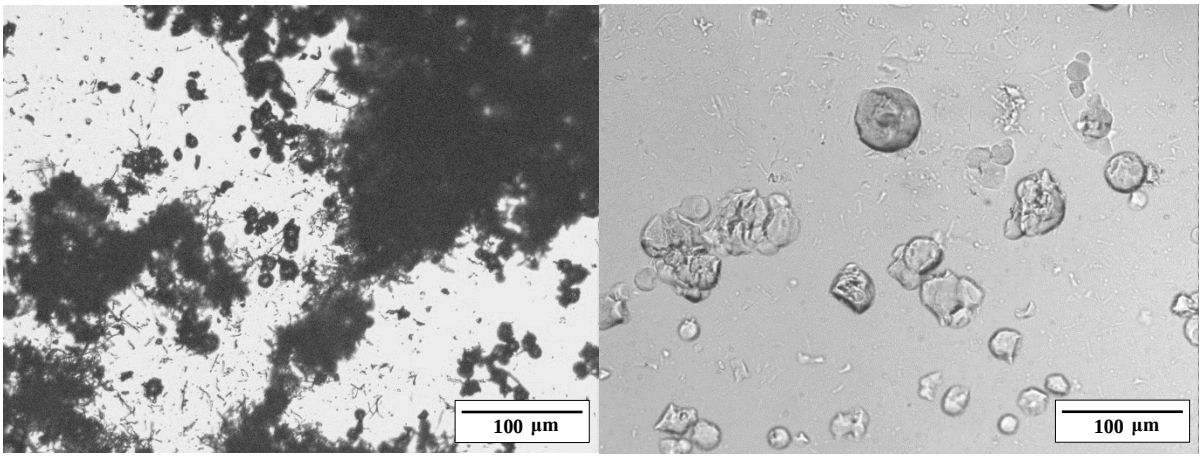

Fig. 21. Potato starch polymer network in dry (left) and rehydrated (right) states

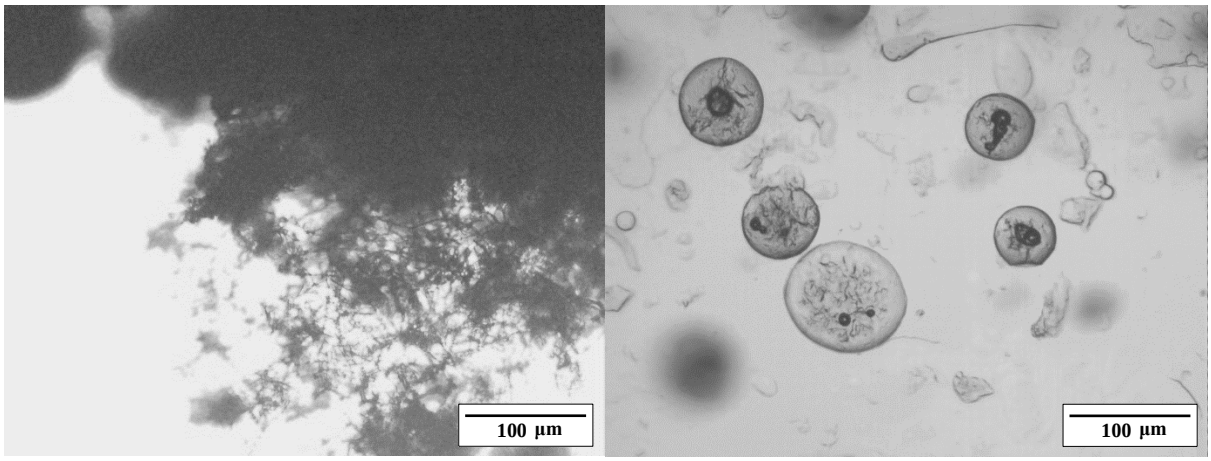

Fig. 22. Barley starch polymer network in dry (left) and rehydrated (right) states

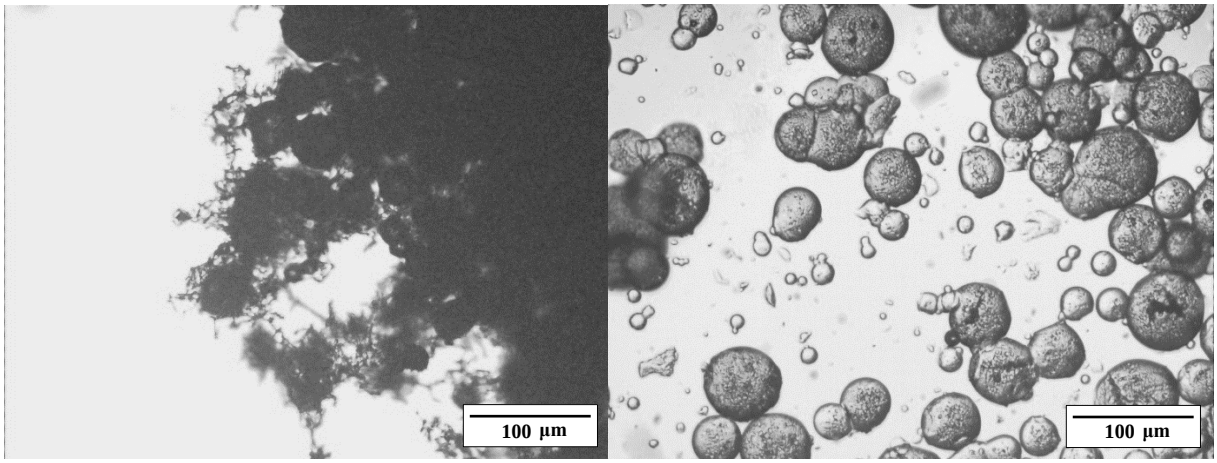

Fig. 23. Corn starch polymer network in dry (left) and rehydrated (right) states

3.3.2. Scanning electron microscopy

SEM images of the dried formulations are presented below in Fig. 24, Fig. 25 and Fig. 26. It was possible to take pictures of microspheres themselves, their surface and slice and the polymer network formed in the process of freeze-drying. It was possible to collect images with 50x,500x and 5000x magnifications in order to properly characterize different aspects and properties of microspheres and compare those properties between the microspheres made of starches from the different botanical sources.

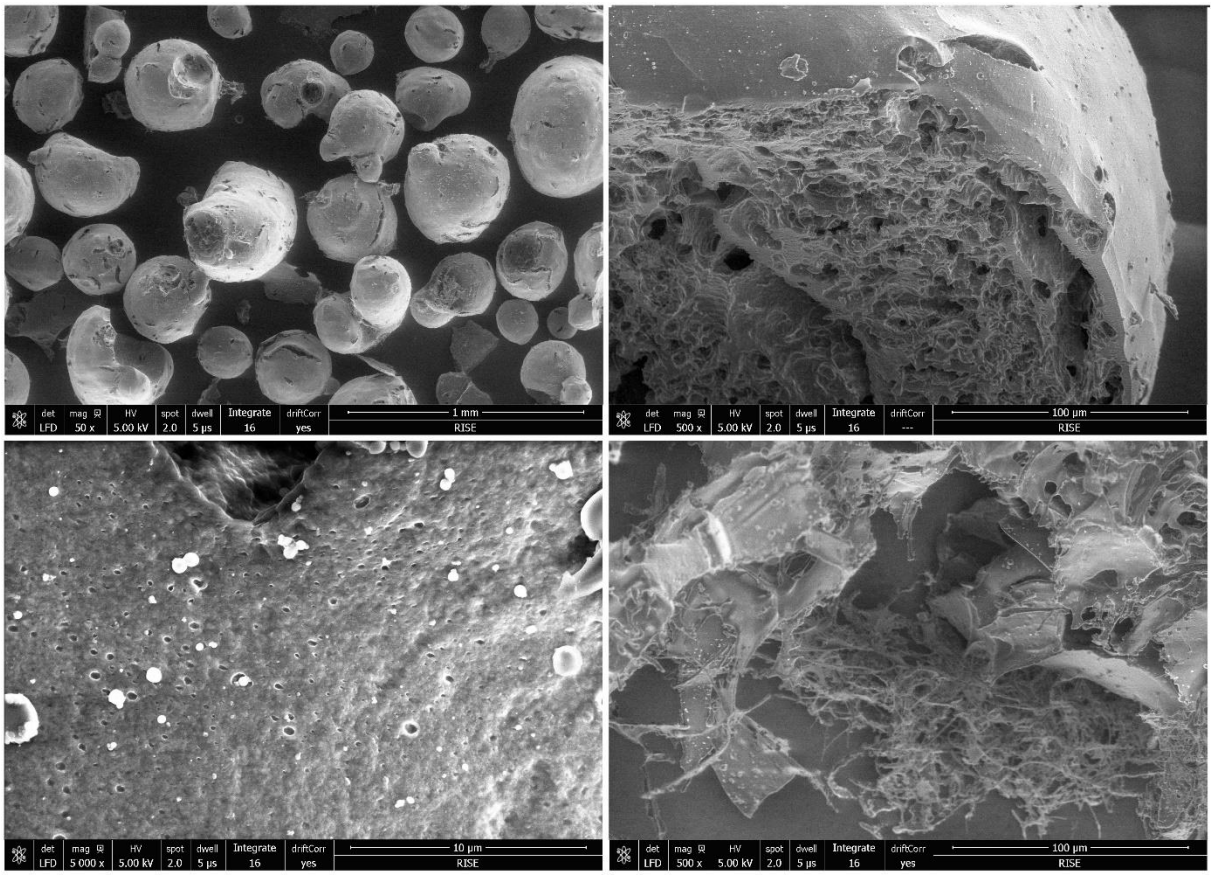

Fig. 24. Barley microspheres SEM images. Top left – free-floating microspheres at 50x magnification, top right – slice and surface of the microsphere at 500x magnification, bottom left – the close-up image of the surface of the microsphere at 5000x magnification and bottom-right – polymer network with microspheres entrapped in it.

It can be observed that microspheres differ in size, porosity, general inner structure and surface structure. Barley starch microspheres were highly porous, while the corn and potato starch ones were more compact and solid. However, the polymer network together with entrapped microspheres looks similar.

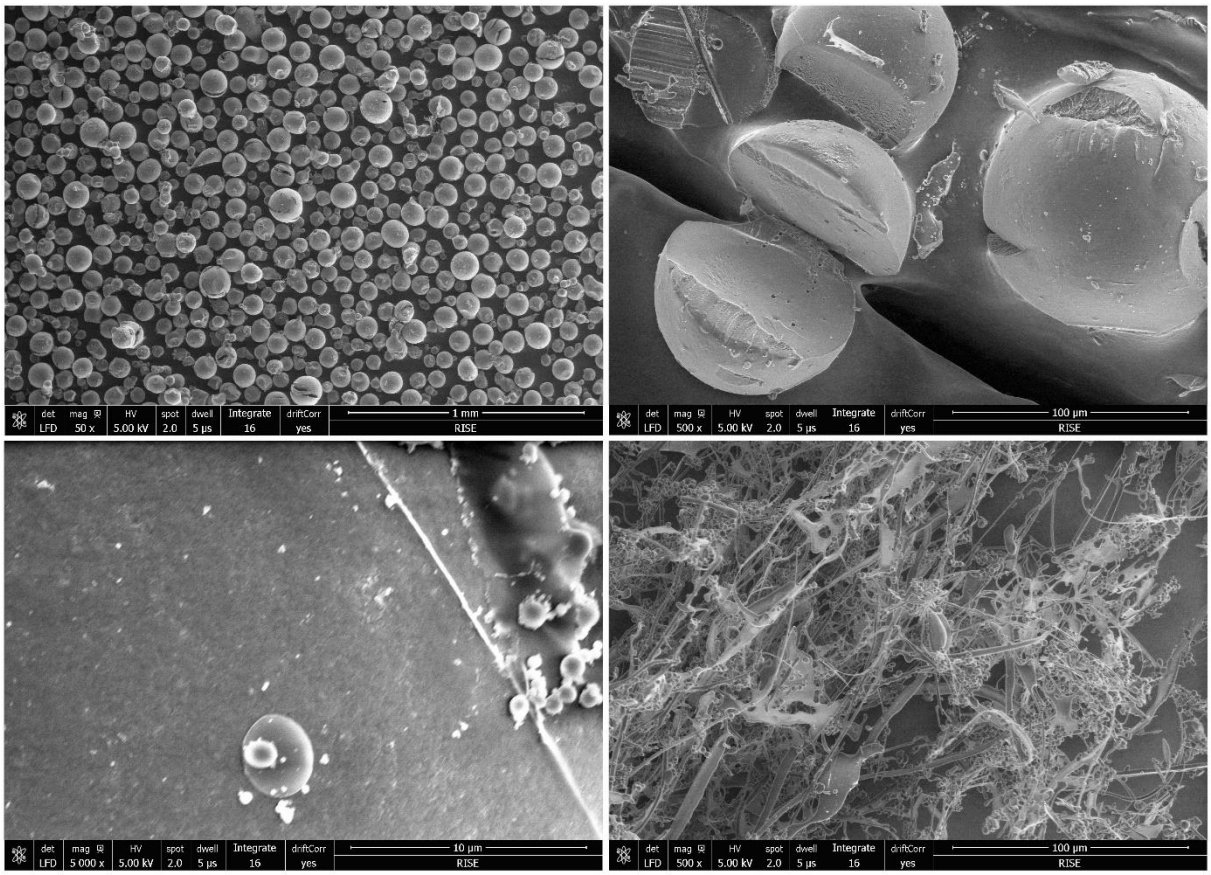

Fig. 25. Corn microspheres SEM images. Top left – free-floating microspheres at 50x magnification, top right – slice and surface of the microsphere at 500x magnification, bottom left – the close-up image of the surface of the microsphere at 5000x magnification and bottom-right – polymer network with microspheres entrapped in it.

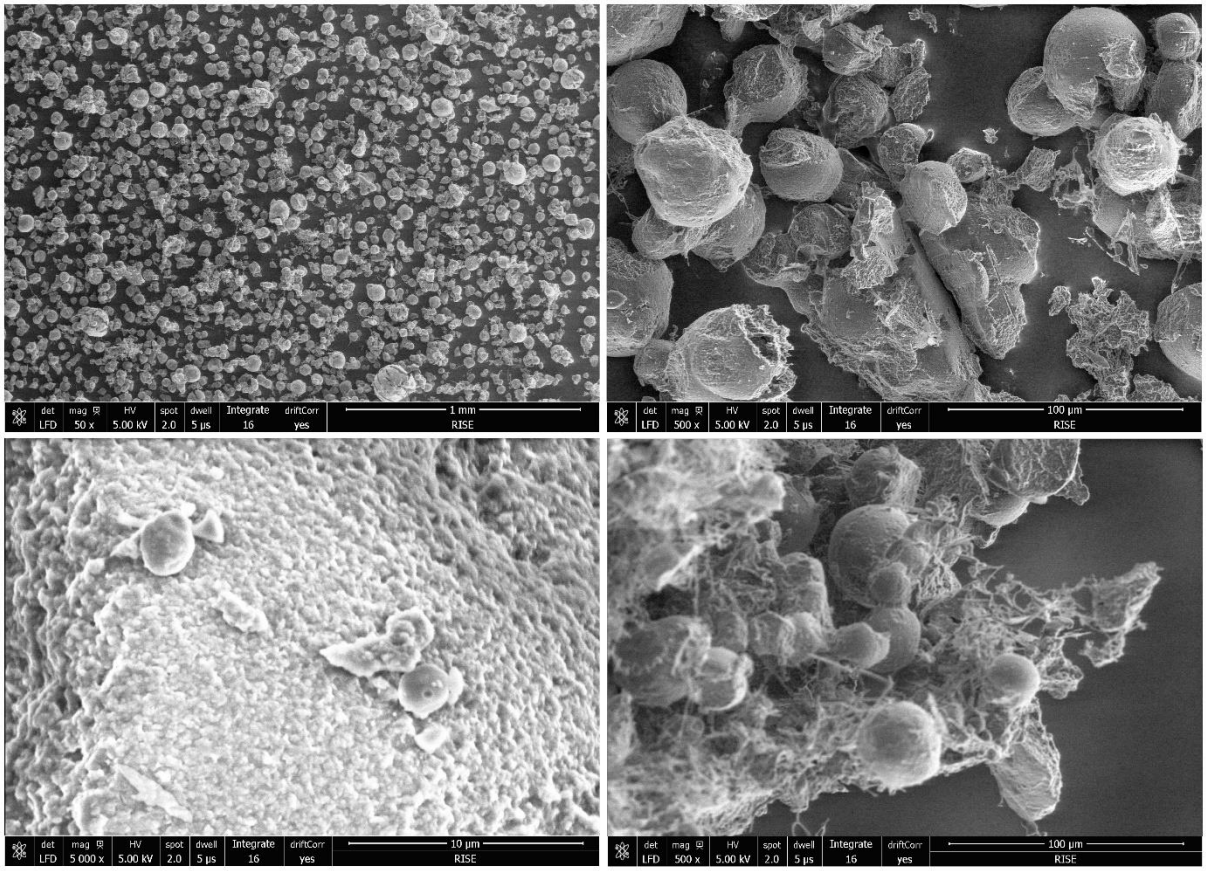

Fig. 26. Potato microspheres SEM images. Top left – free-floating microspheres at 50x magnification, top right – slice and surface of the microsphere at 500x magnification, bottom left – the close-up image of the surface of the microsphere at 5000x magnification and bottom-right – polymer network with microspheres entrapped in it.

3.4. Enzymatic digestion

Results of the enzymatic digestion of different starch microspheres are plotted on graphs versus time, which are presented in Fig. 28 and Fig. 29. During the analysis, a range of cuvettes with an assay that develops color have been prepared and incubated and they are shown in Fig. 27. Graphs represent only the degradation of the microspheres made from native barley and corn starches as the microspheres made from native potato starch were degrading too fast to be measured in the same way as other samples. Additional experiments showed that potato microspheres degrade over time in MilliQ water at 37°C even without the addition of amylase.

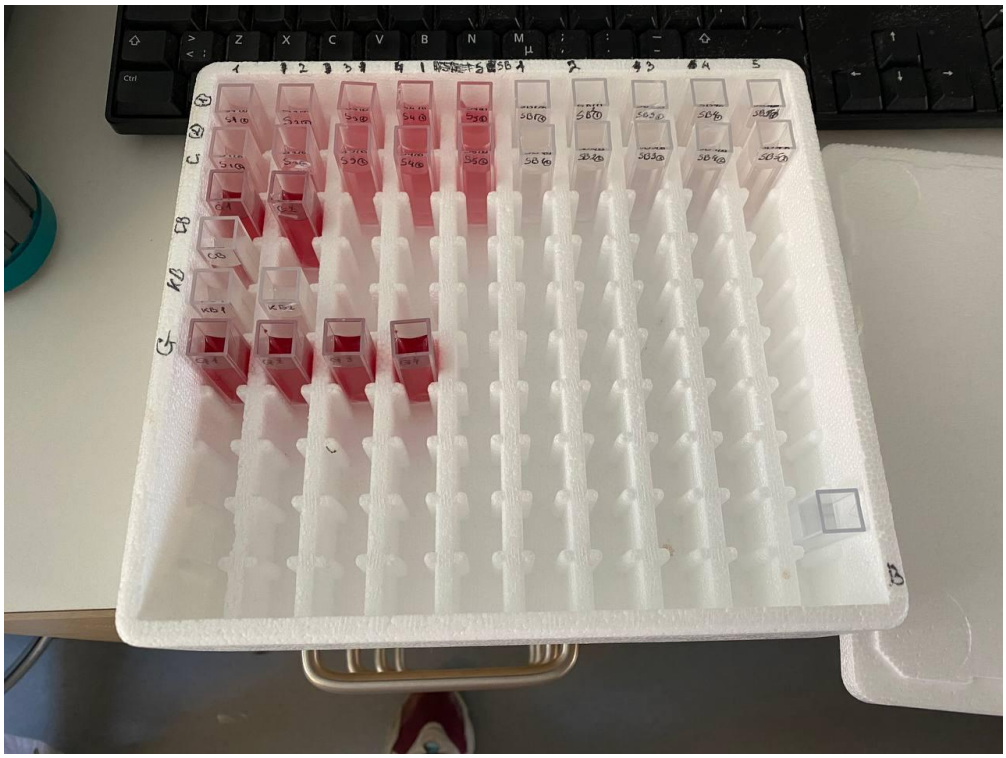

Fig. 27. Final preparations for amylase degradation of corn microspheres assessment after the absorption analysis.

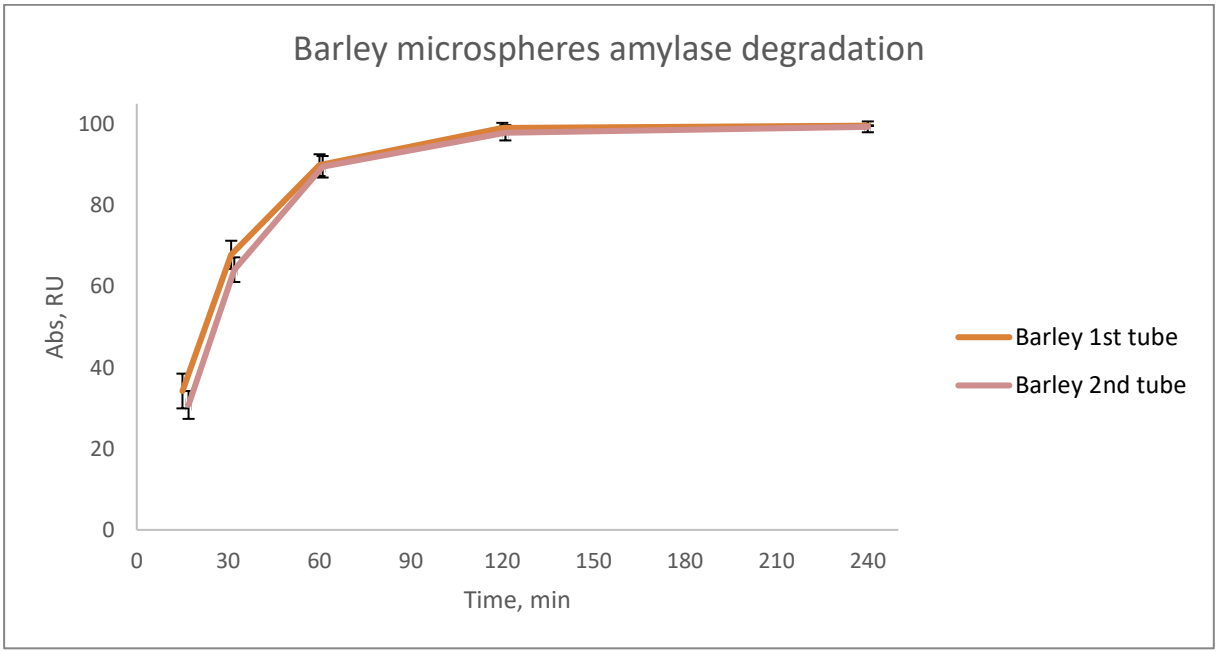

Fig. 28. Amylase degradation of 2 samples containing 100 mg of barley microspheres each plotted against time of incubation.

Both samples of barley microspheres continued to develop color over the time between the 2 absorbance tests and the deviation was added to the graph as the margin lines (Fig. 28). Same margin lines were added to the graphs representing the degradation of corn microspheres (Fig. 29), but the samples with corn microspheres stopped developing the color and the deviation

between the absorbance checks was substantially smaller compared to the deviation of the samples containing barley microspheres.

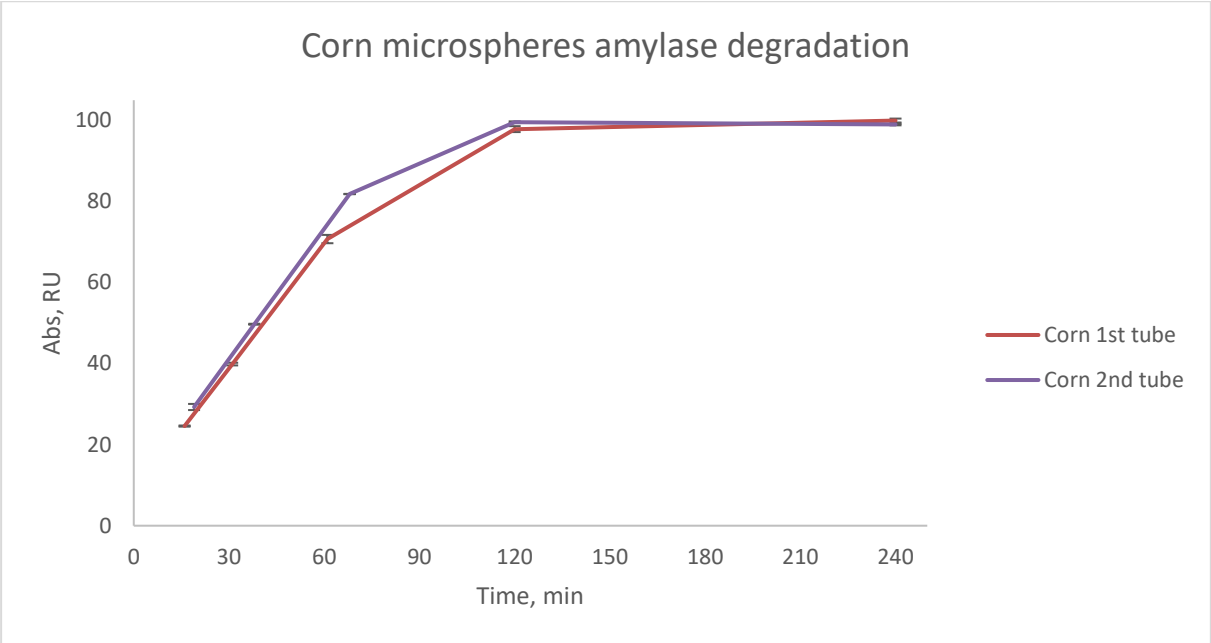

Fig. 29. Amylase degradation of 2 samples containing 100 mg of corn microspheres each plotted against time of incubation.

3.5. DSC

During DSC analysis at least three replicates that gave comparable curves were made. For representation purposes, DSC curves were combined and depicted in Fig. 30. 8 samples were analyzed: freshly prepared microspheres of barley and corn starches with approximately 15% of dry weight, 15% w/w preparations of dry microspheres made of corn, barley and potato that were rehydrated with MilliQ water and 30 % w/w preparation of the same microspheres made the same way. Freshly made potato microspheres were not analyzed due to time limitations and other reasons, that will be touched on in the discussion section.

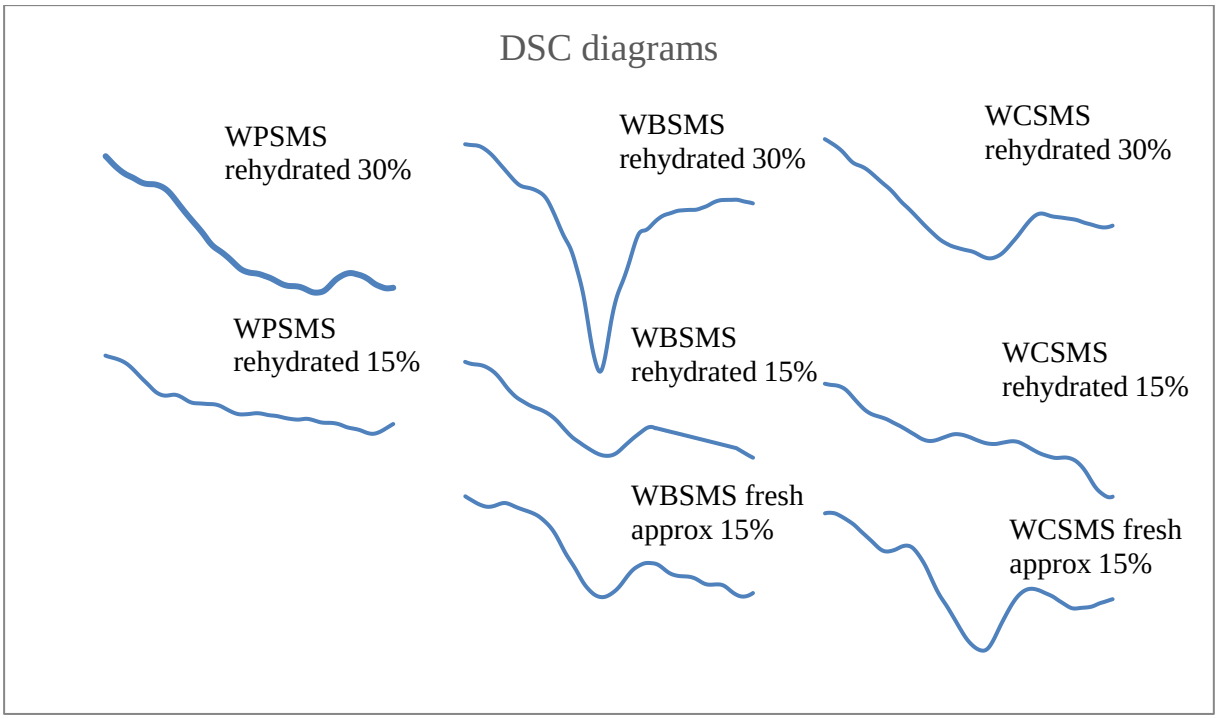

Fig. 30. DSC diagrams of 3 microsphere preparations made of starch with varying biological sources: waxy potato starch microspheres (WPSMS), waxy barley starch microspheres (WBSMS), and waxy corn starch microspheres (WCSMS).

As it can be seen from Fig. 30, microspheres made of starches of various botanical sources behave differently. It was not possible to collect quantitative data of high quality from potato and corn dry microspheres as the signal is too low (15%) or the peaks are too broad (for 30% preparation). Therefore, the qualitative data was only collected and analyzed for the full set of barley microspheres and freshly prepared corn microspheres and is presented in Table 5. When qualitative data point was collected, all three replicates were analyzed and the mean value for each parameter is presented. Freshly prepared corn microspheres have higher Δ enthalpy than freshly prepared barley starch microspheres. Moreover, peak temperatures and temperature intervals for corn starch microspheres are also higher. In addition, the Δ enthalpy for rehydrated barley microspheres is lower than for freshly prepared.

Table 5. DSC mean values for applicable samples

Sample	Onset temp, ℃	Peak temp, ℃	Conc. temp, ℃	ΔT, ℃	ΔH, J/g
Fresh WCSMS	42,82 ± 0,74	52,14 ± 1,21	57,77 ± 1,76	14,46 ± 1,05	7,12 ± 0,23
Fresh WBSMS	43,68 ± 1,29	49,75 ± 0,68	55,52 ± 1,00	11,83 ± 0,69	4,67 ± 0,31
WBSMS dry 15%	43,07 ± 0,22	46,66 ± 0,87	53,02 ± 0,26	9,95 ± 0,48	3,29 ± 0,19
WBSMS dry 30%	44,91 ± 0,86	49,42 ± 0,59	53,62 ± 0,51	8,72 ± 0,80	3,85 ± 0,27

4. Discussion

4.1. Starch treatment

Initially, the method for starch treatment involved neutralization of acid and setting the pH to physiological as any residual HCl can cause damage to the equipment, e.g., lyophilization set up. Therefore, there were attempts to neutralize the acid with a strong and weak base, NaOH and Na2CO3. Whereas the pH was easily and controllably brought to 7.2 when using a strong base, irreversible chemical reaction of said base with starch polymer caused color alterations, which have also been observed in other studies [25]. Subsequently, it was decided to try using a weaker base, but the introduction of weak bases would often result in not controllable pH alterations. Moreover, neutralization in itself would mean the formation of salts in the solution which will precipitate during freeze-drying. This fact might cause problems during the microsphere production, and due to this fact, the method was reworked. This was done by introducing two more washing steps, resulting in 4 washing steps, which substantially reduced acid concentration more than 10^6 times, making it negligible and safe for drying in the said equipment. Additionally, as samples during acid hydrolysis did not heat up higher than 25°C and the gelatinization for native starches start to occur at approximately 50°C on average [10], it was decided not to use the mantle for heat equilibration.

After technique optimization, the first results of acid hydrolysis were received and tested. All 4 samples taken at different time points for both barley and corn starches yielded appropriate values of approximately the same viscosity and rheological profile when compared to reference starch. After gelatinization in the microwave both yielded opaque solutions with the same characteristics. However, potato starch was different, after gelatinization starch suspension became transparent and more viscous compared to the other two samples. This might be explained by either difference in the degree of gelatinization, so there were tests with prolonged microwave treatment for both corn and barley starches, which did not reduce the cloudiness of the dispersions. Another possible explanation is that there might be other molecules connected with the granules, such as lipids, that can alter the transparency. [26]

The degree of gelatinization has also been assessed on the rheometer as after gelatinization shear thickening properties of the starch tend to reduce.[16][17] Due to that Ellis model fit has been used. Such a model is used to study a fluid that has a low Newtonian viscosity region with a transition into the Power Law region. [18] Later on it was found that the set range of parameters was only appropriate for the samples with comparably the same viscosity as the reference as formulations with the same starch to water content exhibited completely different rheological profiles (Fig. 9). However, it was still possible to compare average viscosity after acid hydrolysis, draw conclusions about the appropriate time point of collection and tell if the acid hydrolysis was successful or not.

Another encountered problem was the huge difference in the viscosity of modified potato starch and the misleading outlier presented in Table 3, based on which a theory that potato starch viscosity significantly reduces between 2 and 3 hours of incubation in the described system was made. After incubation of potato starch for 2,5 and 2,75 hours, it was proven that said misleading sample was an outlier and the time for potato starch incubation should be increased. However, only one sample of 16 hours of incubation was produced due to time limitations as the starch modification required 4-5 working days to yield the sample ready for viscosity analysis. Therefore, even though the viscosity of the 16 hour-modified potato starch was not comparable to the reference it was decided to try and make microspheres with it, bearing in mind that they might have different properties in the end.

Another faced issue was that all the efforts to scale up the process and collect a bigger bath of modified starch failed. Up until this point, the reasons for this are unclear, as all critical process parameters were scaled respectively, and the batch size was increased only 2.5 times with a single collection at the 3 hours time point. However, the viscosity of big batch starch was much lower, almost 10 times less compared to the reference sample (Table 3). There were attempts to reduce the concentration of acid in the solution before modification but it did not solve the issue. However, the viscosity of said modified starches, both corn and barley were comparable to 16 hours potato modified starch, which yielded microspheres. Therefore, there was an attempt to prepare microspheres with a big batch of barley and corn modified starch, but after 24 hours of incubation, said starch did not retrograde and there was no rigid microsphere yield. Those batches were discarded as there was no reason to continue experiments with them.

Subsequently, it was decided to combine small batches of modified starches of the same botanical source but collected at different time points, as they had comparably the same viscosity and to proceed with them into further experiments.

4.2. Phase characterization

The general idea for this experiment was to compare the phase behavior of ATPS comprised of starches of varying botanical sources and try to connect the differences with other results. The most important data that we can extract and compare from the phase diagrams shown above in Fig. 11, Fig. 12 and Fig. 13 for corn, barley and potato starches respectively, is

- The general shape of the diagram
- Each preparations phase and water content

It is worth mentioning that the binodal curve was not depicted on the diagrams, mainly because there is no sufficient information to draw it and because it is not needed for the data evaluation. Moreover, the phase diagrams were built on assumptions that it was possible to extract pure phases and that there was only PEG in the PEG-rich phase and only starch in the starch-rich phase (or only negligible amounts of PEG present in the starch-rich phase and only negligible amounts of starch present in the PEG-rich phase) in the observed samples. This limitation could have been addressed by complementary analysis of the separated phases, such as enzymatic quantification of starch in the phases. However, due to time limitations, separate quantification of starch in the phases was not performed. Therefore, the presence of the polymers in both phases cannot be excluded. Nevertheless, the presence of a larger polymer in the opposite phase could be deemed unlikely, based on the high amount of solutes and the different appearance of the dried phases upon ocular observations. However, there might be inclusions of smaller polymer in the opposite phase as the viscosity of the larger polymer is higher and full separation might take longer centrifugation time. As the starch starts to retrograde, it is not possible to allocate more time for centrifugation for proper separation, as retrogradation alters phase behavior. It was assumed that the starch phase contained 0% of PEG and tie lines were built as an approximation of three points on the diagram.

The tie lines represent how the phase separation occurs and the coefficient of those lines dictates the general shape of the phase diagrams. Due to method design, assumptions made and limitations, the coefficients of the lines represented in the phase diagrams acquired in this project slightly deviate from each other for barley and potato starch preparations (Fig. 12 and Fig. 13 respectively) and significantly deviate for 10/30/60 corn starch preparation compared to 6/18/76 and 8/24/68 preparations (Fig. 11). This significant deviation in the 10/30/60 corn starch-PEG ATPS tie line was treated like an outlier (even though two duplicate samples showed very close results for this point on the diagram), and further discussion will not take this tie line into the consideration. However, this sample could have such values of water

content in the starch phase due to the higher viscosity of corn starch, resulting from the higher amount of solutes, making separation of the starch- and PEG-rich phase more problematic.

Small deviations in starch-rich phase content will have a lower effect on the coefficient of the diagrams due to the design of the experiment. The initial point of each tie line (blue, red and green lines) lies on the dilution line (purple dotted lines), like a lever, following the same rule as in physics. The ratio between the shoulders of the tie line can tell us how the deviation in one pure phase content would affect another pure phase content. And as the region of interest lies closer to PEG rich phase, the slight deviation in the calculated and real initial PEG concentration would severely affect the final distribution of the water for the starch-rich phase in the yielding microspheres preparations.

To characterize the phase behavior of those final preparations it was important to get comparable data for the tie line that lies in the closest proximity to those preparations (Table 4) during incubation of the microspheres. For all starches, the red line, representing 8/24/76 preparations, was the closest tie line to the dot representing concentrations of the preparations which would be used for microsphere production. Therefore, the data received from the 8/24/76 is the most relevant for the characterization of the final ATPS preparations.

The initial theory was that the properties of microspheres of different botanical sources would depend on the phase behavior and water content in the phases. As we can follow the red line to yield the information about the phase content in final preparation (Table 4), we can compare this data with microscopic images of the microspheres of different botanical sources at different time points. Phase content of 8/24/76 preparations for barley and corn look quite similar to each other and taking into consideration comparably the same viscosity, microsphere behavior during the incubation of these starches was expected to be similar. However, by comparing microspheres produced from corn and barley starches during incubation, represented in Fig. 16 and Fig. 17 respectively, it was noted that solid corn microspheres have formed after 4hr whereas barley microspheres have not. Moreover, there are differences in size porosity, surface structure (Fig. 24, Fig. 25), enzymatic degradation (Fig. 28 vs Fig. 29) and a number of crystalline structures for fresh samples (Table 5) which will be discussed more in other sections. On the other hand, potato microspheres with different phase content of the same 8/24/76 preparation have comparable size, porosity (Fig. 25, Fig. 26) and DCS data (Fig. 30) for rehydrated dry microspheres when compared to corn starch preparations. Solid potato microspheres have formed at the 4hr time point of incubation (Fig. 15), just as in the corn starch preparations case.

It was noted that there can be a correlation between the viscosity of the starch solution, molecular weight of the polymer and the amount of water the starch phase consists of after centrifugation. As the molecular weight of the starch polymer is a decisive factor, a broad assumption can be made that the amount of the retained water might be connected with the molecular weight of the polymer. In the thesis work of Elsa Roxling [27], the influence of the PEG polymer size on the microsphere preparation was studied, among other things. And it was proven that with PEG 2 000 000 the microspheres did retrograde slower in the cold room environment compared to PEG 20 000, which also may relate to the thought that higher polymer molecules have more structured solvation networks, which do not promote water intake, altering the properties of the ATP.

4.3. Microscopic inspection

Both light and SEM microscopic images gave important insights into the microspheres, that can be connected to the results from other experiments. The general shape of the microspheres is comparable to other examples from the literature, size, however, is different. [8] The size

difference of microspheres prepared from different botanical sources is one of the most straightforward properties that vary, and both SEM and light microscopy prove it (Fig. 18, Fig. 19, Fig. 20, Fig. 24, Fig. 25 and Fig. 26). As on the initial stage, all three starches gave rise to completely same looking ATPS (Fig. 14), differences in final microsphere size can be attributed to two competing processes that are going in parallel: coalescence of the starch vesicles due to low kinetic stability of the ATPS [16] and retrogradation of the starch that results in the rigid structure formation [11] which restricts vesicle coalescence. Therefore, to receive a microsphere of appropriate size both parameters are needed to be taken into consideration when process optimization will be a question.

However, the general idea for doing light microscopy was a fast, ongoing evaluation of the qualities of starch microspheres. Such an approach was useful for, as already mentioned, ATPS incubation characterization and evaluation of rehydration properties. On the other hand, SEM evaluation cannot be done that straightforwardly and was performed on dry samples. The technique has higher resolution and magnification capabilities, which allows for studying such details as porosity/inner structure, and surface structure and assessing the formed polymer network more accurately.

As it was already mentioned after freeze-drying all samples of microspheres have exhibited polymer cotton-like network formation. Light microscopy assessment pointed out that this network contains lots of microspheres entrapped in it and that it is easily dissolved in water (Fig. 21, Fig. 22, Fig. 23) which gives rise to two ideas. There is still no exact information on how this network is formed or which polymer it is made of, either residual PEG left after washing in trace amounts or the broken-down starch polysaccharides. Either way, it was estimated that for both polymers the network was formed probably due to the lack of time for polymers to properly sediment and have been frozen in the solution as is, in the floating state, prior to freeze-drying. However, after studying the SEM images of the network (Fig. 24, Fig. 25, Fig. 26), it was found that starch microspheres, especially those of smaller size are not trapped but rather connected with the network, which gives a conclusion that the network and microspheres have the same origin: polysaccharides from starch. If it would be a PEG network, polymers would repel each other and not form such a system.

Microspheres also have been rehydrated to see how they swell and if they would, how they would burst. The evaluation was continuous and after 10-15 minutes of evaluation, microspheres seemed to stop growing (Fig. 18, Fig. 19, Fig. 20 – right side). It is interesting that microspheres made from potato and corn starch due to swelling significantly increase in size, but barley microspheres do not. Once again, after assessing the SEM images of surface structure and the inner structure of sliced microspheres of all three botanical sources (Fig. 24, Fig. 25, Fig. 26) it was established that barley microspheres have highly porous inner structure, whereas corn and potato are quite solid inside. The surface of barley microspheres also contained a lot of pores, creating more surface, which probably helped water to come inside and interact with the microsphere faster, compared to corn microspheres. Potato microspheres had a non-homogeneous, rough, consisting flake, surface profile, which also might have been helping the water to get inside. Corn microspheres had a very smooth surface with a little number of pores, and it could be assumed that water had trouble penetrating the microspheres. However, even after 10-15 minutes, all three microsphere preparations had a distinct black bubble in the middle of some of the microspheres (Fig. 18, Fig. 19, Fig. 20 – right side). It was not understood, was the air bubble or the core of the microsphere, that did require more time to swell.

Due to the point that, despite having the same viscosity and same phase behavior, barley and corn microspheres had different sizes, porosity and surface structure, a conclusion was made, that the ability of starch to retrograde as well as kinetic stability of the ATPS depends on the

properties of raw material and may vary greatly even between different batches of starches received from the same plant, as the amylopectin to amylose ratio, amount of lipid/protein structures bound to the granule will vary. [2]

4.4. Enzymatic digestion

As the amylase concentration is lower than expected in the duodenum, even though some agitation was added, it is not possible to say the environment in the human duodenum was created. This concentration of amylase was used in order to obtain measurable data for the comparison of kinetic differences. Therefore, the main purpose of the technique was not to mimic the GI tract but to have a sensitive method to investigate differences between corn, barley and potato starch microspheres overall. Two out of three microsphere samples were tested and compared, as during the experiment it was found that the potato starch microspheres degrade over time at 37°C. It was not expected but justifiable, as the properties of the initial material such as viscosity and time of acid treatment were different, which probably substantially change molecular weight and stability in water solutions. And due to the fact that it was not possible to collect reliable data, potato microspheres were removed from this experiment. However, such rapidly dissolving microspheres at body temperature even without the presence of the digestive enzymes might be used in other formulations where such release is desirable (rectal, vaginal, nasal, and other formulations that target mucous membranes as starch has good adhesive properties with mucous [6]) and are interesting for further studies

When comparing corn and barley microspheres' results of amylase degradation (Fig. 28, Fig. 29) it is clear that barley microspheres degraded faster compared to corn. For barley, we can see the degradation of the sample reached 90% at 60 minutes, Fig. 28, whereas for corn this point is somewhere between 60-120 minutes, closer to 120 minutes.

These differences can be explained by differences in microsphere structure as other controlled properties of these preparations are comparably the same. It was assumed that bigger size and higher porosity of barley microspheres help amylase to easily access inner parts of the microsphere to degrade it faster. Swelling constraints for corn and generally more compact and solid structure do the opposite and restrain the amylase to access the inner part and digestion occurs more gradually.

The technique was adopted from the total starch assay kit, which primarily is used to find the concentration of starch in a 100 mg sample, or for quality control purposes during the starch production process. The main purpose of this experiment is the investigation of kinetic properties of starch microspheres when exposed to digestive enzymes and the deliverables are the graphs depicting the relative amount of digested starch rather than a single unknown concentration of the starch in the initial sample. Therefore, there are some possibilities to adjust the technique, which can greatly increase the accuracy of such an experiment. Due to the rapid sedimentation of microspheres, homogenous sample collection was challenging. Instead of incubating 100mg of starch microspheres in one tube with 10 ml of amylase solution and taking samples from it, it was proposed to incubate 5 smaller tubes with 20mg of microspheres dissolved in 2 ml of solution. This addition should eliminate the error in taking aliquots and increase the accuracy of the technique.

4.5. Differential scanning calorimetry

Firstly, the results received during this experiment were highly inconsistent, and the background noise was high. These constraints were not possible to deal with as the equipment used had not been sensitive enough and could not get the stable signal compared to noise for used samples, as usually this equipment is used with higher starch to water concentrations.

Secondly, due to time limitations (mainly) and general non-comparability of acid thinned potato starch and the fact that they dissolve in the solution at 37°C over time, freshly prepared microspheres made from said starch were not analyzed.

As it was already mentioned, only barley microspheres produced readable signals in all three preparations. Corn microspheres produced a good signal only in the fresh state, in the dry state signal received from corn was very comparable to potato microspheres. Assumptions have been made that, either corn and potato microspheres require more time to fully rehydrate and equilibrate with water due to their solid inner structure, compared to the porous inner structure of barley microspheres; or that some irreversible alterations happen with corn samples during freeze-drying, that is not happening with barley microspheres. (Fig. 30) Samples were rehydrated and kept in the crucible for 10-15 minutes prior to investigation, other studies propose to incubate the samples for longer times [8] and it might be helpful for further investigations. Nevertheless, barley starch microspheres Δ enthalpies also decreased upon drying, pointing toward freeze-drying disrupting the ordered structure of the formulations overall.

The process of data interpretation was already discussed in the literature. [8] Narrow peaks with a high signal can be interpreted as the melting of specific crystalline structures occurring. This highlights that there was a high number of crystalline structures of the same nature in the sample prior to DSC. [8] Subsequently, broad peaks with low signal imply that there are impurities, other crystalline structures, etc. Moreover, even though in the observed literature [8], much broader peaks are analyzed, the enthalpy and the peak temperature values for waxy corn starch microspheres (Table 5) are comparable to the values received in the study, for the same type of starch used for microsphere production.

Differences in DSC results between freshly prepared microspheres of barley and corn, smaller enthalpy for barley, in particular, can also be attributed to the difference in structure, as barley spheres look more amorphous in regards to the inner structure, compared to corn ones. (Fig. 24 vs Fig. 25).

5. Conclusion

Starches of all three botanical sources were successfully acid hydrolyzed, but the appropriate viscosity was achieved only for waxy barley and waxy corn starches. For waxy potato starch reference viscosity was not met and it was decided to proceed with a more hydrolyzed version with lower than reference viscosity. Moreover, the results were inconsistent during the scaling-up process.

ATPS based on PEG and acid hydrolyzed waxy starches, which undergone microwave treatment to gelatinize, were produced. Phase behavior for ATPS systems based on all three starches used was studied. Phase diagrams were created according to the data received from PEG and starch-rich phases content. Phase behavior of corn and barley starches were comparably similar. The reasoning for that fact might be the same viscosity profile. As potato starch used in this experiment had a lower viscosity in water solution compared to barley and starch, it had a different, higher water content in the starch phase.

As the data was compared with other properties of the microspheres, the idea that the characteristics of the microsphere production such as the possibility of retrogradation and retrogradation speed as well as the properties of the microspheres such as porosity, surface structure and size would depend on the phase behavior was not proven and more precise studies in this field are required.

Then microspheres from mentioned starches were produced, washed, and freeze-dried successfully. Light microscopy evaluation gave a lot of insights into the process of microsphere preparation and the microspheres themselves. By utilizing SEM, a comprehensive visual characterization of all three produced microspheres, their inner structure, surface and, presumably, polysaccharide network formed during lyophilization, was made and compared to each other. Under current production conditions, acid hydrolyzed waxy corn and waxy barley starches, hydrolysed to the same extent, can generate microspheres of different sizes, morphology, and interior structure. Here, dried barley starch microspheres exhibit porous interiors, while the corn starch microspheres are smaller and display smoother surfaces with a more compact interior.

Enzymatic digestion curves were established during the amylase assay studies. Knowledge about how microspheres would behave in the presence of amylase and the kinetic properties of this behavior was received for barley and corn microspheres. Potato microspheres could not produce representable curves and results for this study were not presented. However, longer acid hydrolysis with potato starch and current production conditions can give microspheres that break down at 37 °C without the presence of the digestive enzymes.

Thermal properties of starch microspheres were assessed by utilizing DSC. As the last stage of retrogradation is recrystallisation, the number of crystalline structures formed can be checked by DSC. The received results were controversial. Method optimization is required. However, the ΔH of freshly prepared corn microspheres was similar to results from other studies about corn microspheres, with more narrow peaks received during this project. It was also possible to compare barley and corn DCS data for freshly prepared microspheres and it seems like corn have more crystalline structures formed, which correlates with microscopy investigation.

6. Future work

Not all initially set aims were reached for this project due to significant time constraints and limitations. Some of those constraints and limitations involved time-consuming experiments with inconsistent results, especially in the initial phase of acid treatment of the starch. Without results comparable to the reference from these experiments it was not possible to continue the further experiment and due to the complexity of acid treatment, it was not possible to do several experiments in parallel. In my opinion, the project was quite massive in itself and could have been possibly divided into three or four projects which would investigate the impact and relationships of only one or two parameters at the moment, with proper focus on technique optimization.

More time and consecutive experiments are needed to optimize the acid hydrolysis for starches of different botanical sources if comparable properties are needed. Moreover, experiments with scaling up the process were not successful and further optimization is required. Results were inconsistent and standardization of the method is needed for reproducibility purposes.

Even though the phase studies were more or less successful, there was one outlier and there is no established technique to tell how water is distributed in these systems. Also, a number of assumptions about phase behavior have been made in this project, that have to be examined more thoroughly.

An optimization for the enzymatic digestion method has already been proposed. Based on the results with the potato starch microspheres, exploring the potential for rapidly dissolving potato starch microsphere formulations may be the focus of future studies.

DSC method requires more consecutive trials, especially on the equipment used in this project. As was suggested during the discussion of the results, as the rehydration properties of microspheres are different, it might be useful for future studies to let microspheres be left at room temperature for a longer time (several hours) for all microspheres to rehydrate.

There are still some unsolved questions about why the polymer network is being formed during freeze-drying, which polymer comprises this network, and can it be used somehow for future studies. Questions about the relations between different parameters and how to tweak the process of microsphere preparation to yield reproducible batches.

As these starch microspheres could be used as protective drug carriers for different APIs, possibilities of encapsulating different kinds of API of the chemical or biological source are of high interest. Studies of how cryoprotectants and excipients, used in such formulations, will affect the process of microsphere preparation are needed. The starch of which botanical source would yield such preparations with the highest quality is also an important question. How to control and which critical properties of raw materials to control needed to be studied as well.

As my work is conducted in collaboration with the researchers from RISE, I hope that the data and insights I have produced will be helpful for them in their future projects.

References

[1] Nasrollahzadeh M, Atarod M, Atarod M, Sajjadi M, Isaabadi Z. An Introduction to Green Nanotechnology. 1st ed. London: Academic Press, an imprint of Elsevier; 2019.

[2] Wang S, Guo P. Botanical Sources of Starch. Starch Structure, Functionality and Application in Foods. 2020:9-27.

[3] Odeniyi M, Omoteso O, Adepoju A, Jaiyeoba K. Starch nanoparticles in drug delivery: A review. Polymers in Medicine. 2019;48(1);41-45.

[4] Mao S, Chen J, Wei Z, Liu H, Bi D. Intranasal administration of melatonin starch microspheres. International Journal of Pharmaceutics. 2004;272(1-2):37-43.

[5] Heritage P, Loomes L, Jianxiong J, Brook M, Underdown B, McDermot M. Novel polymer-grafted starch microparticles for mucosal delivery of vaccines. Immunology. 1996;88(1):162–168.

[6] Hamdi G, Ponchel G, Duchêne D. Formulation of epichlorohydrin cross-linked starch microspheres. Journal of Microencapsulation. 2001;18(3):373-383.

[7] Xia H, Kou T, Liu K, Gao Q, Fang G. Recrystallization kinetics of starch microspheres prepared by temperature cycling in aqueous two-phase system. Carbohydrate Polymers. 2018;198:233-240.

[8] Elfstrand L, Eliasson A, Jönsson M, Reslow M, Wahlgren M. From Starch to Starch Microspheres: Factors Controlling the Microspheres Quality. Starch - Stärke. 2006;58(8):381-390.

[9] Laurentin A, Edwards C. Fiber: Resistant Starch and Oligosaccharides. Encyclopedia of Human Nutrition. 2013;:246-253.

[10] Jenkins P, Donald A. Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study. Carbohydrate Research. 1998;308(1-2):133-147.

[11] Wang S, Li C, Copeland L, Niu Q, Wang S. Starch Retrogradation: A Comprehensive Review. Comprehensive Reviews in Food Science and Food Safety. 2015;14(5):568-585.

[12] Xia H, Li B, Gao Q. Effect of molecular weight of starch on the properties of cassava starch microspheres prepared in aqueous two-phase system. Carbohydrate Polymers. 2017;177:334-340.

[13] Elfstrand L, Eliasson A, Jönsson M, Larsson M, Simpraga A, Thelin B et al. Recrystallization of waxy maize starch during manufacturing of starch microspheres for drug delivery: Influence of excipients. Carbohydrate Polymers. 2007;69(4):732-741.

[14] Pratiwi M, Faridah D, Lioe H. Structural changes to starch after acid hydrolysis, debranching, autoclaving-cooling cycles, and heat moisture treatment (HMT): A review. Starch - Stärke. 2017;70(1-2):1700028.

[15] Wang S, Copeland L. Effect of Acid Hydrolysis on Starch Structure and Functionality: A Review. Critical Reviews in Food Science and Nutrition. 2015;55(8):1081-1097.

[16] Esquena J. Water-in-water (W/W) emulsions. Current Opinion in Colloid & Interface Science. 2016;25:109-119.

[17] Juszczak L, Witczak M, Ziêba T, Fortuna T. Rheological behaviour of heated potato starch dispersions. International Agrophysics. 2012;26(4):381-386.

[18] Ojewumi M, Oyeyemi K, Emetere M, Okeniyi J. Data on the rheological behavior of cassava starch paste using different models. Data in Brief. 2018;19:2163-2177.

[19] Brownlee I, Gill S, Wilcox M, Pearson J, Chater P. Starch digestion in the upper gastrointestinal tract of humans. Starch - Stärke. 2018;70(9-10):1700111.

[20] Murphy D, Davidson M. Fundamentals of light microscopy and electronic imaging. 2nd ed. Hoboken, New Jersey: Wiley-Blackwell; 2012.

[21] Stokes D. Principles and practice of variable pressure/environmental scanning electron microscopy (VP-ESEM). Chichester, U.K.: Wiley; 2008.

[22] Principle of Differential Scanning Calorimetry (DSC) : Hitachi High-Tech GLOBAL [Internet]. Hitachi-hightech.com. 2022 [cited 7 June 2022]. Available from: https://www.hitachi-hightech.com/global/products/science/tech/ana/thermal/descriptions/dsc.html#:~:text=A%20technique%20in%20which%20difference,varied%20in%20a%20specified%20program

[23] Ali S, Kempf W. On the Degradation of Potato Starch During Acid Modification and Hypochlorite Oxidation. Starch - Stärke. 1986;38(3):83-86.

[24] Xia H, Li B, Gao Q. Effect of molecular weight of starch on the properties of cassava starch microspheres prepared in aqueous two-phase system. Carbohydrate Polymers. 2017;177:334-340.

[25] Qin Y, Zhang H, Dai Y, Hou H, Dong H. Effect of Alkali Treatment on Structure and Properties of High Amylose Corn Starch Film. Materials. 2019;12(10):1705.

[26] Domene-López D, García-Quesada J, Martin-Gullon I, Montalbán M. Influence of Starch Composition and Molecular Weight on Physicochemical Properties of Biodegradable Films. Polymers. 2019;11(7):1084.

[27] Roxling E. Starch based formulation for colon delivery of probiotics [master's thesis].Lund (SE): Lund University 2019.

[28] Kim H, Park S, Lim S. Preparation, characterization and utilization of starch nanoparticles. Colloids and Surfaces B: Biointerfaces. 2015;126:607-620.