The stain-potential of novel food ingredients

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Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel „The stain-potential of novel food ingredients“ selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, Februar 2012

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Abstract

Modern foods are produced by combining a complex range of ingredients with specific functionalities like texture agents, health ingredients, sweeteners, flavourings, etc. An evaluation of how such ingredients contribute to staining on their own, or in combination with other food components has not been so far the subject of a serious study. The term stain-potential, as subject of this thesis, refers to the likelihood of stain-formation on textile fabrics, as well as to the available strategies for stain-removal in laundry detergents.

Novel food ingredients are divided into classes according to their functionality in food systems. Several ingredient classes were chosen for further screening, based on the occurrence of properties linked to stain-potential. The ingredient classes selected for investigation included hydrocolloids and starches, fat replacers and health ingredients.

Two different methods on the laboratory scale were used for screening of the target substances. The experimental model for determining stain-potential was based on studies of the enzymatic digestibility of the isolated target substance in solution. The target substances were digested with common detergent enzymes in a model washing system. Suitable analytical methods were employed to characterize the enzymatic reaction. The digestion of the target was compared to the digestion of a traditional ingredient, which served as a reference substance. In this way, it was possible to evaluate whether a novel ingredient is more likely to contribute to staining than a traditional one.

In order to investigate the stain-potential of the target substances under conditions approaching washing as close as possible, a series of empirical tests were designed. In the empirical model, the target substance was formulated into a suitable food matrix. The foodstuff with the target was manually applied as a stain on a piece of cotton fabric, which was consequently washed in a Launder-Ometer® with a commercial laundry detergent. The washing performance was compared to a control, which consisted of stains by the food matrix alone.

Using both screening methods, it could be demonstrated that the modern laundry detergent is well-equipped for the stain-removal of most novel food ingredients. However, several target substances from the hydrocolloid and starches ingredient class were shown to lead to problematic stain-formation. The feasibility of developing novel enzymatic solutions for stain-removal of these ingredients depends on forecasting which ingredient system is likely to represent the dominant hydrocolloid of the future.

Keywords: stain-potential, novel food ingredients, detergent enzymes, model washing system, washing experiments, Launder-Ometer®, laundry detergent, composition of food, stain-removal, manual staining, hydrocolloid of the future

Kurzbeschreibung

Moderne Lebensmittel werden durch die gezielte Kombination mehrerer Inhaltsstoffe mit spezifischer Funktionalität zusammengesetzt, z.B. Verdickungsmittel, Süßstoffe, Aromastoffe. Inwiefern solche funktionellen Lebensmittelinhaltsstoffe zur Fleckbildung an Textilien beitragen, wurde bislang nicht intensiv erforscht. Das Fleckbildungspotenzial, als Thema dieser Arbeit, beinhaltet in diesem Zusammenhang sowohl die Fleckbildung auf einem Textil durch eine Substanz, sowie die etablierten Strategien zur Fleckentfernung mit herkömmlichen Waschmitteln.

Lebensmittelinhaltsstoffe werden gewöhnlich nach ihrer Funktionalität in Klassen eingeteilt. In dieser Arbeit wurden mehrere Substanzklassen für ein Screening des Fleckbildungspotenzials ausgewählt. Die Selektion basiert auf Substanzeigenschaften, die mit der Fleckbildung in Verbindung stehen. Im Fokus der untersuchten Substanzklassen lagen dabei Hydrokolloide, Fettersatzstoffe und gesundheitsfördernde Substanzen.

Zwei unterschiedliche Methoden wurden im Labormaßstab für das Screening der Targetsubstanzen angewandt. Das experimentelle Modell zur Bestimmung des Fleckbildungspotenzials beruht auf einer Untersuchung der enzymatischen Abbaubarkeit der Targetsubstanz. Die Targetsubstanzen wurden mit Waschmittelenzymen in einem Modellwaschsystem umgesetzt. Dabei wurde die enzymatische Abbaureaktion mittels diverser analytischer Methoden verfolgt und charakterisiert. Eine Aussage über das Fleckbildungspotenzial der neuartigen Inhaltsstoffe erfolgte nach einem Vergleich mit dem Abbau von Referenzsubstanzen, die auf traditionellen Inhaltsstoffen basierten.

Um das Fleckbildungspotenzial der Inhaltsstoffe unter möglichst realen Waschbedingungen testen zu können, wurden empirische Versuche durchgeführt. Im empirischen Modell wurde die Targetsubstanz in einer Lebensmittelmatrix formuliert. Das Lebensmittel mit dem Target wurde als Anschmutzung auf Baumwollgewebe aufgebracht. Anschließend wurde das Textil in einem Launder-Ometer® mit einem handelsüblichen Waschmittel gewaschen. Die Wascheffizienz wurde mit einer Kontrolle verglichen, die aus Anschmutzungen der Lebensmittelmatrix ohne Targetsubstanz bestand.

Durch die Anwendung beider Screeningmethoden konnte gezeigt werden, dass moderne Waschmittel für die Fleckentfernung einer Vielzahl an neuartigen Lebensmittelinhaltsstoffen gut geeignet sind. Dennoch erwiesen sich einige Targetsubstanzen aus der Substanzklasse der Hydrokolloide und Stärken als problematische Fleckbildner. Damit sich die Entwicklung neuer enzymatischer Additive lohnt, muss eine Martkanalyse im Bereich der Hydrokolloide für die kommenden Jahren durchgeführt werden.

Stichwörter: Fleckbildungspotenzial, neuartige Lebensmittelinhaltsstoffe, Waschmittelenzyme, Waschversuche, Modellwaschsystem, Launder-Ometer®, Fleckentfernung, zukünftige Inhaltsstoffe

List of abbreviations

Ac	Acetate
APS	Ammonium persulfate
AU	Amylase units
BSA	Bovine serum albumin
ca.	Circa
CAD	Charged-aerosol-detection
CMC	Carboxymethyl cellulose
conc.	Concentration
DH	Degree of hydrolysis
dist.	Distilled
DNSA	Dinitrosalicylic-acid assay
DP	Degree of polymerization
E. C.	Enzyme commission
EFSA	European Food Safety Authority
e.g.	Exempli gratia
et al.	et aliae, “and others”
etc.	Et cetera
Eq.	Equation
EU	European Union
FDA	Food and Drug Administration
FFA	Free fatty acids
fig.	Figure
GRAS	Generally recognized as safe
HM	High methoxyl
HPLC	High-performance-liquid-chromatography
i.e.	Illud est
IFP	Intermediate food product
Inc.	Incorporated
kDa	Kilo Dalton
LM	Low methoxyl
LU	Lipase units
M	Molar mass
MCT	Medium-chain-triglyceride
Mw	Molecular weight
n. a.	Not applicable
NAH	Neoagarohexaose
NAT	Neoagarotetraose

OSA	n-octenyl succinic anhydride
O/W	Oil in water emulsion
PAGE	Polyacrylamide-gel-electrophoreses
PU	Protease units
RI	Refractive index
RP	Reverse-phase
rpm	Rotations per minute
RS	Resistant starch
RT	Room temperature
SALATRIM	Short-And-Long-Acyl-Triglyceride-Molecule
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion-chromatography
TEMED	Tetramethylethylenediamine
t_R	Retention time
TRIS	Tris(hydroxymethyl)aminomethane
U	Units
US	United States
UV	Ultraviolet
vs.	Versus
w/v	Weight per volume
w/w	Weight per weight

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1. Introduction

Throughout history, humans have developed many methods for cleaning their garments, ranging from pounding textiles on rocks in streams to the modern electric washing machine and dry cleaning. The washing process, which has started on the banks of the river, has moved to the private home, with 97 % of German households possessing a washing machine [(1)]. Thus, it can be said that laundering has undergone quite an evolution. The modern laundry process can be best described as the interplay between four elements: washing machine, textile, detergent and stain (see fig. 1.1). Understanding how the washing process is likely to develop in the future, means examining the ways in which each of the elements involved in the process are being transformed.

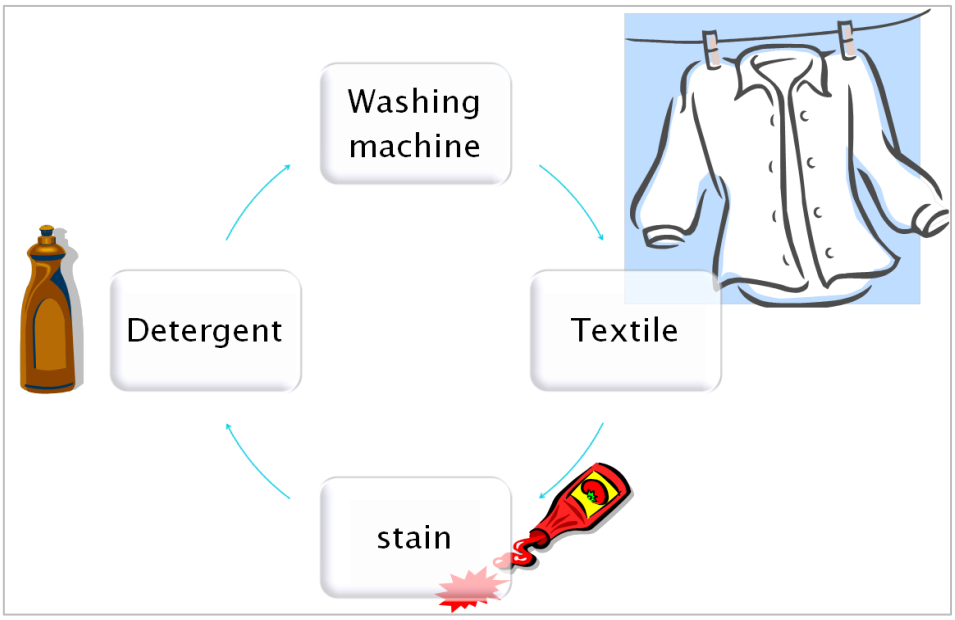

Figure 1.1: The four players in the modern laundry process

The preferred washing equipment of the 21st century is the horizontal-drum washing machine [(2)]. It offers high cleaning performance, combined with gentle fabric care and low water and energy consumption. This is possible due to modern integrated electronic circuitry and fuzzy logic control which allows automatic adjustment of the washing program to the wash load. There are some alternatives to the current technology, e.g. machines which use ultrasound for cleaning, steam for textile refreshment, as well as ozone or silver for hygiene washing. However, these novel approaches are not expected to replace the familiar washing machine in the near future, since they cannot outperform existing technology on the combined basis of cleaning performance, ecological efficiency and cost [(3)].

Modern textiles are made up of a variety of synthetic and natural fibres, and come in a diversity of colours. Interesting developments are to be found mainly in professional and outdoor wear as textiles coupled with membrane technology e.g. Gore-Tex®, Sympatex®. Such clothes are designed to

be light, water- and windproof, while allowing body moisture to escape. Innovations in material science and textile technology can have an impact on the washing process, since these fabrics require special care or different cleaning habits altogether. Examples include textiles with self-cleaning or dirt-repelling surfaces, disposable single-use wear made of biodegradable materials, or textiles from a spray can which are sprayed onto the body and then washed away with water.

A major factor in obtaining good cleaning performance in the washing process is an effective laundry detergent. Today, in the German market alone, there are several hundred different brands of laundry detergents to choose from. These products come in multiple forms (liquid, concentrates, tabs, gels, etc.), sometimes tailored to a specific application (e.g. colour care, jeans, wool). The growing commitment to develop sustainable products and processes is likely to be a significant arena of laundry detergent innovation. This involves further optimization of detergent efficiency per dosage required, the replacement of petroleum-based ingredients with renewable alternatives and the use of enzymes. For instance, novel enzymes with activities at low temperatures have led to the recent development of cold-washing detergents. [(2)]

The fourth player in the washing process, the stain, has often been neglected while considering how the laundry process might change in the future. Of course, the composition of food impacts the type of stains spilled on fabrics. Various technological and socio-economic factors have brought about a transformation of foodstuffs in the last thirty years. Nowadays, foods must meet high standards of safety, be convenient, healthy, tasty, enjoyable, and available at a reasonable price. To fulfil the specific requirements and the variable demands of modern consumers, the food industry must display a huge capacity for innovation [(4)]. Thus, various novel ingredients have appeared on the market. These ingredients allow food manufacturers to create a broad range of products that suit a variety of lifestyles and offer unprecedented consumer choice.

From the perspective of the laundry process, it remains to be investigated whether novel food ingredients currently in development or already on the market play a role in the formation of problematic stains. Such a scenario can stimulate the development of stain removal strategies, often based on optimizing laundry detergent components.

2. Aim and Scope

Developments in the food industry have led to the emergence of various novel food ingredients. The objective of this PhD thesis is to investigate the potential of such ingredients to serve as problematic stains on textiles. The changing composition of foods and its impact on stains is an area of research which has remained largely unexplored. Its relevance extends to the laundry detergent as a player in the washing process, since novel stain types might require novel strategies of stain-removal.

A starting point of this work is a comprehensive analysis of the food industry. Drawing from secondary sources such as scientific journals and industry reports, the theoretical part of the thesis looks into the driving forces and major trends which shape the food industry today. Furthermore, it shows how the market environment has led to the development of many novel food ingredients. These novel ingredients belong to different functional classes (e.g. pigment, hydrocolloid, fat replacer, sweetener, etc.). Some ingredient classes possess properties which are more likely to contribute to staining than others. The aim at the end of the theoretical chapter is to discuss the stain-forming potential of each ingredient class.

In the experimental part of the thesis, several target substances from the following ingredient classes are selected for laboratory testing: fat replacers, hydrocolloids and starches. The stain-forming potential of each target substance is evaluated on the basis of its enzymatic digestibility. The novel ingredient is digested in a model washing environment reflecting typical washing conditions such as corresponding dilutions, alkaline milieu and a detergent enzyme. The breakdown of the target substance is characterized with various analytical methods. If the novel ingredient does not correspond to the specificity of a detergent enzyme, another suitable enzyme is employed for analysis. Whenever possible, the digestion of the target substance is compared to a reference substance, representing a conventional, well-known ingredient with a history of long use.

The next logical step of the thesis is to expand the model of enzymatic digestibility, developed in the laboratory, to include other factors in the formation and removal of stains (e.g. the textile, other food components, mechanical agitation, detergent, etc.), thus coming as close as possible to genuine staining and washing conditions. For this purpose, a series of washing experiments are conceived. These experiments involve the formulation of the target substance within a food matrix, the manual staining of fabrics and washing with a laundry detergent. This empirical approach allows a direct evaluation of how a novel food ingredient impacts stain-formation and also studies consequent removal during the laundry process. The target substances to be screened with the empirical procedure are derived from the ingredient classes of the health ingredients, fat replacers and hydrocolloids.

3. Theoretical background

3.1 The food industry today

As a result of increasing urbanization, the food industry is experiencing a split between the areas of production (e.g. farmlands) and the areas of demand and consumption, located mainly in densely-populated cities. This has led to a transformation in the way food is transported, stored and processed. In order to provide cities with food of uniform quality in a cost-effective manner, the food industry has evolved to resemble manufacturing [5]. Raw materials from agricultural production are refined into ingredients of standardized quality. These tailor-made ingredients or "intermediate food-products" (IFP) are then transported to factories close to the place of consumption and recombined to create the finished food product [6].

Consequently, in contrast to traditional foodstuffs (e.g. cheese, wine, and meat) which are the direct results of processing agricultural raw materials, new food products are made by combining a more complex range of ingredients (e.g. nutrient, flavour, texture modifier, etc.).

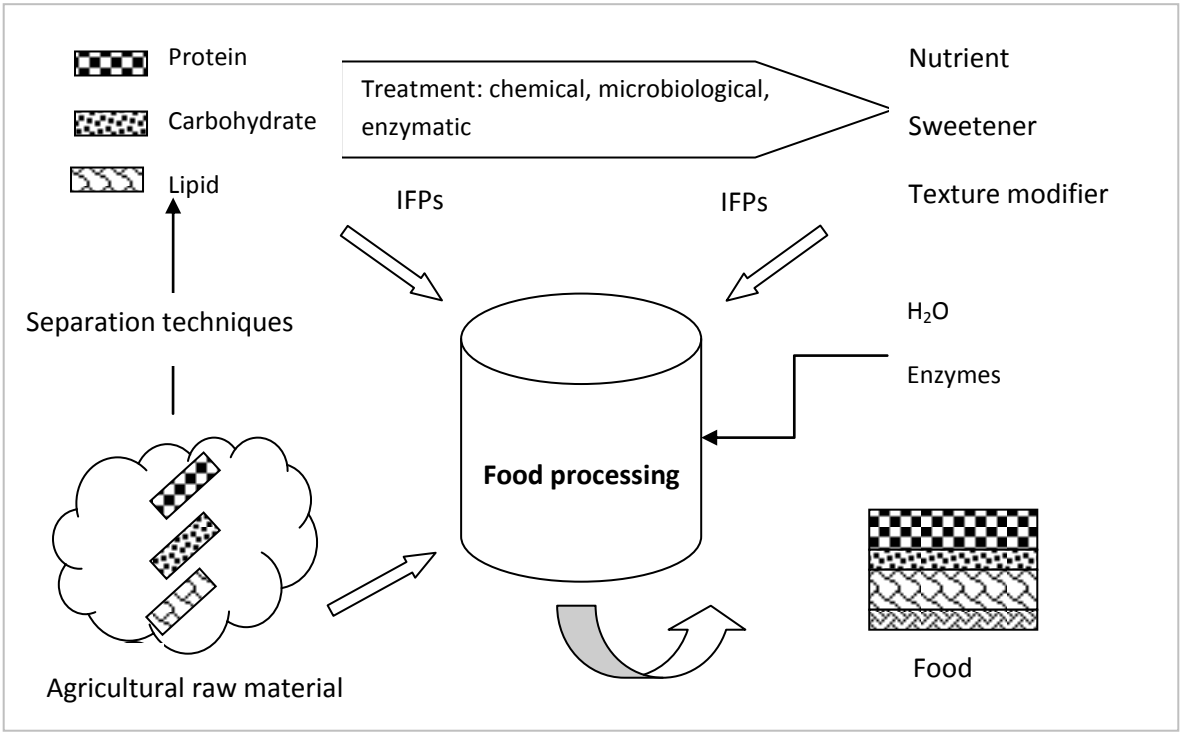

Figure 3.1: Modern food is assembled by combining a range of IFP ingredients by the secondary processing industries

The suppliers of tailor-made ingredients operate on the stage between the agricultural and the food manufacturing industry (see fig. 3.1). IFP's are derived from agricultural, farming and primary food production sources: plant material, dairy products, egg constituents, sea produce, meat, and farming by-products. The raw material is subjected to a refining process by the secondary processing industry. The result is an improved material with distinct functional characteristics (texture agent,

flavouring, etc.). The IFP companies offer their product to the customer, typically together with application methods and services, which are specially suited to the individual requirements of the food manufacturer.

With the exception of some IFP's which are added as nutrients to foodstuffs, most other IFP's are employed typically as food additives. A food additive is defined as a substance, not commonly regarded or used as food in itself, which is added to foodstuffs in order to delay the onset of microbial spoilage, as well as to improve appearance, taste, flavour, or to serve any other technological function in relation to food [(7)]. Food additives can be roughly categorized into two groups: additives that preserve freshness and prevent microbial spoilage, such as preservatives and antioxidants, and additives which promote or amplify the sensory qualities of food, such as texture modifiers, colours, thickeners, sweeteners, flavours and flavour enhancers.

Modern food processing is unthinkable without additives, and the last 50 years have seen the discovery of numerous substances, of both natural and synthetic origin, which can fulfil various functions in foodstuffs. Nowadays there is a rising desire among consumers for "naturalness" and "authenticity", meaning a reduction of the level of additives in foods, or their substitution with natural equivalents: natural flavours, preservatives and colourings [(8)]. For this reason, additives based on IFP ingredients, being derived from agricultural raw materials, are seen as attractive options for the food producer.

IFP ingredients can be classified either according to origin (raw material source) or according to the technological role they fulfil in food systems. In this work, the latter approach is preferred. Nevertheless, it is important to stress that raw material availability plays a major role in the ingredient industry. Common motivators for developing alternative or innovative ingredients are cheaper, locally available resources, rising energy and food prices, or political instability in the region from which a raw material has been traditionally imported [(5)].

3.2 The modern food consumer

Influenced by changing demographics and lifestyles, today's consumers have adopted a different pattern of food consumption, leading to changes in menu planning, food acquisition (shopping), and food preparation (cooking). Traditionally, the food industry has been driven by three main consumer interests: taste, convenience and health. In the 21st century the requirements on all three categories continues to rise [(9)].

3.2.1 Demographics

Consumer behaviour research indicates that the definition of a family is no longer restricted to the traditional nuclear family. A variety of relationships and numbers of people constitute families today. With a greater number of women in the workforce and no substitute at home to prepare meals, family and individual eating habits are changing. Consumers are seeking products that do not require much time, planning or effort to prepare [(9)].

The desire for both simplification and convenience is therefore a significant driving force for the food industry. In order to spend less time and effort preparing meals consumers are effectively "outsourcing" meal preparation [(5)]. The food industry has catered to this need with the development of microwave and frozen meals, ready-made sauces, spice mixtures and instant soups, etc.

This trend has not only necessitated the development of novel ingredients as processing aids, but it has changed the forms and ways in which food is consumed. For example, the growth in eating as a secondary activity (while doing something else, i.e. at the workplace, while watching television, on-the-go) has given rise to the desire for "portability" of food and the growth of "snack" foods. Snacks such as nutritional bars and drinkable yoghurts provide quick nutrition, as well as appetite satisfaction. Snacking itself leads to eating more frequently, in lesser amounts and at more unconventional times [(9)].

The changing demographics of industrialised nations are a major influence on many industries, including the healthcare sector, leisure, and increasingly, the food business. The baby boomer generation is ageing and life expectancy continues to rise. By the year 2050 over 40 % of the population in developed countries will be over 65 years of age (see fig. 3.2 below).

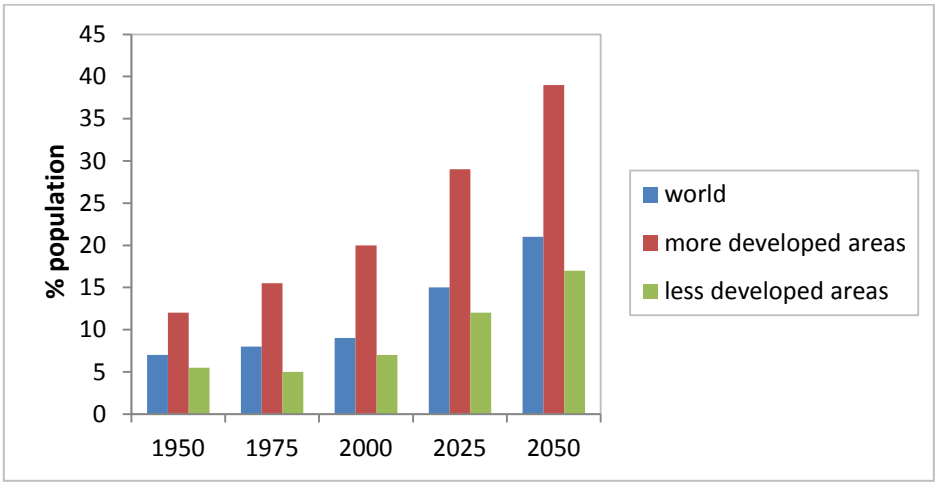

Figure 3.2: Proportion of population aged 60 or over: world and development regions, 1950-2050 [(10)]

The food industry is just beginning to meet the needs of the elderly with special diets. This can be seen in the introduction of functional ingredients and nutraceuticals in many products. In fact, the major buyers of functional foods are the 55+ years old population segment. Foods rich in nutrients which slow the ageing process and/or serve to maintain well-being with advanced age will continue to grow in importance. Ageing brings with it also dampening of the senses. Smell and taste decline. Therefore, food marketers will be enhancing the flavours in products designed for the elderly. As a result, flavours, aromas and pigments will play a significant role in the formulation of foods for older individuals [(11)].

3.2.2 Health and Food

The ageing individual wants to live longer, healthier and more productively. By the 21st century most consumers in developed nations have become increasingly health conscious. Now there is a general recognition of the impact of dietary habits on physical condition and overall health. It is becoming widely accepted that a balanced diet can play a preventive role in the development of a multitude of chronic diseases [(11)].

As a result, interest in all sorts of health foods has grown e.g. soy products, fibre, and whole grains. Health foods have slowly moved into the mainstream. The global trend of healthy eating is presenting a special challenge to the food industry. Food manufacturers should develop products with good taste characteristics, while eliminating ingredients perceived as detrimental to health: saturated fats, trans-fatty acids, refined sugars, high-salt content, etc [(11)]. Other important areas in which the health trend is stimulating ingredient innovation include weight-management products and functional foods [(8)].

3.2.2.1 Weight-management

Obesity is becoming a global health problem of epidemic proportions (see fig. 3.3 on the next page). Currently more than 1.1 billion adults and 10 % of children in the world are classified as overweight or obese [(12)]. This tremendous increase in obesity, aside from a high fat intake, is related to genetic susceptibility and decreased physical activity. In addition, obesity is recognized as an increased risk factor for chronic diseases, including type II diabetes, coronary heart disease and different forms of cancer. Many consumers are interested in products which would help them maintain or lose weight, not only for health benefits, but due to various other reasons, such as the desire for an attractive appearance [(13)]. Since the term “diet” food seems to have negative connotations, most producers and marketers prefer to use the expressions “weight-management” or “weight-control”.

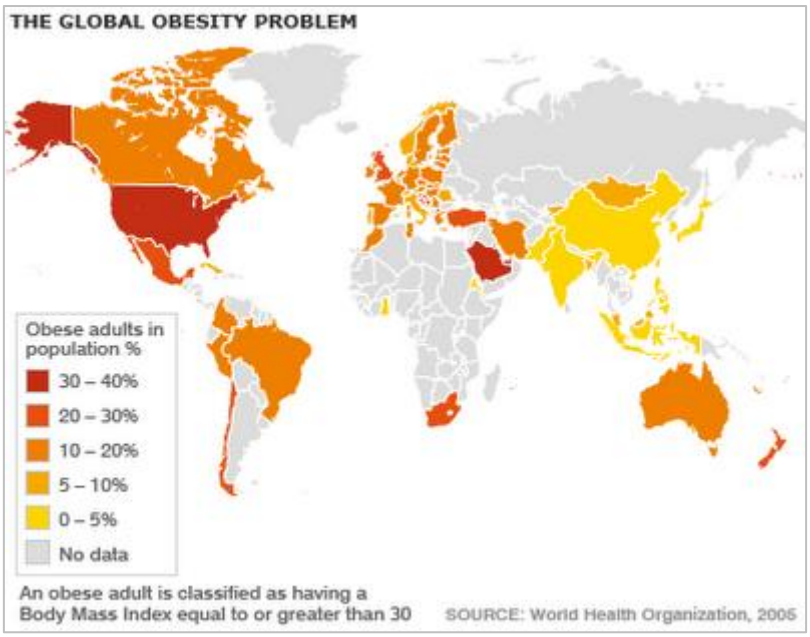

Figure 3.3: Global prevalence of obesity in adults

There is a need to develop food products that help maintain weight at the present low-levels of physical activity. Substances that speed up the process of satiation (so one stops eating sooner) or/and induce longer lasting sensation of satiety (so that one does not feel hungry for a prolonged period of time after eating) may help control weight. Many components of foods influence the feelings of satiety and satiation, as they have an effect on energy metabolism and on hormones related to hunger [(13)].

For example, it is well-known that protein-rich and solid foods have a higher satiating effect, while high-fat foods and liquids have a low satiating efficacy. Some novel ingredients have been introduced to the market, due to their potential ability to help control weight. This could be substances with an effect on noradrenalin and serotonin such as “St John’s wort” (*Hypericum perforatum*) and capsaicin, or substances which influence stomach filling, such as fibre, resistant starch or pectin [(14)].

Most food manufacturers nowadays are expanding their product range to include low-calorie foods. Reducing calories in foods involves “lightening up” or reduction of cholesterol, sugar, salt and animal fat, while adding substances perceived as beneficial to health (dietary fibre, vitamins and minerals, etc.) and novel ingredients which help control weight, such as modified fats. To retain or even improve the appealing sensory qualities of food during the transformation from a traditional to a low-calorie product necessitates the introduction of many food additives [(5)].

For instance, diet foods require texture modifiers to compensate the excess free-water and the loss of mouth-feel resulting from fat and sugar reduction. For this purpose, such foods contain a lot of hydrocolloids such as pectin, xanthan gum, guar, etc. as thickeners and gelling agents. The addition

of flavours and taste enhancers is also essential, since the lack of carbohydrate (sugar) in low-calorie foods means that among others, Maillard products, responsible for aroma, do not form. The sweet taste of food is recreated in turn by bulk or intense sweeteners.

3.2.2.2 Functional food

Many consumers would like to keep a balanced diet, but fail to do so, because they perceive healthy food as not that tasty and/or rather difficult to prepare. This has led to the development of a market for foods, which combine taste with convenience and health. Physiologically-active functional foods were first marketed in Japan in the 1980's. The Japanese Ministry for Health and Welfare issued a series of guidelines for approval of a specific health-related food category "FOSHU" – (foods of specified health use). Products with this label could be marketed carrying established health claims [(15)]. Today, functional food has become a worldwide phenomenon. The global functional food market was worth $ 22.923 billion in 2009 and is expected to grow to $ 27.126 billion by 2015 [(16)].

Functional food is not only intended to satisfy hunger and provide nutrition, but also to prevent diseases and increase physical and mental well-being. It is consumed as part of the normal diet and delivers one or more active ingredients within the food matrix, which have physiological effects and may enhance health. Examples of functional ingredients include probiotics and prebiotics, carotenoids, soy isoflavones, fish oils, polyphenols, etc. [(17)].

The functional ingredient is formulated into a product, which then offers a specific health benefit to the consumer. Important product categories within the functional food segment are beverages, snacks, drinkable yoghurts, and spreads. Examples of functional foods in these categories would include: fibre-enriched fruit beverages, energy-bars, probiotic yoghurts, and cholesterol-lowering margarine. In general, the modern consumer prefers food products that supply health-focused ingredients, instead of having to take supplements [(18)].

As consumers start searching for a more active maintenance of wellness, it is clear that health will become an even greater driving force in the choice consumers make about which foods to buy and eat. Attentive marketers have already begun segmenting consumers based on health condition, instead of age. Products specially suited for a particular health benefit are being developed, i.e. foods for cardiovascular health, foods for blood-sugar balance, foods for joint health, foods for digestion health, etc. It is expected that nutritional individualization with regard to health condition will be a key marketing emphasis in the future. Functional ingredients will therefore continue to play a key role at innovation in the food industry [(4), (18)].

3.3 Stain-formation and stain-removal

Clothes and textiles become soiled through their usage. Any material which is not associated with the textile fabric and/or which is perceived as undesirable to the senses (sight, smell, and touch) is defined as soil. A significant proportion of the soil originates from contact with the human body, as fig. 3.4 below illustrates [19].

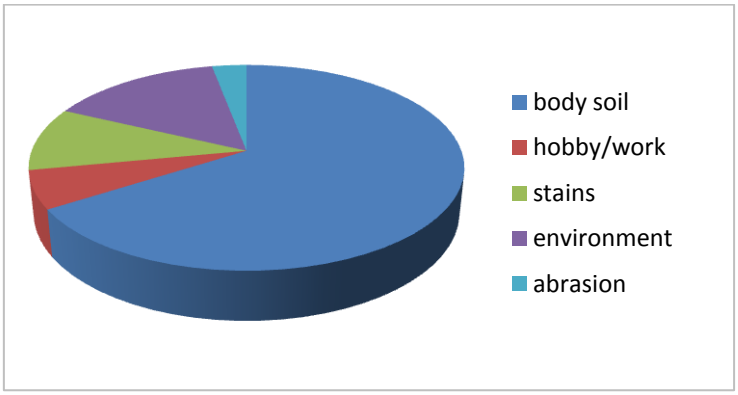

Figure 3.4: The origin of soil in a typical laundry load

The composition of soils is variable and complex. However, it is possible to divide soils into distinct categories according to their chemical properties (table 3.1). Of course, a true soil would consist of a mixture of the materials listed below.

Table 3.1: The components of soils and stains on textiles

Soil type	Examples	Origin
Water-soluble	Salt, urea	Perspiration, urine, food leftovers
Lipids	Triglycerides, waxes	Food, cosmetics, skin oils, mineral oil
Proteins	Gelatine, egg-white	Milk products, keratin from skin, grass, blood
Carbohydrates	Starch, galactomannans	Flour, sauces, thickeners
Pigments	Curcumin, β-carotene	Juice, coffee, wine, fruit and vegetables
Inorganic particles	Metal oxides	Rust, ash, humus, soot

On average, soil makes up 1.3 % (w/w) of a typical laundry load. This is the equivalent of 65 g soil for a 5 kg washing load. Only a small proportion of the soil (typically 20-25 %) is water-soluble. The rest can be removed from the textile by mechanical action or the effect of surfactants. Some soil components (e.g. pigments and proteins) have to be chemically treated by bleaches or enzymes before they can be successfully removed [2].

There are various mechanisms by which the soil material adheres to the textile fabric. These include mechanical entanglement, weak intermolecular forces (e.g. dipole interactions, hydrogen-bonding, van der Waals-forces, etc.) and electrostatic interactions. Adsorption effects and intermolecular interactions are enhanced by a large surface area. As a result, soil particles of 0.2 µm diameter are

very difficult to remove from fabrics, since their small size and large surface area greatly increase the force of intermolecular interactions.

Apart from the size of the soil particles, ageing of the stain also plays a significant role in the stain-formation process. For example, fresh blood stains are easily removed with cold water. Once the stains are older however, the protein denatures and adheres strongly to the fabric. A removal of the stain without the use of proteases is hardly possible. Ageing processes occur in lipid stains as well, mainly through oxidation with atmospheric oxygen.

The stain-formation process is thus dependant on the composition of the soil, the size of particles and their treatment (e.g. ageing processes), and the interaction between soil and textile fabric. The stain-removal process is equally complex; it can generally be divided into three categories: the removal of fat and oily stains, the removal of particulate soil and the removal of hydrophilic stains.

Surfactants are the major players in the stain-removal of fats and oils. They decrease the surface tension of the washing solution leading to complete wetting of textile and oil stain. The surfactants accumulate on the interface and facilitate a rolling-up of the oil droplet away from the textile fibre. Mechanical agitation promotes this process. Once in solution, surfactants help in stabilizing the oil droplets in the washing liquor and prevent their redeposition on the fabric. A simplified model of lipid stain-removal is depicted in fig. 3.5 below.

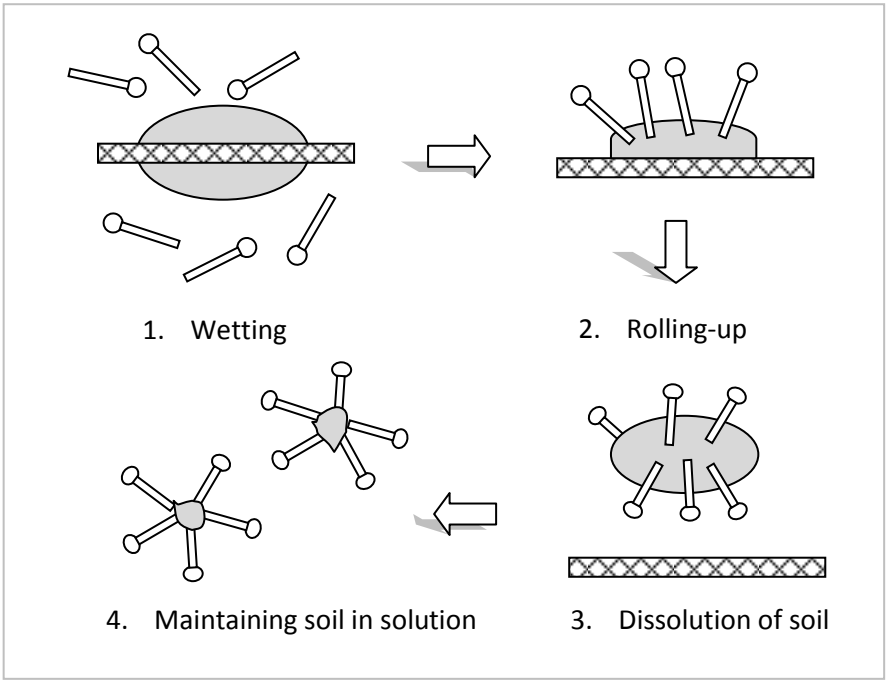

Figure 3.5: The dissolution of an oily stain from a textile fibre by the action of surfactants

A successful stain-removal process involves overcoming the forces with which stain material adheres to a fabric. In the case of particulate matter, the attraction forces are related to the size of the soil particles (weak intermolecular interactions) and to the electrical surface potential between soil and fabric. In an aqueous solution, both soil particles and fabric are negatively charged (see fig. 3.6 below). This surface potential (ζ-potential, *zeta-potential*) is related to the dissociation of surface groups (e.g. carboxylic groups) or to the adsorption of ions. The ζ-potential increases with rising pH, resulting in repulsive forces between soil particles and fabric. This phenomenon is responsible for the observation that alkaline solutions possess mild cleaning effects (e.g. soda solution).

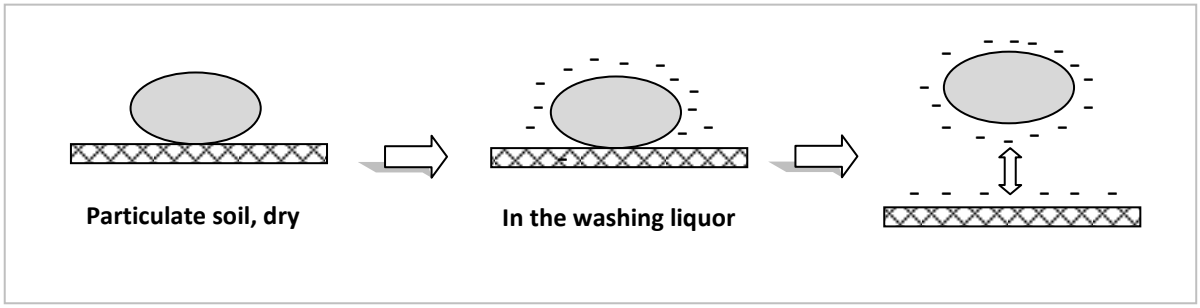

Figure 3.6: A model of stain-removal of particulate matter through electrical repulsion in alkaline solution

Surfactants support the cleaning process further by forming a monolayer around fabric and particle, which enhances the repulsive forces between the two. However, surfactants are not sufficient for effective washing performance if the stain material has strong ionic properties or is very hydrophilic.

Soil material which is strongly polar is removed from fibres with the aid of other detergent components. For instance, calcium-containing soil can be removed by the action of complexing agents or ion exchangers. These decrease the calcium-ion concentration in the washing liquor, leading to a shift in the solubility equilibrium of calcium salts. The calcium from the soil goes into solution leaving cavities in the soil structure, which loosen the deposit and promote its subsequent removal from the textile surface.

Pigments, carbohydrates and proteins are other polar materials which often cause stains. Many pigments can be removed by oxidation with bleaches. Biological macromolecules are treated with enzymes. Detergent enzymes break down their specific substrate into smaller, water-soluble fragments which are easily carried away from the textile fabric by the washing solution. The detergent enzymes commonly found in detergents include proteases, amylases, lipases, mannanases and cellulases.

3.4 The stain-potential of novel food ingredients

As described in the previous chapter, most soils contain a complex mixture of materials. A food ingredient would not contribute to staining on its own, but from within a composite food system. The properties of the ingredients within the foodstuff determine whether difficult staining will occur. A theoretical evaluation of stain-potential involves considering the mechanisms with which soil adheres to fabrics (intermolecular interactions) and the available strategies of stain-removal in laundry detergents (e.g. enzymes). Therefore, it can be inferred that ingredient properties which are likely to influence stain-potential include water-solubility, molecular weight, particle size, polarity, colouration, and enzymatic digestibility.

The vast variety of ingredients which constitute modern foodstuffs can be divided into categories according to the functional role they fulfil in food systems. Table 3.2 presents the major functional classes of ingredients along with typical properties of substances in the group. This classification serves as a starting point in evaluating stain-potential. Nutrients are omitted from the discussion, because they represent traditional ingredients with a history of long use.

Table 3.2: Ingredients classified according to their functionality in food systems

Functional class	Example	Properties related to stain-potential
Sweeteners	Stevia	Low molecular weight, polar, good water solubility
Flavours	Furaneol	Volatile, low molecular weight
Pigments	Monascin	Possess colouration
Hydrocolloids	Gellan	High molecular weight, polar, hydrophilic
Fat replacers	MCT	Lipophilic and hydrophilic ingredients
Health ingredients	Inulin	Prebiotics and fibre relevant due to high molecular weight, polar

Some ingredient classes possess properties which make them improbable candidates for stain-formation. Obvious functional classes which would not contribute to staining include flavours and sweeteners. Flavours are small, volatile molecules applied at very low concentrations in food systems. Novel sweeteners are usually small, polar, water-soluble glycosides or proteins/peptides. These ingredients are unlikely to adhere to fabrics to a significant extent or cause difficult staining.

The stain-potential of the remaining functional classes is more difficult to predict. Pigments might cause problematic staining due to their apparent ability to impart colour. Novel pigments are mainly based on plants or other natural extracts. Since the publication of the Southampton study in 2007 which showed possible links between synthetic colours and hyperactivity in children, demand for natural colours from the food industry has been increasing [(20)]. As of 2010, natural pigments represent more than 38 % of the food colouring market and they continue to replace synthetics in

market share [21]. The rising popularity of natural pigments mirrors the strong interest of modern consumers in food products perceived as “natural” and free of artificial additives. In order to determine the stain-potential of novel pigments based on natural colourings, it is advisable to screen the pigments for their ability to impart stains, as well as to test the capacity of laundry detergents to remove them.

Another ingredient class which should be screened for its stain-potential are the fat replacers. Widespread rates of obesity and interest in weight-management products stimulate developments in this sector. Table 3.3 below presents the type of ingredients from which fat replacers are derived.

Table 3.3: Fat replacers can be classified according to the components they are derived from; fat replacers based on modified lipids are called fat substitutes; fat replacers based on carbohydrate or protein ingredients are fat mimetics

Base component	Examples	Typical applications
Carbohydrate	Maltodextrin, modified starch, gums	Cookies, cakes, salad dressings
Protein	Microparticulated whey protein	Low-fat cheese, ice-cream
Fat	Olestra, SALATRIM, MCT	Snack food, cooking oil

The market is still waiting for an optimal solution for fat replacement [5], [22]. Fat substitutes possess the best technological properties, but are also subject to tighter regulations, due to their controversial health effects. Fat mimetics are safe from a health perspective, but are unable to fulfil the role of lipids in all types of food systems. It is notable that both fat substitutes and fat mimetics might cause “difficult” stains. It remains to be seen whether stains from modified lipids can be efficiently digested by detergent lipase or can be removed by the action of surfactants. Fat mimetics, such as modified starches and microparticulated protein, should also be tested for stain-removal within a model washing system.

Hydrocolloids are complex polysaccharides which are hydrophilic, possess a high molecular weight and are thus likely to contribute to stain formation. The cost of all hydrocolloids has increased in recent years due to rising energy, raw materials, and transportation costs [23]. Nevertheless, hydrocolloid sales keep growing, due to the fact that they are indispensable ingredients for the food processing industry. The use and popularity of a particular hydrocolloid is strongly affected by its availability and the raw material/price situation.

The supply of hydrocolloids is subject to many fluctuations. For example, seaweed shortages in Asia are driving the cost of seaweed hydrocolloids up (alginate, carrageenan, and agar) [24]. Rising energy and fruit costs are increasing the price of pectins. Other factors influencing the hydrocolloid market are political/legal aspects. The ongoing conflict in Sudan, which is the chief global producer of gum

arabic, is driving the research for alternative replacements for this gum [25]. And India, which produces 80-90 % of the world's guar gum, was met with tight European regulations in 2008, when it was revealed that its produce contains dangerously high dioxin levels [26]. All these factors stimulate food manufacturers to think of alternative solutions such as microbial gums or modified starches, which can replace many existing and established hydrocolloid systems.

Another interesting development to consider is that hydrocolloid manufacturers are boosting the levels of typical hydrocolloids (e.g. pectin). This allows the manufactures to position their products as health ingredients [27], [28]. Hydrocolloids are being marketed for their effects as dietary fibre and sometimes even as a prebiotic. A screening of hydrocolloids for their stain-potential should take into account not only typical application levels of these ingredients, but also the higher dosages required for claiming a health benefit.

As large hydrophilic molecules, hydrocolloids cannot be removed from textile fibres by the sole action of surfactants. Strategies of stain-removal typically involve detergent enzymes. Mannanase is included in laundry detergent formulations since 2002, in order to digest guar gum and locust bean gum stains from foods and cosmetics [29]. It is important to watch for developments in the hydrocolloid market, as alternative hydrocolloids or modified starches may replace the dominance of guar gum as most commonly applied texture agent. In such case, other enzymatic solutions for stain-removal might be necessary in laundry detergents.

The health ingredients are a versatile group which includes ingredients as diverse as soy phytosterols, antioxidants, polyunsaturated fatty acids, phospholipids, probiotic bacteria, phytochemicals, prebiotics and dietary fibre. They are included in foods for their health-promoting benefits. Novel health ingredients with stain-potential are likely to be derived from functional carbohydrate ingredients, such as prebiotics and dietary fibre. The high molecular weight and hydrophilic nature of these ingredients might contribute to problematic stain-formation.

Functional classes of ingredients which might contribute to staining due to their properties should be screened by suitable methods for experimental determination of stain-potential. Such methods can include laboratory models of washing systems or might involve empirical tests derived from methods used to measure laundry detergent performance (e.g. Launder-Ometer®). The ingredient classes selected for experimental investigation in this work were the hydrocolloids (including novel starch ingredients), the fat replacers and the functional carbohydrate ingredients. The following chapters present typical examples, molecular structures, modern use and technological specifications of the selected ingredient classes.

3.4.1 Hydrocolloids & starches

Hydrocolloids, or gums, are water-soluble macromolecules exhibiting colloidal properties, i.e. the ability to remain suspended in water under the influence of gravity. Hydrocolloids increase the viscosity of an aqueous solution and can form gels, and are therefore used as food-thickening and gelling-agents. In addition to the primary functionality of thickening/gelling, hydrocolloids often possess valuable secondary characteristics, such as the capacity to act as emulsifiers, fat replacers, whipping agents, stabilizers of suspensions/foams, and encapsulating agents [(30)].

With the exception of some proteins (gelatine and caseinates) which also display colloidal properties, most hydrocolloids are polysaccharide in nature. Hydrocolloids can be classified according to the raw material they are derived from, as shown in table 3.4 below.

Table 3.4: Food hydrocolloids classified according to raw material source

Raw material source	Hydrocolloid examples
Cellulose derivatives	Carboxymethyl cellulose, methyl cellulose
Plant extracts	Pectin, amylose
Seed and root	Guar gum, locust bean gum, konjac gum
Exudates	Gum arabic, gum karaya, gum tragacanth
Seaweed extracts	Agar, carrageenan, alginate
Microbiological	Xanthan gum, gellan gum, pullulan
Animal extracts	Gelatine

One large group of hydrocolloids is derived from plant cell wall material. It includes the soluble cellulose derivatives: methyl cellulose, carboxymethyl cellulose, etc. Another cell wall hydrocolloid is pectin. Pectin is a polymer of galacturonic acid. At certain positions the acid groups are methylated and the degree of esterification influences the properties of pectin.

Another important hydrocolloid class is derived from the glycan exudates of certain ligneous plants. Among the best known include gum arabic, gum karaya and gum tragacanth. Gum arabic, also called acacia gum, is isolated from certain species of the Acacia tree grown in Africa (Sudan). It is the most widely used exudate hydrocolloid and is used as a thickening agent and flavour oil stabilizer.

The galactomannans are also industrially important hydrocolloids. Numerous leguminous grains contain galactomannans with similar structure, but only carob (also called locust bean gum) and guar are used extensively. Guar gum is extracted from the endosperm of the seeds of *Cyamopsis tetragonolobus*, a plant grown in India and Pakistan. This gum is similar to locust bean gum (extracted from the seeds of *Ceratonia siliqua*) and guar gum was originally isolated to compensate the shortage of locust bean gum.

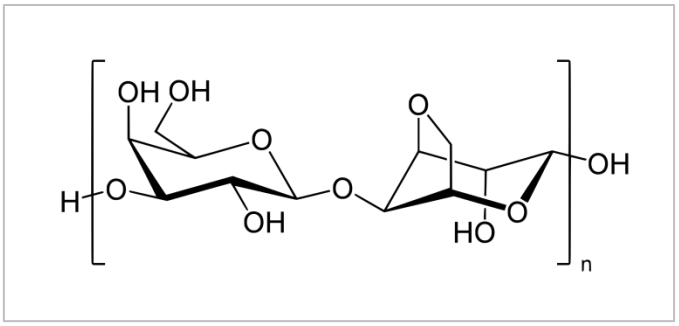

Figure 3.7: Agarose is the principal gelling component in agar. Agarose is a linear polysaccharide composed of alternating residues of α-(1-3)-D-galactosyl-β-(1-4)-anhydro-L-galactosyl units.

Another group of hydrocolloids is produced by extraction from seaweeds or algae. This class includes the carrageenans and agar (both from red algae) and the alginates (from brown algae). The production of hydrocolloids from plants and seaweed suffers from geographical, climatic and political constraints which restrict their availability. As a result, there is a rising interest in microbial gums.

Microbial polysaccharides are produced in a cultivation process and are not subject to climatic or regional limitations [(31)]. The microbial gums currently approved for food use include: xanthan, dextran, gellan, pullulan and curdlan gum. Xanthan, which is produced by the aerobic fermentation of *Xanthomonas campestris*, is by far the most common, due to its unique properties and the low dosage levels required. Curdlan and dextran are less relevant from an industrial point of view, due to their still comparatively high price per unit weight. Gellan is a relatively new gelling agent of microbial origin. It is produced by aerobic fermentation of *Pseudomonas elodea*, and is able to induce gelling at concentrations below 0.05 % (w/v). This is a valuable property, since the lower the amount of gelling agent used, the greater is the impact of the product's flavour and aroma [(5)].

By far the highest proportion of hydrocolloids used in the food industry (> 85 %) is made up of modified and native starches [(32)]. Because of their low cost, wide availability and functionality, starches are not only used as an essential staple food, but also in a range of applications in the food industry [(33)]. The main trends in starch application include the formulation of ready-made meals and sauces, as well as the production of syrup. In addition, the use of starch in health and functional foods is increasing, as they are considered important markets with high growth potential [(34)].

Starch consists of two polymers of glucopyranose - amylose and amylopectin. Their basic structure is presented in fig. 3.8 on the next page. Amylose is a linear molecule containing about 99 % α-(1-4) and 1 % α-(1-6) glycosidic bonds. The average molecular weight of amylose is in the range of 10^5 – 10^6 Da. Amylopectin is a much larger molecule than amylose (10^7 –10^9 Da) and contains extensive branching with ca. 95 % α-(1-4) and 5 % α-(1-6) bonds. Typically, starches contain about 16-35 %

amylose. When the amylose content exceeds 35 %, starches are called amylo-starches. When the amylose content is low (< 15 %), the starches are defined as waxy starches [35].

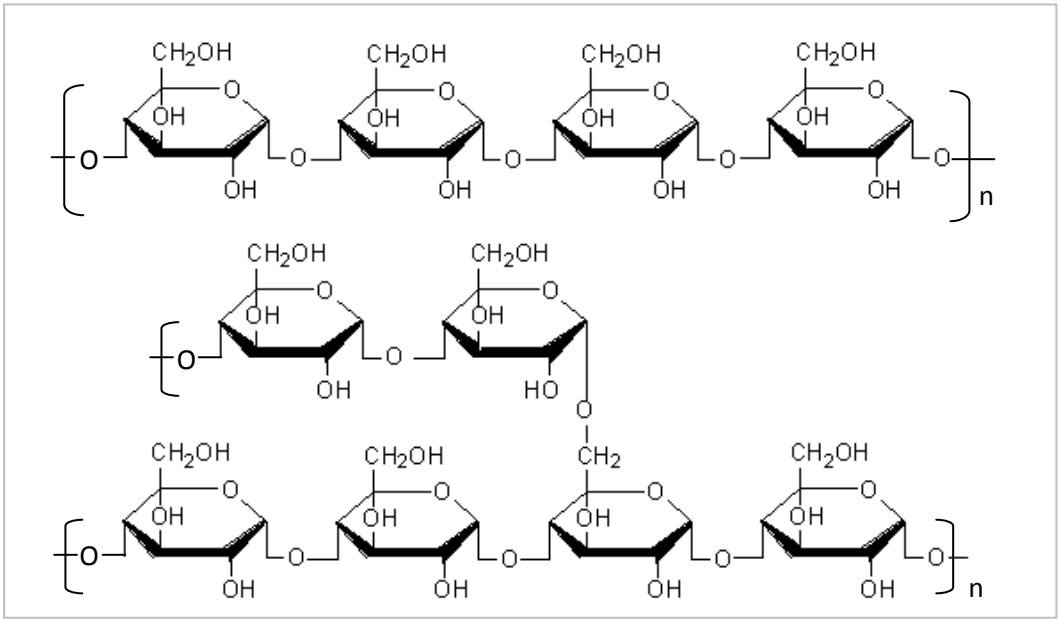

Figure 3.8: Basic structure of amylose (above): α-(1-4) glucan and amylopectin (below): α-(1-6) branching points

Native starches often lack the capacity to provide the desired structure and functionality in food systems. Common limitations of native starches include their excessive viscosity at application levels higher than 6 %, their susceptibility to retrogradation (poor freeze-thaw stability, syneresis) and their lack of process tolerance (low resistance to pH, pressure and temperature) [34]. For this reason, native starches are modified by a variety of physical, chemical and enzymatic methods, thus acquiring interesting traits for specific applications.

Physical modification of starch is accomplished by mechanical shear stress and different forms of drying and heat treatment. Physically modified starches include the pregelatinized and the cold-swelling starches. These provide viscosity immediately without cooking and are therefore popular in instant dry mixes, frozen foods, and convenience products [36].

The chemical modification of starch aims to stabilise the starch for industrial processing conditions or to develop a specific viscosity and gelling profile. Chemically modified starches are treated with oxidation agents, acids, or are subjected to substitution with ester or ether groups. For example, thinned starches provide reduced viscosity for food applications which utilize high starch content. They are produced by partial depolymerisation of the starch molecule through acid hydrolysis or oxidation. Stabilized starch, on the other hand, is typically produced by cross-linking or substitution.

Starch cross-linking is employed to strengthen the structure of the swollen granule upon gelatinization, thus maintaining viscosity during extreme processing conditions. Cross-linking reactions utilize bi- or multifunctional reagents, such as phosphoryl chloride ($POCl_3$) or sodium trimetaphosphate (STMP), capable of forming both intramolecular and intermolecular cross-links between adjacent starch chains [37]. Such cross-linked distarch esters are more resistant to low pH, high temperatures and high shear stress. They are used in highly-processed foods, where they protect the product's texture.

Stabilized starches are also produced in a substitution process by conversion of the hydroxyl groups of the starch molecule into large ester or ether groups with monofunctional reagents [38]. As a result, inter-chain associations are blocked, resulting in more stable pastes and gels. Substituted starches improve shelf-life, freeze-thaw stability and cold storage stability. Monostarch esters include starch acetates, phosphates and succinates. Monostarch ethers provide additional stability to acids and bases, and include hydroxyethyl, hydroxypropyl, and carboxymethyl starches [37], [34].

In some cases, no single type of modification is sufficient to impart the starch molecule with all the properties required for a specific application. Therefore, starches are frequently subjected to a combination of chemical and physical modification processes [34].

Trends for increased fibre consumption have pushed the development of resistant starch (RS) as a health ingredient. Resistant starch undergoes restricted digestion in the small intestine and passes into the colon where it is metabolized by the colon micro-flora. The products are short-chain fatty acids, which are absorbed through the epithelium and are perceived to have multiple health benefits [39]. These include prevention of colon cancer, hypoglycaemic effects, inhibition of fat accumulation, etc. There are four different types of RS presented in table 3.5 below. Generally, changes in the chemical structure of starch (e.g. chemical modification) or changes which make the starch molecule/granule more crystalline tend to reduce the rate of starch digestion [40], [41].

Table 3.5: Classification of resistant starches and corresponding examples

Type	Description	Example
I	Physically inaccessible starch	partially milled seed and legumes
II	Native starch granules, ungelatinized starch	Uncooked potato starch
III	Non-granular retrograded or crystalline starch	Stale bread
IV	Chemical modification with novel bond types	Acetylation, oxidation, etc.

3.4.2 Fat replacers

Fats as food components are responsible for many of the appealing sensory qualities of food: they contribute to creaminess, richness of texture, perception of mouth-feel and aroma, flavour development, palatability, and the feeling of satiety on consumption [22]. At the same time excessive fat intake is associated with higher risk of obesity and coronary heart disease. Many health-conscious consumers are therefore interested in products that contain fat replacers and allow them to follow a low-fat diet without sacrificing taste. Ideally, a fat substitute should be able to mimic the sensory and rheological properties of lipids and at the same time provide fewer metabolic calories [42].

According to their chemical structure, fat replacers can be categorized into two groups – fat substitutes and fat mimetics. The fat substitutes are lipids, derived from conventional triglycerides, which have been structurally modified through a variety of chemical and enzymatic methods, resulting in limited bioavailability. Many fat substitutes are also stable on heating and frying [5]. Table 3.6 presents an overview of typical lipid-based fat replacers.

Table 3.6: Common fat substitutes

Generic or brand name	Composition	Approved for food use
Olestra/Olean	Sucrose polyester of 6 to 8 fatty acids	US only
Sucrose fatty acid esters	Sucrose ester with 1 to 3 fatty acids	US only
Trehalose, raffinose polyester	Carbohydrate polyester with fatty acids	no
Sorbestrin	Polyol polyester with fatty acids	no
Caprenin	C8:0, C10:0, C22:0 fatty acids	yes
MCT	C6 – C10 fatty acids	yes
SALATRIM/Benefat	C2:0–C4:0, C18:0 fatty acids	yes

A well-known modified lipid is the sucrose polyester Olestra® (Procter & Gamble, US). Sucrose polyesters are a mixture of hexa-, hepta- and octa-esters of common sugar with fatty acids of chain length C12 or higher. They have been approved for human use by the FDA since 1996. The appearance, taste and consistency of sucrose polyesters is identical to vegetable oil and it can replace up to 25 % of oil in cooked food and 100 % of oil in fried snacks (potato chips, crackers, etc.). Olestra is not metabolized and not absorbed in the small intestine, which makes it a non-caloric fat substitute [22]. In any case, it is not yet approved for food use in Europe.

Esterifying sucrose with fewer fatty acids (1 to 3), results in molecules which can be digested in the small intestine and are caloric. Sucrose fatty acid esters have hydrophilic and hydrophobic regions in their structure, which enables them to act as surfactants and emulsifiers. They are approved for food use in the US. Esters of fatty acids with other carbohydrates or polyols lead to similar molecules with

emulsifying properties, which can act as fat replacers. Examples include sorbitol, trehalose and raffinose polyesters, which are still under development, and currently not approved for human food use.

Structured lipids, another type of fat substitute, are derived from conventional triglycerides by random transesterification with different short-chain, medium-chain, and long-chain fatty acids. Structured triglycerides reduce the amount of fat available for metabolism, due to their incomplete absorption in the small intestine and the different metabolic pathway of shorter chain fatty acids in the organism [(43)].

Structured lipids are approved for use in the USA, while in the EU only medium-chain triglycerides "MCT" and SALATRIM are allowed. Famous brand names of structured lipids in the USA include Caprenin® (Procter & Gamble, withdrawn from market) and Salatrim (trade name Benefat™, approved by the FDA). Salatrim is also approved in the EU since 2003, as the only modified lipid with "novel food" status [(44)]. Structured triglycerides contain typically at least one long-chain fatty acid (e.g. stearic acid C18:0), and at least one very short chain fatty acid (C2:0, C3:0, or C4:0), randomly attached to the glycerol molecule (see fig. 3.9 below).

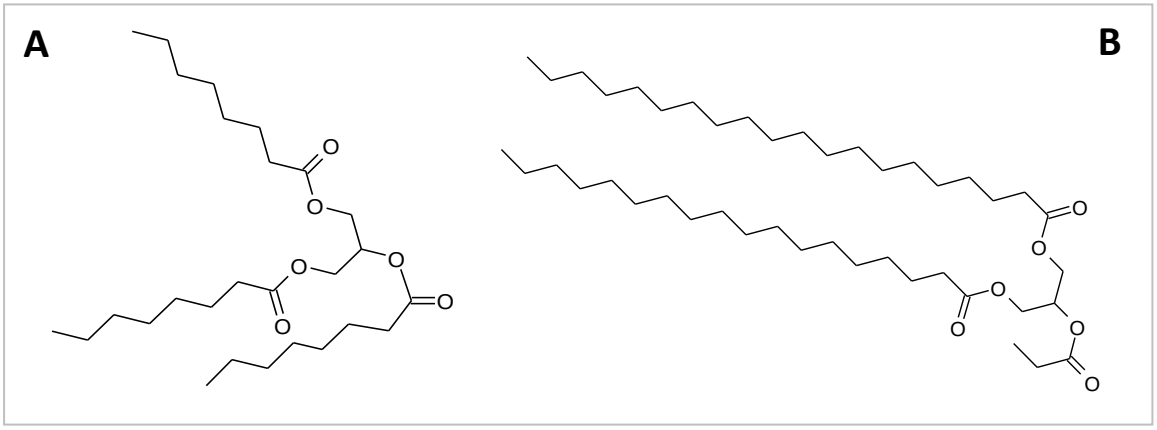

Figure 3.9: Chemical structures of two fat substitutes. A = MCT with three C8 fatty acids and B = Salatrim

The fat mimetics are fat replacers derived from carbohydrates and proteins, which have undergone physical or chemical treatment, enabling them to mimic the organoleptic properties of fat. They offer fewer calories than fats and reduce energy content further by binding high amounts of water. This large excess of water and the fact that mimetics do not carry lipophilic aromas makes it necessary to formulate them into foods together with thickeners, flavours, and emulsifiers. Usually, they are not suitable for cooking, as they lose large amounts of moisture on heating and tend to denature or caramelize at high temperatures [(42)].

Protein-based fat replacers are derived from a variety of protein sources (whey, gluten, soy, etc.) and are produced in a microparticulation process. In this method, the substrate is subjected to moderate

thermal processing, which causes the protein to coagulate into a gel structure, while at the same time applying high shear rates [42], [45]. Normally, heat-coagulated proteins form large particles of gel, perceived as rough or sandy in the mouth. In the microparticulation process, however, the high shear rates reduce the coagulated protein into round, compressible particles of 0.1 – 2.0 µm in diameter, which cannot be distinguished as individual particles by the human tongue [22]. Instead, they are perceived as a creamy, smooth fluid, thus successfully mimicking the mouth-feel of o/w emulsions.

Due to the possibility of thermal denaturation, the use of such protein-based fat mimetics is limited to foods which do not undergo cooking and high temperature treatment (e.g. salad dressings, margarines, frozen desserts, dairy products, etc.). An example of a successfully marketed fat mimetic ingredient is the product Simplesse® (CP Kelco, US), which is a microparticulated whey protein concentrate [46]. Fig. 3.10 below presents a basic overview of the Simplesse microparticulation.

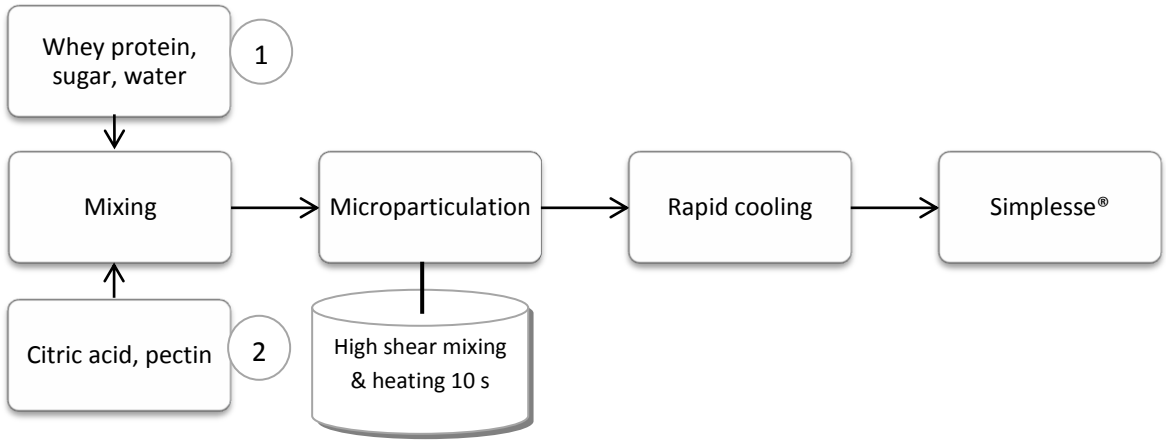

Figure 3.10: A simplified flow-chart of the Simplesse® production process

Carbohydrates can also be used to replace fats, primarily due to their abilities to form gels and to reduce the viscosity of the aqueous phase, which can imitate some of the organoleptic properties of fats. Desired functional and sensory qualities are achieved by using modified starches (acid/enzyme treatment, oxidation, cross-linking, etc.), gums (carrageenan, pectin, guar gum, xanthan, etc.), microcrystalline cellulose, and maltodextrin. Fat replacers of the carbohydrate kind also include polydextrose and other novel dietary fibres, which impart food with smoothness, moistness and density [22], [42].

The type of carbohydrate-based fat mimetic employed in a food system can have a profound influence on the flavour profile of the final product. Starches and cellulose tend to decrease the intensity of the flavour, while contributing some flavour of their own. Gums on the other hand, are used in very low amounts and generally do not tend to mask the taste and flavour of the food system. Most carbohydrate-based ingredients have GRAS status and are approved for food use internationally [5].

3.4.3 Health ingredients: functional carbohydrates

Carbohydrates fulfil various physiological functions in the human organism including serving as a major source of energy, maintaining body temperature, assistance in fat metabolism and acting as a fermentation substrate in the large intestine. In recent years, various novel dietary carbohydrates have been introduced in food applications, especially as ingredients promoting digestive health. Functional ingredients in this category include the non-digestible oligosaccharides added to foods as prebiotics and a diverse group of dietary fibre preparations [47].

In general, dietary fibre is a broad term for plant food components which are not digested by human digestive enzymes. It includes two subclasses – the insoluble and the soluble dietary fibre. Insoluble fibre is the predominant form and includes components of plant cell walls such as cellulose, hemicellulose and lignin. This type of fibre promotes regularity and may reduce the risk of colon/rectal cancer. Soluble fibre is less common and consists of non-cellulosic polysaccharides such as gums and pectins [48]. Soluble fibre is fermented by the gut flora into short-chain fatty acids which are associated with many positive health effects, such as decreased cholesterol levels, enhanced immune function, and improved insulin response [49].

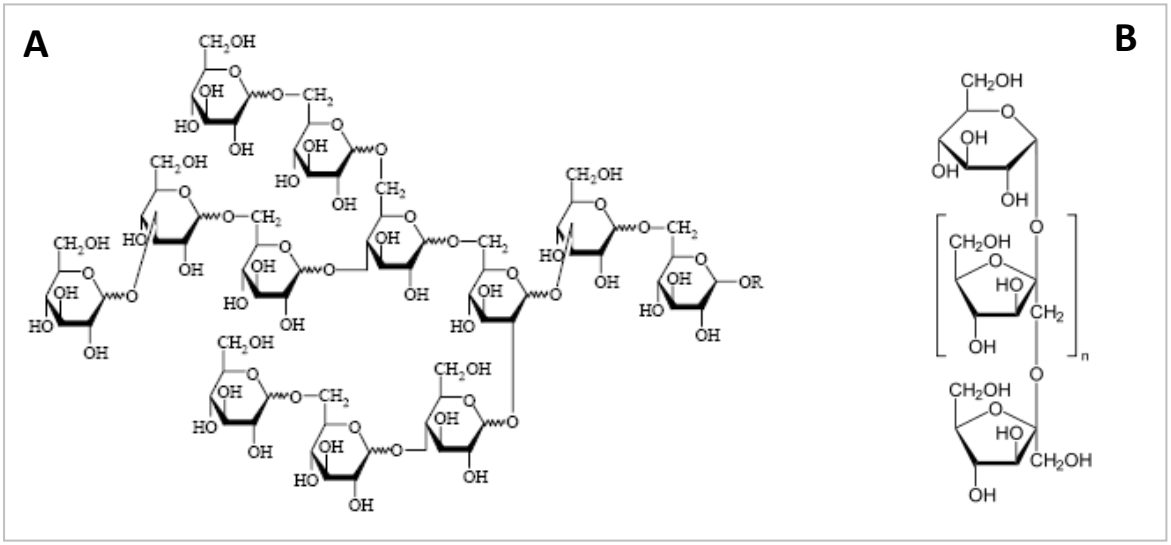

Fig. 3.11: Chemical structures of popular functional carbohydrates; A = polydextrose - a highly-branched, random-bonded polymer of D-glucose (R = H or sorbitol); B = inulin – a fructan with β-(2-1) linkages and a terminal glucose unit

An increased consumption of dietary fibre is believed to be protective against "civilization" diseases such as constipation, colon cancer, gallstones, etc. Since intensive food processing often decreases the dietary fibre content, it has become popular to add purified soluble fibre to foods [50]. There are currently five main types of commercially available dietary fibres from the soluble category: pectin, gums, oat and barley fibre (β-glucan), inulin and polydextrose (structures shown in fig. 3.11 above). Resistant starch can also be included in this group, as it is a type of starch which is fermented in the large intestine and can serve as a type of dietary fibre (see hydrocolloids and starches, chapter 3.4.1).

Apart from the dietary fibres, the probiotics (living microorganisms of *Bifidobacteria*, *Lactobacilli* genera) and the prebiotics are the two other functional ingredients to be found in the gut-health food market. The human gastrointestinal tract contains a complex microbial system comprising more than 400 different bacterial species. The intestinal micro-flora is composed of species believed to benefit the host and others that are potentially pathogenic. The balance of the intestinal ecosystem may be affected by ageing, stress, medication and diet [(51)].

Beneficial endogenous bacteria might be stimulated by providing them with a competitive advantage over other species present in the colonic ecosystem. This can be achieved by prebiotics, which serve as energy sources for selected bacteria [(52)]. Prebiotics are defined as non-digestible food ingredients, which stimulate growth and/or activity of beneficial colon bacteria.

The prebiotics include non-digestible dietary carbohydrates, especially oligosaccharides. They contain fructose (inulin and oligofructose) or galactose (transgalactosylated oligosaccharides and lactulose) and are also called bifidogenic, due to their ability to modify the bacterial composition of the intestinal flora in favour of this genus. In addition to their effect on the gut flora, prebiotics may increase calcium absorption and show dietary fibre properties. Furthermore, prebiotics show interesting technological functionalities such as moisture retention, sugar replacement, fat replacement, and gelling properties [(53)]. Table 3.7 below presents prebiotic ingredients currently available on the market [(50)].

Table 3.7: Overview of commercial prebiotics; G = glucosyl, F = fructosyl, Gal = galactosyl unit, n = number of units

Prebiotic	Chemical structure	Glycosidic bond	DP
Inulin	GF_n	β (2-1)	3 - 60
Oligofructose	GF_n	β (2-1)	2 - 7
Transgalactosylated oligosaccharides	$GGal_n$	β (1-6)	3 - 6
Lactulose	GalF	β (1-4)	2
Soy-bean oligosaccharides	Gal_nGF	α (1-2) (1-6)	3 - 4

Inulin and oligofructose are to date the most thoroughly studied prebiotics, both from nutritional and technological perspective. They are also the only ones widely available for commercial food applications in Europe [(54)]. Nevertheless, the high-value digestive health market is stimulating research in alternative prebiotics (resistant starch, isomalto-oligosaccharides, lactosucrose and xylo-oligosaccharides). Among the most novel ingredients studied for their prebiotic effect are tagatose, pectin, dextrins and larch arabinogalactan.

4. Experimental investigations

4.1 General setup of the model washing system

The stain-forming potential of the target substance was evaluated on the basis of its enzymatic digestibility. For this aim, the target substance was digested with an appropriate enzyme within a model system, resembling washing conditions. The model system consisted of an aqueous buffer (0.1 M phosphate buffer, pH 8), a temperature of 40 °C and mechanical agitation at 150 rpm. Reactions were usually carried out in a 40 ml reaction vessel equipped with a thermostatic mantle and a stirrer. The digestion was started by addition of the enzyme and allowed to proceed for a time frame of 60 - 80 min, as this mimics a typical laundry cycle. Samples were withdrawn at regular intervals from the reaction mixture and the digestion products were analyzed with suitable methods.

The amount of enzyme necessary can be calculated by the dilution resulting when a dose of laundry detergent is added to the total washing liquor. In this work, a total liquor volume of 15 l and a detergent dose of 75 ml are assumed [(2)], resulting in a dilution factor of 200 for each enzyme during washing. The working dilutions for each detergent enzyme in % (w/v) are displayed in the table below. All detergent enzymes used were kindly provided by Novozymes A/S, Denmark.

Table 4.1: Detergent enzymes used in this work and their dilutions

Detergent enzyme	Type	E.C. number	Laundry detergent conc. [%]	Washing liquor conc. [%]
Esperase 8 L	protease	3.4.21.62	1	0.005
Stainzyme 12T	α-amylase	3.2.1.1	0.44	0.002
Lipolase 100	lipase	3.1.1.3	0.2	0.001

Whenever possible, a reference substance was used in a parallel control experiment, as a comparison to the enzymatic breakdown of the target substance. Reference substances are classical ingredients with a history of long use. They often represent an unmodified form of the substrate. For example, native waxy maize starch is a reference substance to modified waxy maize starch.

Sometimes, it was not possible to use a detergent enzyme for analysis, since the target substance was not an appropriate substrate for any of the currently available detergent enzymes. If this was found to be the case, the study of the enzymatic digestibility was carried out with an appropriate enzyme, under optimal conditions for the enzyme under investigation (see fig. 4.1 on the next page).

To conduct an evaluation of the stain-forming potential of the target substance, further methods, such as empirical testing of the ingredient as a component of a true stain, were required.

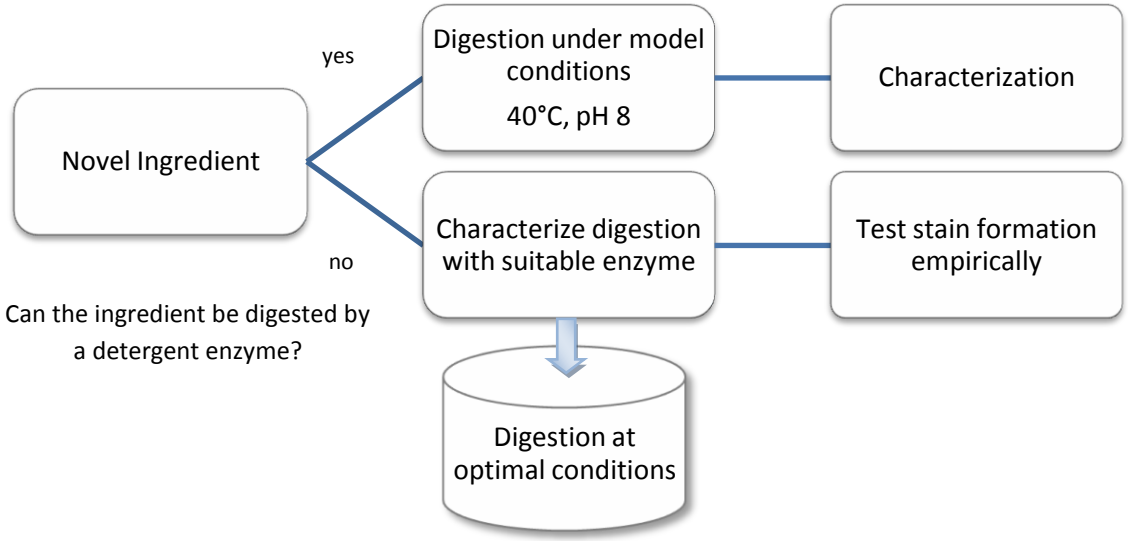

Figure 4.1: Possible experimental paths in the investigation of the stain-forming potential of a novel ingredient

4.2 Hydrocolloids & starches

4.2.1 HPLC methods

The target substances from the "hydrocolloid & starches" group are large polysaccharides. In order to characterize their enzymatic digestion, it was necessary to employ suitable chromatographic methods for separation and quantification of their hydrolysates. Thus, a method for the analysis of molecular weight distribution during enzymatic breakdown was implemented, based on size-exclusion-chromatography (SEC). The final products of hydrolysis expected with the model washing system are oligosaccharides. A high-performance-liquid-chromatography (HPLC) method for oligosaccharide analysis was developed. An overview of the methods is presented in table 4.2 below.

Table 4.2: HPLC methods used in the analysis of polysaccharide digestion

Method	Column	Supplier	Column parameters	Matrix
Oligosaccharide analysis	Polyspher® CH NA	Merck	300 x 7.8; 9 µm	Resin-based
Size-exclusion	BioSep-SEC-S 4000	Phenomenex	300 x 8; 5 µm; 500 Å	Silica-based

The HPLC system used in all experiments consisted of a binary pump Agilent 1200 (Agilent Technologies), a Triathlon autosampler (Spark), a column oven, a Corona CAD detector (ESA Biosciences), and a LaChrom L-7490 RI detector (Merck).

4.2.1.1 Oligosaccharide analysis with Polyspher CH NA

The goal of this chromatographic separation was to develop a simple method for the analysis of carbohydrate hydrolysates, without the need of derivatization or precipitation steps. The Polyspher CH NA column was chosen for this application, because it is optimized for separations of small, water-soluble compounds [55]. Further advantages of this column include the fact that large polymers (undigested polysaccharides) do not bind irreversibly to the matrix and that a simple isocratic elution is possible.

To optimize the separation, a mixture of maltooligosaccharide standards (Supelco) of DP 3-7, as well as maltose and glucose was prepared. This standard mix was injected into the HPLC system and fresh, deionized water was used for the isocratic elution. The major parameters varied for optimization of the separation were flow rate and temperature. Fig. 4.2 below shows the effect of temperature on the separation of the maltooligosaccharide mixture.

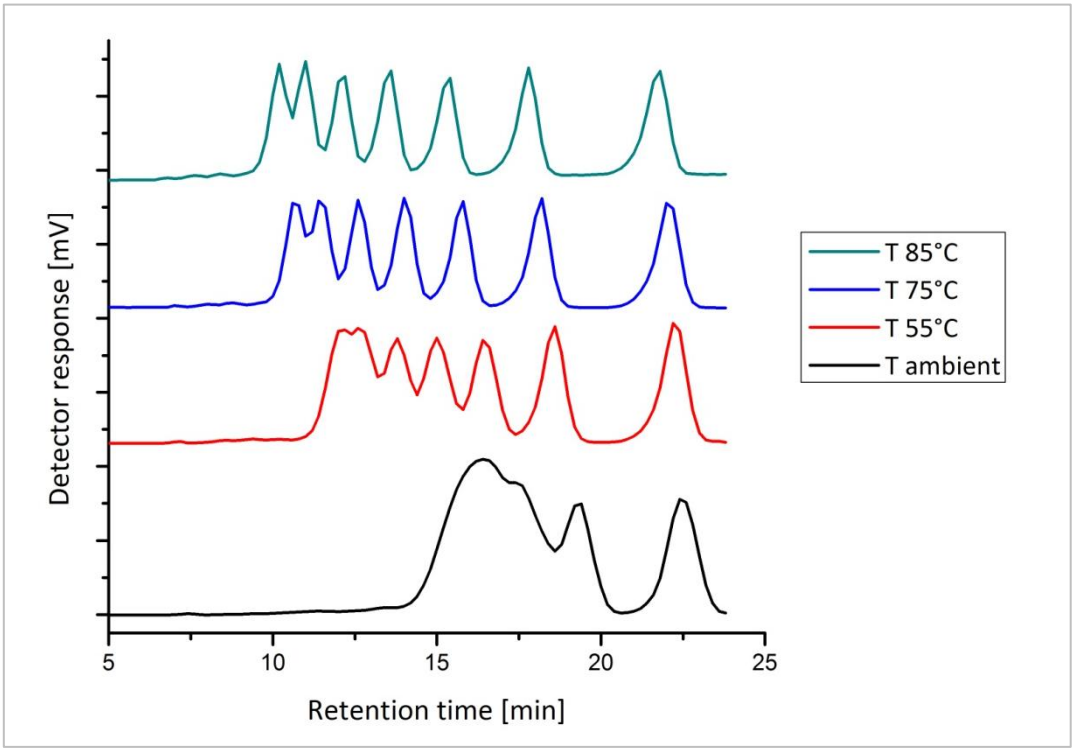

Fig. 4.2: Chromatograms of the standard mixture at different column temperatures. The column temperature increases from the bottom to the top: ambient temperature, 55 °C, 75 °C and 85 °C. Injection volume: 20 µl, flow rate: 0.4 ml/min, standard mix: 5 mg/ml, detection: CAD

As can be seen from fig. 4.2 resolution greatly improves with increasing column temperature. Resolution is defined according to equation 1 (eq. 1) as the ratio of the distance between the retention times t_R of peaks a and b, and the arithmetic mean of the two peak widths, $w_{0.5}$.

$$R = 1.18 \times \frac{t_{Rb} - t_{Ra}}{w_{0.5a} + w_{0.5b}} \qquad \text{(Eq. 1)}$$

At ambient conditions, no effective separation takes place with most peaks overlaying. As the temperature is raised to 55 °C, some peaks can be differentiated from the baseline. The resolution of the first two analytes, which are oligosaccharides with DP 7 and DP 6, is R = 0.46 and therefore still very poor. Additional rise of the temperature to 85 °C improves the resolution of this pair to R = 0.85 with all the remaining peaks exhibiting R-values > 1, indicating that the separation of these oligosaccharides is efficient enough for quantitative analysis. The resolution of the first pair cannot be further improved under these conditions, as higher temperatures lead to deterioration of the column material.

Increasing the column temperature allows a deeper penetration of the sugars into the resin. As a result, mass transfer is enhanced and column efficiency increases. Improved mass transfer is also the reason why slower flow rates improve resolution, as can be seen from fig. 4.3 below.

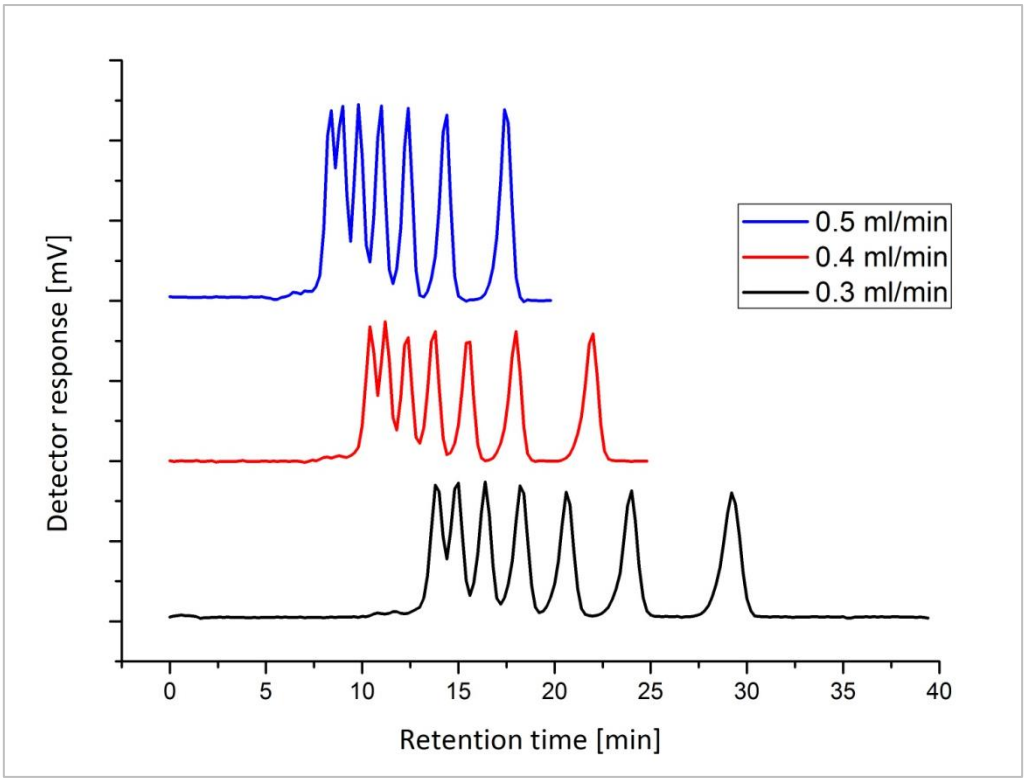

Fig. 4.3: Chromatograms of the standard mixture at different flow rates. The flow rate increases from the bottom to the top. Injection volume: 20 µl, column temperature: 85 °C, standard mix: 5 mg/ml, detection: CAD

Faster flow rates decrease retention time, increase peak height, but lead to poorer efficiency. The chromatogram at 0.3 ml/min flow rate exhibits a fine resolution between all peaks, with resolution of the first two analytes, DP 7 and DP 6, improving to R = 0.96. A further decrease in flow rate was not feasible, not only due to the resulting long analysis times, but also because the CAD requires flow rates above 0.2 ml/min for proper function [56]. As a compromise between measurement time and acceptable resolution, all measurements were consequently performed with a flow rate of 0.4 ml/min. The peak resolution between maltoheptaose and maltohexaose is still acceptable at R = 0.92. The remaining peak pairs exhibit R-values of R > 1.

The Polyspher CH NA column contains a polystyrene-divinylbenzene ion exchange resin. The separation mechanism is based on size-exclusion and ligand-exchange [57]. Oligosaccharides are separated according to size. Smaller sugars can pass into the pores of the cross-linked resin and are retained for longer time periods. Larger oligosaccharides are not retained by the resin as much and elute first. In the case of monosaccharides, ligand exchange is the primary mechanism of separation. This involves the binding of the hydroxyl groups of the sugar to the fixed counterion of the resin. The ligand exchange is affected by the nature of the counterion and by the spatial orientation of the hydroxyl groups. The Polyspher CH NA column has sodium as counterion and can be used for separations of both monosaccharides and oligosaccharides. The specified order of elution can be seen on a chromatogram of the optimized method (fig. 4.4 below), showing the separation of a potato starch hydrolysate.

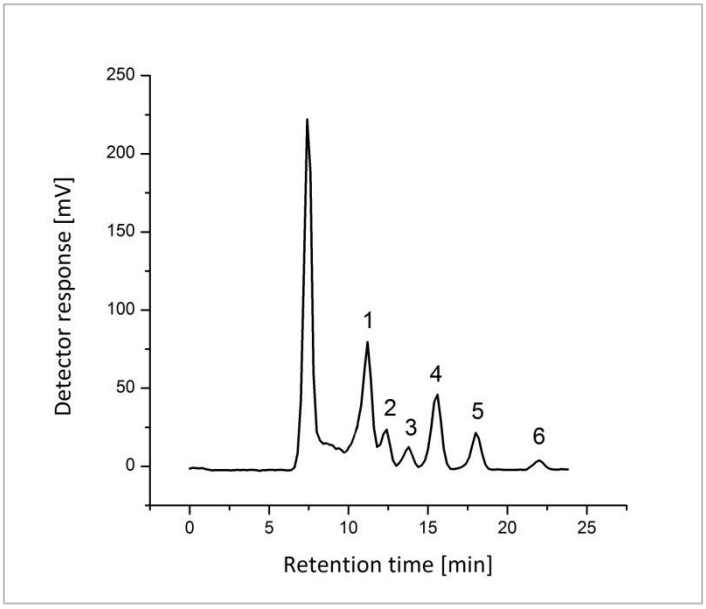

Fig. 4.4: Chromatogram of potato starch after 80 min of enzymatic digestion. Peaks: 1) maltohexaose, 2) maltopentaose, 3) maltotetraose, 4) maltotriose, 5) maltose, 6) glucose. The large peak at 7.5 min is the void volume, containing molecules too large to interact with the resin. Flow rate: 0.4 ml/min, column temperature: 85 °C, injection volume: 20 µl

4.2.1.2 Molecular weight distribution with BioSep-SEC-S 4000

Another important aspect for characterization of the enzymatic digestion was obtaining information on the distribution of average molecular weights resulting from the hydrolysis. For this purpose, a HPLC method based on size-exclusion was implemented on a BioSep-SEC-S 4000 column. This column has a wide fractionation range (5 - 1000 kDa) and is especially suited for the analysis of large molecules due to its large pore size (500 Å). Originally designed for protein applications, the column can also be used for carbohydrate analysis with the use of appropriate standards [58], [59].

To test the performance of the column, dextran standards of defined molecular weight (Fluka) were selected: 12 kDa, 50 kDa, 80 kDa, 150 kDa and 670 kDa. Size-exclusion chromatography separates molecules according to size, but can be used to form an empirical relationship to molecular weight if the standards used have molecular properties similar to the analyte. Dextran is a branched glucan and was chosen to serve as a standard in the development of this method. To calibrate the column, the dextran standards were injected separately. The elution volume (V_e) of each dextran was determined from triplicate measurements at the time at which its absorbance peak was at its maximum. The distribution coefficient (K_d) for each standard was then calculated according to eq. 2, where V_e is elution volume (t_R x flow rate), V_o is void volume, and V_t is the total column volume.

$$K_d = \frac{(V_e - V_o)}{(V_t - V_o)} \qquad \text{(Eq. 2)}$$

A calibration line for the estimation of molecular weight with the BioSep-SEC-S 4000 column was prepared by plotting the K_d values against the log molecular weight (M_w). The line, shown on fig. 4.5 was used in estimating the molecular weight distribution of polysaccharide digests. The V_o was determined as 5.5 ml from the elution peak of blue dextran (M_w = 2000 kDa). V_t was determined as 12.1 ml from the elution peak of glucose.

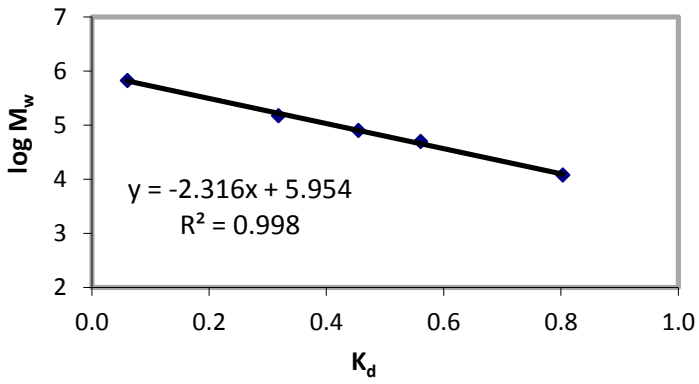

Figure 4.5: Plot of log M_w against K_d for the dextran standards on the BioSep-SEC-S 4000 column

4.2.2 Materials and methods – starches

The starches under investigation, a selection of modified and resistant starches along with their respective reference substances, are presented in table 4.3 below. The tapioca and waxy maize starches were kindly provided by Cargill Inc., US. Eliane™ starch was a gift from Avebe, Netherlands. And the native potato starch is a product of Emsland Stärke GmbH, Germany.

Table 4.3: Overview of the starches tested; native starches serve as the reference substance

Starch product	Description	Functionality
C*ActiStar 11700	Tapioca maltodextrin	Resistant starch, RS3
C*CreamGel 70001	Tapioca native starch	Multifunctional texture agent
C*PolarTex 06719	Waxy maize low cross-linked	Modified starch, E 1442
C*PolarTex 06716	Waxy maize high cross-linked	Modified starch, E 1442
C*Gel 04201	Waxy maize native starch	Multifunctional texture agent
Eliane	Potato waxy starch	Modified starch
Potato starch	Potato native starch	Multifunctional texture agent

Enzymatic hydrolysis

A 0.75 % w/v solution of maize, tapioca or potato starch was prepared in the working buffer of the model washing system (0.1 M phosphate buffer, pH 8). The starch was gelatinized by boiling for 5 min. After cooling, 20 ml of the gelatinized starch suspension were transferred into the reaction vessel. The temperature was adjusted to 40 °C. The enzymatic hydrolysis was started by addition of 10 ml Stainzyme solution [200 AU/g starch]. The concentration of enzyme in the reaction was 22.3 mg/l, corresponding to a dilution of 0.002 % w/v with a protein content of 2.47 % w/w as determined by the Bradford method (see 7.1.3 Bradford assay on page 84).

Samples were withdrawn at regular intervals and the reaction stopped by heating at 99 °C for 15 min. The reaction was characterized by determining the % hydrolysis and by chromatographic methods.

Starch hydrolysis determination

The degree of hydrolysis represents the extent to which the glycosidic linkages in the starch molecule have been hydrolyzed. It is determined according to equation 3 on the next page by measuring the amount of reducing sugar produced by the enzyme, divided by the amount of total sugar in the starch molecule. The release of reducing sugar was assayed by the DNS assay (dinitrosalicylic assay). Maltose sugar served as the standard. The total carbohydrate in starch can be derived after a total

acid hydrolysis of the molecule with subsequent quantification of the sugar produced. For a description of the DNSA and the acid hydrolysis procedure, see appendices 7.1.1 and 7.1.2.

$$\%\ Hydrolysis\ t(x) = \frac{Reducing\ sugar\ t(x)}{Total\ carbohydrate} \times 100 \qquad \text{(Eq. 3)}$$

α-amylase assay

The α-amylase activity was determined by measuring the liberated reducing sugar units from a solution of soluble starch (1 % w/v) in phosphate buffer (0.1 M, pH 8) at 40 °C with the DNSA. One amylase unit (AU) is defined as the amount of enzyme releasing 1 µmol of maltose per minute. The specific activity of the α-amylase Stainzyme was 1777 AU/mg protein.

HPLC analysis

The enzymatic degradation of the starch molecules was characterized with the chromatography methods described at the beginning of this chapter (4.2.1). Size-exclusion chromatography was used to analyze the molecular-weight distribution resulting from the hydrolysis. The profile of oligosaccharides released from the starches was determined on the Polyspher CH NA column. A summary of the method parameters can be found in 7.1.6 HPLC method “oligosaccharide analysis” and 7.1.7 HPLC method “SEC”.

4.2.3 Results and discussion – starches

A first comparison between the target starches and their respective references involved an examination of the degree of total hydrolysis achieved after 80 min reaction time. The results of this investigation are presented in table 4.4 on the next page. The total hydrolysis (in %) is listed below the name of each starch. Under model washing conditions all native (reference) starches were hydrolyzed to ca. 30 %. The modified starch PolarTex 16 and the resistant starch ActiStar showed a lower degree of enzymatic digestion. The waxy potato starch Eliane was hydrolyzed to a similar extent as its reference native potato starch.

The waxy starch Eliane has been produced by plant breeding techniques to contain almost exclusively (ca. 99 %) amylopectin [(60)]. During studies of hydrolysis rate or molecular weight distribution during enzymatic breakdown, no significant differences between Eliane and native

potato starch could be observed. These results will not be shown in this thesis. The "waxy" property of starch does not seem to impact its digestion in a manner very different to a native starch molecule. In contrast, the resistant starch ActiStar, as well as the modified starches PolarTex 16 and PolarTex 19, were digested to a lesser degree than their respective native starches, and will be now discussed in further depth.

Table 4.4: Total hydrolysis in % for each starch; average of a triplicate determination and standard deviation

Source	Target 1	Reference	Target 2
Potato	Eliane	Native	
%	30.8 ± 1.3	30.3 ± 0.8	
Tapioca	ActiStar	CreamGel	
%	16.1 ± 0.9	28.7 ± 0.4	
Waxy maize	PolarTex 16	C*Gel	PolarTex 19
%	23.1 ± 0.8	31.2 ± 1.2	27.6 ± 0.7

ActiStar is a resistant starch; a maltodextrin composed of > 50 % oligosaccharides of chain length 20-35 DP [61]. The property of resistance refers to limited digestibility of this starch with human digestive enzymes. As can be seen from table 4.4, the resistant starch could be digested with bacterial α-amylase. However, the degree of hydrolysis achieved was about half compared to the one of the native tapioca starch CreamGel. This can be seen in further detail from fig. 4.6.

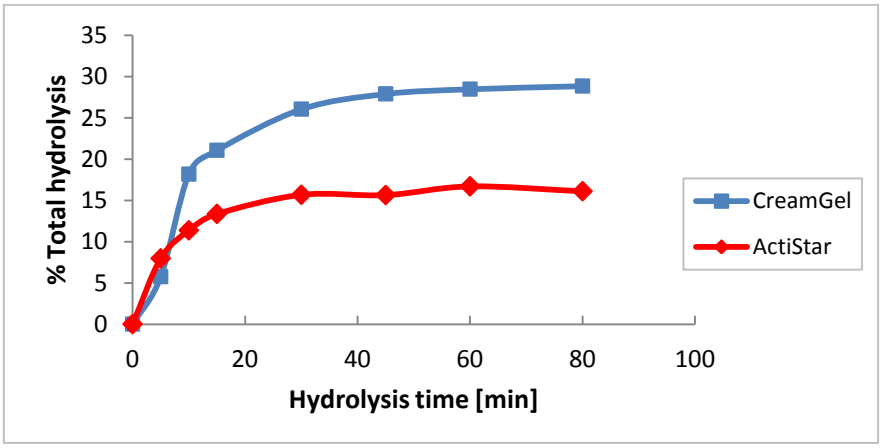

Fig. 4.6: The hydrolysis curves of ActiStar and CreamGel. The points on the graph are averages of triplicate measurements

The enzymatic reaction proceeded swiftly for both starches in the first 5 min of hydrolysis. The digestion of ActiStar started to slow down after 5 min. At 10 min reaction time, ActiStar was digested to 11 %, while the native starch had achieved 18 % hydrolysis. The enzymatic breakdown of ActiStar levelled off at 30 min; CreamGel reached a plateau after 45 min of hydrolysis.

To calculate the different hydrolysis rates, the release of reducing sugars over the course of hydrolysis was examined (fig. 4.7). The maltodextrin with its shorter oligosaccharide chains has, based on the same weight, a higher amount of free reducing ends. Therefore, it showed a high reducing power at the beginning of hydrolysis (t0). The hydrolysis rate for ActiStar, determined by the slope in the first 10 min of hydrolysis, was 0.302 mM*min^{-1}, while CreamGel displayed a rate of 0.480 mM*min^{-1}. The slower rate of hydrolysis of ActiStar can be explained by the shorter chain length of its oligosaccharides. Bacterial α-amylase hydrolyses longer chains of oligosaccharides preferably. K_m values increase drastically with decreasing molecular mass, leading to a slower digestion rate [62].

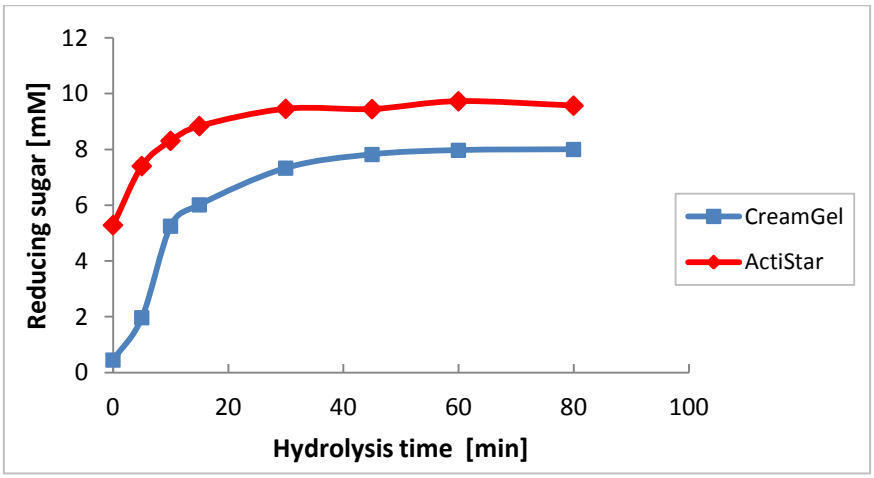

Fig. 4.7: The formation of reducing sugars over the course of enzymatic hydrolysis of the tapioca starches

A further illustration of how the structure of ActiStar impacts its digestion is provided by the results of the SEC analysis (fig. 4.8 on the next page). The average molecular weight distribution of ActiStar before the start of hydrolysis (t0) was 7 kDa. In the first 15 min of the enzymatic reaction, the average molecular weight of the maltodextrin remained constant. In contrast, the native starch CreamGel possessed a large molecular weight of over 1000 kDa at t0, which was quickly degraded down to an average molecular weight of 13 kDa in 5 min. The average molecular weight of the reference starch was continuously reduced by the action of the α-amylase in the first 15 min of hydrolysis.

If the area of the ActiStar peak is being examined, however, it can be shown that despite maintaining a constant average molecular weight, the maltodextrin is being continuously digested by the α-amylase, albeit at a slower rate (see fig. 4.9 on the next page). After 15 min of enzymatic digestion, no signals from any of the tapioca starches could be detected on the BioSep-SEC-S-4000 column. The detection limit of this column is 5 kDa, all molecules with a lower molecular weight co-elute with the elution volume. Both starches were digested to smaller oligosaccharides.

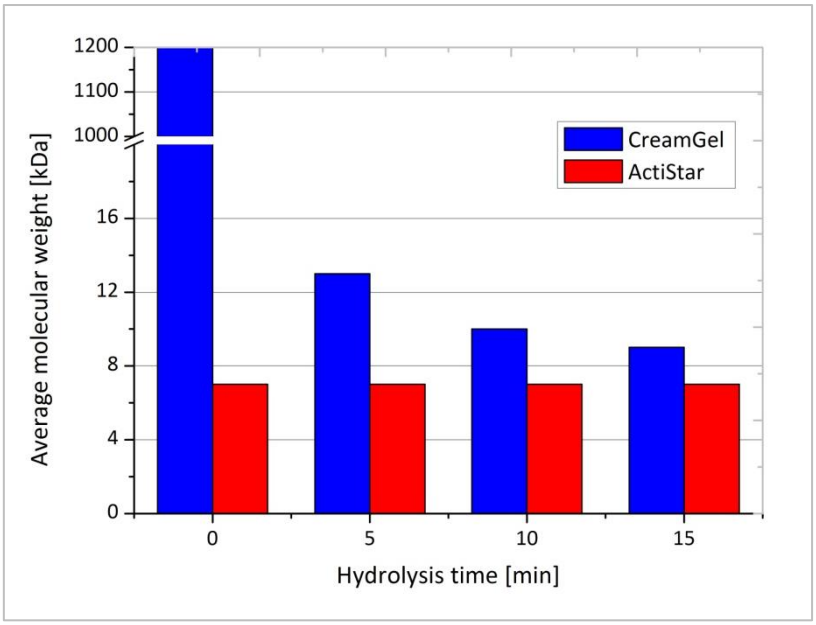

Fig. 4.8: Distribution of the average molecular weight of the tapioca starch molecules during hydrolysis

If the profile of oligosaccharides released from the starches is examined, a common pattern is revealed. In the first 15 min of reaction, oligosaccharides of different chain lengths were quickly released from the starch molecule. The oligosaccharide types produced in the highest amounts include maltohexaose, maltoheptaose and maltotriose. The concentration of maltoheptaose, the largest oligosaccharide under investigation, rose in the first part of the hydrolysis and then dropped at longer reaction times, as maltoheptaose itself became a substrate for α-amylase. This was not observed with the other oligosaccharides since α-amylase displays a lower K_m for long-chain oligosaccharides, which at the given reaction time were present in abundant amounts. This was also the reason why glucose was produced in very small amounts, typically only after 20 -30 min of digestion time.

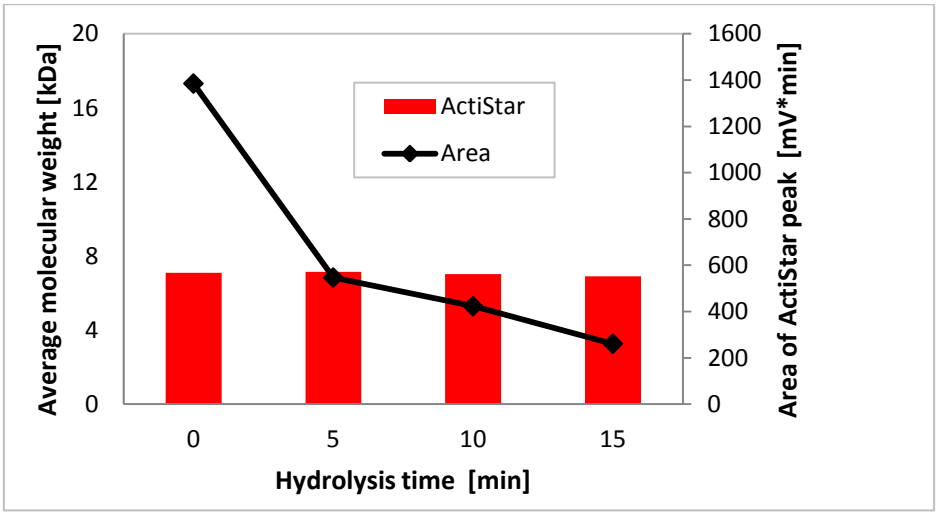

Fig. 4.9: Constant molecular weight, but a decrease of peak area of ActiStar during first 15 min of hydrolysis

This oligosaccharide profile was identical for *all* starches, reflecting the specificity of the bacterial α-amylase Stainzyme. In this work, the graph of CreamGel is shown in fig. 4.10 below, as an example for all target and reference starches under analysis.

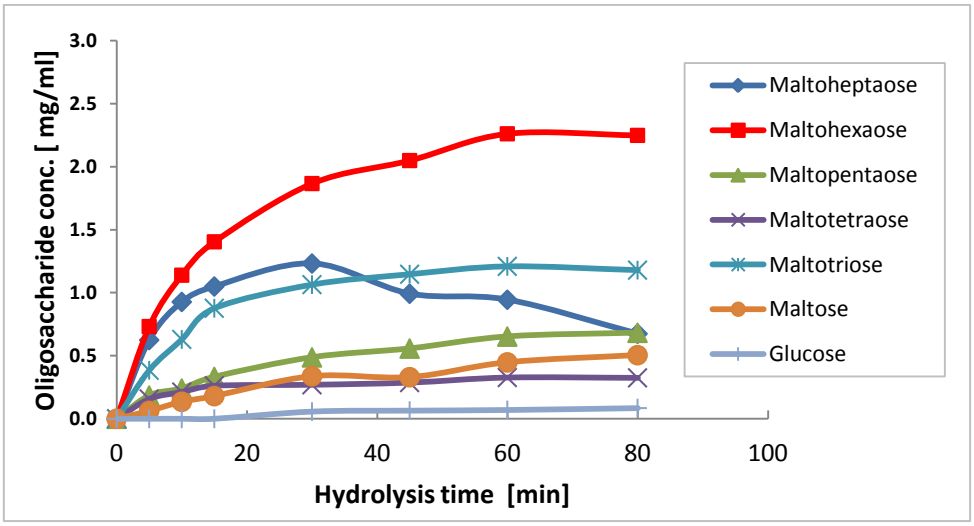

Fig. 4.10: The oligosaccharide profile released by Stainzyme from CreamGel, a native tapioca starch

The differences between target and reference starch is not based on the mechanism of enzymatic digestion, which in all cases follows a similar path, but on the speed of reaction. The divergence between resistant starch and native starch was therefore to be found not in the profile or ratio of oligosaccharides produced, but in the concentrations of oligosaccharides liberated by the enzyme (fig. 4.11).

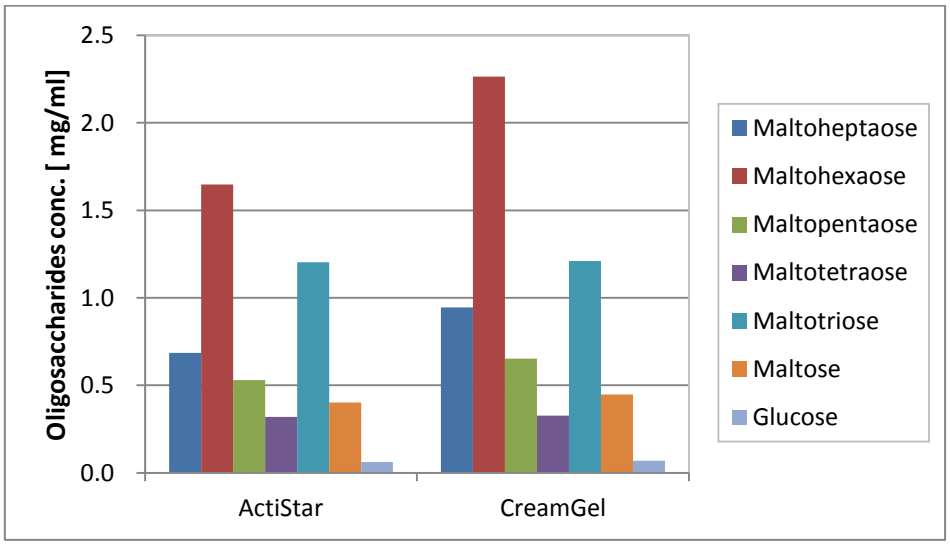

Fig. 4.11: The oligosaccharide profile of ActiStar and CreamGel at t = 60 min of hydrolysis

Based on the same weight, the resistant starch ActiStar generated oligosaccharides of the same type and ratio as CreamGel, however in lower concentration, as fig. 4.11 shows. The difference in

oligosaccharide amount was especially pronounced for the long-chained oligosaccharides with DP7, DP6 and DP5.

The slower enzymatic digestion of the resistant starch ActiStar is based on the higher K_m of bacterial α-amylase for short-chain oligosaccharides. A different explanation must be sought to explain why the modified waxy maize starches PolarTex 16 and PolarTex 19 were digested to a lesser degree in comparison to their native reference form C*Gel. All three starches of the waxy maize type possess large molecular weights of over 1000 kDa. A difference in the enzymatic digestion must therefore be related to the modification of the starch backbone. Indeed, the degree of cross-linking seems to be related to the total hydrolysis achieved under the model washing conditions. PolarTex 16, which is a highly cross-linked starch, was hydrolyzed to a lesser extent (23.1 %) in comparison to its low cross-linked derivative PolarTex 19 (27.6 %) or its native form C*Gel (31.2 %).

The enzymatic digestion displayed a typical course shown in fig 4.12. The reaction proceeded swiftly in the first 5 min of hydrolysis. Differences in digestion were already apparent at this time with C*Gel showing 14 % total hydrolysis, while PolarTex 16 and PolarTex 19 displayed 9 % and 11 % hydrolysis respectively. After 10 min the enzymatic breakdown started to slow down continuously, finally levelling off after 45 min reaction time. The hydrolysis rate, calculated from the release of reducing sugars over time (figure not shown), stood at 0.352 mM*min^{-1} for PolarTex 19, 0.336 mM*min^{-1} for PolarTex 16 and 0.503 mM*min^{-1} for C*Gel. The rate was calculated from the slope of the graph in the first 10 min of reaction.

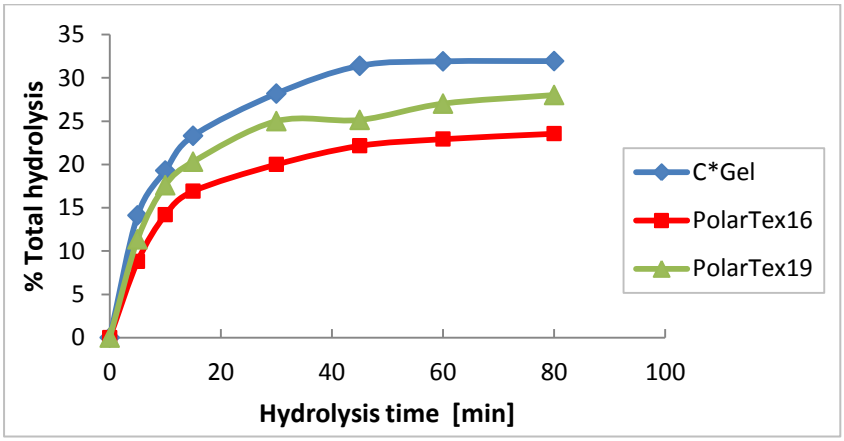

Fig. 4.12: Hydrolysis curves of the waxy maize starches, native and cross-linked forms

The slower digestion of the modified starches is also confirmed by the SEC analysis (see fig. 4.13). At the beginning of hydrolysis (t0), all waxy maize starches have molecular weights exceeding 1000 kDa. The starches were progressively broken down to lower molecular weights by the action of α-amylase.

C*Gel was digested to 14 kDa in 5 min and could not be detected after 30 min of hydrolysis. In comparison, the PolarTex starches showed an average molecular weight of 18 kDa in the first 5 min of reaction and displayed a slower decrease in molecular weight throughout the digestion. Signals from the modified starches could still be detected after 60 min, indicating that the PolarTex starches were not broken down to oligosaccharides of 5 kDa or below, as their native form C*Gel was.

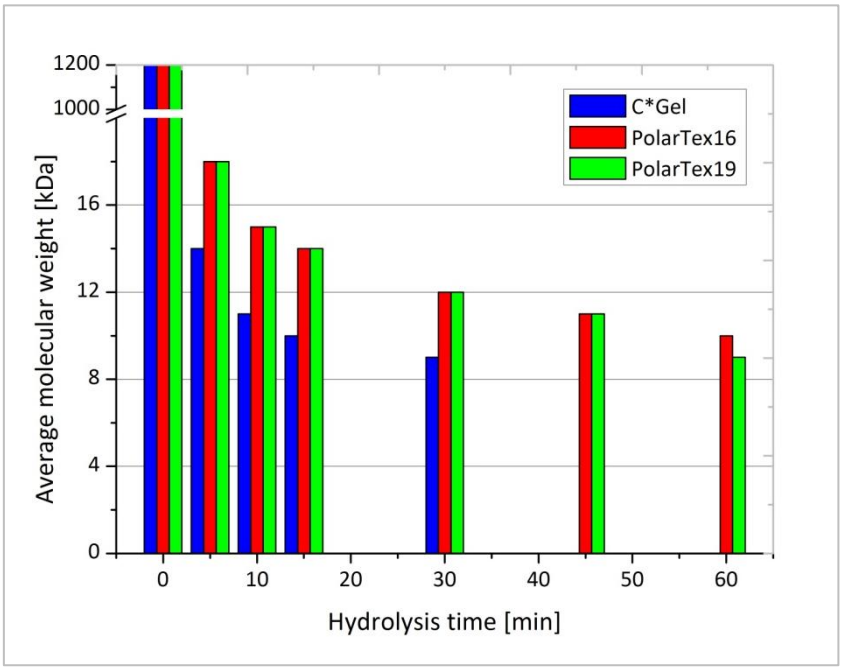

Fig. 4.13: The decrease of average molecular weight of the waxy maize starches during the enzymatic hydrolysis

The lower digestion of the PolarTex starches also resulted in a lower overall production of oligosaccharides. This can be seen in fig. 4.14 on the next page, which shows the oligosaccharide profile of the waxy maize starches after 60 min of hydrolysis. The digestion profiles of the waxy maize starches are very similar to each other, indicating a similar mechanism of enzymatic breakdown. Maltohexaose and other oligosaccharides released from the cross-linked starches are at a lower concentration than the sugars of the native, unmodified form.

The PolarTex starches are dual-modified by cross-linking and substitution with hydroxypropyl groups. It is known from the scientific literature that the modification of starch can impact its digestion. In general, the susceptibility to enzymatic hydrolysis is reduced with increasing cross-linking [(63)]. Other modifications, in particular hydroxypropylation, also lead to lower digestion rates. This phenomenon is caused by the bulky substituent at the C2 of the glucose unit, which sterically hinders the proper positioning of the substrate into the enzyme site. As a result, attacks on adjacent unsubstituted glucose residues are reduced, leading to a lesser ultimate level of starch hydrolysis [(64)].

Studies in the scientific literature have mainly focused on the digestibility of starches with a single modification and are based on the physiological perspective of digestion [65]. In this thesis, dual-modified starch was analyzed under model washing conditions. It could be shown that the PolarTex starches are digested to a lesser degree than their native counterpart C*Gel. Furthermore, the degree of cross-linking also played a role, with the highly cross-linked PolarTex 16 displaying a lower hydrolysis level compared to the low cross-linked PolarTex 19 or the native starch C*Gel.

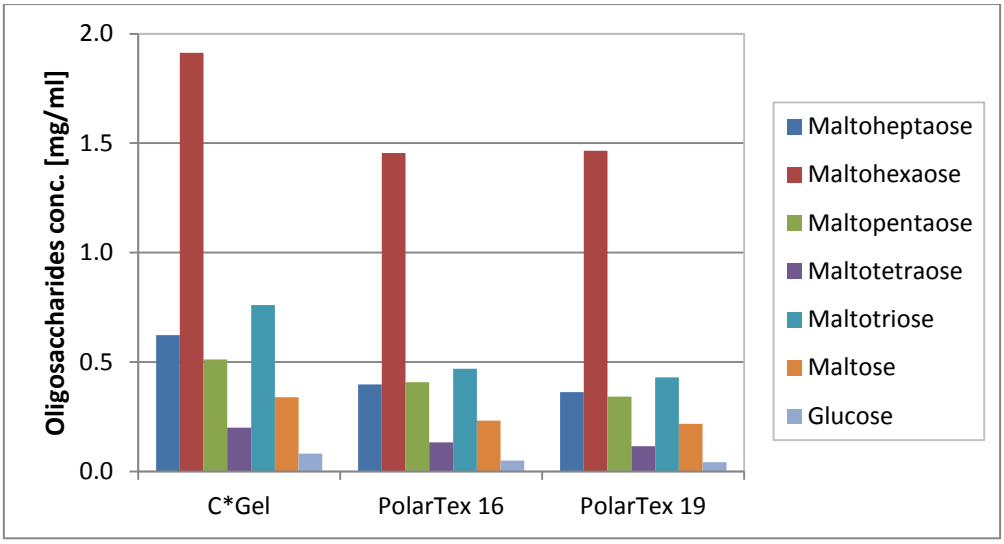

Fig. 4.14: The oligosaccharide profile of the waxy maize starches after 60 min enzymatic hydrolysis with Stainzyme

4.2.4 Summary - starches

Several modern starch ingredients were analyzed under the conditions of the model washing system for an evaluation of their stain-forming potential. The target ingredients under investigation included the resistant starch ActiStar (RS type 3), Eliane (a waxy starch produced by conventional breeding techniques) and two dual-modified starches PolarTex 16 and 19. The digestion of the target starches was compared to the enzymatic hydrolysis of their native forms.

All starches were gelatinized by boiling in the model buffer prior analysis. This was done to mimic the cooking and processing conditions to which starch ingredients are subjected in food systems. It is this "cooked" form of starch, which is likely to be a constituent of stains. Gelatinized starches are also more susceptible to enzymatic hydrolysis due to a destruction of their crystalline structure.

The degree of total hydrolysis obtained for the starches under the conditions of the model washing system ranged from 16 – 30 %. All native starches were digested to ca. 30 %. The target starches were hydrolyzed to a lesser degree, with the exception of Eliane. The waxy potato starch Eliane did not show significant differences in digestion to its native potato form. ActiStar and the PolarTex starches exhibited a slower digestion rate than their reference starches. This was also mirrored in a more gradual decline of molecular weight during enzymatic breakdown. The decreased susceptibility to enzymatic hydrolysis is due to the different molecular structure of the target starches. Based on these observations, a hierarchy of stain-forming potential can be assigned to starch ingredients. A visual arrangement is presented in fig. 4.15 below.

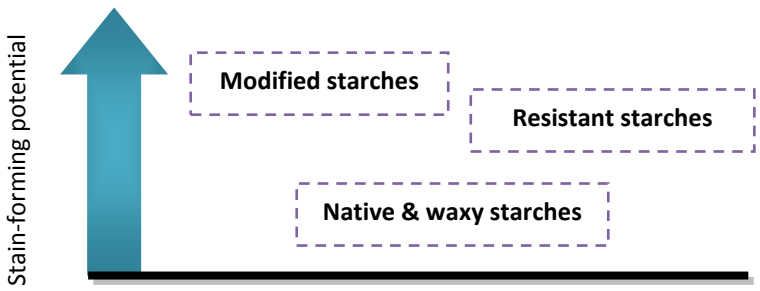

Fig. 4.15: The stain-forming potential of starch ingredients. Resistant starch could be digested to only 16 % in the washing model, but is less likely to contribute to staining due to its lower molecular weight

Both resistant starches and modified starches are slower to digest with detergent amylase and might serve as components of problematic stains. It should be noted, however, that the enzymatic reaction was very fast in the beginning with differences between the starches becoming apparent only after 5 - 10 min. The difficulty in the washing model lays in predicting the molecular size from which the starch will be degraded sufficiently to be completely washed-out.

4.2.5 Materials and methods – agar

Since the target ingredient agar is not a substrate for any of the currently available detergent enzymes, its digestion was studied with a commercial β-agarase E.C. 3.2.1.81 from the marine bacterium *Pseudomonas atlantica* (Sigma-Aldrich). This enzyme cleaves the internal β-(1-4) galactosidic linkages in agarose to produce neoagarooligosaccharides [66]. The β-agarase was dissolved to a concentration of 0.12 mg/ml in a 0.05 M phosphate buffer, pH 6. The substrate agar was purchased from Fluka.

Enzymatic hydrolysis

A solution of 0.5 % (w/v) agar was prepared (0.05 M phosphate buffer, pH 6) by heating to 90 °C for 5 min to dissolve the agar completely. The substrate solution was then maintained above the agar gelling point of 35 °C. For analysis, the agar solution was partitioned into 500 µl aliquots in microtubes. The tubes were placed in a thermal block and the temperature was adjusted to 40 °C. The hydrolysis was started by addition of 50 µl agarase [4.5 U/mg agar] in each microtube. The tubes were shaken at 350 rpm. At the selected analysis times, the tubes were removed from the thermal block and the reaction was stopped by heating at 99 °C for 15 min.

One agarase unit (U) is defined as the amount of enzyme which releases 1 µg of reducing sugar (measured as D-galactose) per minute from 0.5 % w/v agar under the standard assay conditions of 40 C and pH 6. The concentration of enzyme in the reaction was 0.01 mg/ml.

Agar hydrolysis characterization

The agar digestion was characterized by measuring the liberated reducing sugar with the DNSA. The sugar D-galactose served as a standard. The reducing sugar released by the enzyme was divided by the total amount of carbohydrate to determine the % total hydrolysis. Samples from the enzymatic hydrolysis were centrifuged at 12,000 x g, 4 °C for 15 min and the supernatant was filtered through a 45 µm filter. The samples were analyzed on the Polyspher CH Na column (Merck) using the method developed for oligosaccharides. The tetramer neoagarotetraose (NAT) was employed as a standard. Other neoagarooligosaccharides were not commercially available for calibration. For a description of the DNSA, the total carbohydrate determination and the HPLC method, see the appendix chapters 7.1.1, 7.1.2 and 7.1.6 respectively.

Effects of pH and temperature

The enzyme β-agarase was further characterized by determining its T stability and pH optimum. For this purpose several different buffer systems were prepared: 0.05 M citric acid – phosphate buffer (pH 4 and pH 5), 0.05 M phosphate buffer (pH 6, pH 7, and pH 8) and 0.05 M glycine-NaOH buffer (pH 9 and pH 10). The substrate agar was dissolved in the buffers and the activity of the β-agarase was determined according to the standard assay conditions.

To determine the temperature stability of the enzyme, the β-agarase was incubated for 30 min at different temperatures ranging from 20 - 60 °C. The residual activity was then measured according to the standard assay conditions.

4.2.6 Results and discussion – agar

The target substance agar was digested with the enzyme β-agarase at the optimum conditions for this hydrolase – pH 6 and a temperature of 40 °C. The use of a reference substance was considered unnecessary, because the focus of this study was to characterize the target substance with an appropriate enzyme outside the washing context. In this case, it is of interest to characterize the hydrolysis of the substrate and the action of the enzyme, as to make a first general evaluation about the suitability of such an enzyme to remove stains in detergent applications. The curve of the enzymatic breakdown of agar with β-agarase is presented in fig. 4.16 below.

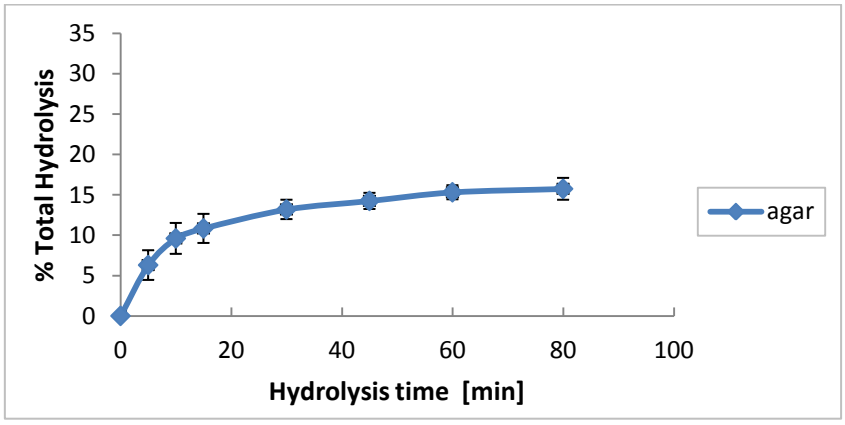

Fig. 4.16: The hydrolysis curve of agar; the points on the graph are the average of triplicate measurements

Agar was digested to 16 ± 1.4 % for a hydrolysis time of 80 min. The hydrolysis rate was calculated from the slope of the rise in reducing sugars in the first 5 min of the reaction (figure not shown). The calculated rate was 0.047 $mM*min^{-1}$.

Samples taken during the hydrolysis were centrifuged at 4 °C. This separates the agar hydrolysate into a high-molecular fraction, which retains the ability to gel at this temperature and a soluble filtrate. The filtrate consists of short-chain neoagarooligosaccharides, which are no longer able to maintain a stable gel network. The soluble fraction was analyzed with the Polyspher CH Na column and the results are presented in fig. 4.17 below.

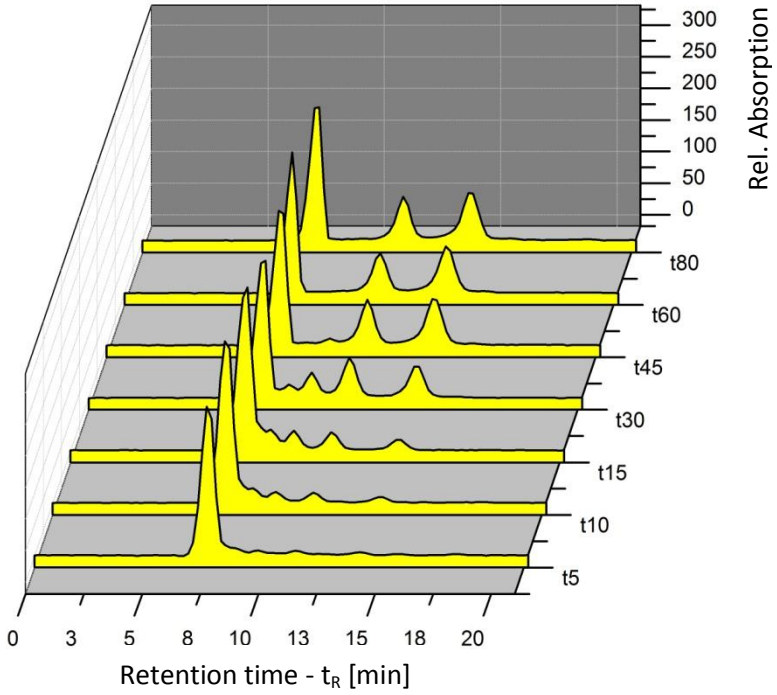

Fig. 4.17: HPLC chromatograms of agar hydrolysis with the enzyme β-agarase to corresponding times of the digestion (sample times shown on the Z-axis)

Common between all chromatograms is the large peak at t_R = 8 min, which consists of all neoagarooligosaccharides too large to be resolved with this column. At the beginning of hydrolysis (chromatograms t5 to t15), several small peaks can be differentiated from the baseline. The peaks at t_R = 11.4 min and t_R = 14.2 min increase in area as the hydrolysis proceeds. The peak at t_R = 14.2 min was identified as neoagarotetraose (NAT) by comparison with the standard. The peak at t_R = 11.4 min was assigned to neoagarohexaose (NAH) by a calibration curve of retention time versus DP of the maltooligosaccharides (see appendix 7.1.8 Correlation DP vs. maltooligosaccharide t_R). The peaks between the injection peak and NAH belong to neoagarooligosaccharides of DP > 6. Their concentration increases in the first 30 min of hydrolysis (chromatograms t5 to t30), only to decline at latter reaction times. At t = 60 min, the neoagarooligosaccharides of DP > 6 could not be detected anymore. They have been digested by the β-agarase. This observation is consequent with literature reports of the action of this enzyme [67], [68]. The β-agarase is known to digest agar by endo-type hydrolysis and to produce NAT and NAH as the predominant products. The tetramer cannot be

hydrolyzed further. This was confirmed by incubating 0.5 mg/ml NAT with 11.3 U agarase for 60 min. No rise in reducing sugar or decrease of peak area in the HPLC could be observed (results not shown).

The release of NAT from agar can also be used to describe the hydrolysis (see fig. 4.18 below). As an end-product of digestion, the tetramer was produced only in minimal amounts during the first 15 min of the enzymatic reaction. At later reaction times the NAT concentration rose considerably, reaching a maximum at 45 min. After this period, no significant rise of the tetramer concentration could be observed.

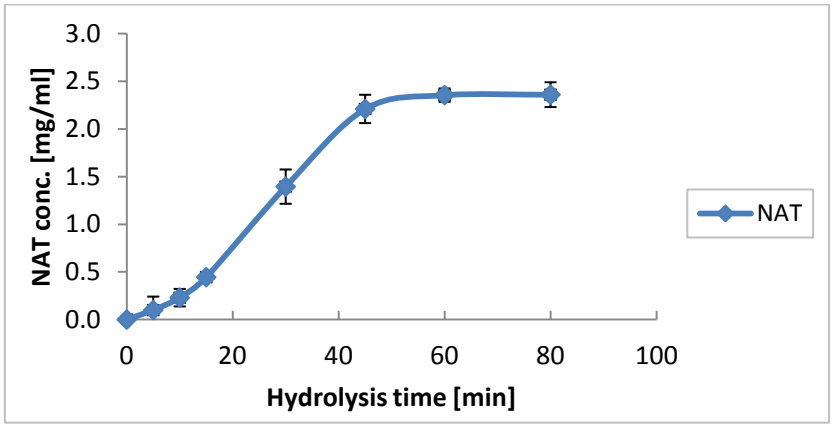

Fig. 4.18: The rise of NAT concentration during the enzymatic hydrolysis of agar

The optimum pH and temperature of β-agarase are 6 and 40 °C respectively. A detergent enzyme should work in alkaline milieu and display a good stability at a temperature of 40 °C. It was therefore of interest to examine the stability of the agarase at 40°C and to check the enzymatic activity at pH values different than the optimum. The results of this investigation are presented in fig. 4.19.

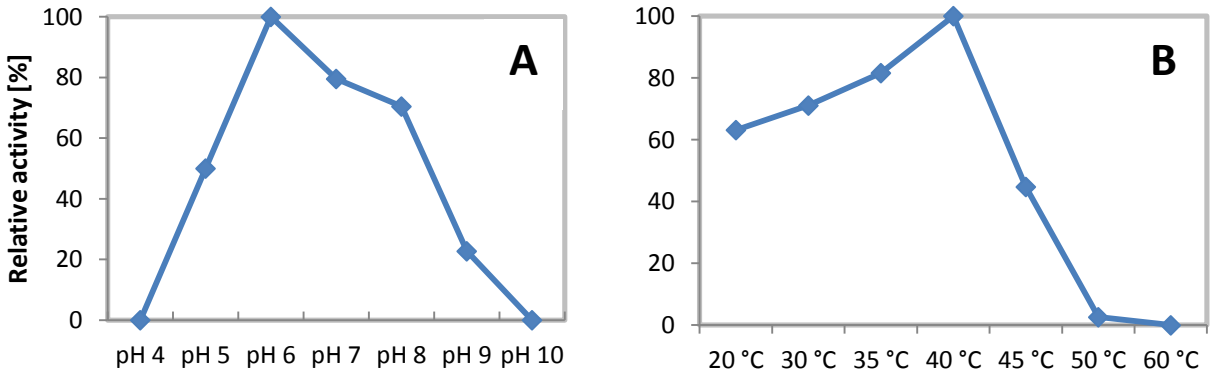

Fig. 4.19: The activity of β-agarase at different pH values (A) and the effect of temperature on β-agarase stability after 30 min incubation (B). 11.3 U agarase were used in each measurement.

The results indicate that β-agarase is a good starting point for the development of a detergent enzyme. Beyond its pH optimum of 6, the enzyme displayed a relatively high activity in the alkaline region, retaining 70.5 % activity at pH 8. No activity could be measured at pH 4 or pH 10. The enzyme

was not able to tolerate high temperatures, displaying only 44.7 % residual activity after incubation at 45 °C. Higher temperatures denatured the agarase. To qualify as a detergent agarase the effect of other reagents, especially detergents, on the activity and stability of the enzyme should be examined. An enzyme which is a good candidate for laundry detergent applications can be further optimized by methods such as protein engineering.

4.2.7 Summary – agar

Employed traditionally in Asian cuisine, agar has a long history of food use as a hydrocolloid. It has been mainly prized as a thickening and gelling agent. Agar was chosen as a target ingredient due to its novel use as an encapsulation matrix for various functional ingredients such as probiotics, antioxidants and vitamins. Furthermore, it finds application as a fat mimetic, satiety agent, and stabilizer in fruit juices [(69)]. In recent years, various patents and publications have appeared which describe the creation of fluid gel drinks with agar micro-particles possessing interesting novel uses and functionalities [(70), (71), (72)]. Due to these advances in agar application, it was included as a target ingredient in this thesis.

Agar displayed 16 % total hydrolysis in 80 min with β-agarase. This enzyme was selected for its specificity for the substrate, but also due to the endo-mode of hydrolysis. The final products of digestion were short-chain neoagarooligosaccharides, which are easily water-soluble. The concentration of NAT, the end-product of agar hydrolysis with β-agarase, reached a maximum within 45 min of reaction. The tetramer concentration did not increase further, indicating that the agar digestion was complete under the assay conditions.

By using only 4.5 U of the enzyme per mg substrate, it was possible to complete the hydrolysis within 45 min. This makes β-agarase an interesting candidate for an enzymatic solution to agar stains. Its good stability in the alkaline region, as well as its optimum activity at 40 °C (the standard laundry temperature) confirms the suitability of β-agarase as a laundry enzyme candidate. Of course, further testing of β-agarase, especially its stability to various laundry detergent components, is necessary for a serious assessment.

In order to be able to make a true evaluation of the stain-potential of agar as a target substance, it is necessary to perform empirical tests with this ingredient as a component of a true stain. For this investigation, please see the washing experiments (chapter 5.3.3 hydrocolloid results).

4.3 Fat replacers

4.3.1 Materials and methods – Simplesse®

The target substance Simplesse®, a microparticulated whey protein concentrate, was kindly provided by its manufacturer CP Kelco, US. The reference substance used in this investigation was whey protein concentrate which has not been subjected to the microparticulation process. It was purchased from Scitec Nutrition, US.

Enzymatic hydrolysis

The whey protein was dissolved in NaOH (0.1 M) to a concentration of 1 % w/v. The protein solution was then diluted with the working buffer of the model washing system (0.1 M phosphate buffer, pH 8) to a final concentration of 0.1 % w/v. The substrate solution (30 ml) was transferred to the reaction vessel of an automatic titrator and the temperature was adjusted to 40 °C. The enzymatic hydrolysis was started by addition of 10 ml Esperase solution [4.31 PU/g whey protein]. The concentration of enzyme in the reaction was 50 mg/l, corresponding to a dilution of 0.005 % w/v with a protein content of 3.88 % w/w as determined by the Bradford method.

The pH of the reaction was maintained by the titrator at pH 8 with addition of 0.1 M NaOH (pH-Stat method). Samples (1000 µl) were drawn at regular intervals and the reaction was stopped by addition of 100 µl HCl (1 M) or heating at 99 °C for 15 min.

Whey protein hydrolysis characterization

The degree of total protein hydrolysis (DH %) was determined from titration data of the pH-stat method. Samples taken during the hydrolysis were analyzed for protein content by the Bradford assay. The enzymatic breakdown of the whey protein was also monitored by gel electrophoresis. The peptide fingerprint resulting from the action of the protease was analyzed by RP-HPLC. For a description of the SDS-PAGE protocol, the Bradford procedure and the peptide fingerprint HPLC method, see the appendices 7.1.5, 7.1.3 and 7.1.9 respectively.

Protease assay

The protease activity was determined by measuring the L-tyrosine released from a casein solution (0.65 % w/v) in phosphate buffer (0.05 M, pH 7.5) at 37 °C with the Folin & Ciocalteu reagent. One protease unit (PU) is defined as the amount of enzyme releasing 1 µmol of L-tyrosine per minute. The

specific activity of the protease Esperase was found to be 1.6 PU/mg. For a description of the protease assay procedure, see the appendix 7.1.4 Protease assay.

4.3.2 Results and discussion – Simplesse®

The degree of total hydrolysis (DH %) represents the extent to which the peptide bonds in a protein have been cleaved. DH % can be expressed as shown in equation 4, where h: peptide bonds cleaved (mmol/g) and h_{tot}: total amount of peptide bonds in a protein (mmol/g).

$$DH = h \times \frac{1}{h_{tot}} \times 100\ \% \qquad \text{(Eq. 4)}$$

The most accurate method for determining h_{tot} , the total amount of peptide bonds in a protein, is by amino acid analysis [73]. Simplesse and its reference contain both 8.8 mmol bonds per g dry weight [74]. The amount of peptide bonds cleaved by the protease (h) can be derived from the base consumption in a pH-stat method. The moles of base used in maintaining constant pH correspond to the amount of peptide bonds cleaved. This can be calculated with equation 5.

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\ \% \qquad \text{(Eq. 5)}$$

B: amount of base (l), N_b: normality of the base (N), α: dissociation constant of the amino bond; α = 1.20 at 40 °C & pH 8, MP: dry weight of the protein (g)

The data from the pH-stat was used for calculating DH %. The results are presented in fig. 4.20 on the next page. The hydrolysis of both proteins was very rapid in the first 5 min of reaction, after which it slowed down in a linear manner without levelling off within 80 min reaction time. In 2 min after enzyme addition, Simplesse was digested to 10 %, while whey protein displayed 7.5 % hydrolysis. The target substance Simplesse achieved a slightly higher degree of total hydrolysis in 80 min (15.4 ± 0.6 %) in comparison to whey protein (14 ± 0.4 %).

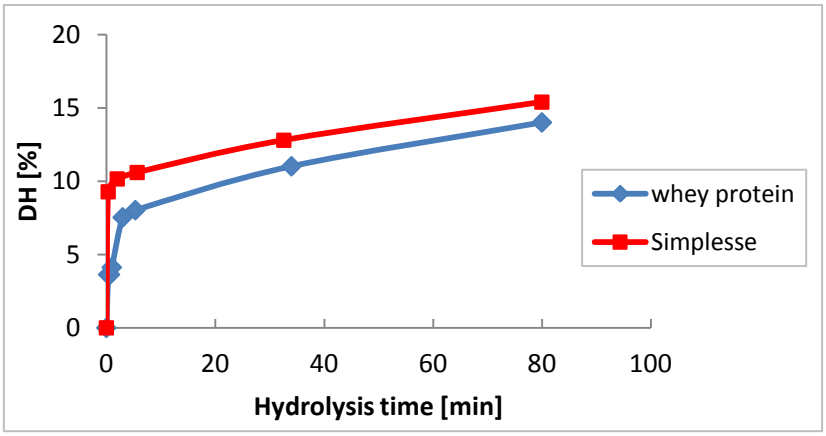

Fig. 4.20: The hydrolysis curves of Simplesse and its reference whey protein

The target substance Simplesse is digested faster by the subtilisin protease and achieves a slightly higher overall degree of hydrolysis. Since both Simplesse and its reference are whey proteins, the difference in digestion must be related to the microparticulation process. Microparticulation coagulates the Simplesse particles on the surface, making them more hydrophobic [(75)]. Subtilisin proteases show preference for hydrophobic residues and digest denatured substrates with preference [(74)]. As a result, Simplesse is hydrolysed at a higher rate. This was also confirmed by the Bradford assay (fig. 4.21), which shows how the protein concentration of both substrates decreases during the enzymatic hydrolysis.

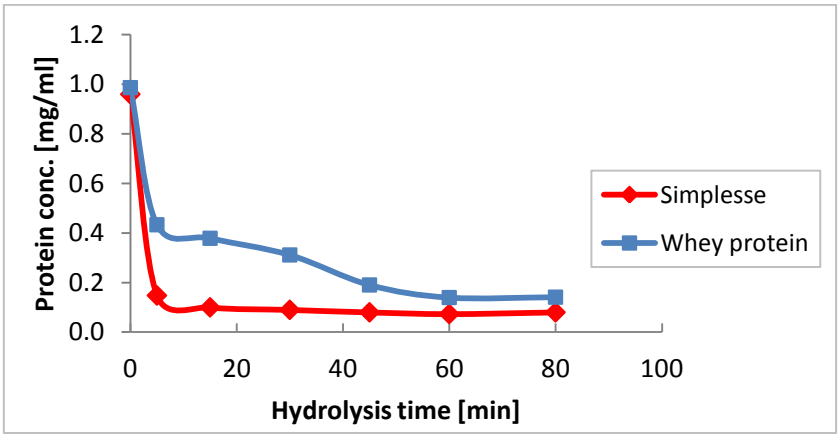

Fig. 4.21: The decrease of protein concentration during proteolysis of Simplesse and whey protein, as measured by the Bradford assay. The points on the graph are the averages of triplicate measurements

The hydrolysis rate was calculated from the slope of the graph in the first 5 min of hydrolysis. Simplesse was digested with a rate of 0.162 mg*ml^{-1}min^{-1}, while the whey protein displayed a rate of 0.111 mg*ml^{-1}min^{-1}. After only 5 min of hydrolysis, the protein concentration of Simplesse fell to 0.15 mg/ml. Further breakdown of protein did not occur at a significant rate; the concentration of Simplesse remained at 0.1 ± 0.011 mg/ml until the end of reaction time.

The proteolysis of the target substance was complete in only 5 min. This was also illustrated by the SDS-PAGE gels of the enzymatic digestion, which are displayed in fig. 4.22.

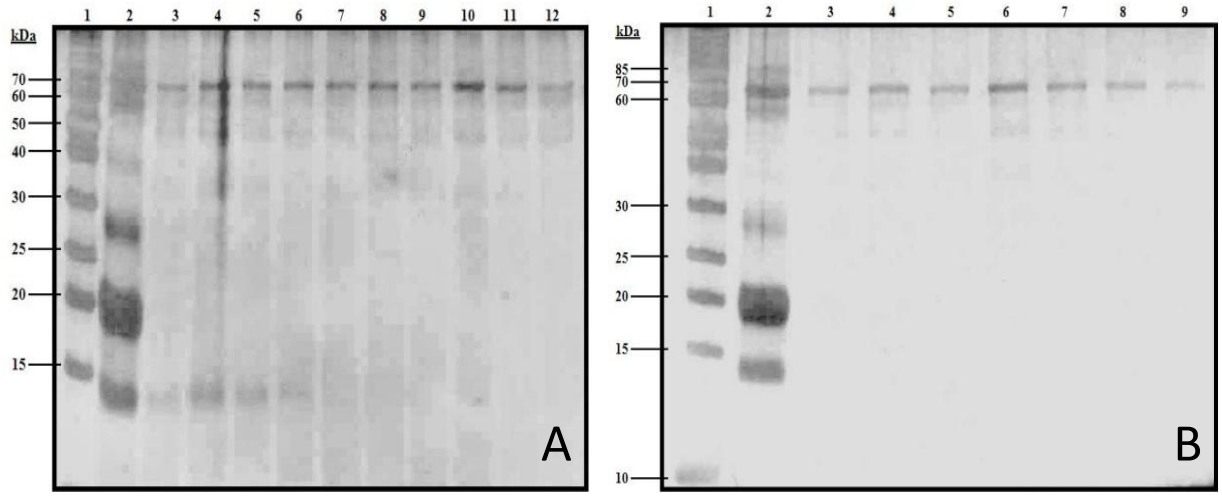

Fig. 4.22: Digestion of Simplesse® analyzed by gel electrophoresis in a 15 % SDS-PAGE gel with silver staining.
Gel A lanes: 1) PageRuler™ Protein Ladder (Fermentas), 2) t= 0 min, 3) to 12) Δ 1 min of hydrolysis, 12) 10 min
Gel B lanes: 1) PageRuler™ Protein Ladder (Fermentas), 2) t= 0 min 3) to 5) Δ 5 min, 6) to 8) Δ 15 min, 9) 80 min.

The protein components of Simplesse before protease digestion can be seen on lane 2 of gels A and B. These include typical whey proteins such as α-Lactalbumin (14 kDa) and β-Lactoglobulin (18 kDa), as well as some proteins carried-over from the casein fraction: casein (27 kDa) and BSA (66 kDa) [76]. After just 1 min of enzymatic hydrolysis (lane 3), the bands of β-Lactoglobulin and casein have disappeared, as they are digested by the protease. The band of α-Lactalbumin (14 kDa) can be seen well into the 4th min of enzymatic hydrolysis. No proteins of the whey protein fraction can be detected after 5 min of digestion, as lane 3 of gel B reveals. The low detection limit of silver staining (100 ng) confirms that none of the whey proteins have remained intact. Simplesse has been completely broken down into smaller peptides. The band of BSA can be seen throughout the hydrolysis until the end of the reaction time of 80 min. BSA is a stable protein possessing sixteen disulfide bridges [77]. This makes it relatively resistant to enzymatic breakdown within the time frame of the experiment.

The results of the gel electrophoresis of the reference substance, ultrafiltered whey protein, were exactly identical to the gels of Simplesse (gel pictures not shown). The reference substance displayed the same protein bands as Simplesse (whey proteins, casein and BSA). The first proteins to be broken down after protease addition were α-Lactalbumin (14 kDa) and casein. No whey proteins could be detected after 5 min of hydrolysis. The BSA band was visible in all lanes until the end of reaction time. The slower rate of protein breakdown could not be observed in any way on the SDS-PAGE gels of the reference substance.

As the SDS-PAGE analysis has shown, the large proteins of the whey protein preparations have been broken down into peptides within 5 min of protease addition. This presents another possibility to compare Simplesse to its reference substance. The peptide fingerprint resulting from the proteolysis was analyzed by RP-HPLC and the chromatograms are displayed in fig. 4.23.

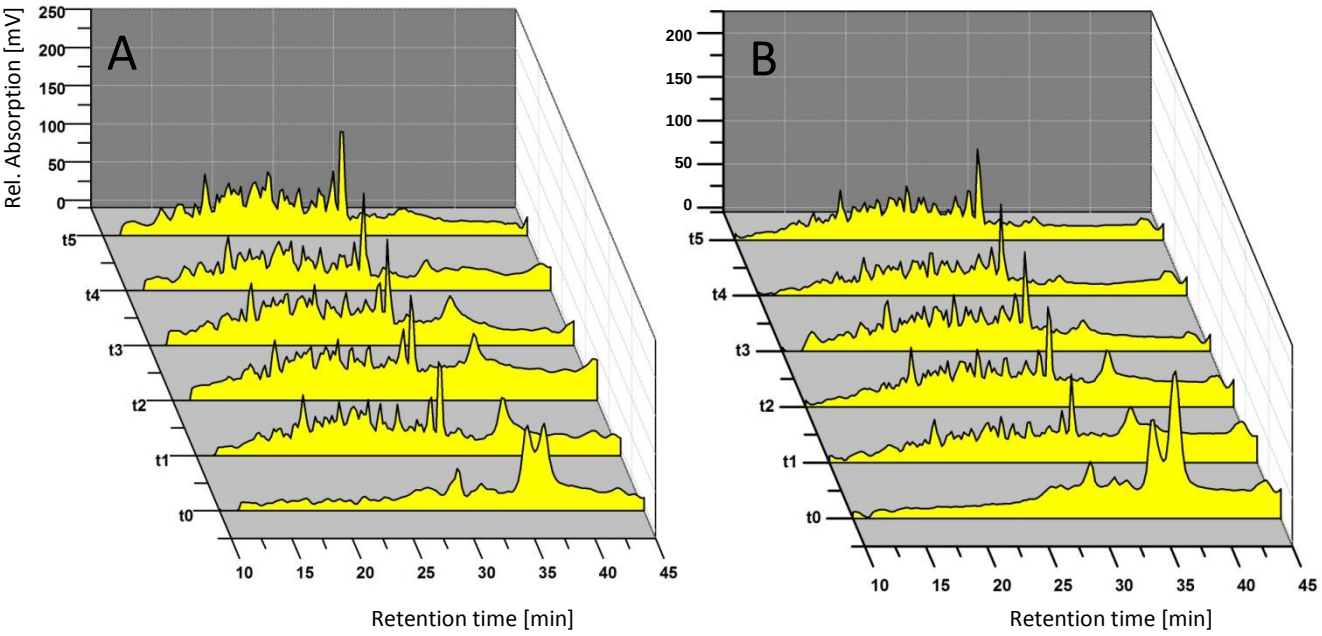

Fig. 4.23: The development of the peptide fingerprint of Simplesse (A) and whey protein (B). The Z-axis shows the reaction time at which the sample was drawn and the hydrolysis terminated.

Characteristic for the undigested substrates is a chromatogram (t0 in both A and B) dominated by two large peaks in the hydrophobic region (> 30 min). Peaks in this region are comprised of proteins of large size which are eluted from the column by higher concentration of organic modifier. The proteins are quickly degraded on the start of hydrolysis, as can be seen from chromatogram t1 on both A and B. The large peaks are diminishing in size, while a pattern of peaks belonging to the peptide fingerprint start to form. After 5 min reaction time (t5), the large proteins cannot be detected anymore.

The fingerprint region is found in the 15 – 30 min segment of the chromatogram and is comprised of small, water-soluble peptides which are the final products of proteolysis of short duration. The pattern of the fingerprint is characteristic of the protein type and the protease used for digestion. It can be used to identify proteins. Indeed, a comparison of the fingerprints A and B in fig. 4.23 reveals that the preparations are identical; they are both derived from whey protein. The microparticulation process does not impact the fingerprint of whey protein. However, the undigested samples show a slight difference in the shape of their peaks in chromatograms (t0). The shape/area of the peaks was

reproducible on multiple injections, indicating that they are typical of Simplesse. Significant changes in the fingerprint after the 5th min of hydrolysis were not observed either for the pattern or for the peak area (fig. 4.24).

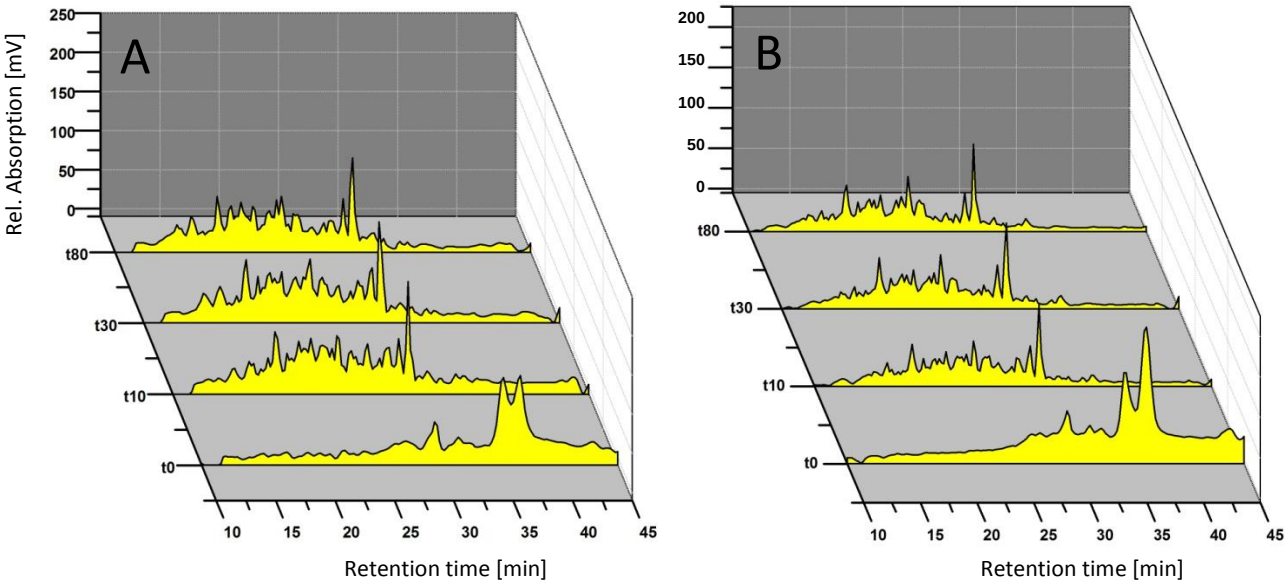

Fig. 4.24: The development of the peptide fingerprint after 10 min reaction time of Simplesse (A) and whey protein (B). The Z-axis shows the reaction time at which the sample was drawn and the hydrolysis terminated.

The results of the HPLC analysis confirm the observations from the SDS-PAGE. The whey proteins in both Simplesse and its reference were broken down within a minute of protease addition. After 5 min of hydrolysis, large protein structures could not be detected on the SDS-PAGE gel or on the HPLC chromatograms. The difference in digestion speed between Simplesse and its reference, which was assessed by the rate of protein breakdown and the pH-stat method, could not be observed by SDS-PAGE or HPLC. The subtle divergence between target and reference could not be assayed by these methods.

4.3.3 Summary – Simplesse®

The microparticulated whey protein Simplesse is a fat mimetic which is used in a variety of dairy products, due to its ability to stimulate the mouth-feel and texture of fat. The target ingredient was digested under the conditions of the model washing system with the detergent protease Esperase. The hydrolysis of Simplesse was compared to the enzymatic breakdown of whey protein which has not been subjected to the microparticulation process.

Simplesse was hydrolyzed to 15 % within the time frame of the model, while its reference achieved DH 14 %. This small, but significant difference in the degree of total hydrolysis is caused by the faster rate of protein breakdown of the target. Both whey protein preparations are identical in composition, as shown by the gel electrophoresis analysis and the peptide fingerprint. The enzymatic digestion was very fast for both ingredients, with the protease cleaving large proteins within a minute of reaction start. After 5 min of hydrolysis all large protein structures were digested to smaller peptides, as confirmed by SDS-PAGE and HPLC.

The slight difference in the hydrolysis rate of Simplesse is due to its microparticulation. This is a physical process which does not change the nutritional quality or composition of proteins [75]. However, the short cooking times and the high shear rates of the process, lead to an increase of surface area and hydrophobicity of the protein particles. As a result, the microparticulated protein becomes more susceptible to enzymatic attack.

Due to its fast digestion, it is unlikely that Simplesse will contribute to problematic stains. The detergent protease hydrolyzes the fat mimetic within minutes to small, water-soluble peptides. Therefore, this target is unlikely to cause stains which are more difficult to wash-out than conventional milk protein stains.

4.3.4 Materials and methods – structured lipids

The structured lipids under investigation are the few ingredients of this type approved for food use in the EU. They are presented along with a short description of their structure in table 4.5 below.

Table 4.5: Target ingredients from the structured lipid category

Product name	Type	Description
Benefat®B	SALATRIM	Mixture of C3, C16, and C18 fatty acid esters of glycerol
Grindsted®MCT 60 X	MCT	Glyceryl tri-caprylate-Caprate

Benefat is a Salatrim (short and long-chain acyltriglyceride molecule) with a melting point of 33 °C, thus being a solid fat at room temperature [78]. Its digestion was compared to other solid fats such as goose lard and coconut butter. Grindsted is a MCT, a medium-chain-triglyceride, with a melting point of 9 °C [79]. The hydrolysis of Grindsted was compared to the enzymatic lipolysis of olive oil. Both fat substitutes were kindly provided by their manufacturer Dansico A/S, Denmark. The reference substances: goose lard, coconut butter and olive oil were purchased from the local supermarket. All

fats were stored in the dark at room temperature in tightly sealed containers. The reference fats were obtained from a single product, which was used in all experiments, to minimize batch-to-batch variations.

Enzymatic hydrolysis

A fat emulsion (O/W) was prepared by homogenizing 3 g fat in 22.5 ml emulsion solution with an Ultra-Turrax for 1 min at 21,500 rpm. The substrate emulsion was diluted with water up to 250 ml and 30 ml of this solution were placed in the reaction vessel of an automatic titrator. The temperature was adjusted to 40 °C and the hydrolysis reaction was started by addition of 10 ml Lipolase solution [86 LU/g fat]. The pH-stat method was used to automatically monitor the pH and maintain it at pH 8 by titrating appropriate volumes of NaOH (0.1 M). The volume of NaOH added to the reaction vessel was recorded and used to calculate the concentration of free fatty acids (FFA) generated by lipolysis.

The concentration of enzyme in the reaction was 10 mg/l, corresponding to a dilution of 0.001 % w/v with a protein content of 4.45 % w/w as determined by the Bradford method. For a description of the emulsion solution composition and the Bradford procedure, see the appendices 7.2.1 and 7.1.3.

HPLC analysis

Samples (100 µl) were withdrawn at regular intervals from the lipid hydrolysis reaction and the reaction was stopped by addition of 1000 µl lipid extraction reagent. This was composed of chloroform and methanol in a 1:1 ratio. This solution was injected (20 µl) and analyzed on a C8 Kinetex column (Phenomenex) with the universal lipid method, using gradient elution and CAD detection. Column temperature was 40 °C and the flow rate was 0.8 ml/min. A triglyceride standard containing a mixture of triglycerides from tricaprylin to tripalmitin (Supelco) was used for the qualitative evaluation of the lipid fingerprint.

For a detailed description of the universal lipid method, a chromatogram of the lipid standards, and the HPLC system configuration, see the appendices 7.1.11, 7.1.10 and 7.2.2 respectively.

Lipase assay

The activity of the enzyme was determined by the titrimetric method using a tributyrin emulsion (5 % w/v) as a substrate [80]. One lipase unit (LU) is defined as the amount of enzyme releasing 1 µmol of butyric acid per minute at 40 °C and pH 8. The specific activity of Lipolase determined by this procedure was 1744 LU/mg.

4.3.5 Results and discussion – structured lipids

The degree of total hydrolysis (%) was determined according to the equation below, by quantifying the free fatty acids (FFA) released during digestion with the pH-stat method, divided by the amount of FFA moles that would be produced if the triglyceride was completely digested. Equation 6 postulates that 2 moles of FFA are produced for each triglyceride molecule [81]. The assumption is valid for Lipolase, which possesses a 1, 3-sn specificity [82]. This indicates that the lipase cleaves the ester bond in positions sn-1 and sn-3 of the triglyceride molecule preferentially.

$$\%\, Hydrolysis = \frac{B \times N_b \times Mw_{\,Lipid}}{ML \times 2} \times 100\ \% \qquad \text{(Eq. 6)}$$

B: amount of base (l), N_b: normality of the base (N), Mw: molecular weight of the lipid (g/mol), ML: weight of the lipid (g)

The molecular weight of each lipid was calculated according to its average fatty acid composition [83]. The values used were: 470.7 g/mol (Grindsted), 875.77 g/mol (olive oil), 642 g/mol (Benefat), 858.1 g/mol (goose lard), 678 g/mol (coconut butter).

The degree of total hydrolysis (%) obtained for each target lipid and its reference is presented in table 4.6. The results shown are the averages of triplicate measurements at least. The % hydrolysis obtained for the duration of 60 min is listed under the name of each ingredient. As can be seen from table 4.6, the target lipids were digested to a higher extent than their respective references. This was observed for both liquid and solid fats. The MCT Grindsted was almost completely digested with 96 % hydrolysis under the conditions of the standard washing system. In comparison, its reference olive oil displayed 29 % hydrolysis for the same time duration.

Table 4.6: The degree of total hydrolysis obtained for the lipids under investigation

Hydrolysis	Target	Reference 1	Reference 2
Liquid fats	Grindsted	Olive oil	
%	95.8 ± 3.0	29 ± 1.7	
Solid fats	Benefat	Lard	Coconut butter
%	52.7 ± 1.3	17.3 ± 0.7	52.9 ± 0.5

The Salatrim Benefat was digested to 53 %, while goose lard was broken down to 17 %. A reference lipid should resemble the target as much as possible in its structure, without itself being a structured fat. Benefat, despite its chemical modifications, was originally derived from coconut oil. For this reason, native coconut butter was included as a second reference in the solid fat category. This plant-based fat achieved a degree of total hydrolysis similar to Benefat with 53 %.

A more comprehensive picture of how the target and reference fats differ in their digestibility is provided by the hydrolysis curves. Figure 4.25 below displays the curves of the liquid fats. Both lipids are digested very fast in the first 5 min of reaction, after which the hydrolysis slows down significantly and proceeds in a linear manner without reaching a stationary level. The difference in digestion rate between the lipids is apparent from the very beginning of reaction. After 2 min of enzyme addition, Grindsted has already been digested to 43 %, while olive oil shows 5 % hydrolysis.

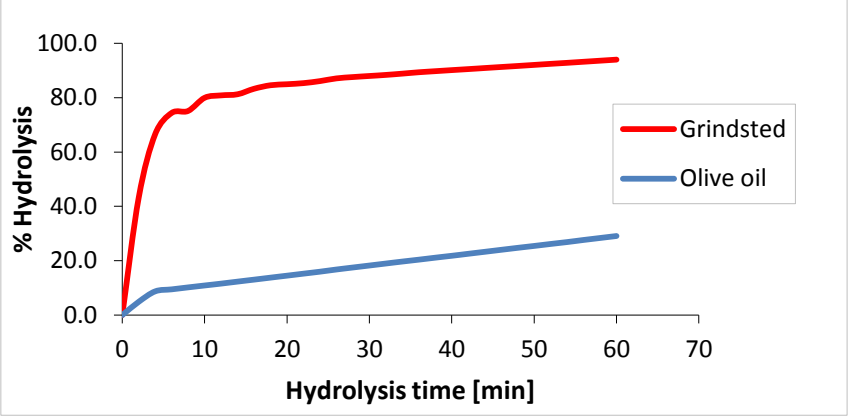

Fig. 4.25: The hydrolysis curves of Grindsted and olive oil

The rate of hydrolysis can be calculated from the rate of fatty acid release from the lipids. This was determined from the slope of the graph in the first 5 min of reaction (figure not shown). The calculated rate for Grindsted was 69.24 µmol*min^{-1}, while the rate for olive oil was almost 14 times lower with 4.98 µmol*min^{-1}.

This difference in digestion rate can be attributed to the mechanism of lipolysis and to the specific structures of the lipids. Lipases are surface-active catalysts operating at the emulsion interface. The products of lipolysis disperse in the aqueous or organic phase, according to their hydrophilic or lipophilic character. Moreover, fatty acids and diacylglycerides possess surface-active properties and tend to accumulate on the oil-water interface, thus blocking access of the enzyme to other triglycerides [82), 84)]. The caprylic acid molecules resulting from the digestion of Grindsted have a higher dispersability in the aqueous phase than the palmitic and oleic acid molecules of the olive oil. As a result, the medium-chain fatty acids migrate into the water medium and do not inhibit the interfacial lipase reaction. The hydrolysis of Grindsted proceeds faster and more efficiently than the digestion of olive oil under the given conditions.

All agents which are active at the emulsion interface have an impact on the rate and extent of lipid hydrolysis [85)]. Under the standard washing model conditions, this was especially observed to be the case for calcium ions. Fig. 4.26 on the next page shows the release of fatty acids on digestion of Benefat emulsions which have been prepared by dilution with deionized or tap water.

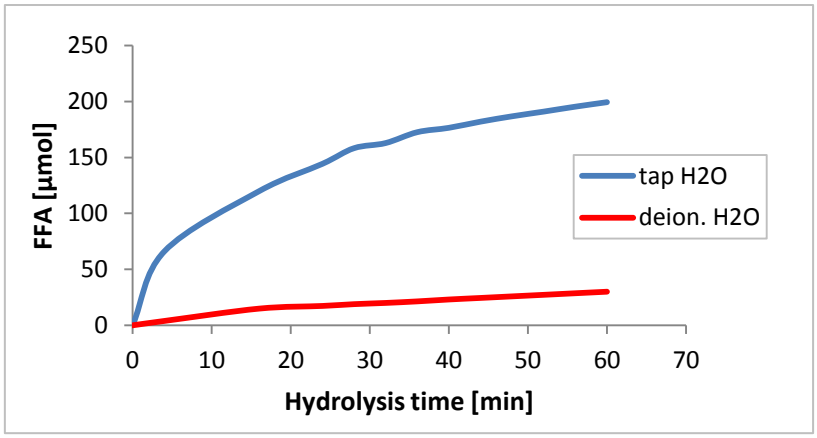

Fig. 4.26: The release of fatty acids from Benefat emulsions diluted with deionized or tap water

The lipolysis rate of Benefat, determined from the slope of the curves in the first 5 min of reaction, was 1.25 µmol*min^{-1} for the emulsion prepared with deionized water and 14.4 µmol*min^{-1} for the emulsion prepared with tap water. This represents more than a ten-fold increase in digestion rate in the presence of calcium ions (water hardness 11.8 °d). All dilutions of emulsions in this work were consequently performed with water from the mains supply.

Calcium ions have the ability to precipitate the long-chain fatty acids which result from triglyceride hydrolysis and accumulate at the oil-water interface. As a result, the lipase has a better access to the emulsified triglycerides [86]. The digestion rate improves significantly. The effect of calcium ions on the rate of lipid digestion is especially pronounced for long-chain triglycerides, because medium- and short-chain triglycerides are more readily water dispersible, and thus do not inhibit lipase by accumulating on the emulsion droplet surface. This can be demonstrated by comparing the digestion of Grindsted emulsions, diluted with deionized and tap water (fig. 4.27). The reaction in both cases was relatively fast. The rate displayed in deionized water was 31.4 µmol*min^{-1}, while the rate in tap water was more than twice as fast with 75 µmol*min^{-1}.

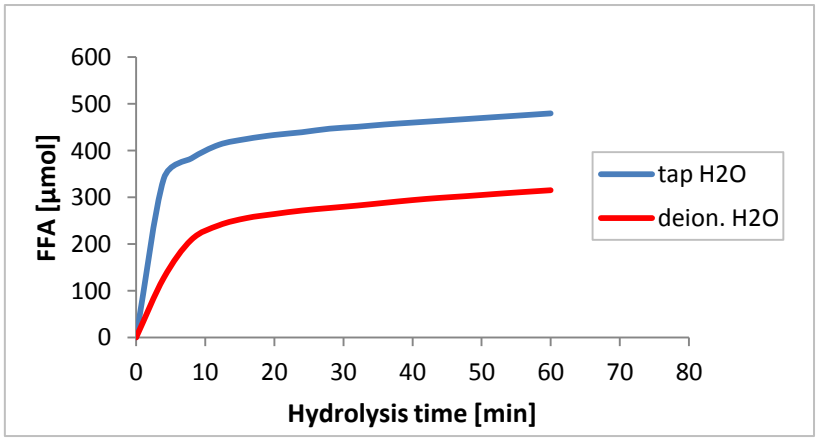

Fig. 4.27: The release of fatty acids from Grindsted emulsions

The same factors influence the digestion of solid fats. Their long-chain fatty acids would accumulate on the droplet interface, inhibiting further lipolysis if not precipitated by calcium ions or by being solubilised in micelles. The digestion of the solid fats under investigation is presented in fig. 4.28.

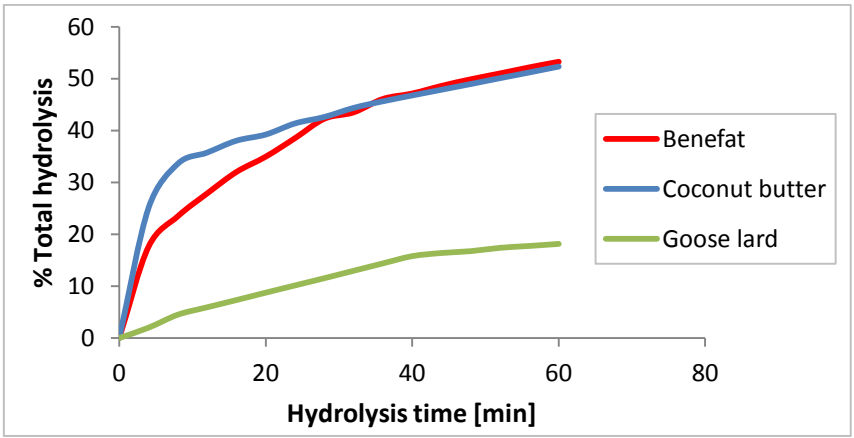

Fig. 4.28: The hydrolysis curves of Benefat, native coconut butter and goose lard

The hydrolysis curves of Benefat and coconut butter are very similar to each other, both in their initial reaction rate and in the degree of total hydrolysis achieved after 60 min. The rate of digestion, calculated from the rise of fatty acid concentration in the first 5 min of reaction (graph not shown), was slightly faster for coconut butter with 20 µmol*min^{-1}. The reaction rate slowed down significantly after 10 min of hydrolysis. In contrast, the initial rate of Benefat was 15 µmol*min^{-1} and the rate slowed down after 5 min reaction time. Benefat carries a short-chain fatty acid (propionic acid) in its structure due to its chemical modification. Coconut butter contains by nature rich portions of medium-chain fatty acids such as caprylic and capric acid in its composition. Due to the good water dispersibility of these fatty acids, the lipolysis reaction of both lipids proceeds fast.

Goose lard, on the other hand, contains high amounts of long-chain fatty acids in its structure, such as oleic, stearic and palmitic acid. When the capacity of calcium ions to precipitate the fatty acids from the emulsion interface is exhausted, the enzymatic reaction would significantly slow down. Indeed, the digestion rate of goose lard was only 1.8 µmol*min^{-1} in the first 5 min of reaction. The reaction rate decreased afterwards and only 17 % total hydrolysis was obtained in 60 min under the conditions of the model washing system.

The enzymatic breakdown of the lipids under investigation was also analyzed by HPLC after a lipid extraction of the samples from the emulsion hydrolysis mixture. The separation was performed on a C8 Kinetex column, which separates the lipids and their hydrolysis products according to polarity. As can be seen from fig. 4.29 on the next page, the chromatogram of Grindsted before the start of

digestion (t0) contains multiple peaks. The peak at 30.1 min was assigned to tricaprylin (glyceryl tri-caprylate-caprate) by comparison with the standard mixture.

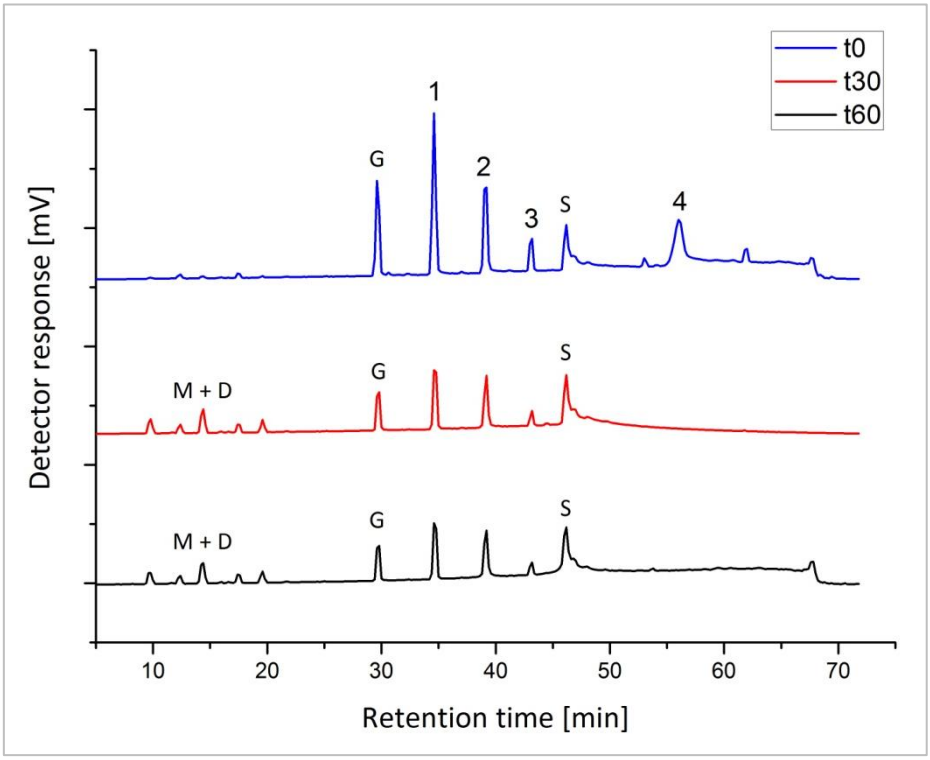

Fig. 4.29: RP-HPLC chromatograms of Grindsted, presented in Y-axis offset (from top to bottom – Grindsted emulsion before enzyme addition at t = 0 min, t = 30 min, t = 60 min of hydrolysis). G is the Grindsted peak (29.9 min); S is solvent peak present in all chromatograms, peaks 1 to 4 are unknown triglycerides, M + D: mono-and diglyceride region.

The rest of the peaks could not be identified. They probably belong to by-product triglycerides from the production process of Grindsted, as they elute in the triglyceride region which extends in the 30-60 min segment of the chromatogram.

As the hydrolysis proceeds, Grindsted and the other triglycerides peaks decrease in area or disappear completely (peak 4). Instead, a series of small peaks in the mono-and diglyceride region (10 - 25 min segment of chromatogram) are easily recognizable. The area of the Grindsted peak decreases rapidly until the 30th min of reaction (t30). After this time, only a minimal decrease in area is observed. This observation correlates with the pH-stat results, which indicate that the Grindsted digestion is largely complete by 30 min of reaction time and the hydrolysis rate slows down considerably.

The chromatograms of the lipolysis reaction are to be perceived as a type of lipid fingerprint, revealing the progress of lipase digestion in a qualitative manner. Due to the unavailability of standards, it is difficult to assign every peak in the chromatogram to a particular reaction product. Nevertheless, since species of different polarity elute in particular segments of the chromatogram, it is possible to examine the lipolysis reaction and its products in a descriptive way.

This was also done for the solid fats under investigation. The chromatograms of the target Benefat are presented in fig. 4.30 below.

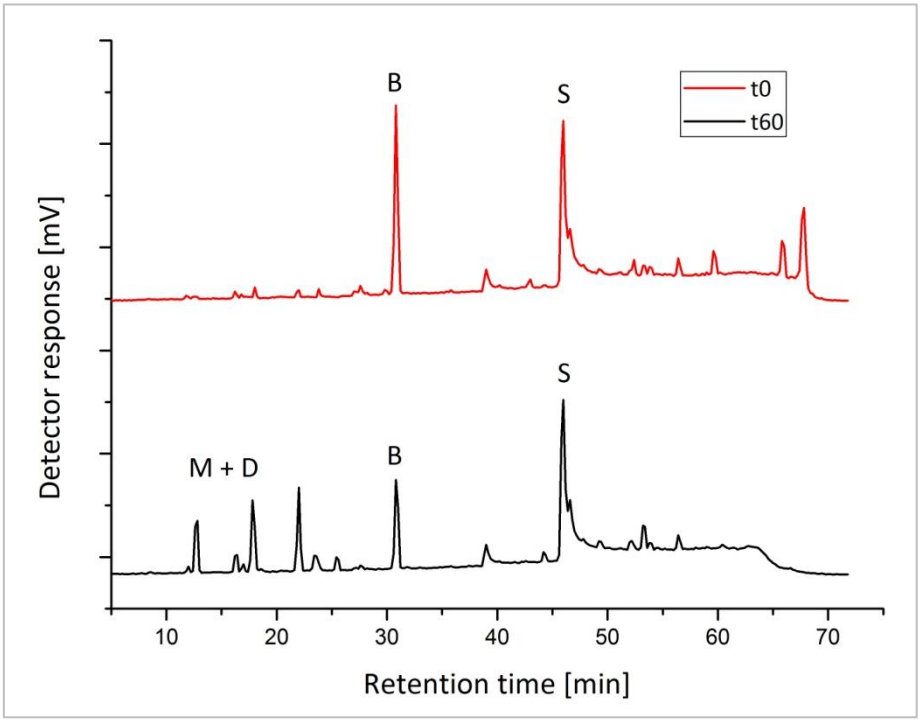

Fig. 4.30: RP-HPLC chromatograms of Benefat at the beginning (t0) and end of reaction (t6o), presented in a Y-axis offset; B: Benefat peak at 31 min; S is the solvent peak present in all chromatograms, M + D: mono-and diglyceride region.

The peak at 31 min retention time was assigned to Benefat after multiple injections, because it is the major peak appearing in the triglyceride region. Triglycerides with long-chain fatty acids like trilaurin (C12) appear in the > 50 min region (see appendix for chromatogram of the triglyceride standards). Benefat, which contains propionic acid in its structure, is less hydrophobic and elutes earlier. As the hydrolysis proceeds, the area of the Benefat peak decreases, while multiple peaks appear in the mono- and diglyceride region of the chromatogram.

Although derived from the same natural source as Benefat, the reference coconut butter displays a different chromatographic profile, as fig. 4.31 on the next page shows. There are multiple peaks in the triglyceride region > 50 min, indicating that the coconut butter is composed of a triglyceride mixture containing predominantly long-chain fatty acids.

The HPLC analysis reveals the difficulty in selecting appropriate references for the study of lipid digestion. Fats from natural sources, like coconut butter, are always composed of complex triglyceride mixtures. Structured lipids have more uniform compositions, as the chromatograms of Benefat and Grindsted reveal.

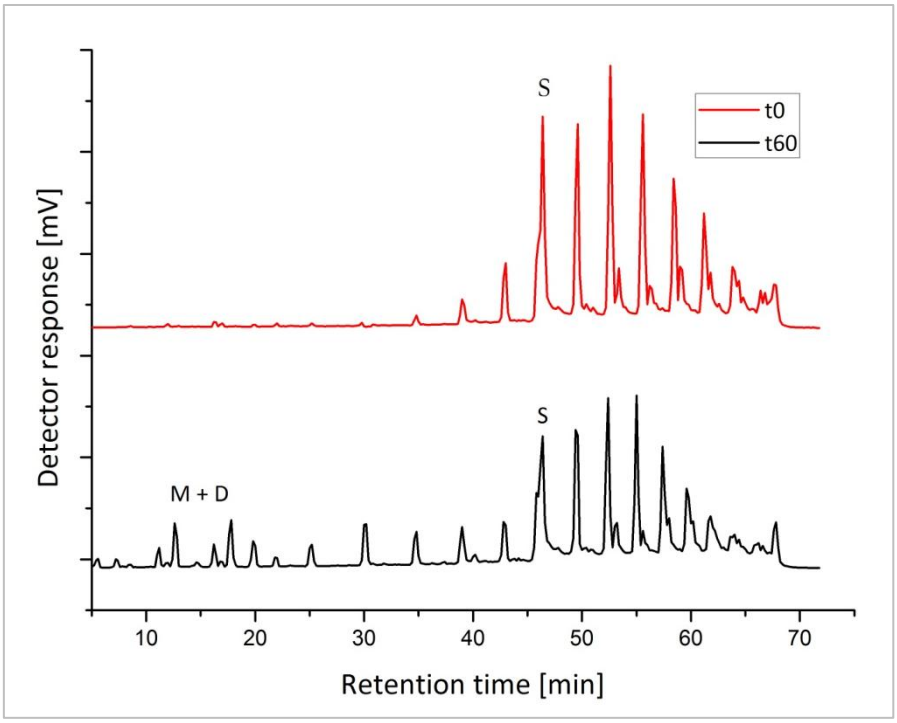

Fig. 4.31: RP-HPLC chromatograms of coconut butter at the beginning (t0) and end of reaction (t6o), presented in Y-axis offset; S is the solvent peak, M + D: mono-and diglyceride region.

The selection criteria for a reference lipid were that it represents an unmodified form of the substrate and has a similar melting point as the target lipid under investigation. However, as the pH-stat experiments show, the crucial factor in the rate and efficiency of lipolysis remains the behaviour of fatty acids at the emulsion interface. Thus, the profile of fatty acids is the single most important factor in evaluating lipid digestibility.

4.3.6 Summary – structured lipids

The enzymatic digestibility of several fats was tested with the model washing system. The target ingredients under investigation were structured lipids approved for use as fat substitutes in the EU. These were the oil Grindsted, which is a medium-chain triglyceride and the solid fat Benefat, which is a Salatrim with Novel Food status. Natural fats with similar melting points as the targets were used as reference substances in the experiments. The degree of total hydrolysis obtained for the lipid ingredients in the model washing system and for a time duration of 60 min ranged from 17 – 96 %.

The fats tested were emulsified in the working buffer of the model washing system prior analysis. The action of the lipase unfolds on the lipid-water interface of the emulsion droplets. It was found that the rate and extent of digestion of the triglycerides depends on the ability of their fatty acids to disperse in the aqueous phase of the emulsion. Therefore, the highest degree of total hydrolysis was obtained for Grindsted, followed by Benefat and coconut butter, which both possess short- and medium-chain fatty acids in their structure. The natural lipids under investigation, olive oil and lard, exhibited lower degree of hydrolysis, due to accumulation of their long-chain fatty acids on the interface and inhibition of lipase action.

The rate of digestion of all lipids was significantly improved when calcium ions from tap water were included in the substrate solutions. Calcium precipitates the long-chain fatty acids from the o/w interface. A further increase in calcium concentration would enhance the digestion rate, however it was considered unfeasible for the washing model to use calcium levels higher than those typically employed in home laundry applications.

Due to the good dispersability of short- and medium-chain fatty acids in the aqueous phase, it is unlikely that structured lipids would contribute to problematic stains. During a true laundry process, fatty acids and other surface-active products of lipolysis would be removed from the interface by detergent molecules. This solubilisation in micelles would ensure that even triglycerides with long-chain fatty acids are digested to a higher extent than the one obtained with the current experimental model. To test this hypothesis, the removal of lipid stains was tested with a laundry detergent under lipase or lipase-free conditions in a series of washing experiments. The results of these empirical investigations are presented in chapter 5.3.2 (results and discussion – fat replacers).

5. Washing experiments

5.1 General procedure of the washing experiments

The experimental model developed for evaluating stain-potential was focused on studying the enzymatic digestibility of the isolated target in solution. While this approach allows a fast screening of an ingredient class, it does not consider many other factors playing an important role in the formation and removal of stains on textiles. A serious assessment of stain-potential should include the effects of the food matrix, the interaction between stain and textile fabric, as well as the action of surfactant molecules and other detergent components during the laundry process. Thus, a series of empirical tests was devised, aiming to investigate staining by the target in a laboratory setting under conditions approaching real washing as closely as possible.

For this purpose, the target ingredient was formulated within a foodstuff, which was used to manually stain a textile fabric. The soiled cloth was washed in a Launder-Ometer® with a commercial laundry detergent. The washed fabric was allowed to dry and was then measured by a photometer to determine the efficiency of stain-removal. The general procedure of the washing experiments is presented in a schematic way in fig. 5.1.

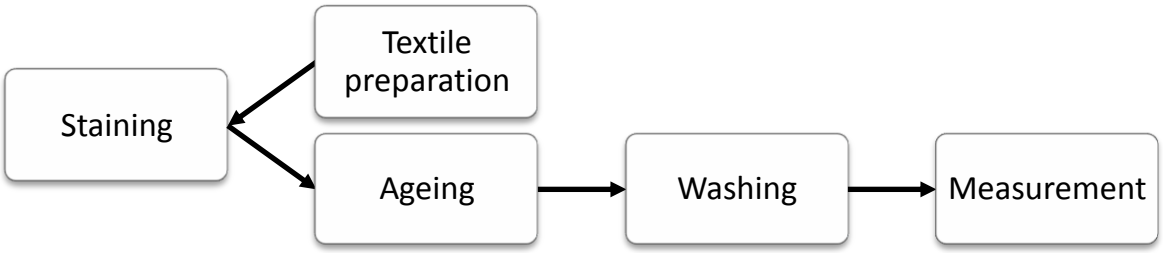

Figure 5.1: The general steps of a washing experiment

The results of the washing experiment were always compared to a control group. In general, this consisted of stains by the food matrix without the target ingredient. Through a comparison of the washing efficiency between target and control, it was possible to examine the effect of the target substance on staining.

Individual food recipes were developed for each target substance. The target substance was formulated at levels typical of its functionality (e.g. inulin was formulated at 3 % w/v, because this is the legal requirement for a "source of fibre" health claim of the product [88]). In addition, the foodstuff had to represent a matrix characteristic for the application of the ingredient (e.g. inulin as a prebiotic is frequently added to juices or joghurt [89]). Last but not least, the food matrix had to

possess a colouration, so as to be able to form a stain which is measurable by a photometer (e.g. plain joghurt does not possess a pigmentation, while a berry fruit joghurt does).

5.2 Materials and methods – washing experiments

The washing and measurement steps of the empirical procedure were performed in the labs of Henkel GmbH at the company headquarters in Düsseldorf, Germany. Henkel kindly provided many materials which were used in the washing experiments such as the pre-washed cotton fabric, liquid laundry detergent and the clamp rings. The stages of the washing procedure are described in further detail below.

Preparation of the textile

All experiments were performed on a white, pre-washed cotton fabric. The textile cloth was cut into strips of 36 x 12 cm, which were also called "test-strips". Circles of 5 cm diameter were drawn on the strips with a pencil. The circles designated the staining area on the fabric. A maximum of four such circles were positioned on the textile strip, as shown on fig. 5.2 below.

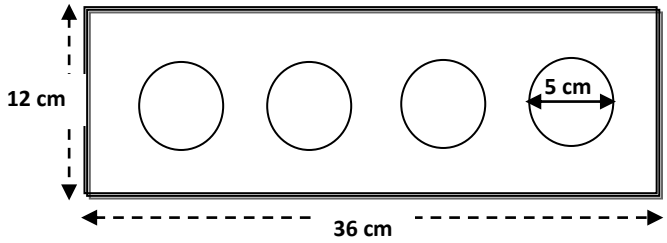

Figure 5.2: The dimensions of a typical textile test-strip used in the washing experiments

Staining

The composition of the stain was defined by recipes, developed individually for each target ingredient. The preparation of the food recipes is described in detail in "the PhD student's cookbook" in the appendix, chapter 7.3. All staining was performed manually, taking care to keep conditions reproducible (e.g. same operator and application of technique). For every stain type, an exactly defined amount was applied uniformly in the stain-circle of the test-strip. The amount of food material necessary for complete staining of the circle area was determined case-by-case in an empirical way. This was an important part of the stain optimization procedure [(90)]. The final amounts used for staining are listed in the manual staining table, appendix chapter 7.3.4.

Solid or viscous stain materials were applied manually on the cloth using gloves. Liquid stains were applied in the middle of the circle by dripping them with a pipette. A clamp ring was used to keep the fabric stretched and taut during application of the liquid stain [91]. The ring was removed after complete drying of the fabric. Figure 5.3 below shows a photograph of typical test-strips, which have been stained with a coloured food matrix in the designated staining area.

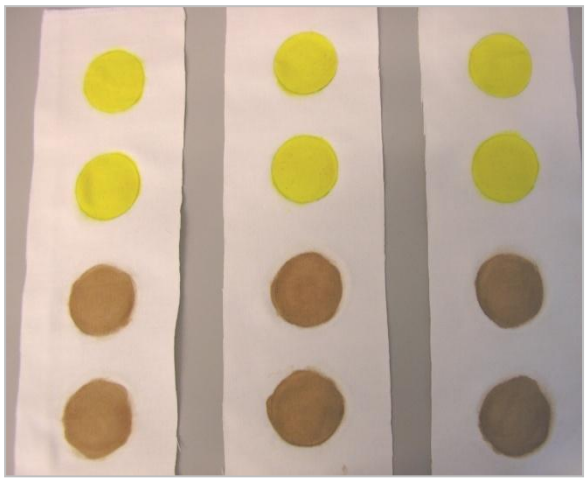

Figure 5.3: Test-strips with freshly applied stain material. The yellow circles are stains from curry sauce with xanthan as a texture agent. The brown circles are stains from chocolate joghurt containing inulin as a prebiotic.

Ageing

The stains were dried at room temperature. Afterwards, they were completely covered by aluminium foil and kept in the dark for seven days. The ageing of the stain mimics typical home laundry conditions. Most clothes are not washed immediately after soiling. Instead, they are kept for some time until a full load for the washing machine has been collected.

Washing

The washing of the test-strips was performed in a Launder-Ometer®. This is a standard machine, specially designed for rapid screening of washing performance on a laboratory-scale at temperatures up to 95 °C [87]. The Launder-Ometer® consists of a mounted reservoir with a total bath volume of 64 l. This tank contains a rotor, which has the capacity to hold twenty steel vessels of 1-l -volume (see fig. 5.4 on the next page). During a washing experiment, the vessels are filled with an appropriate test solution including samples of soiled fabric, i.e. “test-strips”. The vessels are sealed with a gasket and transferred to the Launder-Ometer®. The rotor is driven at a uniform speed of 40 rpm (± 2 rpm). The machine is operated by a control panel, which is typically positioned over the preheating loading table. When in operation, steel spheres are placed in the steel vessels, in order to stimulate

mechanical action during washing. In this manner, the Launder-Ometer® permits the parallel screening of up to twenty experiments in one washing cycle under controlled temperature and mechanical agitation [92].

Figure 5.4: The Launder-Ometer® equipment for screening washing performance (left) with the reservoir compartment open, revealing the rotor holding the steel vessels (right)

To perform a washing experiment, the reservoir compartment of the machine was filled with distilled water. The individual steel vessels were removed from the rotor and placed on a loading table. The containers were opened and filled with laundry detergent. 0.95 g of liquid laundry detergent and ten metal spheres of 10 mm diameter were added to each vessel. Afterwards, the pots were filled with 100 ml distilled water and 100 ml water of 32 °d hardness, resulting in a total hardness of 16 °d for the washing bath.

The weight of every test-strip was adjusted to 16.8 ± 0.5 g with filling fabric. This represents a wash liquor ratio of 1:12. The textiles were added to each vessel by putting the filling fabric in the container first and then placing the test-strip on top. Finally, the vessels were closed tightly and positioned evenly on the rotor in the washing compartment of the Launder-Ometer®. A washing program of 30 min duration with a temperature of 40 °C was started.

At the end of the washing cycle, the containers were opened and their contents emptied over a sieve. The textile fabrics were then immediately subjected to a rinse cycle of 4 x 30 ± 1 s with water from the mains supply in a special rinsing machine. Afterwards, the textiles were centrifuged and dried on a laundry rack. They were ready for measuring on the next day.

Measurement

The stain removal efficiency was determined by measuring the remission of light from the textile fabric. Remission is defined as the percentage of light which is reflected back from a material in comparison to an ideally white sample at a defined wavelength. The remission was determined as the Y-value according to IEC-norm 59D/165/CDV at 420 nm [92]. Measurements were performed with a spectrophotometer. The higher the Y-value, the "whiter" the sample appears to be. Y-values above 88 are perceived as completely white by the human eye.

The arithmetic mean and the standard deviation were calculated from the set of individual samples. A comparison between the target and the control was performed by a student t-test. The t-test is a statistical hypothesis test used to determine whether two means are significantly different from each other [93]. Calculated t-values (t-calc.) above the critical t-value (t-crit.) led to rejection of the null hypothesis (H0) and acceptance of the alternative hypothesis (H1).

Independent sample t-test: comparison between 2 means, unequal variances

Null hypothesis: H0 => $\mu 1 = \mu 2$ t-crit. = 2.45 (N = 6, $\alpha = 0.05$, two-sided)

Alternative hypothesis: H1 => $\mu 1 \neq \mu 2$

$$t_{calc.} = \frac{|\overline{x_1} - \overline{x_2}|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

If t-calc. < t-crit. => H0 accepted

If t-calc. ≥ t-crit. => H0 rejected, H1 accepted

The washing experiments were divided into experimental blocks according to the functional class of the ingredients tested. One additional block studied the effects of direct treatment of the textile fabric with the target. Table 5.1 below presents an overview of the empirical investigations along with a commentary of specifics. Some target ingredients were tested in more than one matrix or at several concentrations typical of their application.

Table 5.1: Overview of the washing experiments performed

Experimental block	Comments
Health ingredients	Stain-potential investigated by empirical tests only
Fat replacers	Additional experiments under lipase-free conditions
Hydrocolloids	Extended to hydrocolloids widely available in the market
Textile treatment	Target applied directly on textile followed by matrix staining

5.3.1 Results and discussion – health ingredients

The health ingredients under investigation were polydextrose and inulin. The target ingredients were not analyzed with the model washing system in the lab, but were directly subjected to empirical testing. Inulin is applied to foodstuffs as a prebiotic or as a source of soluble fibre [(94)]. Therefore, it was formulated at two different concentrations, required for assigning a health claim to the food or beverage product. An amount of 3 % (w/w) can legally carry "a source of fibre" claim, while levels of 6 % (w/w) or above can be labelled as "a high source of fibre" [(88)]. Polydextrose is also a source of dietary fibre, but can also act as a sweetener [(95)]. It provides mouth-feel and body to sugar-free beverages at a concentration of 10 % (w/v).

Inulin was formulated in foods typical for its application - elderberry juice and chocolate joghurt. Polydextrose was tested in elderberry juice. The preparation of the foodstuffs, as well as the amount used for staining, is described in the appendices 7.3.1 and 7.3.4. Table 5.2 displays the results of the washing experiments and their statistical evaluation. The calculated t-value was determined for N = 6 stain samples in each case. The t-critical value (N = 6, $\alpha = 0.05$) in a two-sided test was 2.45.

Table 5.2: Stain-removal efficiency of target health ingredients in typical food and beverages; (IEJ: inulin in elderberry juice, ICJ: inulin in chocolate joghurt, PD: polydextrose, s: standard deviation, H: hypothesis accepted)

	IEJ_3 %	IEJ_6 %	ICJ_3 %	ICJ_6 %	PD_10 %
Average Y	59.4	61.5	79.7	82.8	66.9
Control Y	55.9		75.9		57.6
s	4.0	1.3	1.2	0.6	0.7
t-calc.	1.87	4.64	4.39	8.97	8.02
H	H0	H1	H1	H1	H1

Except for inulin in juice (3 %) which does not differ from the control, all other experiments saw the alternative hypothesis being accepted. The stains with the target substance are significantly different from the stains of the control. If the Y-values are closely inspected, it becomes clear that stains containing the target ingredients are washed-out significantly better than the stains of the matrix alone (higher Y-values). The presence of the target substances also contributed to a texture effect on the matrix, with stains containing the target in higher concentration displaying lower standard deviations between individual stains (more uniform staining material).

The effect of higher washing efficiency in the presence of the target was surprising. Both inulin and polydextrose are carbohydrates which cannot be digested by detergent enzymes. At the washing temperature of 40 °C, they are only partially soluble in water. It was assumed that the molecules of the target bind the pigments of the matrix and bring them in solution. A paper chromatography

experiment was devised in which the migration of juice containing inulin was compared to the spot of inulin-free juice. No differences were observed (results not shown).

Polymers such as carboxymethyl cellulose (CMC) are added to detergents for their soil anti-redeposition effects on cotton textiles [96]. The molecules work by binding the stain particles in the wash solution, thus preventing their renewed adherence to the fabric. Inulin and polydextrose are carbohydrate polymers of smaller size than CMC, but probably exert a similar effect.

5.3.2 Results and discussion – fat replacers

The ingredients tested in this block were the fat replacers already described in the experimental part of the thesis. Since fats and oils often make up soils on their own, it was not deemed necessary to formulate the lipid ingredients into foodstuffs. As a result, the lipids were directly applied on the textile fabric. A dye was used to impart coloration to the fats, in order to render them visible and detectable by a photometer [97]. The stain-removal efficiency of the fat substitutes Grindsted and Benefat was compared to the control lipids olive oil and lard.

The fat mimetic Simplesse is used mainly in dairy products [98]. The microparticulated whey protein was formulated at the level of 3 % (w/w) in low-fat chocolate joghurt. The food matrix without Simplesse served as a control. Details of the recipe preparation, the colouration procedure of the fats, as well as the amounts used for staining are presented in the appendices 7.3.2 and 7.3.4.

The empirical investigation was expanded to study other factors which influence washing efficiency of lipids. For instance, it was of interest to differentiate the contribution of lipase and surfactants to the stain-removal process. For this purpose, the target ingredients and their controls were washed with a lipase-free laundry detergent. The results of this experiment were compared to the washing effects of a test group treated with standard laundry detergent. Thus, it was possible to evaluate the individual impact of lipase enzyme on the washing performance of the fats. For a description of the washing bath under lipase-free and standard conditions, see the appendix chapter 7.2.2.

Another aspect which was examined for its influence on the lipid stain-removal process was the effect of the storage temperature. An experiment was designed in which one lipid test group was aged at 10 °C for a week. The test-strips were transported in a cool box and exposed to ambient conditions just before washing with the Launder-Ometer®. The other test group was stored at room temperature for the same time duration. The stain-removal efficiency between the two groups was compared. Table 5.3 on the next page summarizes the results obtained for the solid fats and the washing experiments with Simplesse.

Table 5.3: Stain-removal efficiency of the solid fats under investigation and Simplesse; (B: Benefat, L+: standard laundry detergent containing lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, GL: goose lard; SCJ: Simplesse in chocolate joghurt, s: standard deviation, H: hypothesis accepted)

	B_L+T+	B_L+T-	B_L-T+	B_L-T-	GL_L+T+	GL_L+T-	GL_L-T+	GL_L-T-	SCJ_3 %
Average Y	83	83	81.5	81.6					79.7
Control Y					83.6	84.1	82.4	81.5	81.2
s	0.4	0.4	0.7	0.6	1.0	0.6	0.7	1.0	1.5
t-calc.									1.99
H									H0

The target ingredient Simplesse, a protein fat mimetic, will be discussed first, as it stands out from the rest of the investigation which was focused on lipid stain-removal. As the calculated t-value reveals, the stains with Simplesse were not significantly different than the ones imparted by the matrix alone. Therefore, the null hypothesis was accepted. The inclusion of Simplesse provides higher protein levels in a product. Nevertheless, the additional protein content did not contribute to problematic staining under the experimental conditions. This result is consistent with the experimental part of the thesis, which described the swift degradation of Simplesse into water-soluble peptides.

All solid fats under investigation displayed high Y-values (> 80) after washing. This indicated a high washing efficiency of the greasy soils. Since the lipids were not formulated into food, a natural fat of similar melting point served as a control. Thus, as table 5.3 shows, goose lard was employed as a control for the modified fat Benefat. A pair-wise comparison of means, as performed by the student t-test, is insufficient to evaluate the additional factors of storage temperature and lipase activity on the overall washing efficiency, without increasing the chance of type I error [(99)]. Hence, a one-sided analysis of variance (ANOVA) with a Tukey test was performed.

The calculated F-value of the ANOVA test for the solid fats was F = 11.8 at a level of significance α = 0.05 and with a probability of $p = 4.7 \times 10^{-8}$. Since the probability is less than the significance level α ($p \leq \alpha$), the null hypothesis was rejected and the alternative hypothesis was accepted. The means of the solid fats treated at various washing conditions are significantly different from each other. A multiple comparison was performed in which the mean of each experiment (comprising lipid type, lipase or lipase-free treatment and storage temperature) was compared to every other mean value. The results of this analysis are presented in a table form (table 5.4) on the next page. A graphical representation of the mean Y-values and their confidence intervals offers the advantage of cluster identification. In this manner, it is easier to identify groups of data which follow a common trend. The solid fats results are displayed visually in figure 5.5 on the next page as well.

Table 5.4: The alternative hypothesis H1: a multiple comparison of the individual means of the solid fats. (B: Benefat, L+: detergent with lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, GL: goose lard, =: no significant difference, +/-: significant difference)

	B_L+T+	B_L+T-	B_L-T+	B_L-T-	GL_L+T+	GL_L+T-	GL_L-T+	GL_L-T-
B_L+T+		=	+/-	+/-	=	=	=	+/-
B_L+T-	=		+/-	+/-	=	=	=	+/-
B_L-T+	+/-	+/-		=	+/-	+/-	=	=
B_L-T-	+/-	+/-	=		+/-	+/-	=	=
GL_L+T+	=	=	+/-	+/-		=	=	+/-
GL_L+T-	=	=	+/-	+/-	=		+/-	+/-
GL_L-T+	=	=	=	=	=	+/-		=
GL_L-T-	+/-	+/-	=	=	+/-	+/-	=	

The mean Y-values of the solid fats can be grouped into four major clusters, as shown in fig. 5.5 below. A glance to the y-axis reveals which washing conditions are shared between the means in the cluster. For both fats, higher stain-removal efficiency was obtained with lipase (clusters 1 & 3) compared to the sole application of surfactants (clusters 2 & 4). No significant differences between Benefat and lard were observed under lipase-free conditions (clusters 2 & 4).

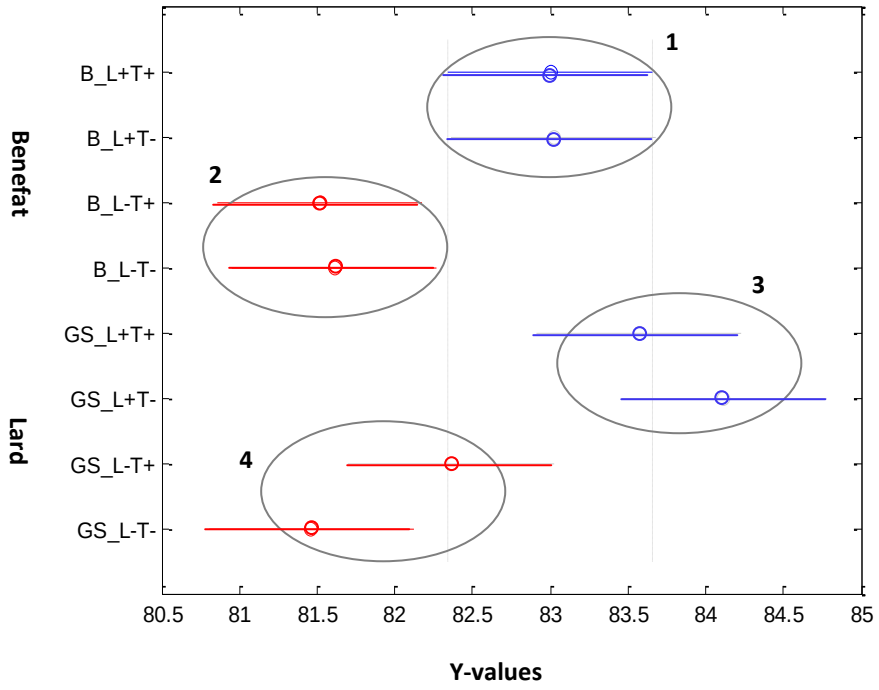

Figure 5.5: ANOVA analysis of the solid fats; the vertical lines represent mean Y-values plus confidence intervals; (B: Benefat, L+: laundry detergent with lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, GS: goose lard)

In contrast, lard was washed-out slightly better than the target Benefat under standard washing conditions with lipase (clusters 1 & 3). The difference, however, is not significant and should therefore be regarded as a mere tendency.

The storage temperature did not impact the stain-removal process of the solid fats in a way the presence of lipase in the laundry detergent did. The ageing conditions had a slight effect on the washing performance of lard (clusters 3 & 4), but not on Benefat, where no differences between cold and ambient treatment of the soils could be observed (clusters 1 & 2).

Table 5.5: Stain-removal efficiency of the liquid fats under investigation; (MCT: Grindsted, L+: standard laundry detergent containing lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, OO: olive oil; s: standard deviation)

	MCT_L+T+	MCT_L+T-	MCT_L-T+	MCT_L-T-	OO_L+T+	OO_L+T-	OO_L-T+	OO_L-T-
Average Y	83.9	84	83.4	83.9				
Control Y					83.5	83	82.5	83
s	0.4	0.3	0.4	0.4	1.1	0.5	0.5	0.4

The liquid fats under investigation were also assayed under lipase and lipase-free conditions, as well as at different storage temperatures. Table 5.5 above lists the results obtained for each experimental group. Grindsted and its control olive oil displayed high washing efficiency with Y-values in the 80's magnitude. The means from the empirical tests were compared to one another with the one-sided ANOVA test. The calculated F-value was F = 6.01 at α= 0.05 and with p = 7.9 x 10^{-5}, which led to a rejection of the null hypothesis and alternative hypothesis approval (since $p \leq \alpha$).

Table 5.6: The alternative hypothesis H1: a multiple comparison of the individual means of the liquid fats. (MCT: Grindsted, L+: standard laundry detergent containing lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, OO: olive oil, =: no significant difference, +/-: significant difference)

	MCT_L+T+	**MCT_L+T-**	**MCT_L-T+**	**MCT_L-T-**	**OO_L+T+**	**OO_L+T-**	**OO_L-T+**	**OO_L-T-**
MCT_L+T+		=	=	=	=	=	+/-	=
MCT_L+T-	=		=	=	=	+/-	+/-	+/-
MCT_L-T+	=	=		=	=	=	=	=
MCT_L-T-	=	=	=		=	=	+/-	=
OO_L+T+	=	=	=	=		=	=	=
OO_L+T-	=	+/-	=	=	=		=	=
OO_L-T+	+/-	+/-	=	+/-	=	=		=
OO_L-T-	=	+/-	=	=	=	=	=	

The stains of the liquid fats, treated at various washing conditions, are significantly different from each other. When a multiple comparison of individual means was performed, (table 5.6 on the previous page) it becomes clear that the statistically significant differences between the values are few and situated at the extremes of experimental conditions. This is best illustrated with a graph of the mean Y-values, as shown in fig. 5.6 below.

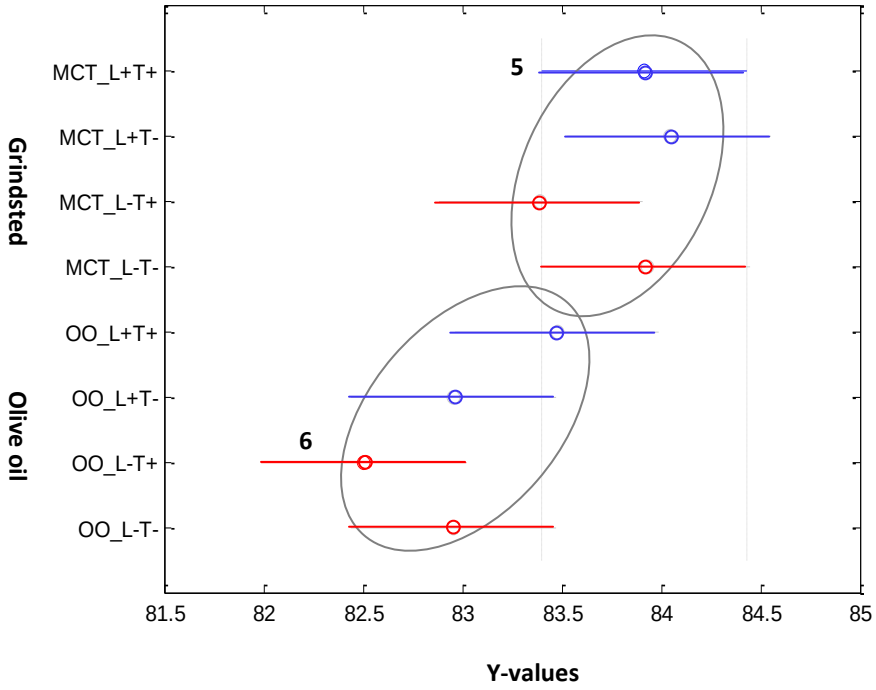

Figure 5.6: ANOVA analysis of the liquid fats; the vertical lines represent mean Y-values plus confidence intervals; (MCT: Grindsted, L+: laundry detergent with lipase, L-: lipase-free laundry detergent, T+: ageing at ambient temperature; T-: ageing at 10 °C, OO: olive oil)

The mean Y-values are situated close to one another, making cluster identification more difficult than in the solid fats case. Nevertheless, the data was divided into two major groups according to lipid type (clusters 5 & 6). For both liquid fats, the action of surfactants alone was sufficient for stain-removal. Indeed, there was no significant difference between the soils washed under lipase-free and standard washing conditions with lipase. The effect of the enzyme was more pronounced when the stains were stored at ambient temperature. In contrast, soils which were kept at 10 °C did not display higher Y-values on lipase treatment.

When the washing efficiency between target and control was compared, no significant differences between Grindsted and olive oil were observed under lipase-free conditions. However, the target Grindsted displayed the tendency to be washed-out slightly better than the control under standard washing conditions.

To conclude, the targets and their controls were tested under a variety of conditions designed to study the washing efficiency of lipids. The ingredients displayed high Y-values after washing, verifying the excellent fatty- stain-removal capacity of the laundry detergent. The presence of lipase enzyme in the detergent formulation improved the stain-removal process in some cases. However, high remission values were obtained for all lipids by the action of surfactants alone, signifying that these are the major players in the lipid stain-removal process. It must be noted that the impact of lipase increases at lower washing temperatures, when surfactants on their own are not as effective [(2)]. All experiments performed in this thesis were carried out at the standard washing temperature of 40 °C.

There are many other factors which affect the stain-potential of lipids. One aspect which was examined in the washing experiments was the influence of the storage temperature. Because differences in stain-removal were observed under otherwise identical washing conditions, it was hypothesized that the storage temperature had an impact on staining. Exposure to higher ambient temperatures is likely to lead to some oxidation of the fats. However, the influence of the storage temperature could not be generalized. Different ageing conditions influence the stain, but the effects are generally too subtle to be significant.

In the experimental part of the thesis it was shown that the enzymatic digestion of lipids is inhibited by accumulation of fatty acids on the emulsion interface. Therefore, natural lipids bearing long-chain fatty acids, such as lard and olive oil, displayed low lipolysis rates with the experimental model washing system. Just as predicted, no such inhibition was observed under washing conditions in a Launder-Ometer®. The surfactants in the laundry detergent would solubilise both the greasy stains and the lipophilic products of enzymatic digestion. To conclude, the removal of stains resulting from novel fat substitutes is just as efficient as the washing performance of ordinary greasy soils.

5.3.3 Results and discussion – hydrocolloids

Various hydrocolloid ingredients were investigated in the washing experiments for an evaluation of their stain-potential. The target substances included relatively novel additions to the food developer's repertoire (e.g. gellan), but also classical hydrocolloids such as gelatine, pectin and guar gum. Some ingredients were included because of novel applications (e.g. encapsulation with agar or carrageenan), others because they have become essential in specific food applications (e.g. gum arabic). The aim of this experimental block was to test a wide range of hydrocolloids and obtain information of their stain-potential by the empirical procedure developed.

Joghurt was selected as a matrix for many of the target ingredients. Hydrocolloids, in their function as gelling agents and thickeners, are frequently added to cultured milk products. They minimize syneresis by reinforcing the natural casein-gel network of joghurt and provide texture to the finished product [30]. The target ingredients formulated in joghurt were agar, gellan, low-methoxyl amidated pectin (LM pectin) and gelatine. The joghurt was processed with elderberry juice and sugar to a joghurt-juice drink, in order to impart the matrix with coloration.

The content of juice was increased in the recipe for high-methoxyl pectin (HM pectin), resulting in a more acidic dairy drink. The creation was named elderberry lassi and was used as a matrix for HM pectin, which typically acts as a stabilizer for casein proteins in the low pH region (3.7 to 4.3) [100]. Since the joghurt matrix was not suitable for the remaining target ingredients, other recipes were developed for formulation.

The galactomannans guar gum and carob gum were employed as thickeners in a ketchup matrix. Carrageenan was formulated in chocolate milk, where it stabilizes casein and keeps cacao particles suspended. CMC was blended in an instant drink powder, where it quickly provides body and mouth-feel, when the drink is hydrated for consumption. Gum arabic served as stabilizer in a juice product. And last but not least, xanthan was formulated in ketchup and in curry sauce for its thickening effects. All details of the recipes preparation, the concentration of the texture agents, as well as the amounts used for staining can be reviewed in the appendices 7.3.3 and 7.3.4.

The results of a first group of hydrocolloids are presented in table 5.7 below. The calculated t-value for all ingredients was higher than the critical value (t-crit. = 2.45), resulting in rejection of the null hypothesis and approval of the alternative hypothesis. This confirms that the washing efficiency between targets and controls is significantly different. The inclusion of some hydrocolloids (carob gum, guar gum, CMC and carrageenan) in the matrix resulted in better washing-out of the stain (higher Y-values). A similar effect was observed with the health ingredients (chapter 5.3.1). Enzymatic action might be responsible for the improved stain-removal of the galactomannans, since mannanase enzyme was present in the laundry detergent formulation.

Table 5.7: Stain-removal efficiency of hydrocolloids in typical foodstuffs; (s: standard deviation, H: hypothesis accepted)

	Agar	Carrageenan	CMC	Carob gum	Guar gum	Gelatine
Average Y	67.8	58.1	71.0	69.6	68.8	68.1
Control Y	69.2	51.2	66.4	64.9		69.2
s	0.5	3.0	1.0	1.3	0.8	0.7
t-calc.	4.57	4.75	5.67	6.82	7.01	3.02
H	H1	H1	H1	H1	H1	H1

Enzymatic action is not always a guarantee for efficient stain-removal, as the results with gelatine reveal. The joghurt stains with gelatine were washed-out poorer than the stains of the control. The additional protein levels of the product led to harder staining, despite the availability of protease in the laundry detergent. The occurrence of such an effect cannot always be predicted. The fat mimetic Simplesse, also a protein, was formulated at a concentration of 3 % (w/v) in the product and it failed to cause significant staining. Gelatine, in contrast, was applied at the modest level of 0.3 % (w/v). However, it must be noted that Simplesse as a whey protein displays excellent digestion characteristics.

The target ingredient agar also contributed to staining at the level of 0.3 % (w/v). Agar is not a substrate for any of the detergent enzymes and is insoluble at the standard washing temperature.

The results of the washing experiments with the remaining hydrocolloids are presented in table 5.8. In the case of gellan and pectin, the null hypothesis was accepted. No differences in stain-removal between target and control were observed. Gellan is similar to agar, for it is insoluble at the washing temperature and it cannot be digested by detergent enzymes. This would generally make it a molecule with high stain-potential. Due to its excellent gelling characteristics, however, it is applied at very low dosage levels in food systems (here 0.03 % w/v). As a result, it did not contribute to stain-formation.

Table 5.8: Stain-removal efficiency of target hydrocolloids in typical food and beverages; (HM: high methoxyl, LM: low methoxyl, XanK: xanthan in ketchup, XanCS: xanthan in curry sauce, s: standard deviation, H: hypothesis accepted)

	Gellan	Gum arabic	Pectin HM	Pectin LM	XanK	XanCS
Average Y	68.9	36.9	71.5	68.7	81.9	81.6
Control Y	69.2	41.2	71.4	69.2	82.6	80.5
s	0.2	0.6	0.3	0.5	1.8	0.35
t-calc.	1.12	8.83	0.41	1.36	0.89	4.09
H	H0	H1	H0	H0	H0	H1

The results with the pectin ingredients were surprising. It is known that pectin causes staining and pectinases for detergent applications are available. However, these were not included in the experimental detergent formulation. The tested pectin ingredients (citrus origin) did not contribute to staining in the single washing performance test.

The alternative hypothesis was accepted for gum arabic. Stains with the target were washed-out with poorer efficiency than the stains of the control (lower Y-values). Thus, gum arabic causes staining, despite good water solubility of the ingredient.

Xanthan was tested in two different matrices, ketchup and curry sauce. The null hypothesis was accepted for the target in ketchup with no significant differences detected between target and control. On the other hand, the alternative hypothesis was accepted for the curry sauce matrix. Curry sauce stains with the target were washed-out significantly better (higher Y-values) than the stains of the sauce matrix alone.

With this observation, xanthan joins a range of hydrocolloids which were found to improve the washing performance of the staining material. The galactomannans, CMC and carrageenan were the other target ingredients in this experimental block which displayed this behaviour. It was hypothesized, that a good solubility of the target at the washing temperature would make it go in solution while binding other components of the stain, thus effectively removing them from the textile fabric. Indeed, xanthan, the galactomannans, CMC and carrageenan are well-soluble at 40 °C and fit this hypothesis. Agar and gellan are the only targets which are insoluble at the washing temperature. Agar was found to contribute to staining, while gellan did not, probably due to the very low levels necessary for its functionality.

The true mechanism of stain-removal by the hydrocolloids is probably more complex. Gum arabic exhibits excellent solubility at all temperatures and yet contributed to staining. The pectin ingredients are also well-soluble at the washing temperatures, but did not cause better washing-out of their stains. Gelatine is a protein, but is also soluble at 40 °C. The composition of the matrix surely also plays a role, with xanthan improving washing efficiency in curry sauce, but not in ketchup.

Anionic polymers such as CMC are added to detergents for their soil anti-redeposition effects on cotton fabrics. Such agents act by adsorption onto the fabric surface, thus sterically inhibiting the redeposition of previously removed particulate matter. Alternatively, they bind soil particles in the washing bath, preventing their renewed adherence to the textile [(96)]. Perhaps the observations made in this thesis are explained by some hydrocolloids acting as soil anti-redeposition agents from within the food matrix itself. This hypothesis can be tested by repeating the experiments with another textile fabric, such as polyester. Anionic soil anti-redeposition agents are generally ineffective on polyester fabrics. Non-ionic polymers are better suited for this role.

Apart from CMC, carrageenan and xanthan are also anionic polymers with good solubility at 40 °C. A way to study their soil anti-redeposition effects would be to test different concentrations of the target in the matrix. The hydrocolloid levels at which improved washing-out of the stain occurs can be determined. The effect should be mainly detected on cotton fabrics and be negligible on fabrics of more hydrophobic nature (e.g. polyester).

5.3.4 Results and discussion - textile treatment

The aim of this investigation was to analyze whether differences in stain-removal occur when the target is directly applied on the textile and is not formulated within a matrix. The target ingredients tested in this way were inulin and xanthan. The washing performance of these targets was already evaluated in previous experimental blocks, including the behaviour in more than one food matrix.

The impregnation of the fabric was performed by dripping an aqueous solution of the ingredient on the test-strip. The cloth was allowed to dry completely, after which it was stained by application of the food matrix. Stains were stored for seven days and washed according to the standard procedure. The controls were performed by dripping deionized water on the fabric, followed by staining with the food matrix. A detailed description of the textile treatment and the materials used for staining can be found in the appendix 7.1.12 Textile impregnation procedure. The results of the investigation are presented in table 5.9 below.

Table 5.9: Stain-removal efficiency of direct treatment of the textile with an aqueous solution of the ingredient; (EJ: elderberry juice, K: ketchup, s: standard deviation, H: hypothesis accepted)

	Inulin_6 % + EJ	Xanthan_0.5 % + K
Average Y	44.9	70.4
Control Y	43.3	66.0
s	1.5	2.2
t-calc.	2.17	4.75
H	H0	H1

The null hypothesis was accepted for the textile treated with inulin solution. The soils on the treated fabric were not significantly different from stains of the matrix alone. In contrast, the results described previously had shown that stains of inulin-containing foodstuffs were always washed-out better than the controls. The textile treated with xanthan confirmed prior observations. Stains on xanthan-treated fabrics displayed higher washing efficiency than untreated test-strips.

Both inulin and xanthan give colourless solutions and do not contribute to staining on their own. It is their interaction with the food matrix which leads to differences in stain-removal. The interplay between target and matrix is based on the ability of the ingredient to dissolve in the foodstuff. Mild heating is required to completely bring inulin into solution. As a result, the ingredient and the matrix do not truly mix and no effect on stain-removal is observed. Xanthan is easily soluble at any temperature. When the ketchup is applied on the fabric, some xanthan on the fabric re-dissolves in the matrix and is able to exert the effects as previously described.

5.3.5 Summary – washing experiments

The washing experiments were a series of empirical tests designed to study the stain-potential of novel food ingredients in an environment resembling true washing conditions on a laboratory scale. For this purpose, the target ingredients were formulated into foods or beverages according to their functionality and application. The food matrix was used to manually stain a white cotton test-strip. The stained textile was washed with a Launder-Ometer® and stain-removal efficiency was determined by remission measurements with a spectrophotometer. The washing performance of the target was always compared to a control, which consisted of the food matrix alone.

A student t-test was used to evaluate whether the stain-removal efficiency of the target and the control are significantly different from each other. The food ingredients tested with the empirical procedure included selected health ingredients, fat replacers approved on the EU market, and traditional, as well as novel hydrocolloids. The washing performance of the target ingredients in comparison to their controls is illustrated graphically in fig. 5.7 below.

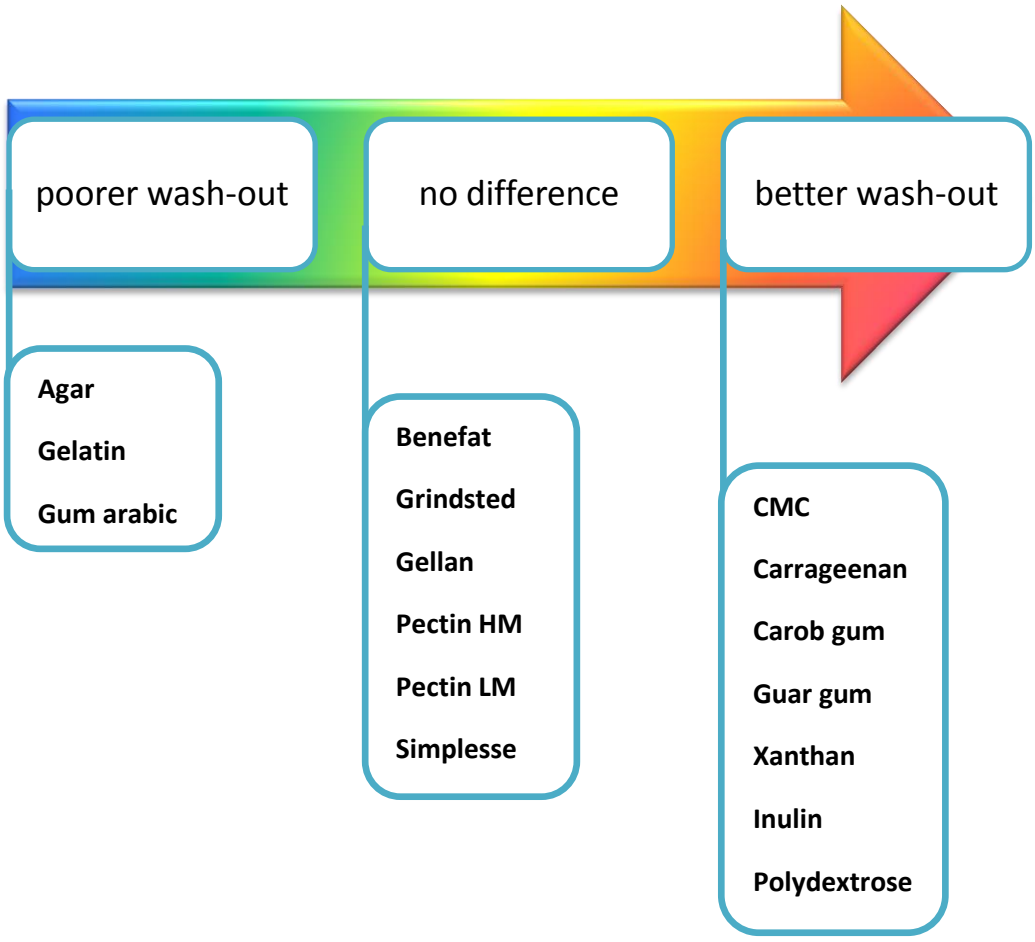

Figure 5.7: Stain-removal efficiency of the target substances in comparison to their controls

The presence of some ingredients in the food matrix contributed to better washing-out of the stain. This effect bears resemblance to the soil anti-redeposition effect of polymers added specially for this purpose to laundry detergents. Some carbohydrate ingredients could apparently act in a similar manner from within the food matrix itself. The ingredients displaying this tendency are all partially or well-soluble in water at the washing temperature. They do not represent substrates for detergent enzymes, with the exception of the galactomannans. The ingredients enhancing the washing efficiency include neutral molecules (inulin, polydextrose, galactomannans), as well as charged hydrocolloids (CMC, carrageenan, xanthan).

Other ingredients did not seem to impact staining by the matrix in any way. All lipids under investigation displayed very good stain-removal efficiency by the action of surfactants alone. The additional protein levels provided by Simplesse did not lead to differences in washing performance. Gellan and both pectin types did not influence staining, unlike other hydrocolloids tested. While pectin is known to contribute to stain-formation, this was not observed in the washing experiments.

The ingredients which did cause more difficult staining were agar, gelatine and gum arabic. The protein gelatine is a classical hydrocolloid, which can be digested by detergent proteases, but contributed to stain-formation due to elevated protein levels in the matrix. The target agar displays characteristics, which classify it as a high-potential stain ingredient. It has a large molecular weight, it is insoluble in water at the washing temperature and it cannot be digested by detergent enzymes. Gellan is another molecule which possesses similar properties, but was not found to influence staining, probably due to its very low levels of application.

Solubility is not a decisive criterion whether a substance will contribute to staining, as gum arabic shows. This hydrocolloid displays excellent solubility in water at all temperatures. Gum arabic is mainly used in beverages as a stabilizer. Commercial products are highly diluted, so that the final concentration of the gum is typically low. The amount of gum arabic tested in this work was the highest concentration found in common applications. However, most products on the market would avoid such levels of the gum, due to the high price of the raw material. Nevertheless, the results show that gum arabic can contribute to difficult staining.

In order to perform the remission measurements, the stain had to possess a colour, which had to be detectable even after washing. Since the target ingredients did not possess pigmentation of their own, they had to be treated with dyes (in the case of lipid ingredients) or formulated within a strongly coloured food matrix. Thus, it was important to differentiate between stain-formation by the target and matrix effects. Apart from the target – control reference frame, this was studied by formulating the ingredient in more than one food matrix type.

Another approach was to treat the textile with an aqueous solution of the target and then to apply the matrix on top. In this manner, the conditions under which the target influences staining could be investigated. However, the determination of stain-formation by the target always remains an indirect method, based on measurement of the food matrix.

Conducting washing experiments is a time-consuming and labour-intensive process. Despite limits to the number of tests which could be performed, the results of this thesis can be used for the foundation of a standard procedure for evaluating stain-potential of novel food ingredients. The optimal analysis method should include a large sample basis (number of stains), in order to eliminate variation due to the sensitivity of the procedure. Using a single operator and identical application technique on a given experimental batch is recommended for maximal reproducibility. Variations also result from the food matrix itself. As a natural product, food is subjected to many fluctuations due to seasonality, processing conditions, raw material availability, etc. It is best to make the recipes oneself if possible, or to use the same product batch in all experiments. As the results with the lipid ingredients have shown, even changes in storage conditions and ambient temperature can lead to different washing performance of the targets. Testing more than one food matrix is also advisable. In this way, the interactions between ingredient and food components can be better understood and generalized. Furthermore, it would be meaningful to analyse the target at different concentrations in the food matrix, as to be able to pinpoint the exact application level, at which an observed effect occurs.

The findings of the washing experiments reveal how difficult it is to predict stain-potential by a sole examination of ingredient properties. Neither good water solubility, nor digestibility with detergent enzymes can guarantee that an ingredient would not contribute to staining. Stain-potential is the outcome of a complex interplay between the target and the components of the food matrix. Therefore, the empirical approach remains absolutely necessary for evaluating stain-potential reliably.

6. Conclusion and outlook

This thesis approached the laundry process from the perspective of how the changing composition of foodstuffs might impact the type of soils found on fabrics. Two different procedures were used to determine the stain-potential of various novel food ingredients. The first method consisted of a model washing system which studied the enzymatic digestibility of the target substance in solution. The second method involved empirical screening of the targets in the form of washing experiments.

Taking enzymatic digestibility as a model for determining stain-potential was justified for most of the target substances which are hydrophilic molecules with large molecular weight such as proteins, starch and polysaccharides. Detergent enzymes serve as well-established tools for the stain-removal of such ingredients. The advantage of the experimental approach is that by developing analytical methods for a particular ingredient class, it is possible to quickly screen a wide range of ingredients for their stain-potential. For example, by developing chromatographic and photometric methods for studying starch digestion, it is possible to study the enzymatic breakdown of a variety of modified starches, native starches and even some polysaccharide gums.

The laboratory screening method provides information how well a detergent enzyme digests a substrate in comparison to a reference substance. This remains a relative assessment of stain-potential. The target must be digested into smaller, water-soluble fragments, which can be easily removed by the washing solution. The experimental model on its own cannot determine the extent to which a target substance should be digested, so that it is able to be successfully washed-out.

The major limitation of the experimental screening of stain-potential, therefore, is that it cannot forecast all effects occurring during true washing conditions. This was especially apparent in the experiments with the modified lipids. The enzymatic reaction was inhibited by accumulation of long-chain fatty acids on the emulsion interface. These effects would not have been observed under true washing conditions, as surfactants would have solubilised the lipolysis products. The enzymatic model is less suitable for the study of modified lipids, since surfactants are the major players in the stain-removal of fats and oils. On the other hand, the use of the experimental model for the study of target lipids is not entirely unreasonable, since lipases are more prominent at lower washing temperatures where surfactants are not as effective. The focus of this work was set on the standard washing temperature (40 °C). However, lower washing temperatures are becoming increasingly important due to reasons of energy efficiency.

In order to be able to evaluate stain-potential of the target substances under conditions approaching “true” washing as close as possible, a series of empirical tests were designed. The strength of the empirical procedure lays in the fact that it considers the multitude of factors influencing stain-

potential – the food matrix, the fabric, mechanical agitation, etc. Washing experiments are surely the most reliable method in the study of ingredient stain-potential. However, empirical screening requires time, specialized equipment and a large amount of labour input. Ingredients should be tested in at least one food matrix type which reflects the functionality of the substance and resembles typical market products. The need to develop suitable and individual recipes for every target substance constitutes a lot of the time and labour effort in this type of analysis. Care should be taken during the interpretation of results, as the procedure is sensitive and displays subtle effects on reproducibility. For example, even slight variation in storage temperature of the lipid targets led to different washing performance.

Both approaches to studying stain-potential, the experimental model based on enzymatic digestibility and the empirical washing model, have their merits and can be used to complement each other. One way to perform an economical and focussed evaluation of a group of targets, e.g. modified starches, would be to start with studies of enzymatic digestibility. In this way, it is possible to screen relatively quickly a large number of molecules for their stain-potential. The target substances which are most likely to contribute to difficult staining, due to their slower enzymatic digestion, can then be specifically subjected to washing experiments.

One strategy of dealing with high stain-potential ingredients is to adapt the detergent enzyme concentration in the formulation. Of course, this concept would only apply to target substances which are substrates for available detergent enzymes. Target substances with high stain-potential are frequently from the hydrocolloid category, for which there are no established detergent enzyme systems. Indeed, the washing experiments in this work have shown that hydrocolloids like gum arabic and agar are likely to constitute problematic stains. There are currently no enzymatic solutions for stain-removal of these ingredients. At this point it is interesting to consider: which circumstances make the development of a targeted enzyme solution to a novel food ingredient worthwhile?

The widespread use of a hydrocolloid gum known to cause staining frequently becomes a motive to introduce enzymatic solutions to stain-removal in detergents. A recent example of this is the development of the detergent enzyme mannanase for dealing with galactomannans, which are used extensively as texture agents in foods and cosmetics. In fact, guar gum is the most widely used hydrocolloid gum worldwide. Would guar remain the dominant hydrocolloid system in the future?

If recent trends are examined, it doesn't seem that this scenario is probable. Guar has been traditionally available to the food industry at very cost effective prices. All this is beginning to change with demand from oilfields pushing the prices for the ingredient at record high levels. The oil and gas industries use guar gum as a lubricant in the process of horizontal drilling, known as "fracking". India,

the major guar gum producer in the world, is increasing production capacity, but prices are expected to remain high and supplies short [101]. As a result, many food manufacturers are considering reformulation with other ingredients such as xanthan or modified starch on the long run.

The supply of other hydrocolloid ingredients is in a similar state, due to climate events and energy prices. Changing temperatures in countries traditionally associated with seaweed production, like Malaysia and the Philippines, have led to failing crop yields. The supply of carrageenan and alginates is affected, as farmers switch to less risky crops. Climate change is also the reason for low citrus fruit yields in Latin America and the US, affecting the supply of pectin ingredients. The hydrocolloid market is characterized not only by shrinking supply, but also by growing demand by industries other than food. For example, hydrocolloids are becoming highly popular in consumer products, e.g. cosmetics, as natural replacements of petroleum-derived chemicals [102].

The dominant hydrocolloid of the future, therefore, is not likely to consist of a single ingredient. Rather, manufacturers are expected to develop food processes which can switch from one ingredient type to another according to the supply/cost situation [103]. Starches, especially starch derivatives, will continue to play a major role in food processing, due to their wide availability and low cost, compared to other hydrocolloids [34]. In Europe, there is a negative perception of chemically modified starch by consumers. Therefore, much attention has been focused on the development of label-free starch ingredients, which usually involve physical modification, e.g. superheated starch [104], annealed starch [34], amylomaltase-treated starch [105], etc. However, no alternative to date has proven capable of eliminating the need for chemically modified starches in the multitude of their applications.

Gums are likely to keep some market share due to their unique properties, especially providing texture while maintaining a complex flavour profile. Important developments to look for include second generation hydrocolloids which are gum blends treated by various chemical and physical methods, in order to impart them with a desired functionality [106]. Examples include OSA-modified gum arabic [107], modified sugar beet pectin [108] and diverse starch-hydrocolloid blends [34].

As this work has shown, the modern laundry detergent is well-equipped for stain-removal of most novel food ingredients. Nevertheless, it is important to watch the market for ingredients with high stain-potential, such as the hydrocolloids and starches. These ingredients will remain relevant in a food industry dominated by cost, convenience and health. Furthermore, the rise of future technologies in food processing, e.g. nanotechnology, will surely influence the way food materials impart stains. Particles below 0.2 µm adhere strongly to fibres and are difficult to wash-out, indicating that edible nano-structures [109), (110] might constitute the next generation of problematic food stain-materials.

7. Appendices

7.1 Methods and protocols

7.1.1 DNSA

The assay [111] is calibrated with a reducing sugar standard in the 0 – 10 mM range. Maltose is recommended for studying starch digestion, while D-galactose is suitable for characterising agar hydrolysates [112]. The following procedure can be used for enzymatic hydrolysis reactions:

- Samples of 150 µl volume are withdrawn from the reaction mixture at specified time intervals
- The reaction is stopped by addition of 300 µl DNS reagent
- The sample is incubated at 99 °C for 20 min in a heating block
- 1500 µl distilled H_2O is added to the microtube and the sample is placed on ice for 20 min
- The absorbance of the sample is measured with a photometer at 540 nm using an appropriate blank

The blank consists of substrate solution to which the enzyme has been added after the DNS reagent. In this case, 300 µl DNS reagent are directly pipetted to 100 µl substrate solution. Afterwards, 50 µl solution of the enzyme is added to the microtube. Measurements should be performed in triplicates at least.
For the calibration, 150 µl of each standard is directly mixed with 300 µl DNS reagent. After that, the standards are treated in the manner described above. The absorbance at 540 nm can be read in suitable cuvettes or in a plate reader after pipetting the sample into the corresponding wells.

7.1.2 Acid hydrolysis procedure

A portion of the polysaccharide to be analyzed (ca. 50 mg) is accurately weighed in a microtube with an analytical balance. 1 ml HCl (2 M) is added to the microtube, after which it is incubated at 99 °C for 2.5 h in a heating block. Consequently, 1 ml NaOH (2 M) is added for neutralisation. The hydrolysate can then be diluted (100x) for quantification by the DNSA or any other suitable method for the determination of sugar.

7.1.3 Bradford assay

The assay is performed in 96-well plates. BSA (or the target protein, if available in pure form) is used as a standard for the calibration curve in the concentration range 100 – 1500 µg/ml. The following protocol can be used for protein hydrolysates after the enzymatic reaction has been terminated by heat treatment or pH shift:

- Pipette 10 µl of each standard or unknown sample into a specified micro plate well
- Add 300 µl of Coomassie reagent to each well and mix with plate shaker for 30 s
- Incubate 10 min at room temperature
- Measure the absorbance at 595 nm with a plate reader

Subtract the average blank measurement from the measurements of all other standards and samples. Prepare a calibration curve by plotting the measurements for each standard vs. its concentration in µg/ml. Use the curve to determine the protein conc. of an unknown sample.

7.1.4 Protease assay

The assay is calibrated with L-tyrosine standards in the range of 0 – 0.04 mM. The following procedure can be used for determining the activity of a protease preparation:

- The protease is diluted to a suitable activity in 10 mM NaAc buffer (pH 7.5), containing 5 mM CaAc
- 1ml of the preparation is added to 5 ml casein 0.65 % (w/v) in 0.05 M phosphate buffer, pH 7.5
- The reaction is allowed to proceed for exactly 10 min at 37 °C in a water bath
- The reaction is stopped by addition of 5 ml trichloroacetic acid (110 mM)
- The test tube is mixed and incubated for further 30 min at 37 °C
- Filtration of the tube contents through a Whatman filter paper # 50

The filtrate is used for the further procedure which involves the colour development with the Folin & Ciocalteu reagent. The standard solutions of L-tyrosine are treated in an identical manner.

- 2 ml of the filtrate or standard is mixed with 5 ml Na_2CO_3 solution (0.5 M)
- 1 ml of the Folin & Ciocalteu reagent is added and the test tube is incubated at 37 °C for 30 min
- The test tubes are allowed to cool to RT and the absorbance is read at 660 nm in suitable cuvettes

One protease unit is defined as the amount of enzyme which will produce the colour equivalent to 1 µmol of tyrosine per minute at pH 7.5 at 37 °C.

7.1.5 SDS PAGE

The electrophoretic separation of the protein samples is performed using a 6 %-SDS-polyacrylamide stacking gel and a 12 %-separation gel. The PAGE is carried out in a vertical electrophoretic chamber using Tris-Glycine-SDS (TGS) as running buffer. The protein samples are mixed 1:1 with SDS sample buffer, then heated at 95 °C for 5 min, centrifuged at max speed for 1 min, and finally loaded on the gel together with a protein marker. Gels are run first at 100 V for 30 min until the stacking gel is passed and then at 200 V for 1 h. For the silver staining procedure the following protocol can be used:

- The gel is laid in the fixing solution for 30 min, after which it is washed with dist. H_2O twice
- The gel is laid in Farmers reducer for 2.5 min
- The gel is washed for 5 min with dist. H_2O or until it is colourless
- The gel is laid in 0.1 % (w/v) $AgNO_3$ for 30 min and then rinsed with dist. H_2O for 30 s twice
- The gel is rinsed with Na_2CO_3 solution (2.5 %)
- The gel is laid in a bath containing 100 ml Na_2CO_3 solution (2.5 %) and 400 µl formaldehyde
- Wait until yellow-brown bands begin appearing
- The reaction is stopped by laying the gel in 5 % HAc for 10 min

7.1.6 HPLC method "oligosaccharide analysis"

This is a method for the separation and quantification of oligosaccharides based on size-exclusion and ligand-exchange. The method was developed on a Polyspher CH Na column (Merck). Summary of the parameters:

- Column oven temperature: 85 °C
- Mobile phase: ultrapure H_2O
- Flow rate: 0.4 ml/min
- Detection: CAD

7.1.7 HPLC method "SEC"

This is a method for analyzing the distribution of average molecular weights resulting from enzymatic hydrolysis, based on size-exclusion chromatography. The method was implemented on a BioSep-SEC-S 4000 column (Phenomenex). The method parameters in brief:

- Column oven temperature: RT
- Mobile phase: 0.1 M $NaNO_3$
- Flow rate: 1 ml/min
- Detection: RI
- Injection volume: 20 µl

7.1.8 Correlation DP vs. maltooligosaccharide t_R

Figure 7.1 depicts a calibration curve constructed by plotting retention time on the Polyspher column versus the degree of polymerisation of the maltooligosaccharide standards. This curve can be used to determine the approximate DP of neoagarooligosaccharides from their retention times.

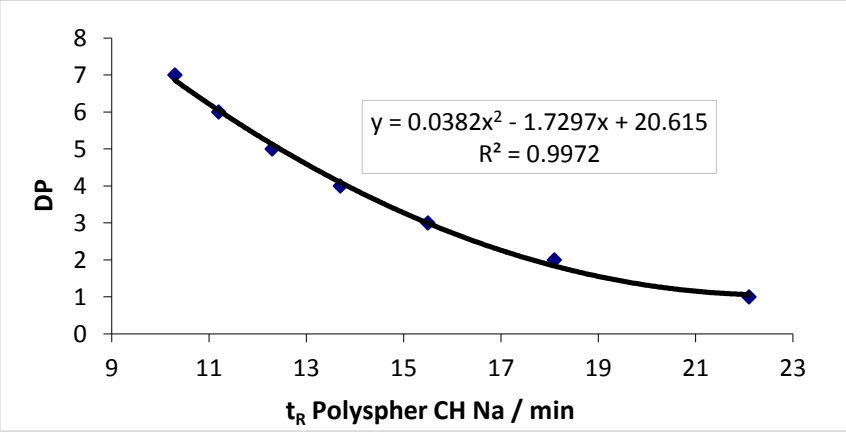

Figure 7.1: Calibration curve for calculating the DP of oligomer products separated by the Polyspher column

7.1.9 HPLC method "peptide fingerprint"

This method is used to separate protein digests into a unique pattern called "the peptide fingerprint". The separation is based on RP-HPLC and is performed on a C12 Jupiter™ Proteo column: 4 µm, 250 x 4.6 mm (Phenomenex) with gradient elution. The method parameters include:

- Column oven temperature: 37 °C
- Flow rate: 0.4 ml/min
- Detection: UV @ 214 nm
- Injection volume: 50 µl

Gradient elution is performed using a binary mobile phase system consisting of:

- Eluent A: 0.1 % TFA in deionized H_2O
- Eluent B: 0.1 % TFA in CH_3CN
- Gradient: 1.3 % eluent B/min

7.1.10 Chromatogram of the lipid standards

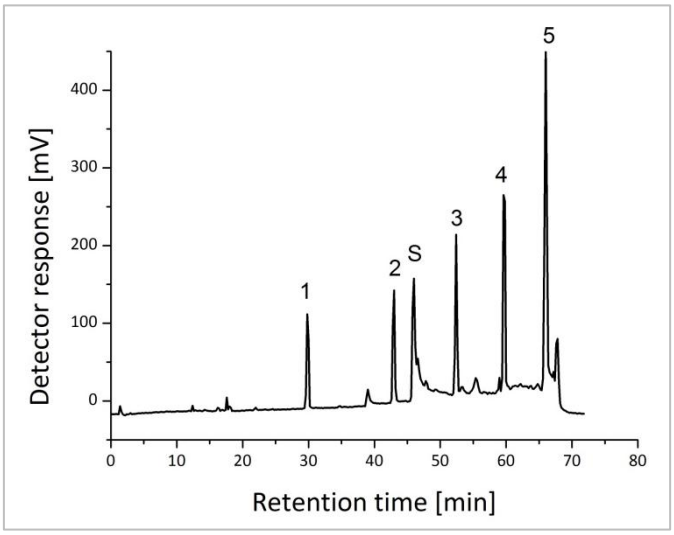

Fig. 7.2: Chromatogram of the lipid standards on the C8 Kinetex column; peaks: 1) Tricaprylin C8 (29.9 min), 2) Tricaprin C10 (43.4 min), 3) Trilaurin C12 (53.1 min), 4) Trimyristin C14 (60.6 min), 5) Tripalmitin C16 (67.7 min), S: solvent peak

7.1.11 HPLC method "universal lipid"

This method is used to separate a mixture of lipids. The separation is based on RP-HPLC and is performed on a C8 Kinetex column: 2.6 µm, 150 x 4.6 mm, 100 Å (Phenomenex) with gradient elution. The parameters in brief:

- Column oven temperature: 40 °C
- Flow rate: 0.8 ml/min
- Detection: CAD
- Injection volume: 20 µl

Gradient elution is performed using a binary mobile phase system consisting of:

- Eluent A: CH_3OH, H_2O, HAc (750:250:4)
- Eluent B: CH_3CN, CH_3OH, THF, HAc (500:375:125:4)
- Gradient: 0 – 70 % B to 46 min, 70 – 90 % B to 60 min, 90 % B to 65 min, 0 % B from 65.1 to 72 min

7.1.12 Textile impregnation procedure

An aqueous solution of the target substance is prepared in a suitable concentration. The solution is dripped with a pipette onto the staining area of the test-strip and the textile is left to dry completely at RT. Afterwards, a defined amount of the food matrix is applied on top of the staining area. The rest of the procedure follows the established operation mode of the washing experiments. The material and amounts used in this work:

- Textile: 1000 µl xanthan solution (0.5 % w/v) → drying → 1 g ketchup without additives
- Textile: 1000 µl inulin solution (6 % w/v) → drying → 400 µl elderberry juice

7.2 Materials

7.2.1 Reagents and buffers

Coomassie reagent:	25 mg Coomassie Blue G-250, 12.5 ml ethanol (95 %), 25 ml H_3PO_4 (85 %), ad 250 ml with dist. H_2O; store at 4 °C
DNS reagent:	Solution A: 3.15 g $Na_2S_2O_5$, 125 g NaK-tartarate, 167 ml 4.5 % NaOH => heat Solution B: 4.40 g dinitrosalicylic acid in 457.5 ml H_2O => gentle heating Mix solution A & B, keep in a dark bottle, use within 6 months
Emulsion solution:	3 g gum arabic, 1.46 g NaCl, 0.688 g KH_2PO_4, 6.375 g Na_2HPO_4, 270 ml glycerol, adjust pH to 8, ad 500 ml with dist. H_2O
Farmers reducer:	10 mg of $K_3[Fe(CN)_6]$ and $Na_2S_2O_3$ ad 100 ml H_2O
Fixing solution:	80 ml HAc, 400 ml dist. H_2O, 400 ml ethanol
Phosphate buffer:	[0.1 M, pH 8 at 40 °C] 1.38 g KH_2PO_4, 12.75 g Na_2HPO_4, adjust pH to 8.05 at RT, ad 1 L with H_2O
SDS sample buffer:	20 mM Tris-HCl, 2 mM EDTA, 5 % SDS, 0.02 % Bromophenol blue, 10 % glycerol, and 10 % β-mercaptoethanol
Separation gel (12 %):	2.92 ml acrylamide (40 %), 1.56 ml bisacrylamide (2 %), 2.8 ml Tris (1.5 M), 1 ml SDS (1 %), 1.76 ml H_2O, 20 µl TEMED and APS
Stacking gel (6 %):	731 µl acrylamide (40 %), 390 µl bisacrylamide (2 %), 760 µl Tris (1.5 M), 0.3 ml SDS (1 %), 3.82 ml H_2O, 10 µl TEMED and APS
TGS running buffer:	25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3

7.2.2 Washing bath fat replacers

Standard conditions	**Lipase-free conditions**
90 ml lipase-free detergent solution	90 ml lipase-free detergent solution
(0.95 g liquid laundry detergent in 90 ml H_2O)	(0.95 g liquid laundry detergent in 90 ml H_2O)
100 ml H_2O with 32°d	100 ml H_2O with 32°d
10 ml lipase solution [Lipex 0.2 %]	10 ml dist. H_2O

7.2.3 Equipment

Apparatus	Name, company, headquarters
Analytical balance	R 160P, Sartorius-Stedim Biotech, Göttingen
Automatic titrator	DL 50 Graphix, Mettler Toledo, Greifensee
Bench centrifuge	Centrifuge 5415 R, Eppendorf AG, Hamburg
Electrophoresis system	Mini-Protean Tetra Cell, Bio-Rad, München
Heating block & stirrer	IKA Combimag RCO, IKA Werke GmbH, Staufen
pH-Meter	691, Metrohm AG, Filderstadt
Photometer	Multiskan Spectrum, Thermo Fischer Scientific, Waltham
Thermal block	Thermomixer comfort, Eppendorf AG, Hamburg
Ultrapure water system	Arium 611, Sartorius-Stedim Biotech, Göttingen
Ultrasonic bath	Sonorex Super 10P, Bandelin GmbH, Berlin
Ultrasonic homogenizer	Ultra-Turrax T25 Basic, IKA Werke GmbH, Staufen
Water bath	Julabo 5B, Julabo Labortechnik GmbH, Seebach
<u>HPLC system carbohydrates & lipids</u>	
Autosampler	Triathlon, Spark, Holland
Binary pump	Agilent 1200, Agilent Technologies, Santa Clara
CAD	Corona, ESA Biosciences, Chelmsford
RI detector	LaChrom L-7490, Merck , Darmstadt
<u>HPLC system proteins</u>	
Autosampler	Triathlon, Spark, Holland
Binary pump	L-6200 A, Merck Hitachi
UV detector	L-7400, Merck Hitachi
<u>Washing experiments</u>	
Centrifuge	CENTRI 772 SEK, Thomas Electronic GmbH, Hamburg
Photometer	Minolta CR 200, Minolta, Osaka
Rinse machine	Henkel AG & Co. KGaA, Düsseldorf
Washing equipment	Launder-Ometer®, Atlas Electric devices, Chicago

7.3 The PhD student's cookbook

7.3.1 Recipes health ingredients

Fibre-enriched elderberry juice [inulin]

- Inulin (e.g. from dahlia tubers, Fluka)
- Elderberry juice (e.g. Voelker Bio Holundersaft)

1. Weigh the required amount of inulin (3 - 6 % w/v preparation) with a balance
2. Suspend the powder in the juice and heat carefully to 60 °C while stirring for dissolving the inulin
3. Cool the juice to RT. Then it can be used for staining. Mildly heated juice without inulin serves as a control

Sugar-free elderberry juice [polydextrose, E 1200]

- Polydextrose (e.g. Nutriose®, Roquette)
- Sugar-free juice (e.g. Voelker Bio Holundersaft)

1. Weigh a suitable amount of polydextrose (for a 10 % w/v preparation) with a balance
2. Suspend the powder in the liquid and stir for complete dissolution. Use the juice for staining
3. Juice without polydextrose can serve as a control

Prebiotic chocolate joghurt [inulin]

- Inulin (e.g. from dahlia tubers, Fluka)
- Whole milk (3.5 % fat)
- Chocolate cream (e.g. "Dunkles Geheimnis", Rewe)
- Lactobacillus culture (e.g. *Lactobacillus bulgaricus*)

1. Bring the milk to boiling temperature and add the inulin powder to the hot milk while stirring
2. 3.5 g inulin (3 % w/v inulin joghurt) or 7.3 g inulin (6 % w/v inulin joghurt) are added pro 100 ml milk
3. Allow the milk to cool down to 38 °C
4. Inoculate the milk with e.g. 1 tea spoon joghurt (3.5 % fat) containing living bacterial culture
5. Ferment the milk for a minimum of 12 h at 38 °C until a pH of approximately 4.3
6. The inulin joghurt is then stored at 4 °C until it is required for staining

In order to impart colouration to the joghurt, it is processed with chocolate cream. For this purpose:

7. 4 g chocolate cream are mixed 20 g inulin joghurt (ratio: 1 g cream / 5 g joghurt)
8. The mixture is homogenized (e.g. Ultra-Turrax 15 s at 21,500 rpm)
9. The chocolate joghurt can be used for staining

The required inulin concentration (3 % w/v or 6 % w/v joghurt) results after dilution with the chocolate cream. The control is a chocolate joghurt produced in an identical manner (for reproducible results it is recommended to use the same batch chocolate cream and inoculation joghurt), but without the addition of inulin to the milk.

7.3.2 Recipes fat replacers

Low-calorie chocolate joghurt [Simplesse®]

- Microparticulated whey protein (e.g. Simplesse®, CP Kelco)
- Skim milk (1.5 % fat)
- Diet chocolate cream (e.g. "mct-basis-plus Schokocreme", Dr. Schär GmbH)
- Lactobacillus culture (e.g. *Lactobacillus bulgaricus*)

1. Bring the milk to boiling temperature and add the protein powder to the hot milk while stirring
2. 3.5 g Simplesse® are added pro 100 ml milk
3. Allow the milk to cool down to 38 °C
4. Inoculate the milk with e.g. 1 tea spoon joghurt (1.5 % fat) containing living bacterial culture
5. Ferment the milk for a minimum of 12 h at 38 °C until a pH of approximately 4.3
6. The low-calorie joghurt is then stored at 4 °C until it is required for staining

In order to impart colouration to the joghurt, it is processed with chocolate cream. For this purpose:

7. 4 g chocolate cream are mixed 20 g joghurt (ratio: 1 g cream / 5 g joghurt)
8. The mixture is homogenized (e.g. Ultra-Turrax 15 s at 21,500 rpm)
9. The chocolate joghurt can be used for staining

The final concentration of Simplesse® amounts to 3 % w/v and results after dilution with the chocolate cream. The control is a chocolate joghurt produced in an identical manner, but without the addition of Simplesse®.

Colouration procedure lipids

The lipid-soluble dye Sudan blue was used to impart colouration to the oils and fats under investigation. The dye was mixed with the lipids in the concentration 0.01 % w/w. This imparts an intensive blue colour to the fats, which enables them to be used for stain screenings.

7.3.3 Recipes hydrocolloids

Joghurt with 2.5 % fat without additives [control]

- Whole milk (3.5 % fat) 49.0 % v/v
- Skim milk (1.5 % fat) 49.0 % v/v
- Dry milk (e.g. Bio-Magermilchpulver, Heirler) 2.0 % w/v
- Lactobacillus culture (e.g. *Lactobacillus bulgaricus*) as required

1. For the production of ca. 200 ml cup-set joghurt, mix 98 ml whole milk and 98 ml skim milk
2. Add 4 g dry milk powder and dissolve by stirring
3. Heat the milk to pasteurize it and then allow it to cool down to 38 °C

4. Inoculate the milk with e.g. 1 tea spoon joghurt (2.5 % fat) containing living bacterial culture
5. Ferment the milk for a minimum of 12 h at 38 °C until a pH of approximately 4.3
6. The joghurt is then stored at 4 °C until it is required for staining

In order to impart colouration to the joghurt, it is processed into a **yoghurt-juice beverage**. For this purpose, it is best to take a strongly coloured juice like elderberry or blackcurrant. The following recipe can be used:

- Homemade joghurt 68 % w/w
- Dry milk (e.g. Bio-Magermilchpulver, Heirler) 3.0 % w/w
- Strongly coloured juice (e.g. elderberry juice) 29 % v/w

7. 1.75 g dry milk powder is dissolved in 35 g homemade joghurt by stirring
8. 15 ml elderberry juice is added to the joghurt and homogenized (e.g. Ultra-Turrax 15 s at 21,500 rpm)
9. The resulting joghurt-elderberry-drink can be used for staining

Joghurt with 0.4 % w/v agar [agar, E 406]

The recipe is identical to the one described above for the control. For the production of 200 ml cup-set joghurt:

1. Dry blend 960 mg agar with 4 g milk powder.
2. Stir together 98 ml whole milk with 98 ml skim milk
3. Add the powder to the milk and heat for pasteurization. Allow to cool to 38 °C and inoculate
4. Incubate at 38 °C for 12 h at least and keep refrigerated afterwards
5. For staining, the agar joghurt is processed into a joghurt-juice drink as described above

The final concentration of agar, **0.33 % w/v** results after dilution with the juice.

Joghurt with 0.4 % w/v gelatine [gelatine, E 441]

The recipe is identical to the one of the control. For the production of 200 ml cup-set joghurt:

1. Dry blend 960 mg gelatine with 4 g milk powder.
2. Stir together 98 ml whole milk with 98 ml skim milk
3. Add the powder to the milk and heat for pasteurization. Allow to cool to 38 °C and inoculate
4. Incubate at 38 °C for 12 h at least and keep refrigerated afterwards
5. For staining, the gelatine joghurt is processed into a joghurt-juice drink as described above

The final concentration of gelatine, **0.33 % w/v** results after dilution with the juice.

Joghurt with 0.04 % w/v gellan [gellan, E 418]

The recipe is identical to the one of the control. For the production of 200 ml cup-set joghurt:

1. Dry blend 96 mg gellan with 4 g milk powder. Stir together 98 ml whole milk with 98 ml skim milk
2. Add the powder to the milk and heat for pasteurization. Allow to cool to 38 °C and inoculate
3. Incubate at 38 °C for 12 h at least and keep refrigerated afterwards
4. For staining, the gellan joghurt is processed into a joghurt-juice drink as described above

The final concentration of gellan, **0.03 % w/v** results after dilution with the juice.

Joghurt with 0.18 % w/v low-methoxyl amidated pectin [LM pectin, E 440ii]

The recipe is identical to the one of the control. For the production of 200 ml cup-set joghurt:

1. Dry blend 432 mg LM pectin (e.g. Unipectine™ AYS 407 C, Cargill) with 4 g milk powder.
2. Stir together 98 ml whole milk with 98 ml skim milk
3. Add the powder to the milk and heat for pasteurization. Allow to cool to 38 °C and inoculate
4. Incubate at 38 °C for 12 h at least and keep refrigerated afterwards
5. For staining, the pectin joghurt is processed into a joghurt-juice drink as described for the control

The final concentration of LM pectin, **0.15 % w/v** results after dilution with the juice.

Elderberry lassi with 0.4 % w/v high-methoxyl pectin [HM pectin, E 440i]

- HM pectin (e.g. Unipectine™ AYD 250, Cargill) 0.4 % w/v
- Sugar 4 % w/w
- Dry milk (e.g. Bio-Magermilchpulver, Heirler) 5 % w/w joghurt
- Homemade joghurt without additives (2.5 % fat) 54 % w/w
- Strongly coloured juice (e.g. elderberry juice) 18 % w/w
- Water up to 100 %
- Citric acid (50 % w/v) as required

1. For the production of ca. 100 ml lassi, dry blend 0.4 g HM pectin and 4 g sugar
2. Add the powder to 23.6 ml water at 70 °C while stirring
3. Heat the solution to 85 °C for complete dissolution of the HM pectin and then cool to 15 °C
4. Homogenize 2.7 g milk powder in 54 g homemade joghurt containing no texture agents
5. Mix 18 ml juice, the pectin solution and the joghurt at 15 °C and homogenize the mixture
6. Set the pH at 4.2 with the citric acid solution and use the lassi for staining.

The control consists of a lassi drink prepared in a similar manner, but without HM pectin added to its recipe.

Chocolate milk with 0.03 % w/v carrageenan [carrageenan, E 407]

- Carrageenan 0.03 % w/v
- Sugar 1.0 % w/v
- Cacao 3.0 % w/v
- Dry milk (e.g. Bio-Magermilchpulver, Heirler) 1.0 % w/v
- Whole milk (3.5 % fat) 95 % w/v

1. For the production of ca. 300 ml chocolate milk, dry blend 90 mg carrageenan with 3 g sugar and 9 g cacao
2. Dissolve 3 g milk powder in 285 ml whole milk by stirring
3. Add the dry blend ingredients to the whole milk with agitation and heat for pasteurization
4. Cool and store refrigerated.
5. The chocolate milk can be used for staining in this form.

Chocolate milk without carrageenan serves as a control in the washing experiments.

Instant drink with CMC [CMC, E 466]

- CMC 0.75 % w/w
- Sugar 18.0 % w/w
- Cacao 20.0 % w/w
- Dry milk (e.g. Bio-Magermilchpulver, Heirler) 61.25 % w/w
- Water 1:5 w/v

1. Dry blend 12.25 g dry milk, 3.6 g sugar, 4 g cacao and 150 mg CMC
2. The powder is made into a drink by addition of 100 ml water, which can be used for staining

The final CMC conc. in the beverage is **0.15 % w/v**. A drink made without CMC addition serves as a control.

Juice with gum arabic [gum arabic, E 414]

- Gum arabic
- Strongly coloured juice (e.g. elderberry juice)

1. Weigh a suitable amount of gum arabic for a 40 % w/v preparation with a balance
2. Suspend the powder in the liquid and dissolve by mild heating and constant agitation
3. Cool the juice to RT, after which it can be used for staining immediately

Juice without gum arabic addition serves as a control.

Ketchup with carob gum [E 410] and guar gum [E 412]

- Carob gum or guar gum
- Ketchup without texture agents (e.g. bio-ketchup, Rapunzel)

1. Weigh the amount of gum required for a 0.5 % w/w ketchup preparation with a balance
2. Add the powder to the ketchup and homogenize at 21,500 rpm for 5 min. Use the ketchup for staining.

The ketchup without additives serves as a control. For reproducible results, it is recommended to use the same ketchup batch in all experiments.

Ketchup with xanthan [xanthan, E 415]

- Xanthan gum
- Ketchup without texture agents (e.g. bio-ketchup, Rapunzel)

1. Weigh the amount of gum required for a 0.3 % w/w or a 0.5 % w/w ketchup preparation with a balance
2. Add the powder to the ketchup and homogenize at 21,500 rpm for 5 min. Use the ketchup for staining.

The ketchup without additives serves as a control. For reproducible results, it is recommended to use the same ketchup batch in all experiments.

Curry sauce with xanthan [xanthan, E 415]

- Xanthan gum
- Curry spice
- Butter
- Crème fraiche

1. Weigh the amount of gum required for a 0.3 % w/w or a 0.5 % w/w preparation with a balance
2. Melt 5 g butter in a frying pan, add 5 g curry spice and fry for 1 min
3. Add 200 g crème fraiche to the pan while stirring and cook the sauce until it starts to boil
4. Let the curry sauce cool down to RT. Then, add the xanthan gum and homogenize at 21.500 rpm for 5 min
5. The curry sauce with xanthan should display a rich consistency and can be used for staining.

Curry sauce without xanthan addition serves as a control. Commercial curry sauces are not recommended for performing washing experiments, as they usually contain some amounts of xanthan gum.

7.3.4 Manual staining table

Target substance	Matrix	Concentration	Staining amount
Agar	Joghurt-juice-beverage	0.33 % w/v	1 g
Carob gum	Ketchup	0.5 % w/v	1 g
Carrageenan	Chocolate milk	0.03 % w/v	950 µl
CMC	Instant drink	0.15 % w/v	950 µl
Benefat	n. a.	n. a.	0.7 g
Gelatine	Joghurt-juice-beverage	0.33 % w/v	1000 µl
Gellan	Joghurt-juice-beverage	0.03 % w/v	1000 µl
Grindsted	n. a.	n. a.	300 µl
Guar gum	Ketchup	0.5 % w/v	1 g
Gum arabic	Juice	40 % w/v	800 µL
Inulin	Chocolate joghurt	3 % and 6 % w/v	1 g
Inulin	Elderberry juice	3 % and 6 % w/v	400 µl
Lard	n. a.	n. a.	0.7 g
Olive oil	n. a.	n. a.	400 µl
Pectin HM	Juice lassi	0.4 % w/v	950 µl
Pectin LM	Joghurt-juice-beverage	0.15 % w/v	1000 µl
Polydextrose	Elderberry juice	10 % w/v	400 µl
Simplesse®	Chocolate joghurt	3 % w/v	1 g
Xanthan	Curry sauce	0.3 % and 0.5 % w/v	0.7 g
Xanthan	Ketchup	0.3 % and 0.5 % w/v	1 g

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Curriculum vitae

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Education

2008 – 2012	Doctoral research at the Institute of Technical Chemistry Leibniz University Hannover; Supervisor: Prof. Dr. Thomas Scheper Doctoral thesis title: "The stain-potential of novel food ingredients"
2005 – 2008	Leibniz University Hannover, graduate course "Life Science" M. Sc. – Master of Science with a final grade (A) – excellent Thesis title: "The digestion of functional ingredients with detergent enzymes" Majors: bioprocess engineering and chemistry of natural products Minors: bioinformatics and molecular biology
2002 – 2005	Reutlingen University, undergraduate course "Chemistry with Marketing" B. Eng. – Bachelor of Engineering with a final grade (1.6) – good Thesis title: "Alternative substrates for the fermentation of *S. cerevisiae*"
1998 – 2002	PIS International School, Cairo, secondary school IGCSE (International General Certificate of Secondary Education) A-levels: Biology (A), Chemistry (B), English Literature (A)

Work experience

2008 – 2011	Research assistant at the Institute of Technical Chemistry, Leibniz University
2004 – 2005	Internship at the protein analysis labs of Solvias AG, Basel

Research interests

Food ingredients, industrial enzymes, HPLC method development and implementation, enzymatic hydrolysis, food of the future, functional food, relevance of the changing food industry to non-food sectors like detergent industry and cosmetic industry, sustainable development, futurism

Some words of appreciation and gratitude

Sitting in front of the final version of my thesis, I glance over the formatting, the figures and the tables. The curry sauce recipe on page 95 calls to my attention. It is excellent with steamed cauliflower. Despite the fatal stains which result if this creation is transferred onto your garments, I recommend it best served warm. And yet, if I have to write down one last recipe in this work, it should be the recipe of my doctorate.

One should begin with a good kitchen. Above all, this should be a place where one enjoys spending time and where one is given plenty of space to try working on one's own ideas. In this context, I am very thankful to my supervisor, Prof. Dr. Thomas Scheper, who has provided the impulse for this project, as well as the means and the room to carry it out. Thank You, Thomas, for making TCI such a nice place to work!

The real cooking of the thesis itself always starts from a base of professionals which share their know-how, support and resources. I was fortunate to have a number of such people on my side during my doctoral studies, both at home in the TCI, and at the Henkel KGaA headquarters in Düsseldorf.

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Any doctoral thesis ingredients list is incomplete without a portion of fresh, high-quality colleagues. The more kilograms of them you add to your recipe, the better off you are. Even Monday mornings have meaning when you are looking forward to seeing their sleepy faces. Whether it is singing in the lab while doing your pipetting, going to the Mensa Stammtisch, discussing experimental results, asking who's-last-seen-that-chemical-and-where?, comparing theories or just smiling and exchanging a joke while walking down the corridor – these are the people which create the special TCI atmosphere. As our (informal) motto states "TCI – allein DAS ist schon ein Grund zum Feiern!" Listing all the people which have brightened my workdays is hardly possible. Nevertheless, there are several which deserve honorary mention: Tonya (for never-ending humour), Anne S., Anne G., Isabelle, Steffen, Daniel S. (for Life Science & Sons and beyond), Steffi H. (for sharing a laboratory and song refrains), and my bachelor student Semra Alemdar (who was my trustworthy helper with the whey protein ingredients).

By the time we have proceeded to the “berüchtigte Terrasse”, some colleagues have turned into accomplices and even friends. Special thanks goes to my coffee club (especially Daniel L. and Mike) with whom I have collected sunshine, caffeine, plans for world domination and crazy entrepreneur ideas. I am jolly glad for the many special moments shared on the dance floor (Anne S., Pierre), the Feierabendbierchen, the public viewings at football championships, the organization of mad hat events and the countless informal and merry activities I have experienced in the TCI-access all areas.

The next additions to the recipe of this doctorate are all flavourings and spices. These are the things, which might appear to some to be secondary in importance, due to the fact that they are added in various minute amounts, but their inclusion to a dish is essential for its nourishment, harmony and enjoyment. (Ask anybody who has tried eating a meal without salt.) My sugar and spice were my dear fellows from the theatre with whom I shared many playful scenes, meaningful dialogues, and regular doses of complete lack of seriousness. I am also deeply grateful to the Friday temple, where my sisters move their bodies and their hearts. They have always helped me dance my head behind, so I can restore my sanity and strength.

My decision to pursue a doctoral degree at the Leibniz University was intricately linked with turning Hannover into a place I can truly call home for the last few years. For somebody like me with deep nomadic roots it is quite an achievement to stay so long in one place! I want to say big thank you to you people who make me feel at home here: Mike, Ruby and my roommates in Linden.
And as for the other part of home, it definitely belongs to my family. I am grateful to my Mom and Dad, for their unwavering support in every way imaginable, for being the stable bow that shoots forth the arrow, for always being there for me when I need them.

And You, my dear Schwest, are the special kind of zest. Thank You for being my constant champion, for opening windows to novel lands and showing me where the horizons run, and most of all, for letting a sun rise in my heart when You are there. It makes me sing, dance and be happy.

There is one last spice, without which, this period of my life would have lacked its peculiar flavoring.
It is the kind of the Quín-Cha-Mòkua-Che. I found him in a golden room beyond the IAESTE door. As a key master of the realm, he gave me a bunch of keys leading to places I didn’t suspect I could reach. They are pockets of parallel μ-worlds, generators of supercritical steam and springs restoring manna and finally, I am grateful he shows me how to lie down in the sunset and look fearlessly at the Ocean.