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DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Characterisation of acetylated inulin as potential excipient for the production of 5-amino salicylic acid loaded pellets“

verfasst von / submitted by

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Kurzfassung

Chronisch entzündliche Darmerkrankungen umfassen hauptsächlich Morbus Crohn und Colitis ulcerosa. Sie sind gekennzeichnet durch schubweise oder kontinuierliche Entzündungen der Darmschleimhäute. Die Behandlung dieser Krankheiten erfolgt durch hochdosierte oral verabreichte Arzneistoffe wie Glucocorticoide, Immunsuppressiva und Aminosalicylate. Das am häufigsten verabreichte Aminosalicylat ist 5-Aminosalicylsäure (5-ASA), auch Mesalazin^{INN} genannt, von dem Patienten 3-4 g täglich einnehmen müssen. 5-ASA wird bereits in den oberen Abschnitten des Gastrointestinaltraktes absorbiert. Da der Arzneistoff seine volle Wirkung aber nur direkt im Colon entfalten kann, muss er geschützt werden, um dieses auch erreichen zu können.
Präbiotika wie Inulin, können sich ebenfalls positiv auf den Krankheitsverlauf von chronisch entzündlichen Darmerkrankungen auswirken. Neben der Anwendung als klassisches Präbiotikum ist auch eine Verwendung als Trägermaterial für das Drug Delivery ein aktuelles Ziel der Forschung. Inulin ist allerdings sehr gut wasserlöslich und daher als Trägermaterial für 5-ASA nicht gut geeignet, da der Arzneistoff bereits im Magen freigesetzt werden würde. Um diese Tatsache zu umgehen, kann Inulin chemisch modifiziert werden, wodurch die Lipophilie gesteigert und eine geringere Wasserlöslichkeit erreicht werden kann. Dadurch kann es zur Verkapselung von 5-ASA verwendet werden und so ein zielgerichteter Transport zum Ort der Entzündung im Colon ermöglicht werden.
Das Ziel dieser Arbeit ist die Acetylierung von Inulin und die Charakterisierung der Derivate in Abhängigkeit vom Acetylierungsgrad. Außerdem sollen Pellets aus 5-ASA und acetyliertem Inulin hergestellt werden und der Einfluss des Acetylierungsgrades auf die Charakteristika der Pellets beschrieben werden.

Diese neue Kombination eines aktiven Arzneistoffes und eines Präbiotikums kann sich vorteilhaft für Patienten auswirken. Einerseits wird dadurch der zielgerichtete Transport als auch die Freisetzung des Arzneistoffes am Ort der Entzündung ermöglicht, wodurch es andererseits wiederum möglich ist, die Dosis und damit mögliche Nebenwirkungen zu senken. Zusätzlich kann der mögliche probiotische Effekt die Bereitschaft der Patienten steigern, das Arzneimittel gerne einzunehmen.

Abstract

Inflammatory bowel diseases are characterised by relapsing or continuous progressive inflammations of the mucosa of the lower parts of the gastrointestinal tract (GIT). These include Crohn's disease and ulcerative colitis. The treatment is mainly based on inhibiting the inflammation with orally administered drugs, like glucocorticoids, immunosuppressive drugs or amino salicylates. The most commonly used amino salicylate is 5-amino salicylic acid, which is already resorbed in the upper parts of the GIT. In order to be efficacious it must reach the colon and therefore needs protection. Moreover, patients have to take high doses of more than 3-4 g per day.
Furthermore, prebiotics like the oligofructose inulin may have a beneficial effect on the course of inflammatory bowel diseases. Inulin is highly soluble in water and is therefore modified by acetylation to increase its lipophilicity. Acetylated inulin can then be used as a carrier material for 5-ASA and also enables a targeted delivery to the site of inflammation.

The aim of this thesis is the acetylation of inulin and characterisation of acetylated inulin as an encapsulation material for 5-ASA. Moreover, the impact of the degree of acetylation on the properties is investigated. Another aim is to form pellets with acetylated inulin and 5-ASA and, to examine the influence of the degree of acetylation on the pellets.

The combination of an active pharmaceutical ingredient and a prebiotic may increase patients compliance. In addition, the dose as well as possible side effects could be reduced because of the targeted transport and release of the drug in the colon at the intended site of action.

My sincere thanks go to all of you who have supported me throughout the years of my studies!

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My parents, brothers, partner and friends for constantly supporting and motivating me in all these years.

List of Abbreviations

°C	degree Celsius
µg	microgram
µL	microlitre
5-ASA	5-amino salicylic acid
AcN	acetonitrile
ATR-FT-IR	attenuated total reflection – Fourier transformation – infrared spectroscopy
CD	Crohn's disease
cm^{-1}	wave number
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DP	degree of polymerisation
EE	encapsulation efficiency
g	gram
GIT	gastrointestinal tract
HPLC	high pressure liquid chromatography
IBD	inflammatory bowel disease
IL	interleukin
k	capacity factor
L	litre
LC	loading capacity
M	molar concentration
mAUC	mean area under the curve
mbar	millibar
MeOH	methanol
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mmol	millimole
M_n	number-average molecular weight
M_w	the weight-average molecular weight
nm	nanometre
NMR	nuclear magnetic resonance
P	partition coefficient
ppm	parts per million
rpm	rotations per minute
SEC	size exclusion chromatography

TNF α	tumour necrosis factor α
UC	ulcerative colitis
xg	centrifugal force

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1 Project definition

1.1 Scope of the thesis

In order to allow for drug delivery of 5-amino salicylic acid in the colon, acetylated inulin is chosen to protect it from absorption in the upper parts of the gastro-intestinal tract. The aim of this diploma thesis is to form pellets of 5-ASA and acetylated inulin.

In contrast to inulin, acetylated inulin is not characterised as potential auxiliary agent for pharmaceutical preparations yet. Here, two different types of inulin are acetylated and compared. The impact of the degree of acetylation on the properties of acetylated inulin is investigated.

Pellets formed are examined regarding the influence of the different levels of acetylation on pellet formation and loading capacity. Furthermore, pellets are optimised in order to increase pellets size, stability and content of 5-ASA.

1.2 Research questions

- ➔ What is the impact of the degree of acetylation on the characteristics of acetylated inulin on pellet formation?
- ➔ How do the properties of the different levels of acetylated inulin affect pellet size, stability and 5-ASA content?
- ➔ How can pellets be optimised to increase the loading capacity of 5-ASA as well as size and stability?

2 Introduction

2.1 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are inflammatory diseases of the GIT located in different regions, which show exacerbations and remissions or chronic progression. The two main diseases are ulcerative colitis (UC) and Crohn's disease (CD). Patients display clinical symptoms such as abdominal pain, diarrhoea, intestinal bleeding and extra-intestinal inflammatory processes (Aktories et al., 2017).

The aetiopathology of IBD is complex and therefore not yet completely understood (Mutschler et al., 2013). It is assumed that the interaction between genetic predispositions like mutations in the NOD2 (nucleotide-binding oligomerisation domain-2-) gene, environmental factors such as smoking, nonsteroidal antirheumatic drugs or vitamin D deficiency, the composition of microbial flora in terms of quantitative and qualitative changes or a reaction of the immune system to parts of the microbiome and reactions of the immune system are some of the main causes (Dignass et al., 2019; Mutschler et al., 2013). Both diseases show a response of the innate immune system (Dignass et al., 2019). In addition Crohn's disease is characterised by a TH1- and TH17-cell mediated immune reaction, whereas in ulcerative colitis TH2 cells are activated (Dignass et al., 2019; Mutschler et al., 2013) .

2.1.1 Crohn's disease

Crohn's disease particularly concerns young adults and is characterised by chronic recurring periods of relapses and remissions (Smith & Morton, 2017). It is a chronic though discontinuous inflammatory disease that may occur in any region of the GIT from mouth to anus, but it is most commonly located in the terminal ileum and the proximal colon (Mutschler et al., 2013).

Significant for Crohn's disease is the transmural inflammation, meaning that each layer of the intestinal wall from mucosa to the muscle layer is affected (Mutschler et al., 2013). This may cause obstructions and scarring after an acute attack may also contribute to the narrowing of the gut lumen (Smith & Morton, 2017). Lymphocytes and plasma cells infiltrate the mucosa and furthermore epithelioid cell granuloma may form (Mutschler et al., 2013).

Another characteristic is the formation of enteric fistula between different intestinal loops or between intestine and the skin (enter cutaneous fistula), which particularly occur in perianal regions (Smith & Morton, 2017). There may also arise gastrointestinal bleedings and

diarrhoea, which subsequently may lead to iron deficiency, anaemia and weight loss (Mutschler et al., 2013; Smith & Morton, 2017). Due to the long term damage of the gut mucosa, patients also have a higher risk of developing cancer (Smith & Morton, 2017).

2.1.2 Ulcerative colitis

Ulcerative colitis is a chronic inflammation of the superficial intestinal mucosa layers, which begins at the rectum and continuously spreads to proximal segments of the colon. This disease has a continuous progressive or intermitting course, with unpredictable exacerbations as well as remissions. It especially affects patients at the age of 20-40 years. A characteristic hallmark is the formation of crypt abscesses by the accumulation of granulocytes in the crypts (Mutschler et al., 2013).

In addition, epithelial cells in the crypts show signs of degeneration and mucosal ulcerations may occur(Smith & Morton, 2017). The damaged mucosa is not able to resorb enough water and ions, which leads to massive and often bloody diarrhoea (Mutschler et al., 2013; Smith & Morton, 2017).

Figure 1 shows Crohn's disease and ulcerative colitis compared to each other with their different inflammatory patterns in the intestine. Beyond that effects on the intestinal wall are also shown.

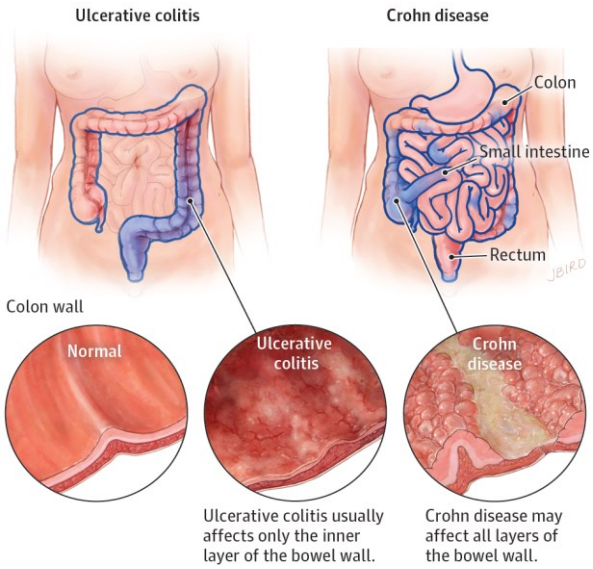

Figure 1: Comparison of the inflammatory pattern of ulcerative colitis and Crohn's disease (Jin, 2014).

2.1.3 Treatment of inflammatory bowel disease: the role of 5-amino salicylic acid

Since the aetiology of inflammatory bowel disease is not identified, there is no causal therapy available. Therefore, the standard procedure is inhibiting the inflammation to reduce symptoms and recurrence rate (Aktories et al., 2017).

The therapeutic decisions are depending on the disease's activity, possible complications like fistula forming or extra intestinal symptoms and previous medication. Approximately half of the Crohn's disease patients and up to one third of ulcerative colitis patients suffer from the chronic form, so they need to be treated with corticosteroids or immunosuppressive drugs. In case of an intermitting course of the disease and long remissions, they only have to be treated therapeutically during an acute attack (Dignass et al., 2019).

Glucocorticoids can be administered orally (protection from gastric juice is needed), rectally, parenterally or even topically. They are immunosuppressive and possess a high anti-inflammatory potential. Unfortunately, they also have severe side effects. That is why budesonide is often orally given: due to the gastric juice resistant capsule, the drug is released in the distal ileum or the proximal colon. After the resorption, it has a high first-pass effect of about 90% and therefore the side effects are not distinctive (Aktories et al., 2017).

The active substances azathioprine, methotrexate, 6-mercaptopurine, ciclosporin and tacrolimus as well as the monoclonal antibodies vedolizumab and the TNF-α-blockers also have an immunosuppressive mode of action. However, due to the potential severe side effects they are only used for second line therapy (Aktories et al., 2017).

Mesalazine (Figure 2), olsalazine (Figure 3) and sulfasalazine (Figure 4) are amino salicylates and often used in the treatment of ulcerative colitis. Olsalazine consists of two molecules of mesalazine, which are linked by an azo bridge, whereas the azo compound sulfasalazin consists of the sulphonamide sulfapyridine and mesalazine. Sulfasalazine and olsalazine are degraded in the colon, so that the active form of these two substances is also mesalazine (Aktories et al., 2017).

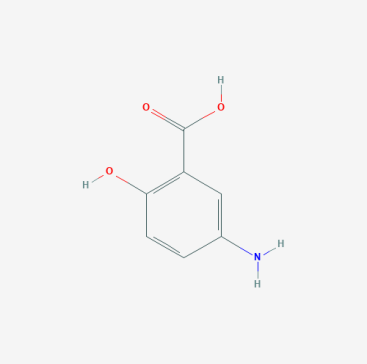

Figure 2: Chemical structure of mesalazine (*PubChem*, 2020a)

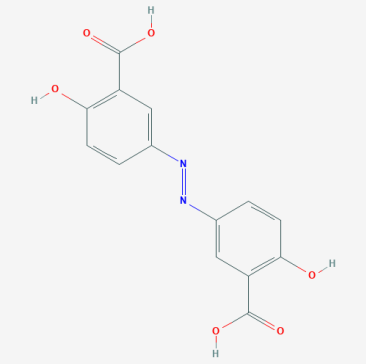

Figure 3: Chemical structure of olsalazine (*PubChem*, 2020b)

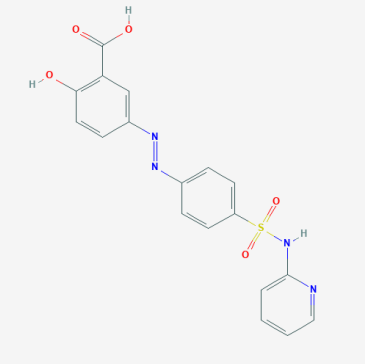

Figure 4: Chemical structure of sulfasalazine (*PubChem*, 2020c)

Mesalazine also called mesalamine or 5-amino salicylic acid (5-ASA) exerts only an antiphlogistic effect if there is direct contact with the colonic mucosa. Primarily, the peroxisome-proliferator-activated receptor γ (PPARγ) is activated by 5-ASA, which stops the activity of inflammation promoting transcription factors like the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) through its function as nuclear transcription factor. Moreover, proinflammatory cytokines (IL-1, IL-6, TNF-α) cannot be synthesised and certain signal transduction pathways as well as the induction of cyclooxygenase-2 and the nitric oxide synthases are blocked (Aktories et al., 2017).

After oral administration, 5-ASA is already absorbed in upper small bowl regions. Ulcerative colitis and Crohn’s disease especially affect lower parts of the gastrointestinal tract, so there would be no effect of 5-ASA. Therefore the substance needs to be protected until it reaches the lower parts of the intestine (ileum and colon) (Mutschler et al., 2013).

2.2 Inulin

Inulin, a α-D-glucopyranosyl-[α-D-fructofuranosyl](n-1)D-fructofuranoside) (Figure 5), is a mixture of oligo- and polysaccharides, that consists of linear chains of fructosyl groups with a terminal glucose unit. The fructose units are linked through an β(2→1) glycosidic bond, whereas there is an α-D-glucopyranosyl bond between fructose and the glucose (Barclay et al., 2010).

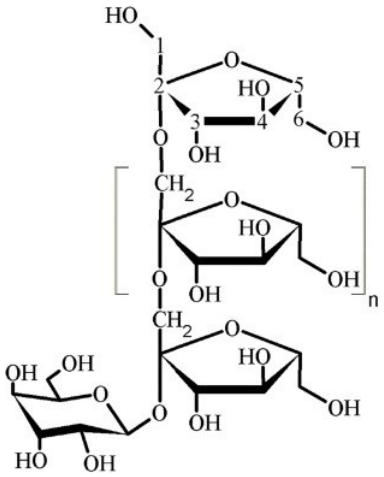

Figure 5: Chemical structure of inulin (Tripodo & Mandracchia, 2019).

Inulin can be extracted from various vegetables, like roots from chicory (*Chicorium intybus*), Jerusalem artichoke (*Helianthus tuberosus*) or dahlia tuber (genus: dahlia) (Tripodo & Mandracchia, 2019). The degree of polymerisation (DP), giving the number of monomeric units in the macromolecule, of inulin isolated from plants usually is relatively low (<200) in comparison to bacterial inulin, with a DP from 10000 to over 100000 (Barclay et al., 2010; Franck et al., 2006). In addition, inulin from bacteria has a high degree of branching of about 15%, whereas plant inulin is less or hardly ever branched up to 1-2% (Franck et al., 2006).

2.2.1 Inulin and its potential as prebiotic

The human gastrointestinal tract is colonized by a wide variety of bacteria, which mainly feed on fibre. Prebiotics are fibres that improve the composition of the intestinal microbial flora and promote bacteria growth or rather the metabolic processes. Among these are also oligofructose-polymers respectively inulin. Inulin is a prebiotic that especially promotes reproduction of bifidobacteria in the bowel, leading to a favourable impact on food utilisation, the transit time and absorption of nutrients and minerals as well as an increased resistance to pathogens (Sticher et al., 2015).

Inulin-type fructans are soluble, but the β(1→2) linkage cannot be hydrolysed by human digestive enzymes in the gastrointestinal tract, because they can only cleave α-glycosidic bonds (Jain et al., 2014; Kelly, 2009). Therefore, after oral intake only a small percentage of inulin is split by acidic hydrolysis in the stomach before it reaches the colon almost undamaged, where it is degraded by the gut microbiota by aid of the enzyme inulinase (Jain et al., 2014; Sticher et al., 2015). In the process short chain fatty acids, lactate and gases like carbon dioxide or hydrogen arise and due to the bacterial gas evolution higher intakes

in humans of more than 12 g per day may lead to flatulence (Kelly, 2009; Roberfroid, 2005; Sticher et al., 2015).

2.2.2 Inulin and its use for colon drug delivery

“Drug delivery” describes the approaches, formulations, technologies and systems for transporting a pharmaceutical compound in the body. Consequently, the specificity of the drug effect is increased and the risk for systemic side effects is reduced. In recent years, colon drug delivery has become more important as a local therapy for the treatment of bowel diseases like Crohn’s disease or ulcerative colitis. The formulation has to pass the upper sections of the gastrointestinal tract nearly unchanged and should only be degraded finally in the colon to elicit specific effects in this area (Walz et al., 2018).

In search for new suitable encapsulation materials for drugs, many polymers including polysaccharides such as inulin have been studied. The advantage of inulin is that it is not degraded by human enzymes and hence it reaches the colon almost unaffected, where it is metabolised by bacteria. However, since native inulin is easily soluble in water, it cannot be used to encapsulate pharmaceutical active ingredients (Walz et al., 2018).

Moreover, inulin microspheres release in phosphate buffer (pH 7.4) large amounts of the encapsulated drug within a few minutes (Poulain et al., 2003). In order to decrease the hydrophilic character of inulin to be applied as an encapsulation material for the colon targeted transport, it has to be modified (Walz et al., 2018). The hydroxyl groups of inulin are replaced by hydrophobic moieties like acetyl residues (Poulain et al., 2003; Walz et al., 2018). Thus, the hydrophilicity of acetylated inulin is reduced and microparticles prepared from acetylated inulin show an extended release profile (Jain et al., 2014; Walz et al., 2018).

3 Materials and methods

The materials required for acetylation of inulin, the characterisation and the subsequent pellet production are listed in Table 1.

Table 1: List of chemicals used for acetylation and characterization of native inulin as well as pellet production.

chemical	manufacturer	specifications
5-amino salicylic acid	Cayman Chemical, Ann Arbor, Michigan USA	
acetic anhydride	Sigma Aldrich, Steinheim, Germany	≥99%, ReagentPlus®
acetone	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
acetonitrile	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	ROTISOLV® HPLC Gradient Grade
butanone	Honeywell Research Chemicals	≥99 %, ExtraPure, ACROS Organics™
butyl acetate	Merck KGaA, Darmstadt, Germany	anhydrous, ≥99%,
dimethylsulfoxide	Merck, Darmstadt, Germany	max. 0.03% water
distilled water	Produced by the University of Vienna	
ethyl acetate	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	≥99.5%, Ph. Eur., extra pure
formamide	Merck, Darmstadt, Germany	p.a.
fructose	Sigma Aldrich, Steinheim, Germany	≥99%, HPLC: suitable
hydrochloric acid	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	ROTIPURAN® 37 % fuming
inulin Alfa Aesar®	Thermo Fischer (Kanel) GmbH, Karlsruhe, Germany	
inulin Carbosynth®	Biosynth Carbosynth, Berkshire, United Kingdom	
methanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	≥99,5 %, Ph.Eur., reinst
N,N-dimethylformamide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	ROTIPURAN® ≥99,8 %, p.a., ACS, ISO
propyl acetate	Sigma Aldrich, Steinheim, Germany	99%
sodium acetate-trihydrate	Fluka Chemie GmbH, Switzerland Sigma Aldrich Chemie GmbH, Germany	≥99.0% (NT), puriss. p.a. ACS

sodium chloride	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	≥99,5 %, p.a., ACS, ISO
sodium hydroxide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	≥97 %, p.a., ACS
sorbitol	Merck, Darmstadt, Germany	European Pharmacopoeia (EP) Reference Standard

3.1 Chemical modification of native inulin

3.1.1 Size exclusion chromatography

Two different types of inulin, one purchased from Alfa Aesar® (derived from dahlia tubers) and the other from Carbosynth® (isolated from chicory roots) are characterised regarding the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) by size exclusion chromatography (SEC). Using this data the degree of polymerisation (DP) of native inulin is also calculated.

For the analysis an Agilent 1260 Infinity Series chromatography system (Agilent Technologies, Santa Clara, CA) equipped with a Wyatt DAWN HELEOS II MALLS detector (Wyatt technology) and a PL Aquagel-OH MIXED-M, 8 µm, 7,5 x 300 mm column (Agilent Technologies, Santa Clara, CA) is used. The system is calibrated with a BSA standard using distilled water as an eluent. The injection volume is 100 µL and the mobile phase flow rate is 0.3 mL/min at 40 °C.

3.1.2 Chemical modification by acetylation

Native inulin is modified by acetylation according to Wu & Lee (2000) (Wu & Lee, 2000) with some modifications. In order to reach different levels of acetylation the amount of acetic anhydride added is varied. Table 2 shows the amount of added acetic anhydride to achieve different degrees of acetylation. The relation of inulin to acetic anhydride is chosen such that one hydroxyl group from the native inulin is exposed to ~3 molecules of acetic anhydride (level of acetylation 1).

Table 2: Amount of acetic anhydride [mL and mmol] for the acetylation of 5 g native inulin from Carbosynth® to yield different degrees of acetylation.

level of acetylation	acetic anhydride [mL]	acetic anhydride [mmol]	inulin [g]	inulin [mmol]
1	25.0	264.5	5	1.4
2	22.5	238.1	5	1.4
3	20.0	211.6	5	1.4
4	17.5	185.2	5	1.4
5	15.0	158.7	5	1.4
6	12.5	132.3	5	1.4
7	10.0	105.8	5	1.4
8	7.5	79.4	5	1.4

For acetylation 5 g of native inulin are dissolved in 50 mL dimethylformamide (DMF) in a round bottom flask and then 0,066 g sodium acetate-trihydrate are added as a catalyst. The round bottom flask is mounted on a rotary evaporator (40 °C, 250 mbar, 100 rpm) and the reaction is set for 24 hours.

For further experiments, only acetylated inulins with acetylation level 1 and 6 were required and so the synthesis was scaled up to increase the output. After dissolving 10 g inulin in 100 mL DMF and adding 0.132 g sodium acetate trihydrate, the corresponding volumes of acetic anhydride are added according to Table 3. Due to the different molecular weights of native inulin from Alfa Aesar® and from Carbosynth®, the required volumes of acetic anhydride are calculated accordingly.

Table 3: Amount of acetic anhydride [mL and mmol] for the acetylation of 10 g native inulin from Alfa Aesar® and Carbosynth® to yield acetylation level 1 and 6.

level of acetylation	acetic anhydride [mL]	acetic anhydride [mmol]	inulin [g]	inulin [mmol]
Carbosynth level 1	50.0	528.9	10	2.8
Carbosynth level 6	25.0	264.5	10	2.8
Alfa Aesar level 1	54.7	571.3	10	3.0
Alfa Aesar level 6	27.3	288.8	10	3.0

The acetylated inulin is precipitated with at least five volumes of distilled water and then centrifuged (Sorvall LYNX 6000 Thermo Scientific with a FIBERLITE F21-8x50y rotor and 40 mL Nalgene™ tubes with sealing cap) at 15 °C and 38758*xg* for 15 minutes. For larger batches, the same centrifuge is used with the BIOFlex HC rotor and 1000 mL tubes at 10 °C and 7068*xg*. After centrifugation, the supernatant is discarded and the pellet is dissolved in acetone, followed by precipitation with at least five volumes distilled water and centrifugation to produce a pellet once again. This purification step is repeated three times and finally the pellet is dried in a drying oven at 40 °C for 24 hours.
Three batches of level 1 and level 6 of both types of inulin are synthesised according to the procedure described above.

3.2 Characterisation of native and acetylated inulin

3.2.1 Attenuated total reflection – Fourier Transformation – Infrared Spectroscopy

Spectra of inulin are recorded before and after acetylation in an attenuated total reflection – fourier transformation – infrared (ATR-FT-IR) spectrometer (Tensor 27, Bio-ATR I tool, Bruker Optics, Ettlingen, Germany) equipped with a liquid nitrogen cooled mercury cadmium telluride detector. First, a spectrum without a sample for setting the baseline is recorded, and then dry powder samples are placed directly on the zinc selenide ATR crystal. For each sample 32 scans are acquired in double sided mode with a resolution of 4 cm^{-1} in the range of 4000–850 cm^{-1}. Qualitative data analysis of absorbance spectra as well as determination of peak positions are performed using OPUS 5.5 software (Bruker Optics, Germany).

3.2.2 Nuclear magnetic resonance spectroscopy

^{1}H Nuclear magnetic resonance (NMR) spectra of each batch of acetylated inulin are recorded using a Bruker Avance III 400 spectrometer operating at a frequency of 400 MHz. For spectral recording acetylated inulin is dissolved in $CDCl_3$ and tetramethylsilane is used as internal standard. The degree of acetylation is estimated from its ^{1}H NMR spectrum based on the publication from Jain et al. (2014) (Jain et al., 2014) with minor changes. It is calculated by using the ratio of integrals of resonance peaks at ~1.9–2.4 ppm and ~3–6 ppm corresponding to the three methyl protons of acetate (acetylation) and the seven protons of the fructose skeleton according to equation (1) and (2) described in the publication cited

above. Thus the number of acetyl groups per fructose unit as well as the degree of acetylation for each level of acetylated inulin is calculated.

$$\text{Acetyl groups per fructose unit} = \frac{(A_{1.9-2.4}/3)}{(A_{3-6}/7)} \quad (1)$$

$$\text{Degree of acetylation} = \frac{\text{Acetyl groups per fructose unit}}{\text{Number of OH groups per fructose unit}} \times 100 \quad (2)$$

3.2.3 Size exclusion chromatography of acetylated inulin

For SEC analysis of acetylated inulin, the samples are dissolved in dimethylsulfoxide (DMSO), which is also the mobile phase. The column used is a MZ-Gel SDplus 10E5 Å 300x8.0 mm GPC/SEC-Column (MZ Analysentechnik, Mainz, Rheinland-Pfalz). The chromatography system, the detector and the method for analysis are the same as described in chapter 3.1.1.

3.2.4 Density

The density *ρ* is defined as the mass of a substance per cubic centimetre and depends on the pressure and temperature of the surrounding area. Most solids and fluids marginally expand upon heating and shrink only a little under pressure so that they do not need to be included in the calculation of the density *ρ* (equation (6)) (Tipler & Mosca, 2009).

Here, the density *ρ* is determined with a pycnometer (10 mL, Borosilicate glass 3.3. DURAN®, Lez Laborglas GmbH & Co. KG, Wertheim, Germany) at room temperature (22±1 °C). First the tare weight of the pycnometer is determined with a precision scale (Sartorius AG, Göttingen, Germany), then it is filled with dimethyl sulfoxide (DMSO) and weighed again. On the basis of this data and the specific density of DMSO at a certain temperature, the exact volume in the pycnometer is calculated.

A solution of acetylated inulin in DMSO with a concentration of 8°mg/mL (precisely weighed) is prepared. Then the clean and dry pycnometer is filled with this solution and weighed. By multiplying the concentration per millilitre of this solution with the calculated volume of the pycnometer, it is possible to calculate the amount of acetylated inulin in the pycnometer. The density is then calculated according to equations (5) and (6) (acetylated inulin is referred to as InAc).

$$\text{Volume of InAc } [m^3] = \frac{(\text{tare weight [g]} + \text{InAc [g]}) - \text{pycnometer with InAc solution [g]}}{\text{Density of DMSO } [g/m^3]} \quad (5)$$

$$\text{Density of InAc } [\text{g/m}^3] = \frac{\text{Amount of InAc } [\text{g}]}{\text{Volume of InAc } [\text{m}^3]} \quad (6)$$

Each batch of acetylated inulin is tested two times. The density of native inulin is determined with the same method, but using distilled water instead of DMSO.

3.2.5 Melting point

The melting point of acetylated inulin is determined using the Stuart SMP30 Melting Point Apparatus (Stuart, Cole-Parmer, Staffordshire, UK) and melting point tubes open at one end. The melting point tube is filled with sample up about 1 cm height and then slowly heated up at 0.1 °C steps at a rate of 0.5°C per minute. The temperature display as well as the transition of the tested substance from solid to liquid are observed through the integrated magnifying lens. The temperature range of phase transition is recorded and the procedure is repeated two times.

3.2.6 Partition coefficient - Log P

The distribution of a substance between two immiscible phases such as octanol and water is described by the partition coefficient as a measure of lipophilicity of a substance. Amongst un-dissociated compounds, *P* is the quotient of the concentration in 1-octanol and the concentration in water (equation 3)(Steinhilber et al., 2010).

$$\text{P} = \frac{[\text{g}]_{octanol}}{[\text{g}]_{water}} \quad (3)$$

Usually the distribution is given as log *P* and compounds with a log *P* above zero are higher soluble in octanol, whereas substances with a log *P* less than zero are more easily soluble in water (Steinhilber et al., 2010).

The determination of log *P* is performed adhering to the OECD guideline for testing of chemicals, number 117, using the high-pressure liquid chromatography method (OECD, 1989).

Substances with a known log *P*, that are structurally related to the acetylated inulin, are used as reference compounds; here acetone, butanone, ethyl acetate, propyl acetate and butyl acetate are used. Following the OECD guideline, the inert substance formamide is used to determine the dead time of the HPLC column NUCLEODUR® C18 gravity column, 5 µm

(C18, 4.6x150 mm, Macherey-Nagel, Germany). As eluent, a mixture of 75% methanol and 25% distilled water is used.

10 µL formamide as well as 10 µL of each of the reference substances are diluted with 990 µL eluent. Then a mixed standard of these solutions is prepared from 200 µL of each diluted substance.

The elution profile is recorded with an UltiMAte3000 HPLC system (Thermo Fisher Scientific, Waltham, USA) equipped with a photodiode array detector PDA-100 at 30 °C and a flow rate of 1 mL/min for 6 min. 10 µL of the mixed standard and formamide are injected and detected at the respective absorption maxima of the compounds at 210 nm (formamide, ethyl acetate, propyl acetate, butyl acetate) and at 265 nm (acetone, butanone).

Applying this method, the amount of substance dissolved in octanol or water is determined from the capacity factor *k*. The capacity factor *k* is defined as the ratio between the time an analyte is retained on the stationary phase and the time it is retained in the mobile phase. It is calculated according to the equation (4), with the retention times (referred to as t_R) and the dead time (t_0).

$$k = \frac{t_R - t_0}{t_0} \tag{4}$$

A calibration graph is drawn from the calculated log *k* and the given log *P* of the reference substances.

To determine the retention time of the acetylated inulin, 40 mg are dissolved in 1 mL eluent and processed by HPLC as stated above. After calculating the *k*-factor of each acetylated inulin according to equation (4) and inserting log *k* of this factor into the equation of the calibration graph, log *P* is determined. Log *P* of each level of Inulin Alfa Aesar® and Inulin Carbosynth® is determined and experiments are carried out by at least three independent measurements.

3.2.7 Water solubility

The determination of the water solubility of acetylated inulin is based on the OECD guideline for the testing of chemicals number 105 (OECD, 1995).

First, the saturation concentration of the substance in distilled water estimated in a preliminary test. About 100.0 mg of the powdered solid sample are put into a 10 mL glass-stoppered measuring cylinder and increasing volumes of distilled water (from 1 mL to 10 mL) are added and incubated for 24 hours on a laboratory shaker. As the substance is not

dissolved the procedure is continued in a 100 mL measuring cylinder and stepwise addition of up to 100 mL of distilled water.

The main test is run in triplicate: three 100 mL volumetric flasks with 150 mg of acetylated inulin and 100 mL of distilled water each are used. The volumetric flasks are shaken 30 times per minute in a water bath at 30 °C The first volumetric flask is taken out of the water bath after shaking for 24 hours at room temperature, the next one after 48 hours and the last one after 72 hours.

The amount of acetylated inulin dissolved in distilled water is determined at 21 °C applying the spectrophotometric method described by Saengkanuk et. al. (Saengkanuk et al., 2011). First, 40 mL of each of the three solutions are centrifuged (Sorvall LYNX 6000 Thermo Scientific with a FIBERLITE F21-8x50y rotor and 40 mL Nalgene™ tubes with sealing cap at 30145 x *g* at 20 °C for 15 minutes. Then the dissolved acetylated inulin is hydrolysed under acidic conditions to yield the individual fructose and glucose units: 20 mL of the supernatant are acidified to a pH value <2 with 0.1 M hydrochloric acid and left in the drying oven at 97 °C for 45 minutes. After cooling to room temperature the pH of the samples is set at 7 by addition of 0.01 M and/or 0.1 M sodium hydroxide solution. The neutralised hydrolysates are filled up with distilled water to 25.0 mL. 150 µL neutralised hydrolysate, 100 µL 0.01 M sodium periodate solution, 5mL 0.02 M citrate buffer pH 6.0 and 4.6 mL distilled water are mixed in a glass tube and incubated for 5 minutes at room temperature. Sodium periodate oxidises the monosaccharides at pH ~6. Then 150 µL 0.1 M potassium iodide solution are added and the reaction mixture is incubated for 5 minutes. Potassium iodide reacts with excess of sodium periodate the colourless solution becomes yellow. The intensity of the colour reflects the number of oxidised fructose units: a higher number yields only a small amount of sodium periodate that can react with the potassium iodide and to a pale yellow, whereas a low number produces a more intense colour. In addition, the formed complex is not very stable. Therefore, the sample tubes always have to be tightly sealed throughout the last incubation period and the time interval has to be constant until finishing the spectrophotometric measurement at 350 nm with a Tecan INFINITE M200 plate reader (Tecan Trading AG, Switzerland). As positive control and for standard calibration, fructose is dissolved in distilled water and treated the same way as described above.

This method is an indirect quantification, because in fact not the sample itself but excess of unused reagent is determined. Moreover, the amount of fructose is determined and not the acetylated inulin. Therefore, the amount of fructose has to be divided by the DP of each acetylated inulin (Saengkanuk et al., 2011).

3.3 Preparation of 5-ASA loaded pellets

3.3.1 Pellet preparation and optimisation

In order to prepare 5-ASA loaded pellets 500 mg of acetylated inulin and 20 mg of 5-ASA are dissolved in 500 µL of DMSO in a 2.0 mL Eppendorf tube. The tube is placed in an ultrasonic bath for 5 minutes. Then the viscous solution is filled into a 1 mL disposable syringe (Injekt®-F, B. Braun Melsungen AG, Germany) and slowly pressed through a 0.40 x 20 mm cannula (Sterican® Gr. 20, B. Braun Melsungen AG, Germany) into a beaker containing about 500 mL of distilled water and slowly moved by a magnetic stirrer. For preparation of round and well-formed pellets each drop has to separately hit the water surface from a distance of about 10 cm.

The precipitated pellets are incubated in an Erlenmeyer flask in about 100 mL of distilled water on the laboratory shaker overnight. After decanting the distilled water, the pellets are washed three times with 100 mL of distilled water and shaken on the laboratory shaker for 10 minutes each. The pellets are transferred into a glass petri dish, frozen at - 80 °C and lyophilised.

To improve size and stability of the pellets, different non-solvents are tested (Table 4). The solutions are prepared by dissolving the required amount of substance, also given in Table 4 in 100 mL of distilled water. The corresponding solutions are then also used to perform the purification steps.

Table 4: Different non-solvents for pellet production.

Non-solvents for pellet production
0.9% NaCl solution
15% NaCl solution
20% NaCl solution
30% NaCl solution
10% sorbitol solution
20% sorbitol solution
30% sorbitol solution
40% sorbitol solution
50% sorbitol solution
60% sorbitol solution
70% sorbitol solution

In order to increase the amount of active pharmaceutical ingredient per pellet, 500 mg of acetylated inulin as well as 500 mg of 5-ASA are levigated with 700 µL of DMSO in a porcelain mortar and pestle. The trituration is transferred into a 2.0 mL Eppendorf tube and placed in an ultrasonic bath for 45 minutes at a temperature of about 50 °C. The pellets are produced as described above, except that the end of the cannula is positioned about 5 cm above the water surface to generate spherical pellets. Figure 6 shows the position of the cannula and pellet formation out of the trituration, whilst Figure 7 displays curing of the spherical pellets.

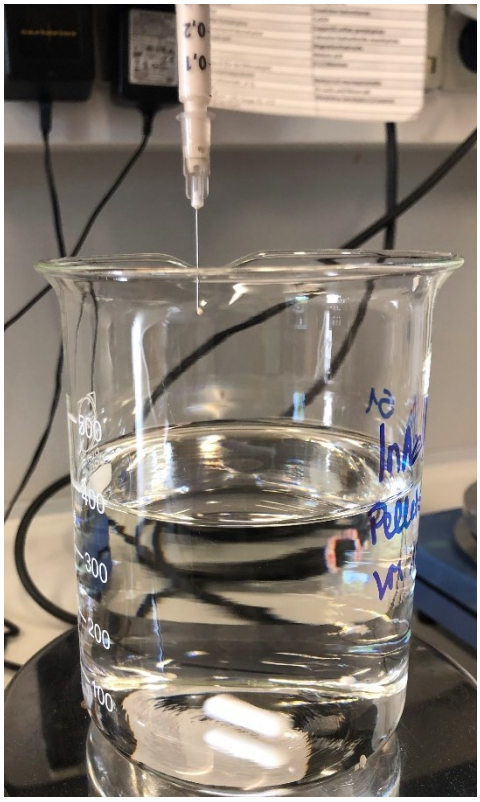

Figure 6: Pellet production from a dispersion of levigated 5-ASA, acetylated inulin in DMSO by use of a 0.4 mm cannula.

Figure 7: Curing of the pellets in distilled water on a magnetic stirrer.

3.3.2 Determination of 5-ASA loading capacity and encapsulation efficiency

For determination of the pellets loading capacity (LC), a spectrophotometric as well as a HPLC method are applied.

For each pellet batch, 10 individually weighed pellets in 2 mL Eppendorf tubes are dissolved in DMSO. Then the samples are diluted so that the expected 5-ASA content fits into a previously established calibration curve of 5-ASA in DMSO ranging from 0.078 mg/mL to 0.001 mg/mL. The spectrophotometric quantification is carried out using the same plate reader as described in chapter 3.2.7 at the absorption maximum of 5-ASA in DMSO at 366 nm.

The 5-ASA content is determined by HPLC as described in chapter 3.2.6. The mobile phase consists of 75% distilled water pH 3.0, 12.5% methanol (MeOH) and 12.5% acetonitrile (AcN). Individually weighed pellets with a lower drug-content are dissolved in 1 mL of the mobile phase whereas those with a higher content are each dissolved in 1 mL DMSO and then diluted with the mobile phase. The sample volume is 10 µL, the flow rate is 0.4 mL/min and 5-ASA is detected at the absorption maximum 300 nm with a photodiode array detector (PDA-100) at 30 °C column temperature. For analysis and quantification the Chromeleon 7.1-software is used. A calibration graph of 5-ASA dissolved in DMSO and diluted with the eluent is established.

The LC represents the percentage of 5-ASA in relation to the total weight of each pellet containing acetylated inulin and 5-ASA. It is determined by dividing the calculated 5-ASA content by the weight of the pellet and multiplying the result by 100. Another important value is the encapsulation efficiency (EE) which gives the percentage of the amount of added 5-ASA that is actually entrapped into the pellets. It is calculated by dividing the loading capacity by the maximum possible loading capacity and finally multiplying by 100.

4 Results and discussion

4.1 Characterisation of native and acetylated inulin

Different degrees of acetylated inulin were prepared by varying the amount of acetic anhydride added for the chemical synthesis. The less acetic anhydride used the smaller the obtained amount of acetylated inulin and, most important, the lower the degree of acetylation.

Table 5 indicates the yields of level 1 to 8 of acetylated inulin starting from 5 g of native Carbosynth® inulin. Level 8 represents the lowest degree of acetylation, but most probably due to high hydrophilicity the product could not be precipitated and even at level 7 the yield was very low. For comparison, Table 6 displays the yield of level 1 and level 6 acetylated inulin from Carbosynth® and Alfa Aesar® starting from 5 g native inulin.

Table 5: Yields of the different levels of acetylated inulin made from Carbosynth® inulin.

level of acetylation	**yield [g]**	**yield [%]**
level 1	3.2	64.0
level 2	2.6	52.0
level 3	2.6	52.0
level 4	1.7	34.0
level 5	1.2	24.0
level 6	0.2	4.0
level 7	0.02	0.4
level 8	-	-

For further experiments and comparative studies, only level 1 and 6 of acetylated inulin were used. Level 1 was chosen, because it shows the highest degree of acetylation and level 6 was the type with low degree of acetylation and at the same time with enough yield for the economic performance of the experiments.

Table 6: Yields of level 1 and 6 of acetylated inulin from Carbosynth® and Alfa Aesar®.

level of acetylation	native inulin	yield [g]	yield [%]
level 1	Carbosynth®	5.9±0.9	59±9
level 6	Carbosynth®	1.5±0.2	30±2
level 1	Alfa Aesar®	4.8±0.8	48±8
level 6	Alfa Aesar®	2.7±0.6	27±6

As shown in Table 6, acetylation level 1 yields higher amounts of acetylated inulin than at level 6 probably due to the lower hydrophilicity and thus preferred precipitation with water. Moreover, Carbosynth® inulin gained higher amounts of acetylated inulin than those from Alfa Aesar® probably due to the different sources of the inulin used. Alfa Aesar® has its origin from Dahlia whereas that from Carbosynth® derives from chicory.

4.1.1 Interpretation of ATR-FT-IR spectra

AT-FT-IR spectroscopy is a qualitative method for a fast check of whether acetylation of native inulin has taken place and performed successfully.

Carbohydrates like oligofructose are examined in detail and the recorded IR-spectra show many similarities. Due to the configuration of the bonds between C-O, C-H and C-C groups and the geometry of the hydroxyl groups these spectra are also very specific (Grube et al., 2002).

In general, the region below 900 cm^{-1} is called the fingerprint region, which shows characteristic bands, but they are superimposed with other signals (Grube et al., 2002; Petkova et al., 2018). Coupled C-C, C-O stretching as well as C-O-H, C-O-C deformation modes are located in the range of 1200 and 900 cm^{-1}, whereby Carbohydrates provoke an intensive and rather broad band at ~1080 cm^{-1}, due to the *v* (CC) and *v* (CO) vibrations (Grube et al., 2002). Carbohydrates elicit few but sharp bands between 3000-2700 cm^{-1} due to asymmetric C-H stretching of CH_2 and symmetric C-H stretching of CH_3 (Grube et al., 2002).

Figure 8 shows the FT-IR spectra of native inulin from Alfa Aesar® compared to level 1 and level 6 whereas Table 7 lists characteristic vibrations of native and acetylated inulin analysed by FT-IR spectroscopy.

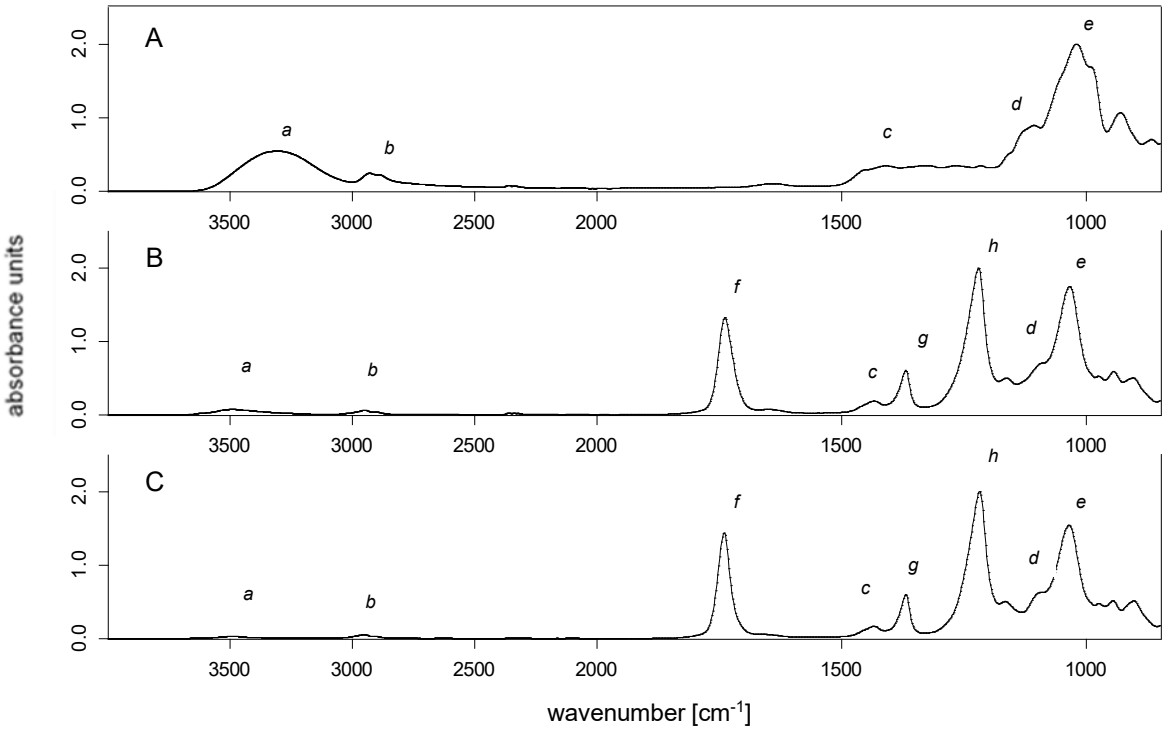

Figure 8: ATR FT-IR spectra of native Alfa Aesar® inulin (A) and the corresponding acetylated inulin with a degree of acetylation of 2.4±0.1 (B) and 2.9±0.1 (C), respectively.

Table 7: Characteristic peaks in FT-IR spectra of native and acetylated inulin.

native inulin	
~3300 cm^{-1}; *(a)*	stretching vibrations of the hydroxyl groups (νO-H)
2929 cm^{-1}; *(b)*	νC-H vibration
1438 cm^{-1}; *(c)*	νC–O–H bending vibrations
~1080 cm^{-1}; *(d)*	νC-C and νC-O stretching peaks
~1030 cm^{-1}, *(e)*	νC-O-C stretching peaks
acetylated inulin	
~1740 cm^{-1}; *(f)*	νC=O
~1370 cm^{-1}; *(g)*	νC-H of methyl group, bending peak
~1220 cm^{-1} ; *(h)*	νC-O, bending peak

The spectrum of native inulin (Figure 8 A) exhibits at ~3300 cm^{-1} a band caused by stretching vibrations of OH-groups. The band at ~2929 cm^{-1} and the shoulder nearby at ~2900 can be

assigned to the asymmetric and respectively symmetric stretching vibrations of CH_2-groups (Grube et al., 2002; Petkova et al., 2018). By acetylating native inulin, the OH-groups are replaced by acetyl groups and therefore these peaks are flattened as well as slightly shifted to higher wavenumbers (Figure 8 B,C).

Moreover, the spectrum of native inulin exhibits C-C and C-O stretching peaks at ~1080 cm^{-1} and another band at ~1030 cm^{-1} that can be assigned to the C-O-C deformation mode of the bonds in the pyranose ring (Grube et al., 2002). These bands are also observed in the spectrum of acetylated inulin in addition to the band at ~1740 cm^{-1} which derives from vibrations of the carbonyl group (Robert et al., 2012). Furthermore, the spectrum of acetylated inulin shows a band at ~1370 cm^{-1} caused by the bending vibration of the CH_3 group and a band at 1220 cm^{-1} characteristic for the bending vibration of the C-O group of the acetyl residue (Poulain et al., 2003).

Thus it is concluded that the process of acetylation is a well-established one.

4.1.2 Confirmation of the degree of polymerisation via NMR spectra

NMR spectroscopy is an important method to elucidate the structure of organic compounds. In this work, ^1H NMR spectra are the basis to determine the degree of acetylation and they were interpreted according to Jain et al., 2014 (Jain et al., 2014). The spectra of acetylated inulin from Alfa Aesar® level 1 and level 6 are shown in Table 9 and Figure 10 respectively.

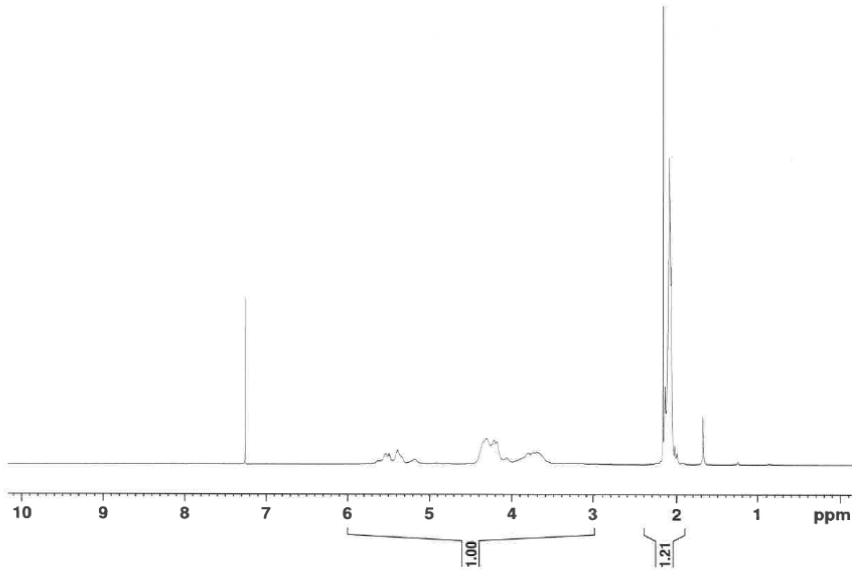

Figure 9: ^1H NMR spectrum of acetylated inulin from Alfa Aesar® level 1.

The peaks in the range between 3-6 ppm are characteristic for the skeleton protons of the fructose units of inulin. The spectra were normalised by setting the integral of these peaks at 1 for each sample. The three hydrogen protons of the methyl group of the acetyl side chain provoke peaks at 1.9-2.4 ppm. For acetylation level 6 (Figure 10) the integral of these peaks was considerably smaller than that for level 1 (Figure 9).

The number of acetyl groups per fructose unit and the degree of acetylation were calculated from the equations described in chapter 3.2.2 and the results are shown in Table 8. Alfa Aesar® level 1 possessed the highest degree of acetylation with 94.9±2.8% corresponding to 2.9±0.1 acetyl groups per fructose unit. Carbosynth® level 1 was not that highly acetylated and the degree is similar to Alfa Aesar® level 6, corresponding to 2.5±0.1 and 2.4±0.2 acetyl groups per fructose unit, respectively. Acetylation level 6 with Carbosynth® inulin yielded only 2.0±0.1 acetyl groups per fructose unit and 66.7±1.0% acetylation.

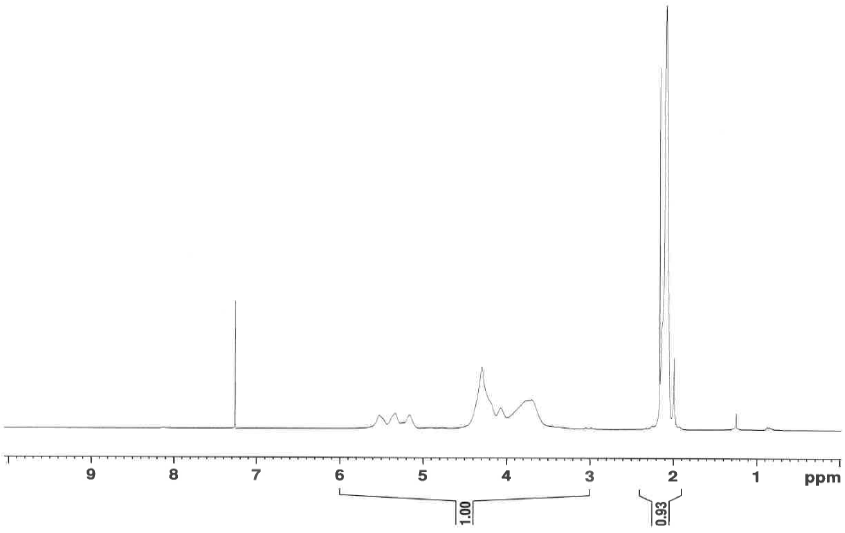

Figure 10: ^{1}HNMR spectrum of acetylated inulin from Alfa Aesar® level 6.

These results are in accordance with those from Robert et al. (2012) (1.6 and 2.1 acetyl groups per fructose unit), Poulain et al. (2003) (1.8 acetyl groups per fructose unit) and Walz et al. (2018) (0.3 to 2.1 acetyl groups per fructose unit), who varied the ratio of native inulin to acetic anhydride too. Especially results reported by Walz et al. (2018) were curious: acetylated inulin precipitated in water possesses 1.8 to 2.1 acetyl groups per fructose unit, whereas purification by dialysis yielded inulins with lower degrees of acetylation down to just 0.3 acetyl groups per fructose unit. On the other hand, the yields even were higher. Therefore, it seems to be possible to gain high amounts of lower acetylated inulin via dialysis, which could be interesting for pellet formation.

Table 8: Degree of acetylation and number of acetyl groups per fructose unit of inulin from different origin.

native inulin	level of acetylation	degree of acetylation [%]	acetyl groups per fructose unit
Alfa Aesar®	level 1	94.9±2.8	2.9±0.1
Alfa Aesar®	level 6	81.1±5.3	2.4±0.2
Carbosynth®	level 1	84.0±2.3	2.5±0.1
Carbosynth®	level 6	66.7±1.0	2.0±0.1

In addition, Table 9 shows the degree of polymerisation and the number of acetyl groups per fructose unit of each acetylation level from Carbosynth® inulin. Accordingly, the stepwise reduction of acetic anhydride added reduces the degree of acetylation. Moreover, level 7 shows the lowest degree of acetylation corresponding to 1.8 acetyl groups per fructose unit.

Table 9: Degree of acetylation and number of acetyl groups per fructose unit acetylated inulin from Carbosynth®.

inulin from Carbosynth®	degree of acetylation [%]	acetyl groups per fructose unit
level 1	86.3	2.6
level 2	84.8	2.5
level 3	82.4	2.5
level 4	81.7	2.5
level 5	73.9	2.2
level 6	65.3	2.0
level 7	61.4	1.8

4.1.3 Size exclusion chromatography – determination of molecular weight

The average molecular weight of native inulin was determined by size exclusion chromatography and is given in Table 10. The DP, the number of monosaccharide units in inulin, was calculated from the known molecular weight of fructose and glucose. The molecular weight and the DP of native inulin from Carbosynth® was found to be slightly higher than that from Alfa Aesar®.

Table 10: Molecular weight and degree of polymerisation of native inulin types.

native inulin	M_w [g/mol]	degree of polymerisation
Carbosynth®	3609±200	20±1
Alfa Aesar®	3300±622	18±3

In order to detect a possible change in DP due to acetylation and purification processes, acetylated inulins were subject of SEC. Thus, the M_w determined by SEC was compared to the calculated M_w (Table 11). The calculated M_w considers the DP of each type of inulin as determined by SEC and the weight and number of the acetyl groups of each level as determined by NMR.

SEC chromatograms of native Alfa Aesar® and Carbosynth® inulin and corresponding acetylated inulins are shown in Figure 11.

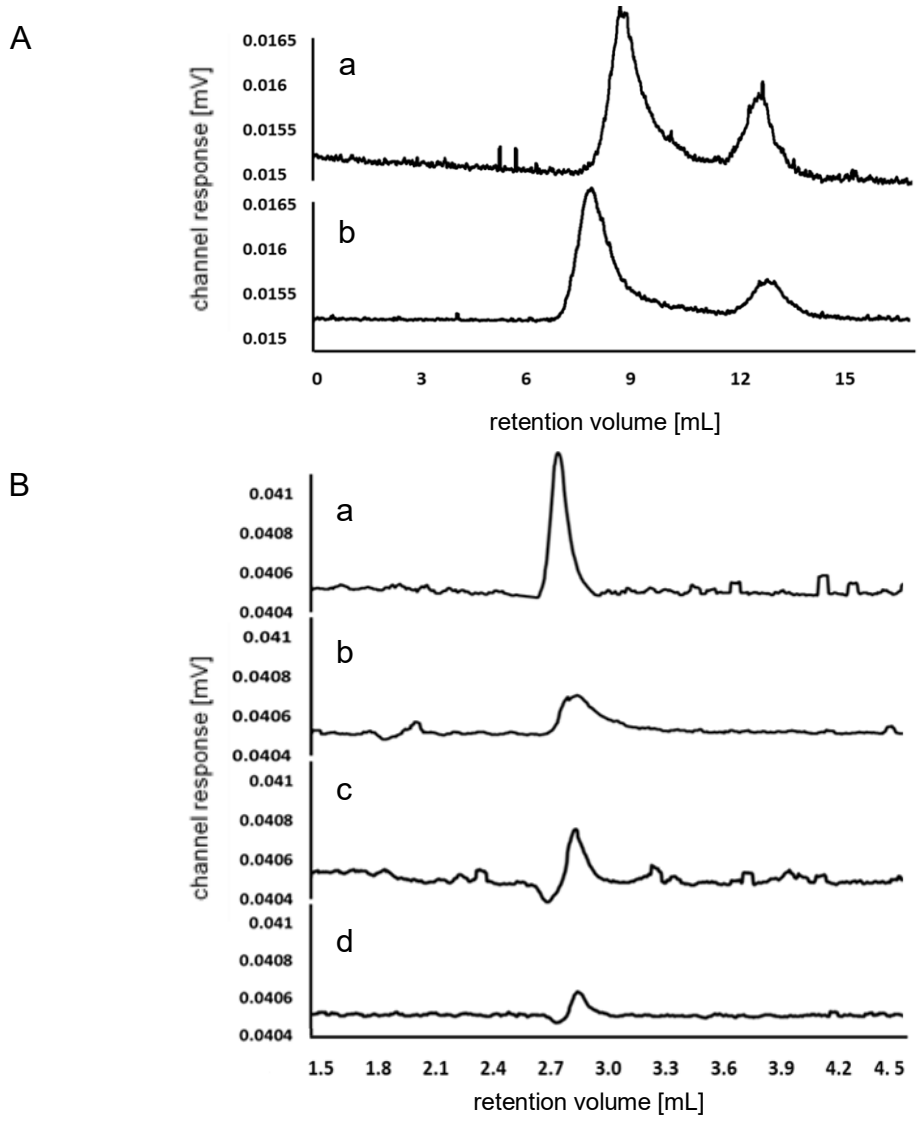

Figure 11: Chromatogram of native and acetylated inulins detected by MALLS and RI detector.
A: native inulins – Alfa Aesar® with retention time (RT) =24.14 min (a) and Carbosynth® with RT=23.04 min (b).
B: acetylated inulin Alfa Aesar® level 1 with RT=8.89 min (a) and level 6 with RT=9.10 min (b), acetylated inulin Carbosynth® level 1 with RT=9.24 min (c) and level 6 with RT=9.11 min (d).

There is a greater difference between the measured and the calculated M_W of acetylated inulin from Alfa Aesar® compared with that from Carbosynth® (Table 11).
The mean measured M_W of Carbosynth® level 1 is 159°g/mol higher than the calculated M_W and for level 6 the difference is 44 g/mol. The measured values for Carbosynth® are greater than the calculated ones and thus the DP remains unaffected. However, in case of Alfa Aesar® level 1, the calculated M_W is greater than the mean measured one and for level 6 it is even quite higher than level 1. These differences are broad and the measured M_W are

smaller than the calculated ones, but quite small in comparison to the molecular weight of inulin. In addition, the DP of Alfa Aesar® inulin is 18 with a high standard deviation in contrast to inulin from Carbosynth® with a DP of 20 and low standard deviation. Inulin is a natural product and not every single molecule possesses the same DP, but it is rather a mixture of molecules with different DP varying from oligosaccharides to polysaccharides. Therefore, the DP determined is just an average of all these molecules with varying numbers of building units. Consequently, there are always differences between the measured and the calculated M_W, but this variation is no hint towards fragmentation during the chemical synthesis.

Table 11: M_W, M_N and the difference between calculated and measured M_W of acetylated inulins.

acetylated inulin	M_W [g/mol]	M_N [g/mol]	M_W mean [g/mol]	M_w calculated mean [g/mol]	difference measured/calculated Mw [g/mol]
Carbosynth® level 1	5268	5212	5240±63	5081	159
Carbosynth® level 1	5168	5206			
Carbosynth® level 1	5284	5194			
Carbosynth® level 6	5056	4958	4947±94	4991	44
Carbosynth® level 6	4868	4885			
Carbosynth® level 6	4967	4958			
Carbosynth® level 6	4897	4929			
Alfa Aesar® level 1	4544	4366	4448±85	4690	242
Alfa Aesar® level 1	4416	4291			
Alfa Aesar® level 1	4384	4300			
Alfa Aesar® level 6	5036	5062	5006±42	4609	397
Alfa Aesar® level 6	4976	5004			
Alfa Aesar® level 6	4670	5040			

4.1.4 Density and Melting point

Important parameters for the characterisation of chemical compounds and subsequently also for the process of the pellet production are the density as well as the melting point.

Densities of native and acetylated inulins are listed in Table 12. At a density > 1 g/cm^3 the inulins and their derivatives sink in water due to their higher density than water of 0.9982067 g/cm^3 at 20°C.

Table 12: Density of native and acetylated inulin types.

inulin	density ρ [g/cm^3]
Alfa Aesar native	1.58±0.01
Alfa Aesar® level 6	1.44±0.02
Alfa Aesar® level 1	1.28±0.06
Carbosynth® native	1.61±0.04
Carbosynth® level 6	1.51±0.09
Carbosynth® level 1	1.43±0.03

Native inulin from Carbosynth® has a higher density than that from Alfa Aesar®, most likely because of the higher M_W and corresponding DP. Moreover, native inulin has a higher density than the acetylated one. Carbosynth® level 6 possesses the highest density, followed by Carbosynth® level 1 and Alfa Aesar® level 6. The latter two have a different DP, but their degree of acetylation is very similar and so that their densities are found within the same range. Alfa Aesar® level 1 inulin is almost completely acetylated and has the lowest density. Thus, the greater the degree of acetylation the lower is the density. This phenomenon is due to high number of hydroxyl groups of native inulin that form intermolecular hydrogen bonds (Barclay et al., 2010). The number of interacting OH-groups is reduced by acetylation. Thus, the number of hydrogen bonds is reduced leading to a decreased density.

The melting range of native inulin from Carbosynth® as well as from Alfa Aesar® is between 158 °C and 165 °C according to the manufacturers.
Table 13 lists the melting points of acetylated inulin and there is a coherence with the degree of acetylation: Alfa Aesar® level 1 has the highest degree of acetylation and at the same time the lowest melting point of the four derivatives. Once more Alfa Aesar® level 6 and Carbosynth® level 1 are in the same range. Inulin with the lowest degree of acetylation (Carbosynth® level 6) displays the highest melting point.

Table 13: Melting points of acetylated inulins.

acetylated inulin	melting point [°C]
Alfa Aesar® level 1	78.9±1.3
Alfa Aesar® level 6	86.3±1.9
Carbosynth® level 1	84.2±0.3
Carbosynth® level 6	95.5±0.0

These characteristics can also be correlated to the decreasing number of hydrogen bonds with concurrently increasing degree of acetylation. The loss of OH-groups leads decreases the melting point of acetylated inulin down below < 100 °C, which is an important parameter for pellet production.
These melting point values are similar to those of Wu & Lee, (2000) and Jain et al., (2014). According to Jain et al. (2014) the melting point of native inulin with a DP of ~30 is 173.22 °C, whereas that of 54% acetylated inulin was 81.82 °C. Wu & Lee (2000) reported the melting point of a native inulin to be 172-176 °C and after acetylation it significantly decreased to 87-92 °C, which was also determined with a capillary apparatus like that described in here.

4.1.5 Log P and water solubility

Log P and water solubility were determined in order to assess whether pellets made of acetylated inulin would pass the upper parts of the GIT unaffected and reach the colon intact or not.

With the method applied (chapter 3.2.6), the log *P* of native inulin could not be determined as appropriate reference substances are not available. But the reference material for acetylated inulin is available. The manufacturers of the inulin types did not determine the log *P* either, but PubChem (*PubChem*, 2020d) stated for native inulin with a molecular weight of about 6200 g/mol a computed XlogP3 of -70.2. Therefore, *P* is ~$10^{-70.2}$ which means that 1 part inulin is dissolved in 1-Octanol and at the same time $10^{70.2}$ parts inulin are dissolved in water. This implies that native inulin is highly soluble in water and nearly insoluble in organic solvents. The solubility characteristics are also stated in the safety data sheets of inulin from both manufacturers.
Figure 12 displays the calibration graph used to determine log *P* of acetylated inulin, which was generated by plotting the log *k* values of each reference substance on the y-axis and the log *P* values on the x-axis. The individual values are given in Table 14.
From the retention time of each acetylated inulin, first the log *k* was calculated and then inserted for y in the linear equation of the calibration graph and so *P* as well as log *P* were determined. The acquired values are shown in Table 15.

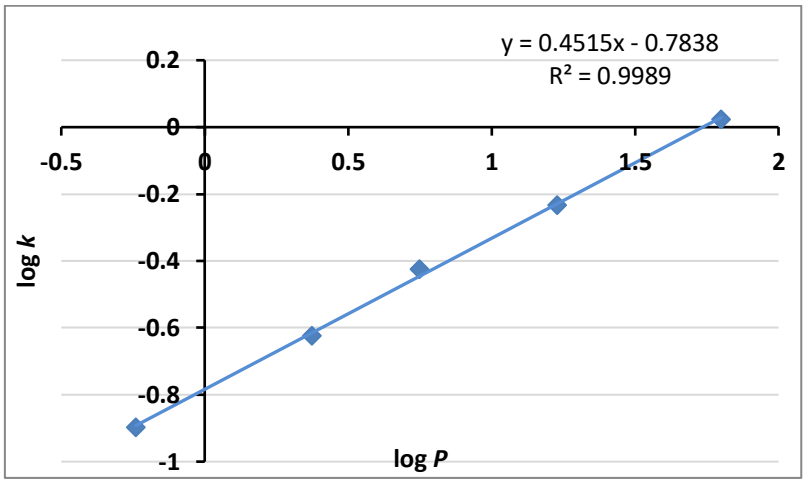

Figure 12: Calibration graph for the determination of log *P*.

Table 14: log *k* and log *P* of the reference substances for the calibration graph.

Reference substances	log *P*	log *k*
acetone	-0.24	-0.89661579
butanone	0.374	-0.62208262
ethyl acetate	0.75	-0.42412294
propyl acetate	1.23	-0.23345781
butyl acetate	1.8	0.02441775

Alfa Aesar® level 1 has a log *P* of ~1.7 and respectively *P* is ~49, which implies that it is 49 times more soluble in organic compounds than in water. Level 6 of Alfa Aesar® and level 1 Carbosynth® are found to be very similar again, but less lipophilic than Alfa Aesar® level 1. The slightest lipophilic acetylated inulin is Carbosynth® level 6 with log P of 1.3 and a 20 times better solubility in organic solvents.

Table 15: log *P* and *P* values of acetylated inulin.

acetylated inulin	Log *P*	*P*
Alfa Aesar® level 1	1.69±0.01	49.2±0.3
Alfa Aesar® level 6	1.58±0.02	37.9±1.4
Carbosynth® level 1	1.57±0.01	37.3±0.4
Carbosynth® level 6	1.30±0.01	19.8±0.2

These results show clearly that in contrast to native inulin, acetylated inulin is hardly soluble in water. This fact is mainly caused by the CH_3-group of the acetyl side chain which reduces the probability of hydrogen bond formation and thus solubility. In contrast, the carbonyl group is and remains rather hydrophilic. Therefore, the higher the number of acetyl moieties per molecule, the greater is the lipophilicity and therefore the log *P*.

To validate these results, the water solubility of acetylated inulin was additionally investigated in detail.
The test was performed adhering to the OECD guideline for the testing of chemicals 105 as described in chapter 3.2.7. The amount of dissolved inulin was quantified according to Saengkanuk et al., 2011, which is also described in chapter 3.2.7.
According to the United States Pharmacopeia (*The United States Pharmacopeia, USP 30-NF 25*, 2007) and the British Pharmacopeia (*British Pharmacopoeia*, 2009) a substance is (nearly) insoluble in water if less than 0.1 g per litre water are dissolved (Savjani et al., 2012). Figure 13 illustrates the percentage of the initial quantity of each level of acetylated inulin that was dissolved in water. In each case, less than 3% of acetylated inulin were dissolved corresponding to less than 5 mg per 100 mL although 150 mg acetylated inulin had been used initially. All in all, acetylated inulin can be considered as water insoluble as less than 0.05 g acetylated inulin per litre were dissolved.

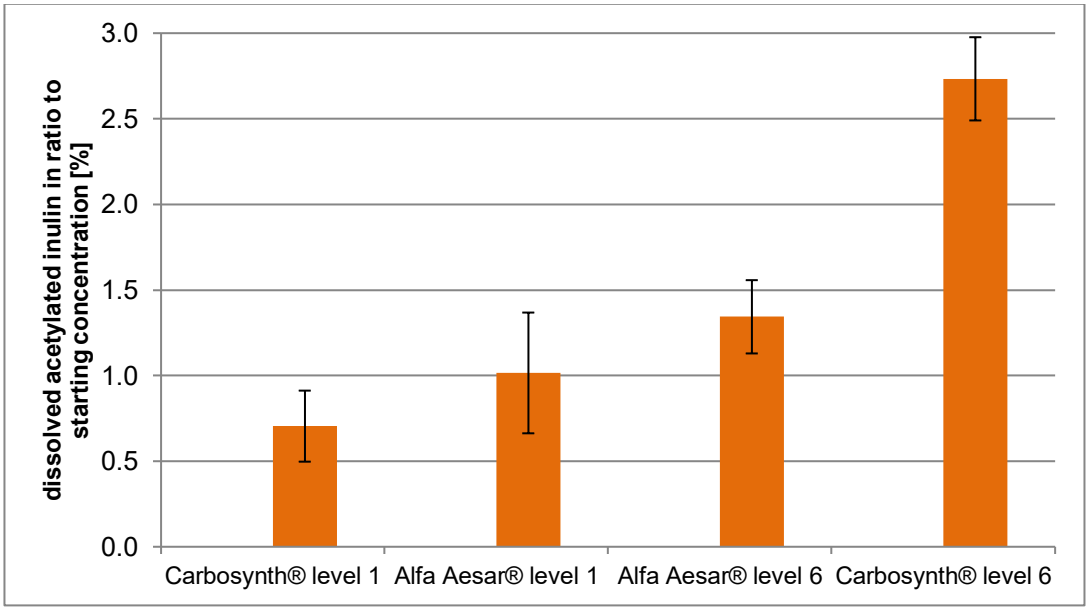

Figure 13: Water solubility of acetylated inulins presented as percentage of dissolved InAc related to the initial concentration.

The largest quantity of acetylated inulin was dissolved in water in case of inulin with acetylation level 6 from Alfa Aesar®. The aqueous solubility was about 2.7 % of the initial quantity due to the lowest level of acetylation as well as the lowest log *P*. The values of the other three acetylated inulin derivatives are in the range of 0.7 % to 1.3 % and their standard deviations overlap significantly. Out of this reason it is not possible to precisely specify which derivative of those three exhibited the highest or lowest percentage of dissolved acetylated inulin.

Nevertheless, it should be considered that determination of log P yields more significant results and determination of water solubility only served to confirm these results.

4.2 Drug entrapment efficiency and loading capacity of 5-ASA InAc pellets

Pellets prepared by dissolving 20 mg of 5-ASA and 500 mg of acetylated inulin in 500 µL DMSO (see chapter 3.3.1) are spherical and about 2 mm in diameter as illustrated in Figure 14. Furthermore, Figure 14 shows the cross section of a pellet made from Alfa Aesar® level 1. The yellowish core of 5-ASA is surrounded by a white coat of acetylated inulin. Table 16 displays the characteristics of pellets prepared from Alfa Aesar® inulin level 1 with maximal LC of about 3.85 %. The pellets range in weight from 1.7 mg to 2.8 mg and their 5-ASA content is between 0.02 mg and 0.04 mg as determined spectrophotometrically described in chapter 3.3.2. This means that the LC is 1.1 % to 1.4 % corresponding to an EE of just 30 % to 36 %.

Figure 14: Size and composition of a pellet from acetylated Alfa Aesar® level 1.

Table 16: LC and EE of pellets prepared by dissolving acetylated inulin Alfa Aesar® level 1 and 5-ASA in DMSO.

	pellets weight [mg]	**area under the curve [mAU]**	**5-ASA content [mg]**	**LC [%]**	**LC_{MAX} [%]**	**EE [%]**
A1	2.03	13.3518	0.024	1.20	3.85	31.10
A2	2.00	13.1114	0.024	1.19		31.02
A3	1.80	11.1742	0.020	1.13		29.51
A4	1.74	11.8337	0.022	1.24		32.27
A5	2.79	21.7463	0.039	1.40		36.49

Due to the fact that the oral daily intake of patients is 3-4 g 5-ASA for the treatment of UC or CD, unfortunately, the loading capacities of the pellets achieved by this methodology are not sufficient to reach the therapeutic window. Appling these dosage forms efficiently would require oral intake of enormous amounts of pellets. Therefore, the production process was optimised in order to increase the encapsulated amount of 5-ASA.
Initially, the aim was to increase the EE as well as to reduce the pellet size to make them more compact. Therefore, different non-solvents as drop-in media were tested (listed in Table 17).

Table 17: Different non-solvents and their effect on pellets on pellet production.

Non-solvent	**pellet formation (yes/no)**	**shape**
distilled water	yes	spherical
0.9% NaCl-solution	yes	spherical
15% NaCl-solution	yes	oval
20% NaCl-solution	yes	oval
30% NaCl-solution	no	platelets
10% Sorbitol-solution	yes	spherical
20% Sorbitol-solution	yes	spherical
30% Sorbitol-solution	no	platelets
40% Sorbitol-solution	no	platelets
50% Sorbitol-solution	no	platelets
60% Sorbitol-solution	no	platelets
70% Sorbitol-solution	no	platelets

Only three of the non-solvents under investigation yielded spherical pellets i.e. 0.9 % NaCl-solution, 10 % sorbitol-solution and 20% sorbitol-solution, but not all pellets were well-shaped. Moreover, the pellets seem to be moderately stable and tend to break easily. Thus none of the media tested are superior to distilled water. Furthermore, distilled water is inexpensive and easily available and therefore it was used for further experiments.
Pellets were then optimised by triturating 5-ASA and acetylated inulin with DMSO in order to increase the loading with 5-ASA per pellet. The quantity of 5-ASA in proportion to acetylated inulin was gradually increased until the ratio was 50/50 as described in chapter 3.3.1.

Table 18 gives an overview of different batches of pellets prepared by the trituration method with acetylated inulin level 1 and 6 from both manufacturers with a maximum possible LC of 50 %.

Table 18: 5-ASA content, LC and EE of different batches of pellets prepared by the trituration method.

acetylated inulin	pellet weight [mg]	shape	LC_{max} [%]	5-ASA content per pellet [mg]	LC [%]	EE [%]
Carbosynth® level 1	2.2±0.1	spherical	50	1.0	45.0±1.6	90.0±3.1
Carbosynth® level 1	2.3±0.1	spherical	50	1.1	45.8±1.1	91.6±2.2
Carbosynth® level 6	2.5±0.1	non-uniform	50	1.2	47.3±1.3	94.6±2.5
Carbosynth® level 6	2.0±0.3	non-uniform	49.9	0.8	41.5±3.0	83.2±6.0
Alfa Aesar® level 1	2.2±0.1	spherical	50	0.8	37.3±2.1	74.7±4.3
Alfa Aesar® level 1	2.0±0.2	spherical	50	0.8	41.3±4.5	82.6±8.9
Alfa Aesar® level 6	2.3±0.1	spherical	50	1.1	46.1±1.5	92.2±3.0
Alfa Aesar® level 6	2.4±0.0	spherical	50	1.1	44.9±1.1	89.9±2.2

According to the results the pellet weight has not really changed in comparison to the pellets prepared by the previously applied method and is in the range of 2.15±0.64 mg. However, a difference is that with the naked eye core and shell cannot be distinguished so that 5-ASA is either diffusely distributed throughout the pellet or a very thin shell of acetylated inulin covers the core of 5-ASA. Except for pellets from Carbosynth® level 6 which are rather shapeless and angular (Figure 15 D) all pellets are spherical and well-shaped (Figure 15 A, B, C). Additionally the diameter of the pellets remained rather constant being 1.9 mm.

The 5-ASA content ranges between 0.8 mg and 1.2 mg and is the 20-40 fold quantity of 5-ASA in pellets prepared with the dissolution method. Thus, the LC ranges from 37.3±2.1% to 47.3±1.3% corresponding to an EE of 74.7±4.3% up to 94.6±2.5%. The lowest LCs of 37.3±2.1% and 41.3±4.5% corresponding to an EE of 74.7±4.3% and 82.6±8.9%, respectively, was obtained with Alfa Aesar® level 1 which has the highest degree of acetylation. Once again in the same range are Alfa Aesar® level 6 and level 1 from Carbosynth®: all pellets have a LC in the range of 44.9±1.1 % to 46.1±1.5% and so the EE amounts to 89.9±2.2% - 92.2±3.0%. Among all batches the pellets made from Carbosynth® level 6 exhibit the highest LC and EE as indicated by 47.3±1.3% and 94.6±2.5% respectively, but another batch of pellets from Carbosynth® level 6 with a LC of 41.5±3.0% and an EE of 83.2±6.0% is just similar to pellets from Alfa Aesar® level 1.

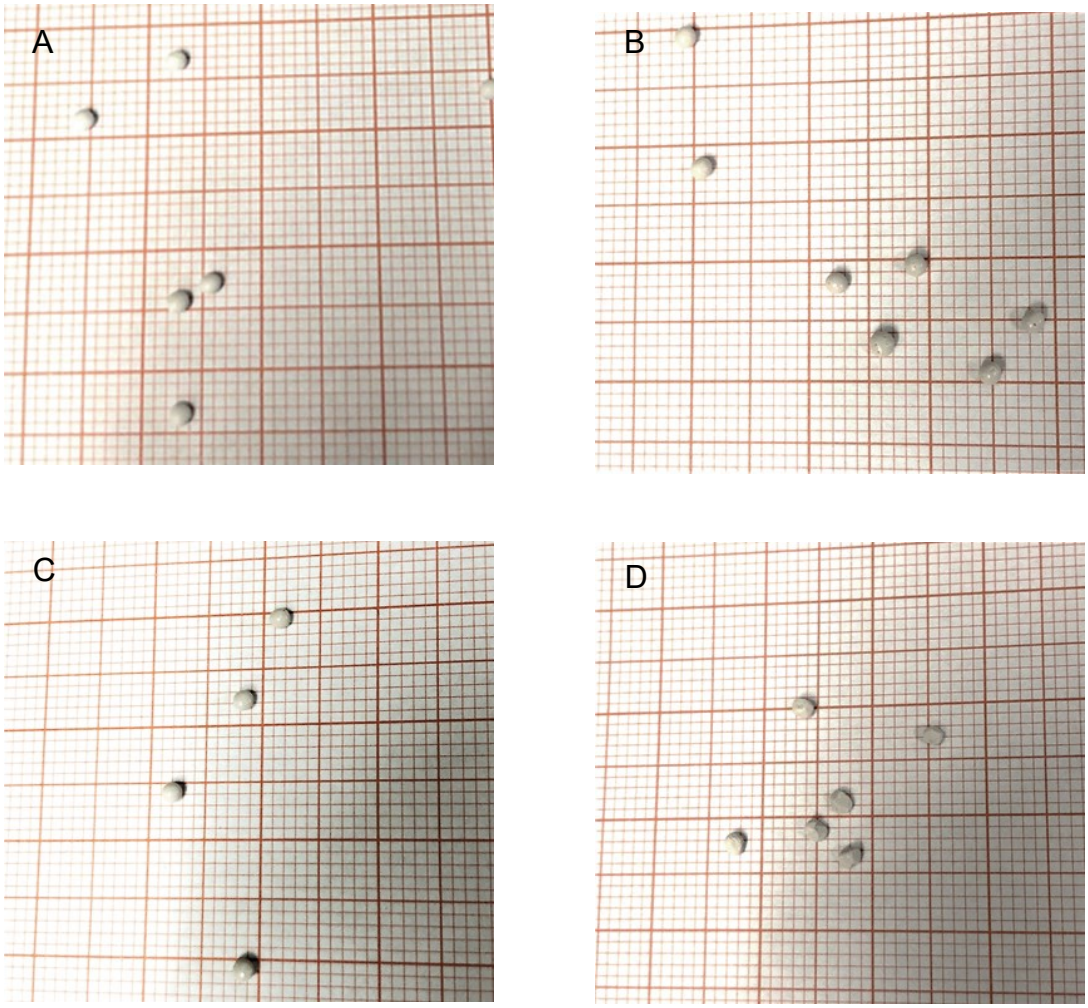

Figure 15: Appearance of pellets prepared from different types of acetylated inulins. Pellets made from acetylated inulin Alfa Aesar® level 1 (A), acetylated inulin Alfa Aesar® level 6 (B), acetylated inulin Carbosynth® level 1 (C) and acetylated inulin Carbosynth® level 6 (D).

These results imply that the degree of acetylation and the resulting properties of acetylated inulin considerably impact the pellet characteristics in terms of surface, loading capacity, encapsulation efficiency and appearance. Pellets prepared with inulin with highest degree of acetylation, respectively the most lipophilic one, contain the lowest amounts of 5-ASA. Carbosynth® level 6 with the lowest degree of acetylation yields contradictory results, but due to the tendencies of the other levels, it is the type enabling the highest content of 5-ASA. Further experiments are definitively required in terms of increasing the 5-ASA loading as well as improving pellet shape.

5 Conclusion

In this diploma thesis, to enable the specific intestinal delivery of 5-amino salicylic acid, pellets out of two different types of acetylated inulin were produced. Two different types of inulin were compared, one from Alfa Aesar® with a degree of polymerisation of 18±3 and one from Carbosynth® with a degree of polymerisation of 20±1. The acetylation process of inulin was successful and different degrees of acetylation could be obtained by gradual reduction of the quantity of acetic anhydride added. Inulin from Alfa Aesar® was higher acetylated than that from Carbosynth® applying this method. Higher yields could be obtained upon acetylation of 10 g native inulin for level 1 (Carbosynth® 5.9±0.9 g, Alfa Aesar® 4.8±0.8 g) than for level 6 (Carbosynth® 1.5±0.2 g, Alfa Aesar® 2.7±0.6 g), which may be due to the different origin. Size exclusion chromatography results confirm that inulin was not fragmented throughout the acetylation and purification process.
The degree of acetylation determined the characteristics of inulin. Thus, inulin with a lower degree of acetylation has a higher density and melting point than higher acetylated inulin. Moreover, acetylated inulin has a high log *P* and is nearly insoluble in water. The higher the degree of acetylation the higher the log *P*. Although the two types of inulin compared are of different origin and possess different degrees of polymerisation as well as molecular weight, they display the same characteristics at the same degree of acetylation.

Acetylated inulin is a proper carrier material for 5-amino salicylic acid and pellets made of 5-amino salicylic acid and acetylated inulin described in here, are about 1.9 mm in diameter and have a weight in the range of 1.7 mg to 2.4 mg. Pellets prepared by dissolving 5-amino salicylic acid and acetylated inulin in dimethyl sulfoxide consist of a core of 5-amino salicylic acid and a shell of acetylated inulin. In contrast, in pellets prepared by trituration of 5-amino salicylic acid and acetylated inulin the drug is diffusely distributed all over the pellet. The latter method, however, yielded highest 5-amino salicylic acid -loadings amounting to 0.8 mg to 1.2 mg 5-aminosalicylic acid per pellet as compared to only 0.02 mg to 0.04 mg 5-amino salicylic acid in case of dissolving 5-amino salicylic acid and acetylated inulin. Accordingly, the encapsulation efficiency of pellets prepared by trituration was 75% to 95% in comparison to 30% to 36% in case of the dissolution technique. Among the different drop-in media under investigation distilled water emerged most appropriate because of its abundance and inexpensiveness.

All in all, future research will focus on elucidation of the prebiotic benefits of acetylated inulin. Moreover, further chemical modification of inulins with varying degrees of acetylation is envisaged.

New treatment strategies for the therapy of inflammatory bowel diseases are emerging by applying fully characterised and modified prebiotics as biocompatible and non-toxic excipients, which also would open up new combination therapies.

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Im Rahmen der vorliegenden Diplomarbeit konnte zur Publikation “Chemically modified inulin for intestinal drug delivery – a new dual bioactivity concept for inflammatory bowel disease treatment” (Hufnagel et al., 2020) maßgeblich beigetragen werden