Thylakoid membranes from spinach

Effect of processing on their function as appetite suppressing ingredient

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DOCTORAL DISSERTATION

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| Western diet contains a high proportion of palatable food, rich in refined carbohydrates and fat, that subsequently increases the risk of overconsumption and possibly leads to overweight and obesity. Therefore an approach to strengthen the inherent satiety for fat can be used to prevent overconsumption.  Thylakoid membranes isolated from the chloroplast of green leaves have been found to reduce the rate of lipolysis by inhibiting lipase/co-lipase. Administration of thylakoids to animals and humans reduced food intake and body weight as well as affected appetite-regulation hormones.  When a product for consumers shall be formulated based on a plant extract with appetite suppressing abilities, the material must maintain the functionality during industrial processing and storage. Heat treatment is often applied to assure the microbiological safety of the product, and to dry the plant extract into powder form increases the shelf life and flexibility in applications while at the same time also reduces transport and handling costs. The present thesis aims to investigate the effect of processing on thylakoids’ physicochemical properties and subsequent physiological functions of the appetite-suppressing ingredient.  Heat treatment reduced the ability to inhibit lipase/co-lipase *in vitro.* Heat treatment also affected chlorophyll content and the thylakoids’ ability to stabilize the oil-water interface in oil-in-water emulsions.  Drum drying, spray drying and freeze drying were investigated. Drying at higher temperatures decreased the emulsifying capacity of the thylakoids as well as reducing lipase-inhibiting effect. Deterioration processes was initiated during dehydration and continued during storage. Moisture in surrounding air had a definite effect on chlorophyll degradation with highest degradation rate in high relative humidity. Emulsifying capacity of thylakoid powders was impaired after storage and the reduction was accelerated at higher relative humidities. Spray-dried thylakoid powder had the highest chlorophyll content and the highest emulsifying capacity of all powders investigated. The mild heat treatment during dehydration preserved the chlorophyll content but was severe enough to partly inactivate degradation enzymes. Of the studied techniques, spray drying was concluded to be the most suitable drying technique with respect to functionality of the resulting powder after processing and storage.  A correlation between characteristics and functionality of the thylakoids was established both in aqueous solution and in powder formula. Chlorophyll content, green colour and lightness of powders and ability to stabilize the oil-water interface were correlated to lipase-inhibiting capacity. This opens possibilities to partly replace the enzymatic activity measurement method with the less time consuming and more cost efficient spectrophotometric method and emulsion-based model in screening processes, for example during process optimization.  In conclusion, the thesis contributes with knowledge about the effect of processing and storage on thylakoid products’ functionality. Thus the results can be applied in production of a more standardised functional food ingredient with optimized and predicted appetite reducing properties. | | |
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*To Valter*

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# Abstract

Western diet contains a high proportion of palatable food, rich in refined carbohydrates and fat, that subsequently increases the risk of overconsumption and possibly leads to overweight and obesity. Therefore an approach to strengthen the inherent satiety for fat can be used to prevent overconsumption.

Thylakoid membranes isolated from the chloroplast of green leaves have been found to reduce the rate of lipolysis by inhibiting lipase/co-lipase. Administration of thylakoids to animals and humans reduced food intake and body weight as well as affected appetite-regulation hormones.

When a product for consumers shall be formulated based on a plant extract with appetite suppressing abilities, the material must maintain the functionality during industrial processing and storage. Heat treatment is often applied to assure the microbiological safety of the product, and to dry the plant extract into powder form increases the shelf life and flexibility in applications while at the same time also reduces transport and handling costs. The present thesis aims to investigate the effect of processing on thylakoids’ physicochemical properties and subsequent physiological functions of the appetite-suppressing ingredient.

Heat treatment reduced the ability to inhibit lipase/co-lipase *in vitro.* Heat treatment also affected chlorophyll content and the thylakoids’ ability to stabilize the oil-water interface in oil-in-water emulsions.

Drum drying, spray drying and freeze drying were investigated. Drying at higher temperatures decreased the emulsifying capacity of the thylakoids as well as reducing lipase-inhibiting effect. Deterioration processes was initiated during dehydration and continued during storage. Moisture in surrounding air had a definite effect on chlorophyll degradation with highest degradation rate in high relative humidity. Emulsifying capacity of thylakoid powders was impaired after storage and the reduction was accelerated at higher relative humidities. Spray-dried thylakoid powder had the highest chlorophyll content and the highest emulsifying capacity of all powders investigated. The mild heat treatment during dehydration preserved the chlorophyll content but was severe enough to partly inactivate degradation enzymes. Of the studied techniques, spray drying was concluded to be the most suitable drying technique with respect to functionality of the resulting powder after processing and storage.

A correlation between characteristics and functionality of the thylakoids was established both in aqueous solution and in powder formula. Chlorophyll content, green colour and lightness of powders and ability to stabilize the oil-water interface were correlated to lipase-inhibiting capacity. This opens possibilities to partly replace the enzymatic activity measurement method with the less time consuming and more cost efficient spectrophotometric method and emulsion-based model in screening processes, for example during process optimization.

In conclusion, the thesis contributes with knowledge about the effect of processing and storage on thylakoid products’ functionality. Thus the results can be applied in production of a more standardised functional food ingredient with optimized and predicted appetite reducing properties.

# List of publications

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

1. Montelius C, Gustafsson\* K, Weström B, Albertsson PÅ, Emek SC,   
   Rayner M, and Erlanson-Albertsson C. Chloroplast thylakoids reduce glucose uptake and decrease intestinal macromolecular permeability. *Br J Nutr*. 2011;1-9.
2. Östbring K, Rayner M, Sjöholm I, Otterström J, Albertsson PÅ, Emek SC, and Erlanson-Albertsson C. The effect of heat treatment of thylakoids on their ability to inhibit *in vitro* lipase/co-lipase activity. *Food Func*. 2014;5:2157-2165.
3. Östbring K, Rayner M, Albertsson PÅ, and Erlanson-Albertsson C. Heat-induced aggregation of thylakoid membranes affect their interfacial properties. *Food Func.* 2015;6:1310-1318.
4. Östbring K, Sjöholm I, Sörenson H, Ekholm A, Erlanson-Albertsson C, and Rayner M. Characteristics and functionality of appetite reducing thylakoid powders produced by three different drying processes. Submitted to *J Sci Food Agri.*
5. Östbring K, Sjöholm I, Erlanson-Albertsson C, and Rayner M. Effects of storage conditions on chlorophyll degradation in thylakoid powders produced by different drying methods. Manuscript.

\* Surname before marriage. Current surname is Östbring.

**Additional publications**

* Rayner M, Emek SC, Gustafsson\* K, Erlanson-Albertsson C, and Albertsson PÅ. A novel emulsifier from spinach with appetite regulation abilities. *Procedia Food Science*. 2011;1;1431-1438.
* Stenblom EL, Montelius C, Östbring K, Håkansson M, Nilsson S, Rehfeld JF, and Erlanson-Albertsson C. Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women. *Appetite*. 2013;68:118-123.

\* Surname before marriage. Current surname is Östbring.

**List of contributions**

1. Karolina Östbring together with Caroline Montelius performed the experiments, took an active part in the evaluation of the results and wrote a major part of the paper.
2. Karolina Östbring designed the study together with the co-authors, performed most of the experimental work, evaluated the results and wrote a major part of the paper.
3. Karolina Östbring designed the study together with the co-authors, performed the experimental work, evaluated the results and wrote a major part of the paper.
4. Karolina Östbring designed the study, performed most of the experimental work, evaluated the results and wrote a major part of the paper.
5. Karolina Östbring designed the study, performed the experimental work, evaluated the results and wrote a major part of the paper.

# Abbreviations and symbols

AOAC Association of Official Agricultural Chemists

ATP Adenosine triphosphate

AUC Area under the curve

aw Water activity

Awavelength Absorbance at indicated wavelength

Ca Initial concentration of emulsifying agent (mg/ml)

CCK Cholecystokinin

Chl *a* Chlorophyll *a*

Chl *b* Chlorophyll *b*

d32 Surface-weighted diameter (μm)

d43 Volume-weighted diameter (μm)

DDpH Drum-dried powder produced by the pH method

DDpH+M Drum-dried powder produced by the pH method with addition of maltodextrin

DG Diacylglycerol

DGDG Digalactosyldiacylglycerol

DM Dry matter, wet base (g/g)

DMSO Dimethyl sulfoxide

EC Emulsifying capacity (m2/mg)

EI Emulsification index

Fd Ferredoxin

FDw Freeze-dried powder produced by the water method

FFA Free fatty acids

FITC-dextran Fluorescein isothiocyanate labelled dextran

GI tract Gastrointestinal tract

GLP-1 Glucagon-like peptide 1

LDL cholesterol Low density lipoprotein cholesterol

LHC I and II Light harvesting complex I and II

MG Monoacylglycerol

MGDG Monogalactosyldiacylglycerol

NADPH Nicotinamide adenine dinucleotide phosphate

NaTDC Sodium dodecyl sulphate (g/L)

Papp Apparent permeability coefficient (cm/s)

PCy Plastocyanin

PLS1 Partial least square 1

PS I and II Photosystem I and II

PYY Peptide YY

R2 Coefficient of determination

RH% Relative humidity (%)

RI Refractive index

S Specific surface area (m2/m3 dispersed phase)

SCFA Short chained fatty acids

SDw Spray-dried powder produced by the water method

SEM Scanning electron microscopy

TEM Transmission electron microscopy

TG Triacylglycerol

Ve Emulsion volume (ml)

α Average lipase/co-lipase activity

φ Disperse phase volume fraction

φ Lipase/co-lipase inhibiting capacity

Γs Surface load (mg/m2)

# Populärvetenskaplig sammanfattning

Övervikt och fetma ökar i hela världen och fortfarande vet ingen exakt varför vi äter mer än kroppen behöver. En rådande teori är att den västerländska dieten till stor del innehåller ”supervälsmakande” mat med mycket socker och fett som godis, glass, chips och feta såser. Sådan mat ökar risken för överkonsumtion och kan därför leda till övervikt och fetma. Att öka mättnadseffekten av det fett som finns i maten är därför en strategi som kan användas för att förebygga överkonsumtion.

Thylakoidmembran isolerade från gröna blad har visats fördröja fettspjälkningen i tarmen. Thylakoider hämmar enzymerna lipas och co-lipas som är ansvariga för nedbrytning av fett i tarmen. Fördröjd fettspjälkning ger utökad mättnadskänsla för att maten stannar i tarmen under längre tid. I försök på djur och människor har thylakoider visats hämma hungersignaler och öka mättnadssignaler. Den minskade aptiten ledde till minskat födointag och dokumenterad viktnedgång.

När ett växtextrakt med aptitdämpande effekt ska göras till en produkt för konsumenter måste extraktet genomgå en rad industriella processer som värmebehandling och torkning, samt lagring. Naturligtvis måste den aptitdämpande effekten bevaras i så hög grad som möjligt genom dessa behandlingar, för att skapa den utlovade mättnaden hos konsumenten. Avhandlingens syfte är att undersöka vilken effekt industriell bearbetning och lagring har på thylakoidernas aptitdämpande egenskaper. När man känner till vilken effekt olika värmebehandlingar, torkmetoder och lagringsbetingelser har, kan man skräddarsy produktionen av thylakoider så att slutprodukten dämpar aptiten så mycket som möjligt.

Men varför måste växtpreparatet genomgå industriell bearbetning överhuvudtaget? Gröna blad som växer på öppna fält är ofta täckta av mikroorganismer som bakterier, jäst och mögel som kan vara farliga för människor. Mikroorganismerna kan också förstöra produkten under lagring vilket minskar hållbarheten. Vid värmebehandling dör mikroorganismerna och produkten blir säker att äta. Torkning till pulver ökar användningsområdena av växtpreparatet. Det kan t.ex. blandas in i hälsodrycker, såser, marinader, yoghurt, bars och flingor.

Värmebehandling minskade thylakoidernas förmåga att hämma enzymerna lipas/co-lipas och orsakade nedbrytning av det gröna färgämnet klorofyll. Värmebehandling minskade också thylakoidernas förmåga att stabilisera gränsytan mellan fett och vatten i emulsioner. Emulsioner användes som förenklat modellsystem i stora delar av avhandlingen. Thylakoidernas uppgift i kroppen är att fästa på fettytor i tarmen och genom det hämma lipas/co-lipas vilket skapar utökad mättnad. Ett enkelt och snabbt sätt att mäta thylakoidernas förmåga att fästa på en fettyta är att tillverka en emulsion (jmf majonnäs). Ett bra thylakoidmembran med hög yt-aktivitet klarar av att stabilisera små fettdroppar medan ett skadat thylakoidmembran förlorar sin yt-aktivitet och lyckas bara stabilisera stora droppar.

Tre olika torkmetoder utvärderades: valstorkning (105°C i ca 30 sek), spraytorkning (72°C i ca 3 sek) och frystorkning (20°C i 7 dagar). Torkning vid högre temperaturer orsakade nedbrytning av klorofyll samt minskade den emulgerande förmågan hos thylakoidmembranen. Torkning vid högre temperaturer minskade också förmågan att hämma lipas/co-lipas. Från dessa försök var frystorkning därför den bästa torkmetoden.

Ett pulver måste klara lagring. Thylakoidpulver torkade med valstorkning, spraytorkning och frystorkning lagrades i olika luftfuktighet under åtta månader. Det visade sig att de försämringsprocesser som startats vid torkningen fortsatte under lagring. Hög luftfuktighet under lagring orsakade kraftig nedbrytning av klorofyll. Pulvren tappade sin gröna färg och blev istället brunaktiga. Pulvrens emulgerande förmåga minskade också efter lagring och försämringen accelererades av högre luftfuktighet. Thylakoidpulver bör därför förvaras så torrt som möjligt, gärna i lufttäta förpackningar för att behålla funktionaliteten. Spraytorkat pulver hade den högsta klorofyllhalten och den högsta emulgerande förmågan av alla pulver efter lagring. Den milda värmebehandlingen under torkning bevarade det mesta av klorofyllet men värmebehandlingen var samtidigt tillräckligt kraftig för att inaktivera klorofyll-nedbrytande enzymer som finns naturligt i thylakoider. Spraytorkning är därför den lämpligaste torkmetoden av de undersökta metoderna, som ger ett pulver med hög funktionalitet efter industriell bearbetning och lagring.

Ett samband mellan thylakoidernas fysikaliska kännetecken och funktionalitet kunde fastställas. Klorofyllinnehåll, grön färg, ljushet och förmåga att stabilisera gränsytan mellan vatten och olja var starkt korrelerat till thylakoidernas förmåga att hämma lipas/co-lipas. Analysen av lipas/co-lipas är dyr och tar lång tid. Med hjälp av de funna sambanden kan enkla, snabba och billiga mätmetoder som klorofyllanalys eller analys av grön färg användas för att få en uppskattning över hur bra thylakoiderna hämmar lipas/co-lipas. Speciellt i början av ett utvecklingsprojekt, när mängder av faktorer ska justeras, är det praktiskt att inte behöva göra omständliga analyser på alla prover.

Sammanfattningsvis bidrar avhandlingen med kunskap om hur industriell bearbetning och lagring påverkar thylakoidernas funktionalitet. Resultaten kan användas i produktion av en mer standardiserad och kontrollerad mervärdesingrediens med optimerade aptitdämpande egenskaper.

# Aims and hypothesis

The general aims of the thesis are to investigate how the active structure and subsequent physiological functions of thylakoids are affected by processes such as heat treatment, drying and storage conditions. Since it is not feasible to conduct human studies to evaluate every single effect of processing parameters, other functional parameters must be found and used instead. In previous studies, thylakoids’ ability to inhibit pancreatic lipase/co-lipase *in vitro* has been well correlated to results in human studies. The *in vitro* pH stat model is complex and another aim of the thesis is to find other directly measureable physicochemical parameters that indicated changes in the supramolecular structure during processing and storage (Fig 1). These parameters can possibly be correlated to the ability to inhibit pancreatic lipase/co-lipase *in vitro* with the possibility to replace the pH stat method in screening processes.

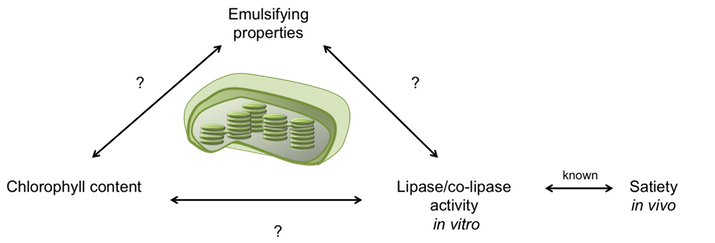


Figure 1. An aim of the thesis was to identify physicochemical parameters that indicated changes in the supramolecular structure of the thylakoids post processing. Correlations between lipase/co-lipase inhibition capacity and possibly identified parameters would be used to partly replace the complex pH stat method in screening processes.

Below are the individual aims and hypothesis of the papers included in the thesis.

* In Paper I the aim was to study the *in vitro* passage of marker molecules of different size across the rat intestinal wall in the presence of thylakoids. The hypothesis was that thylakoids would affect the passage of marker molecules, and permeability of the intestinal wall.
* In Paper II the aim was to investigate if thylakoids after heat treatment, still could inhibit pancreatic lipase/co-lipase *in vitro* and if a correlation to chlorophyll degradation could be established. The hypothesis was that thylakoids would aggregate by exposure to heat and thereby display less available surface. The limited exposed thylakoid surface would decrease the ability to interact with the lipid droplet surface, which in turn would lead to reduced ability to block lipase/co-lipase from its substrate.
* In Paper III the aim was to investigate if the reduced ability to inhibit lipolysis by heat-treated thylakoids (demonstrated in Paper II) could be linked to the thylakoids’ interfacial properties and ability to stabilize oil-in-water emulsions. The hypothesis was that since lipolysis is mainly an interfacial process, the thylakoids interfacial properties must be central. If the thylakoid membranes’ surface activity was reduced by heat it must be reflected by both impaired ability to inhibit lipolysis as well as impaired ability to stabilize oil-in-water emulsions.
* In Paper IV the aim was to investigate the influence of three different drying methods (freeze drying, spray drying and drum drying) on the physicochemical properties and functionality of resulting thylakoid powders. A second aim was to study the relation between characteristics and functionality of thylakoids in powder form. The hypothesis was that spray drying would generate thylakoid powder with highest functionality due to small particle size, relatively mild heat treatment and exposure of hydrophobic parts during drying, yielding a powder particle with hydrophobic parts turned outwards. Chlorophyll content and ability to stabilize oil-water interface were hypothesized to be correlated to the lipase-inhibiting capacity as demonstrated for aqueous solutions in Paper II and III.
* In Paper V the aim was to investigate how the powder characteristics of thylakoids dried in three different ways were affected by moisture exposure from different surrounding relative humidity in the air during storage. A second aim was to investigate if the thylakoids’ functionality is altered by exposure to moisture. The hypothesis was that the thylakoid powder would absorb water, and be subjected to loss of colour due to enzymatic degradation known to be accelerated at high relative humidity. Chlorophyll is known to be important for the internal membrane structure, therefore it was hypothesized that the powder with greatest loss of colour would display the greatest loss in functionality.

# Delimitation of thesis

The thesis includes experiments performed within different areas, namely isolation of thylakoids, processing and formulation, role in digestion (*in vitro*) and thylakoids effect on intestinal permeability *in vitro* in animal studies (Fig 2). Other areas such as human studies, animal studies *in vivo,* effect of proteolytic enzymes of thylakoids and binding studies between thylakoids and other substances was outside the scope of the thesis. These parts are covered by other co-workers in the research group.

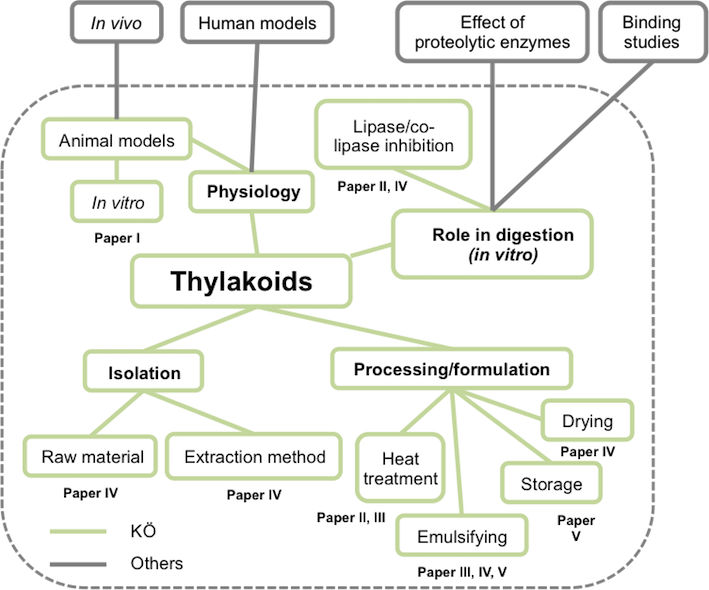


Figure 2. Delimitations of thesis. Areas indicated by green surrounded by the dotted line are included in the present thesis. Areas excluded by the dotted line are covered by other members of the research group.

# General introduction

Thylakoid membranes isolated from spinach have previously been demonstrated to increase satiety signals and enhance satiety in humans. The overall aim of the thesis was to characterize the thylakoid membranes and optimize production and processing of thylakoids for the purpose of retaining the active component through the unit operations. The loss of the active component can thus be minimized and a potent appetite-suppressing ingredient can be produced. This was done by combining an engineering approach with knowledge from the thylakoids’ function in the plant and the thylakoids’ function in the digestion process.

The increasing prevalence of overweight and obesity is well established, and there is an urgent need for practical interventions. The incidence of obesity has doubled globally since 1980 and in 2014, more than 1.9 billion adults, 18 years and older, were overweight (WHO°311). In Sweden, approximately half of the adult population suffers from overweight or obesity, and nearly 20% of children 7-9 years old are overweight1. Although numerous environmental and genetic factors contribute, the development for overweight and obesity is a consequence of energy intake exceeding energy expenditure.

The Western diets contain high proportions of palatable food, rich in refined carbohydrates and fat, which is obesity promoting. A strategy that can strengthen the satiety signals from fat is therefore a promising approach.

Thylakoid membranes isolated from green leaves have been demonstrated to reduce appetite in animal and human models by prolongation of the lipolysis2-4. The presence of lipids in the distal ileum increase the gastrointestinal transit time, stimulates release of gastrointestinal hormones, inhibits pancreatic and gastric acid secretion and reduces appetite by a negative feedback mechanism called the ileal brake5. Administration of thylakoids suppressed food intake and body weight and elevated levels of the satiety hormone CCK in mice3. Thylakoids administrated in human studies induced loss in body weight, reduced LDL cholesterol levels and increased the satiety hormones GLP-1 and CCK4,6.

In order for thylakoids to be formulated into a potent appetite-reducing ingredient that can be incorporated in food, several challenges in large-scale production must be addressed. A heat treatment unit operation needs to be implemented to eliminate potential harmful pathogens, and drying thylakoids to powder formula opens possibilities in terms of longer shelf life, transportation economy and flexibility in applications. A powder with appetite reducing abilities must also retain the functionality during storage to assure the effect. This thesis evaluates how the active macromolecular structures and subsequent physiological functions of thylakoids are affected by processing (such as heat treatment and drying) and storage. The overall intent is to contribute with knowledge that can be applied in production of a standardised functional food ingredient with optimized appetite reducing properties.

# Background

## The structure and function of thylakoid membranes within plants

All forms of life on earth use energy for growth and maintenance. Green plants, algae and certain types of bacteria capture energy from sunlight and convert it into chemical energy in a process called photosynthesis. The word photosynthesis comes from the greek *phos* (light) and *synthesis* (putting together). Photosynthesis is the conversion of CO2 and H2O into carbohydrates and O2. The photosynthesis occurs in two stages. In the first stage, the light dependent reaction, light is captured and NADPH and ATP are generated. In the second stage, the light-independent reaction, NADPH and ATP are used to reduce CO2 and produce carbohydrates. In higher plants the photosynthesis takes place in specialized organelles called chloroplast. Chloroplasts contain three types of membrane systems: an outer membrane, an inner membrane and a network system of thylakoid membranes (Fig 3).

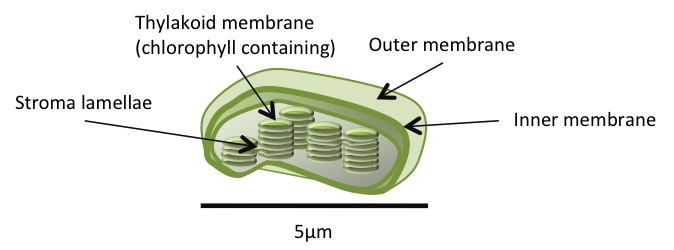


Figure 3. Schematic illustration of the chloroplast with thylakoid membranes indicated. Illustration drawn by Magnus Hillman.

Thylakoids are arranged in stacked structures called grana and unstacked structures called stroma lamellae both of which are located in the stroma. The thylakoid structure is complex and the membrane network contain more than hundred different proteins (extrinsic and intrinsic), pigments (chlorophyll *a* and *b*, carotenes, xanthophylls), membrane lipids (galactolipids, sulfolipids, phospholipids), plastoquinones, tocopherols and phylloquinones7,8.

The thylakoid membrane houses all biological structures involved in solar energy conversion: light harvesting proteins, reaction centres, electron-transport chains and ATP-synthase (Fig 4). The main extrinsic membrane complexes are plastocyanin (PCy) and ferredoxin (Fd) and the main intrinsic membrane protein complexes are photosystem I (PS I) with light harvesting complex I (LHC I), photosystem II (PS II) with light harvesting complex II (LHC II), cytochrome b*6*f and ATP synthase9.

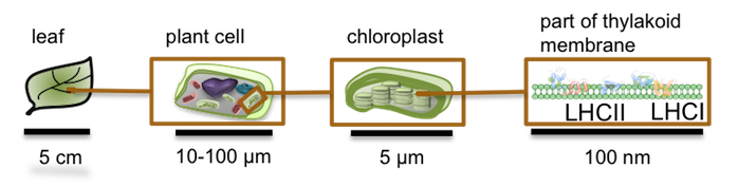


Figure 4. Overview of the structural hierarchy of thylakoid membranes with relative sizes indicated. LHC II and LHC I = light harvesting complex II and I.

During photosynthesis chlorophyll molecules attached to the photosystems I and II captures photons from sunlight, which excites electrons inside the chlorophyll molecule (Fig 5). The excited electrons are used to transfer the electrons from H2O to a quinone and simultaneously evolve O2, which diffuses to the atmosphere. The proton gradient is used to drive the synthesis of ATP. The electrons are further transferred to the Cyt-b*6*f allowing a flow of electrons to PCy and finally through the photosystem I that leads to reduction of NADP+ to NADPH. The light dependent reaction of the photosynthesis thereby uses sunlight and water to produce NADPH and ATP by use of chlorophyll molecules and integral membrane proteins located in the thylakoid membrane. The light-independent reaction aims to reduce CO2 to carbohydrates in a process called the Calvin cycle which require the newly formed NADPH and ATP.

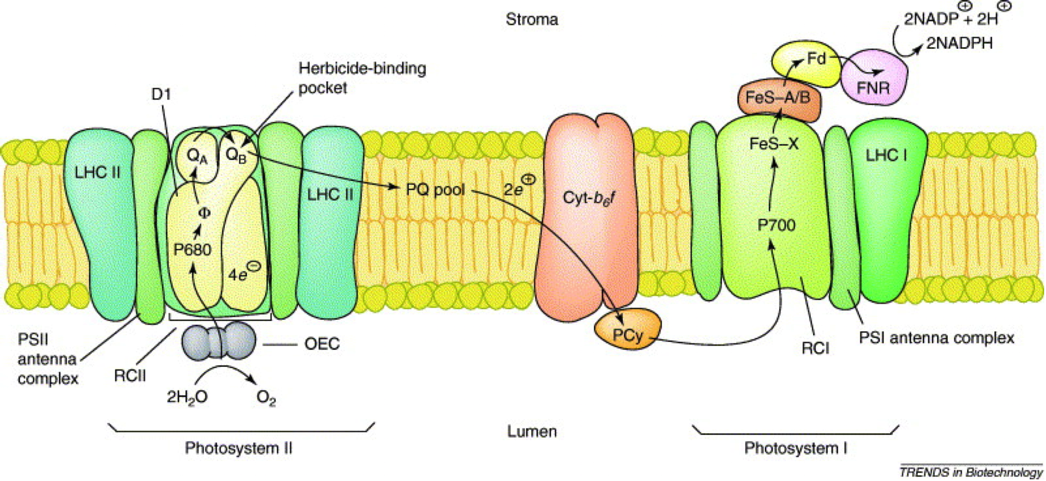


Figure 5. A schematic representation of the electron flow from water oxidation to the reduction of NADP+. Illustration adapted from Giardi et al (2005)9.

The thylakoid structure is complex and the membrane display a heterogeneity in surface charge as well as exposure of hydrophobic and hydrophilic areas along and across the membrane10. The charge heterogeneity is thought to contribute to the stacking of thylakoids. The membrane exposes positively charged groups in certain regions especially the stroma lamellae10,11 (connective structure linking the granum, Fig 3) and negatively charged side groups enriched at the granum surface10. Differences in ratio between negatively charged and neutral lipids along the thylakoid membrane may also contribute to the heterogeneity10. However, the isoelectric point for the thylakoid membrane is 4.7 and thus negatively charged in physiological pH. Thylakoid membranes remains stacked (Fig 3) when dispersed in medium with high ionic strength whereas they unstack when dispersed in medium with low ionic strength12.

All biological membranes contain intrinsic membrane proteins exposing hydrophobic groups oriented towards the membrane lipids. The photosystem I and II are the largest membrane protein complexes in the thylakoid membrane and accounts for almost 70% of the thylakoid mass7. The photosystems are housing the light harvesting complexes LHC I and LHC II with associated chlorophylls (Fig 6). The X-ray structure of LHC I and II has been reported as trimers built up by hydrophobic helices with attached pigments such as chlorophyll *a* and *b*13. It has been shown that chlorophyll *a* separates the helices inside the monomers whereas chlorophyll *b* separates the different monomers from each other. By this arrangement the pigments act to prevent aggregation of the hydrophobic helices inside the LHC I and II and are thereby supporting the structure14.

In a series of previous studies it has been demonstrated that thylakoid membranes prolong lipolysis by inhibition of pancreatic lipase/co-lipase in a dose-dependent way *in vitro*2. The inhibition of lipase/co-lipase activity *in vitro* was further investigated *in vivo*, and found to induce satiety in rat2 and mice3 as well as in humans6,15. Thylakoids reduce the lipase/co-lipase activity by up to 80% by primarily adsorbing to the oil-water interface and thereby hindering the enzyme complex to reach its substrate2,16. Below follows a description of the human lipid digestion in the absence of thylakoids, to provide a background of the dietary lipids route from oral ingestion to storage in adipose, cardiac and skeletal muscle tissue.

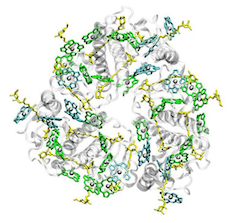


Figure 6. Schematic picture of the light harvesting complex II structure in the thylakoid membrane with chlorophyll *a* (green), chlorophyll *b* (turquoise) and carotenoids (yellow) indicated. Chlorophyll *a* and *b* stabilize the hydrophobic regions inside the LHC II. Illustration adapted from Wikimedia commons.

## Lipid digestion and lipolysis

### Composition of dietary fat

The main function of the gastrointestinal tract is to convert energy from the food and make it accessible for the bodys’ cells, as well as provide the cells with substrate needed for growth. The Western human diet contains approximately 40 energy % fat mainly composed by triacylglycerols (TG). Also a large number of lipid-containing compounds such as phospholipids, sterols, vitamin A and vitamin E are present in the food17. Lipid digestion is a complex coordination between lingual, gastric, intestinal, biliary and pancreatic functions. Lipid digestion and absorption lasts for 16-24 hours after food intake if no food is consumed after the initial meal18. The human digestive system is remarkably effective at digesting and absorbing most lipids. This is probably due to the evolutionary drive to maximize the energy intake where fat has the highest caloric value of all nutrients.

The dietary lipids in the human diet are mostly present in emulsion form, i.e. lipid droplets dispersed in an aqueous phase such as milk, ice cream, mayonnaise and sauces. This arrangement increases the available lipid surface in the intestine and facilitates the enzymatic lipid digestion and absorption. Lipid digestion is a highly efficient biological processes in healthy individuals with >95% of the lipids consumed being absorbed19.

The lipid fraction of the human diet is mainly composed of TG (95%). The TG structure is three fatty acids esterified to a glycerol backbone. TGs cannot be absorbed by the enterocytes in the intestine, but need to be hydrolysed prior to absorption20. Digestion is started in the stomach (approximately 15%) facilitated by gastric lipase expressed by the chief cells21. The acidic stomach environment is required for the gastric lipase, which is considered to be an extremophilic enzyme22. Pre-digestion creates hydrolysis products that increase the solubilisation of TG as well as binding of co-lipase to lipase in a later stage of lipid digestion (demonstrated by Erlanson-Albertsson and Larsson in 1986).

The main digestion of TGs takes place in the upper part of the intestine. When dietary lipids reach the duodenum, they stimulate secretion of the hormones cholecystokinin and secretin. The hormones, in turn, causes contractions of the gallbladder and bile salts are secreted via the bile duct. Bile salts have an unconventional structure compared to other surface-active agents (phospholipids, proteins etc.) in the gastrointestinal tract. Bile salts are derived from cholesterol and have a steroid structure of four rings with a side chain terminating in carboxylic acid in which either a taurine or a glycine can be conjugated. This structure results in a molecule with hydrophobic and hydrophilic regions which is thought to lay relatively flat onto the oil-water interface23 (Fig 7).

The lipid surface must be maximized in order to increase the efficacy of the enzymatic digestion process. Lipid droplets are divided into smaller droplets by muscle contractions in the stomach and by movements by the pyloric sphincter connecting the stomach with the duodenum. Several bio-surfactants such as phospholipids together with bile salts secreted from the bile together combat coalescence of the created lipid droplets.

Enzymes required for digestion are mainly secreted from the pancreas. Pancreatic juice contains several digestive enzymes such as lipases, proteases, amylases and nucleases24. Enzymes are delivered into the small intestine where ingested macronutrients are hydrolysed.

Lipases are water-soluble but have affinity for hydrophobic surfaces. Thus lipase has affinity for the interface created between the aqueous environment and the complex oil phase composed by dietary lipids. Almost all dietary TGs and DGs segregate into the core of the emulsion particle, which is covered by a monolayer or multilayer of polar lipids, phospholipids, fatty acids and small amounts of cholesterol. Additionally the surface is coated with denatured dietary proteins, oligosaccharides and bile salts21.

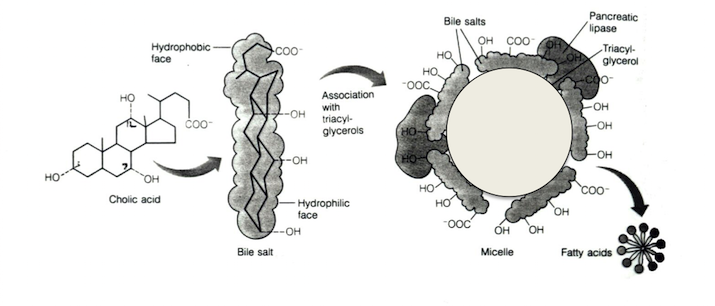


Figure 7. The hydrophobic surface of the bile salt molecule is associated with triacylglycerol (represented by a circle), and the polar surface of the bile salt is facing towards the aqueous solution. This allows association with pancreatic lipase/co-lipase, hydrolysing triacylglycerols to free fatty acids, which are released in much smaller micelles. The micelles are then absorbed through the intestinal wall. Illustration adopted and modified from educational material from Campbell University.

### Bile salts facilitate lipolysis

Lipase can bind to the oil-water interface on its own, but the adsorption and thus the activity is inhibited in the presence of bile salts, phospholipids or proteins with higher surface activity25-27. To solve this problem, lipase has a cofactor, co-lipase, which due to electrostatic interactions with bile salts, is able to adsorb onto a bile salt dominated interface (Fig 7). Co-lipase also binds to lipase in a 1:1 molar ratio28, hence enabling the adsorption of lipase onto the oil-water interface in the presence of bile salts21. Co-lipase alone has no enzymatic activity. In the absence of co-lipase, steatorrhea is present29. Due to the structure of bile salts (described above), they cannot pack onto the surface as efficiently as conventional surfactants. Conventional surfactants are amphiphilic molecules arranged on the oil-water interface with the hydrophilic head group facing the aqueous phase and the hydrophobic tail facing the lipid phase, thereby stabilizing lipid droplets in aqueous solution. Bile salts, with its different structure, can however adsorb into monolayer probably due to ionic interactions with the head group of the adsorbed surfactant. Bile salt will therefore not fully displace the adsorbed surfactant layer but will disrupt it enough to allow lipase/co-lipase to adsorb to the interface23. When the enzyme complex lipase/co-lipase has adsorbed onto the oil-water interface, it hydrolyses ester linkages in TGs, producing one sn2-[monoacylglycerol](http://en.wikipedia.org/wiki/Monoglyceride) and two [fatty acids](http://en.wikipedia.org/wiki/Fatty_acid).

### Lipid transport within the lumen

The hydrolysis products of dietary lipids (two FFAs and one MG) are poorly soluble in water. There is a need for displacement of the products from the interface and to pack them into water-soluble micelles23 to facilitate transport of the hydrolysed lipids from the aqueous medium in the lumen to the intestinal wall for further absorption. Hence mixed micelles are created by combining bile salts, FFAs, MGs, DGs lysophospholipids and cholesterol. The micelles are small and can readily diffuse through the aqueous medium to the enterocytes of the intestinal wall30. Thus the bile salts display two main functions in the lipolysis: to disperse the oil into small particles, and to displace and pack hydrolysis products in micelles for further transport and absorption through the intestinal wall23. The last step is crucial for allowing lipolysis to continue.

### Absorption and transport of lipids in the lymphatic system

When the micelles reach the enterocytes, the FFAs become protonated and leave the mixed micelle to diffuse through the lipid bilayer membrane where after FFAs and MGs are resynthesized to TGs. Short free fatty acids (C4-C12) are amphipathic and soluble in water and can cross the intestinal wall without protonation31. The exact mechanism for how lipid digestion products are transported through the intestinal wall is not clear. The traditionally assumed mechanism suggests that all digestion products passed the apical membrane by simple diffusion. Later theories state that the transport is facilitated by specific binding proteins to the endoplasmic reticulum where the lipid digestion products are resynthesized32. After absorption, the TGs are packed together with cholesterol and proteins into a transport lipoprotein called chylomicrons (Fig 8). The chylomicrons are exported to the lymphatic system for further transport to the adipose, cardiac and skeletal muscle tissue. The TGs are again hydrolysed and free fatty acids are absorbed by the cells. When a large portion of the TGs inside the chylomicron is released, the chylomicron remnant is transported to the liver. An overview of fat digestion, absorption and storage in the human body can be seen in Fig 8.

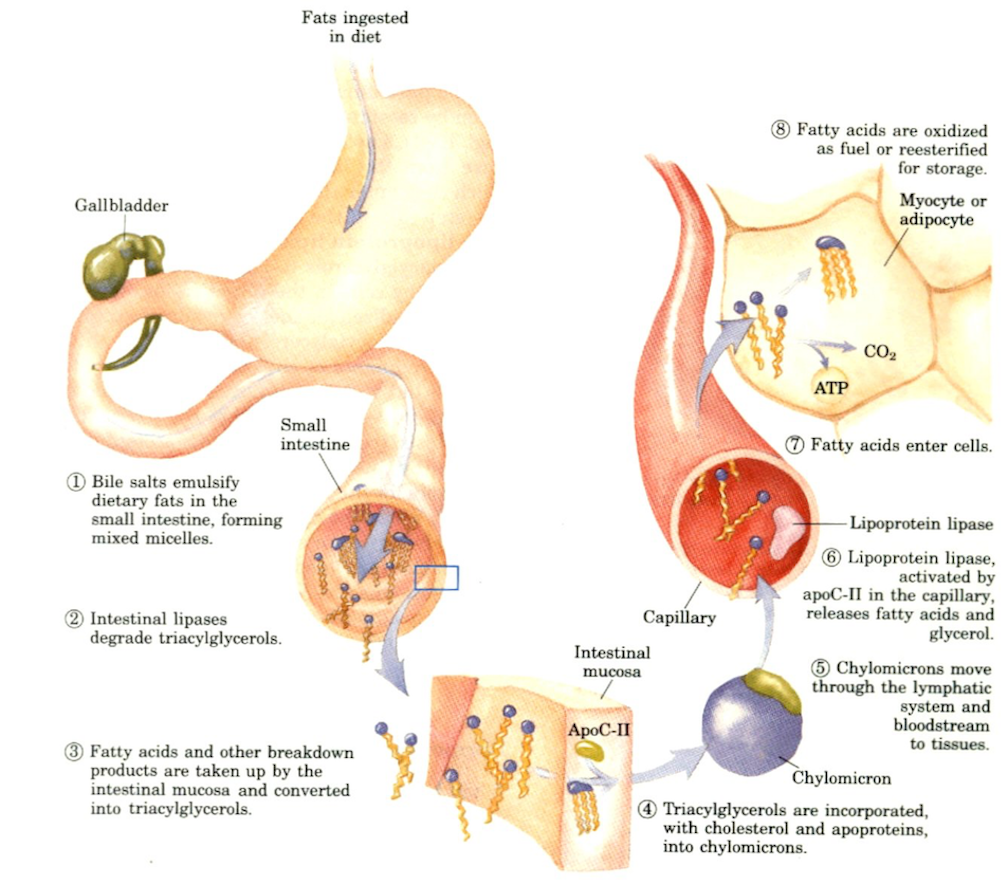


Figure 8. Overview of fat digestion, absorption and storage in the human body. Illustration adopted from educational material from Campbell University.

## Appetite regulation and historical development in strategies to modulate lipolysis

Obesity is a disease characterized by an imbalance between caloric intake and energy expenditure. The pharmaceutical industry has invested many efforts in producing antiobesity drugs. Since dietary lipids represent the major source of caloric intake, the inhibition of fat digestion is an interesting approach for reducing fat absorption33. During the last decades a lot of effort has been invested in the searching for substances that can potently reduce fat digestion with minimized side effects. The present thesis is a contribution to this ongoing work, and in the following section the different approaches are reviewed.

### Appetite regulation and ileal brake

Appetite is regulated by both neural and hormonal signalling. Physical factors are distension of the stomach and sensorical properties of the ingested food such as taste and texture. When food has entered the stomach, neural inputs are sent to the brain, which results in secretion of several satiety hormones such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1)34. These hormones are signalling that food has been ingested and that nutrients are absorbed. CCK is secreted in the small intestine specifically in presence of proteins and fat and stimulates the release of pancreatic enzymes needed for digestion35. CCK also decreases the intestinal motility and increases satiety36. GLP-1 is secreted by the intestine and is stimulated by all macronutrients but is especially elevated by fat37. GLP-1 delays gastric emptying and gut motility with increased satiety as consequence38. Upon fasting, when energy is needed, other hormones such as ghrelin are raised which stimulates hunger. Ghrelin is secreted from the gastric mucosa and stimulates gastric emptying39 signalling that more energy is needed and the hormonal cascade is starting over again.

Thus the gastrointestinal (GI) tract plays an essential role in regulation of food intake. The entry of nutrients into the ileum (distal part of the intestine) activates a negative feedback mechanism called the ileal brake5. The inhibitory effect of ileal brake is a result of neural and hormonal signals affecting the proximal parts of the intestine. Furthermore gastric emptying is delayed by exposure of the ileum for fats and fatty acids. Delaying of lipid digestion and absorption in the proximal part of the intestine allows lipids to progress to the ileum and is therefore an important factor for strengthening postprandial satiety.

### Orlistat – an irreversible lipase inhibitor

The only clinically approved pharmacological approach to modulate lipolysis present on the European market today is Orlistat, a lipid digestion inhibitor obtained from an actinobacterium. Orlistat acts by inhibiting pancreatic lipase irreversibly with reduction of triacylglycerol absorption as consequence. Long-term administration of Orlistat accompanying restricted diet has been shown to reduce body weight significantly compared to diet alone. Disadvantages include adverse effects as steathorrhea, bloating, and faecal incontinence, leading to reduced patient compliance. The gastrointestinal side effects are due to the irreversible inhibition of pancreatic lipase. Triacylglycerols remain unhydrolysed in the intestine and pass further down the gastro intestinal tract to colon where the lipids are released in the fecals causing steathorrhea. The effect of Orlistat on satiety and fullness is disputed. Damci et al found elevated levels of GLP-1 after Orlistat administration in obese patients with Type 2 diabetes40. Goedecke et al found no effect of Orlistat on CCK levels and appetite ratings after Orlistat treatment of healthy subjects41. Other reports reduced postprandial secretion of the gastrointestinal hormones CCK42,43, PYY43,44 and GLP-143,45 after administration of Orlistat to healthy subjects. Suppression of the satiety hormones may elevate appetite sensations leading to increased food intake and must be considered as a potential side effect of Orlistat.

### Dimaele – a transient synthetic lipase inhibitor

Due to the side effects of Orlistat, there has been an interest in the search for new substances, aiming to find an approach to reversibly inhibit pancreatic lipase concomitant with increased levels of satiety hormones. Dimaele (dimethylaminoethyldodecylether), a positively charged lipid compound was found to inhibit lipase/co-lipase *in vitro*46. When administered to rats fed a high-fat diet, the food intake was reduced in a dose dependent manner. The compound gave no steathorrhea as was demonstrated for Orlistat as a positive control. Instead, the secretion of pancreatic lipase and colipase was greatly stimulated, to compensate for the impaired lipolysis. The satiety hormone CCK was elevated compared to control. The elevated secretion of pancreatic lipase and co-lipase during treatment with dimaele was suggested to occur through the stimulation of CCK. Small intestinal satiety is largely mediated through CCK mechanisms. There is also evidence that fatty acids and monoacylglyerols are more satiating than the unhydrolysed triacylglycerols. Thus a compound that reduced the rate of lipolysis without leaving unhydrolysed lipids in the intestine may be a more satiating compound compared to a compound that leaves triacylglycerols in the intestine, like Orlistat. The trials with dimaele did not progress into human models due to concerns about the post absorption phase. Suspicions about what would happen with digestion products of dimaele systemically were raised and a potential risk could not be excluded.

### Galactolipids – an unstable transient natural lipase inhibitor

A candidate of natural origin that could reversibly inhibit lipase, and thereafter self be digested was regarded as a top candidate in the search for a new approach to modulate lipolysis. Galactolipids isolated from pea seedlings was reported to inhibit pure lipase/co-lipase in the presence of bile salts47. The hydrolysis rate was greater for MGDG compared to DGDG. Chu et al confirmed DGDG to inhibit lipase/co-lipase to a larger extent compared to MGDG48. The large headgroups of DGDG together with molecular packing at the oil-water interface was suggested to be an important factor for modulating the rate of lipolysis. However, when galactolipids isolated from pea seedlings were incubated in human pancreatic juice *in vitro*, lipase inhibition was not induced to the same extent as was demonstrated for pure lipase/co-lipase47. Andersson et al suggested the possibility that pancreatic juice contain other types of galactolipid-specific lipases, which induce digestion of galactolipids reducing their lipase-inhibiting effect47. Olibra, an emulsion composed of palm oil emulsified with hydrophilic galactolipids were demonstrated to delay GI transit time when delivered directly into the GI tract of healthy subjects49. Olibra was administered to healthy subjects via oral intake in a 12 weeks randomized, double-blind, parallel trial. Administration of Olibra decreased sensation of hunger but failed to exert a consistent effect on food intake, appetite regulation, body weight or body composition50.

### Thylakoid membranes – a stable transient natural lipase inhibitor

The search continued after materials rich in galacolipids that could remain undigested in the GI tract long enough to delay lipolysis. Thylakoid membranes isolated from green leaves are known to be rich in galactolipids. Albertsson et al demonstrated that thylakoid membranes isolated from spinach inhibited up to 80% of the pancreatic lipase/co-lipase activity *in vitro*2. In a series of investigations it was demonstrated that the active part of the thylakoids membrane inhibiting lipase/co-lipase were surprisingly not galactolipids, but the hydrophobic proteins one such being the alpha helices inside LHC I and LHC II2. Another advantage with these membranes was the stability against proteolytic hydrolysis. To evaluate the resistance towards enzymatic breakdown, thylakoids were treated with pepsin, trypsin, gastric and pancreatic juice. In all cases, several of the proteins in the thylakoid membrane were degraded within 30 min, while the main parts of the pigment-protein complexes were resistant for hours51. Emek et al suggests that pigments closely bound to membrane helices of the thylakoid membrane proteins protected these from digestion. The resistance to enzymatic attacks was further elevated when oil was emulsified with thylakoids prior to digestion. The effect of thylakoids in animal and human studies has been investigated in numerous studies. Mice fed a thylakoid-enriched high-fat diet for 100 days had suppressed food intake, body weight gain and body fat as well as elevated CCK levels compared to high fat-fed control mice3. Thylakoids administrated to rats fed a high-fat diet for two weeks suppressed food intake, reduced blood lipids and raised CCK2.

The results were confirmed in human studies. Administration of oil emulsified by thylakoids was later reported to induce loss in body weight, reduce LDL cholesterol and increase the satiety hormone GLP-1 in a three months study in voluntary overweight women4. Thylakoids has also been shown to suppress hunger motivation and increase CCK levels in an acute meal study in overweight women6. The main mechanism for appetite regulation is suggested to be prolongation of lipolysis due to thylakoids attached to the oil-water interface hindering lipase/co-lipase to reach their substrate16. The inhibition of lipolysis is not persistent as demonstrated as absence of lipids in the fecals52.

### Other natural inhibitors of pancreatic lipase

Many other medicinal plants may provide safe, natural and cost-effective alternatives to synthetic drugs in prolonging lipolysis. Thus hundreds of extracts from plants, fungi, algae and bacteria are being evaluated worldwide, to find a natural source for lipase inhibitors. Most of the common compounds found in different plant species with lipase inhibitory effects are polyphenols, saponins or terpenes. The molecular structures link them together; they are all characterized by conjugated aromatic rings.

Many plant extracts rich in polyphenols such as peanut shells53, tea plant extract54,55 and cocoa tea extract56 have ben reported to inhibit pancreatic lipase *in vitro*. Administration of extracts from peanut shells reduces body weight in rats fed a high fat diet53. Black tea polyphenols suppressed postprandial hypertriglyceridemia in a dose-dependent manner in rats54 and prevented increases in body weight and adiposity in mice fed a high-fat diet55. A single oral administration of cocoa tea extract produces an inhibition of plasma triacylglycerides in olive-oil loaded mice and triolein-loaded rats56.

Saponins belongs to the family of secondary metabolites that occur in a wide range of plant species. Compounds rich in saponins have been isolated from different parts of the plants including the roots, rhizomes, stems, bark, leaves, seeds and fruits to evaluate inhibitory effect on pancreatic lipase. A saponin mixture extracted from the seeds of the Japanese horse chestnut was reported to have a strong inhibitory effect on pancreatic lipase57. In mice fed a high-fat diet, saponin extract from the chestnut seeds suppressed the increase in body weight, adiposity, and increased the triacylglycerides in the feces, whereas it reduced plasma tracylglycerides after oral administration58,59. Saponins from ginseng roots have been shown to reduce plasma triacylglycerols and suppress expected weight gain in mice fed a high-fat diet.

Terpenes are the primary constituents of the essential oils of many types of plants. The pharmacological interest for triterpenes (three terpene units in the molecule) has increased during the last two decades demonstrating beneficial properties such as wound healing, antibacterial, anti-inflammatory and antitumoral effects, combined with low toxicity60. Triterpene extract from bark of birch have also been shown to display an inhibitory effect on pancreatic lipase61.

Lipase inhibitors of plant origin also include certain proteins such as those from soybeans62 or wheat germ63. Other basic plant proteins such as protamine64 and poly-lysine65 also display lipase inhibitory effect homologous to amphiphilic proteins like ovalbumin and beta lactoglobulin found in milk66. The proteins are thought to act by desorption of lipase from its substrate due to a change in interfacial quality67.

The plant extracts with lipase inhibitory effect are promising but only a few have proceeded to human studies. Ginseng extract rich in saponins decreased circulating levels of cholesterol and triacylglycerides but did not display any effect on weight in an 8 weeks study in human68. Administration of green tea extract rich in polyphenols for 6 weeks lowered body weight in moderately obese subjects by stimulating thermogenesis and increasing energy expenditure69. For all extracts more data are needed to define effects, optimal dose required and mechanisms of action. Also, possible side or toxic effect must be examined before entering human trials.

## Processing and formulation

All plant extracts have to be processed and formulated, in order to be used as hygienically safe and convenient products by consumers.

### Heat treatment

All foods produced for human consumption must be microbiologically safe, i.e. the growth of microorganisms must be kept under control to prevent food poisoning. Potentially harmful bacteria can grow under beneficial circumstances and be a threat to both product quality and human health. The most common way of controlling microbial growth in the food industry is by heat treatment. The food material is heated to a certain temperature and is kept at the specific temperature for a certain amount of time. The combination of temperature and time required to reduce microorganisms to safe levels differ among foods. Milk is pasteurized at 72°C for 15 seconds whereas vegetable juice often is pasteurized at higher temperature and longer holding times typically 90°C 15 seconds (carrot juice) and 90°C 30 seconds (orange juice). Several factors determine the temperature and time required such as the microbiological load in the start material, pH and water activity. Most microorganisms grow slowly at acidic pH and at low water activity (availability of free water which can be utilized by microorganisms, enzymes etc.), hence can lower processing temperatures and times be used under these circumstances. The expected shelf life of the product is also considered when designing the heat-treatment set up. A long shelf life requires a harsher product heat treatment to assure a low microbiological load.

Spinach leaves grows on open fields and is microbial contaminated, as all plant materials in contact with soil. Mesophilics, psychrotrophics and pseudomonaduceae are commonly present on spinach leaves, as well as enterobacteriaceae, micrococcaceae, coliforms, lactic acid bacteria and yeasts70.

Heat treatment reduces most pathogens except spores. For spore elimination, sterilization (121°C for 15 min) must be used. Although necessary, heat treatment has to be carefully designed to achieve the microbial safety but with minimal quality loss. Quality losses associated with heat treatment are degradation of proteins, colours and micronutrients as well as taste. Heat treatment of green leaves such as spinach71, mint72 and coriander72 induces chlorophyll degradation.

Furthermore, the photosynthesis is known to be the most heat-sensitive physiological reaction in the plant cell73. The colour changes from bright green to olive brown when the magnesium ion in the chlorophyll molecule is substituted by two hydrogen ions74. Also, it was shown that heat treatment of isolated thylakoids membranes induces aggregation. Aggregation of thylakoid membranes subjected to heat has been reported for plants *in vivo* as well73. The aggregation is hypothesised to be a defence mechanism for the leaf. Heat is in nature associated with sunlight. When an excess of sunlight is available, the plant must exudate the energy surplus to avoid severe damage. Aggregation of the light harvesting complexes (LHC I and LHC II) in the thylakoid membrane facilitates exudation of excess energy73. Heat also induces release of a macromolecule called plastoglobuli, which are associated to carotenoids, aiming to take care of free radicals in the cell. Thus the plant cell exposed to heat is less protected from free radicals as peroxidase, which induces structural rearrangements in the protein complex where chlorophylls are attached73.

Heat treatment of plant material is therefore a balancing between enough heat treatment to assure elimination of pathogens in the plant material to microbiological safe levels, but at the same time as mild treatment as possible to minimize loss of product quality.

In cases where heat treatment is not applicable there are other microorganism reducing techniques that can be applied. Dry product as herbs is often irradiated to reduce pathogens75. This method has several advantages, but is not commonly accepted by the public in Europe and is therefore not allowed for most food products. Other eliminating methods can be fermentation or disinfection by ethanol. Another method is inoculation of bacteria as lactic acid bacteria. The health beneficial lactobacilli are then used as biocontrol agents to prevent growth of pathogens and funghi76.

### Drying

Formulating plant extracts in powder form by drying increase the number of suitable product applications. The powder formula offers several advantages such as long shelf life and flexibility in suitable applications as health drinks, sauces, marinades, yogurt, nutrition bars and cereals. Powders are easy to dose in processes and both transport and handling costs can be significantly reduced compared to liquid containing formulas. Drying is a mass transfer process where water is evaporated from a solid, semi-solid or liquid material. The removal of water decreases the water activity, thus less free available water for microorganisms and chemical reactions. Open air-drying using sun and wind has been used as preservation strategy for food as meat, fish and fruit since ancient times.

Drying is a common unit operation in the food and health product industries and it is well known that drying processes can affect the quality of the product. Drying can induce various changes in physical, chemical and biological characteristics such as protein denaturation, changes in colour and structure. Undesirable biochemical reaction can also develop during drying such as deterioration of aroma compounds or degradation of nutritional substances. When handling high-value foods, the choice of preservation method may be crucial77. In the present thesis drum drying, spray drying and freeze drying of thylakoid membranes has been evaluated.

*Drum drying* is commonly used in production of baby gruels78, confectionary ingredients79 and starches80 (Fig 9). The drum is heated internally by saturated steam and the product is spread on the outer surface (100-120°C) and dries quickly (less than one minute). The product is scraped from the surface and can be further milled to obtain a free-floating powder80. Advantages include low production costs and high throughput, but a major drawback is severe quality losses in the final product caused by the high temperatures used in drum drying81.



Figure 9. Illustration of a drum dryer in industrial scale adapted from Huaxiadrying.

*Spray drying* is widely used in a variety of different commercial application areas such as the production of milk powders82, instant drinks for vending machines83, and biotechnology products such as enzymes84 (Fig 10). The method has several advantages including rapid drying and high throughput due to continuous operation85. The liquid feed is pumped through a nozzle with an atomizer, which in turn creates small droplets meeting a stream of pre-heated air inside the drying chamber. The droplets are rapidly dried (few seconds) due to the large created droplet surface area and efficient heat and mass transfer between the droplets and pre-heated air85. The feed flow rate, dry matter content in feed, and atomizer speed affect the powders’ physical properties such as particle size, sphericity and moisture content85,86. However, due to the relatively high temperature and abundance of air, there may be some loss of product quality, especially with respect to vitamins, aromas and colour87.



Figure 10. Illustration of a spray dryer in industrial scale adapted from Mozoro.

*Freeze drying* is a drying process based on sublimation of ice from a frozen product under reduced pressure77. Freeze drying is considered as a gentle drying process where most of the chemical and microbiological deterioration processes are inhibited due to the absence of liquid water and low temperature characteristic of freeze drying. However, the disadvantages are high energy consumption, high production costs and low throughputs due to batch processing87,88 which limit freeze drying to heat sensitive high value products where the additional cost can be justified. Though, it must be emphasised that freeze drying should not be regarded as an expensive drying process in all cases. The energy spent in the freeze drying process becomes insignificant when it comes to high value raw materials such as instant coffee and berries. If the freeze drying gives a reasonable added value to the products or if its keep the high-value it should be worth consideration77.

### Emulsions and emulsion models

Thylakoids are amphiphilic with hydrophobic and hydrophilic domains. This supramolecular structure enables thylakoids to stabilize the oil-water interface of, for example emulsions.

An emulsion consists of two immiscible liquids. One of the liquids is dispersed as small droplets in the other liquid. In food the two liquids are usually oil and water89. Though the definition of emulsions is wider and can include other liquids as well. The mean diameter of the dispersed droplets in food emulsions are typically 0.1 – 100 μm. Emulsions are classified according to the distribution of the different phases. Oil droplets dispersed in water is referred to as oil-in-water emulsions e.g. milk, cream, ice cream, mayonnaise, beverages and soups. Similarly is water droplets dispersed in oil is referred to as water-in-oil emulsions e.g. margarine and butter. The material that makes up the droplets is usually referred to as “dispersed phase” whereas the surrounding liquids is referred to as “continuous phase”.

Conventional emulsions are thermodynamically unstable due to the unfavourable contact between oil and water, and the phases will separate over time. Therefore, creation of emulsions that are kinetically stable over a period of time for practical use in the food industry (e.g. weeks or months) requires a stabilizer to avoid coalescence. Surface-active emulsifiers are amphiphilic and can adsorb to an interface with the lipophilic part heading towards the oil phase and the hydrophilic part heading towards the water phase. This arrangement decreases the surface tension, which facilitate disruption of emulsions droplets during homogenizing, which aids in the formation of emulsions containing smaller droplets. An emulsion with smaller droplets is generally more stable compared to an emulsion with larger droplets due to lower movement velocity such as creaming or sedimentation depending on the nature of the system. Emulsifiers also increase steric hindrance or electrostatic repulsion, which further increase the stability of the emulsion. Stabilizing agents used in food emulsions are divided in low mass surfactants and high-mass surfactants90. Low mass surfactants (e.g. monoglycerides, lecithins and fatty acids) are very mobile at the interface and are particularly efficient in reducing the interfacial tension. High mass surfactants cover proteins (e.g. whey protein, caseinate, soy protein, egg protein) and polysaccharides (e.g. gum arabic, modified cellulose and starches)91.

Some surfactants have been shown to induce allergies and are therefore not suitable for incorporation in food. Surfactants are not specific but can induce changes in the selective permeability of both skin and tight junctions in the intestinal wall. Unfortunately this facilitate paracellular uptake of allergens92. Chassaing et al demonstrated that two common food emulsifiers (carboxymethylcellulose and polysorbate-80) induced alterations in the gut microbiota, allowing growth of bacteria with mucolytic effect93. The mucosa layer covering the epithelial cells lining the intestine was reduced in thickness, causing increased intestinal permeability. The altered microbiota composition also induced low-grade inflammation and metabolic syndrome in mice. Therefore it is of uttermost interest to find new non-allergenic emulsifiers, which do not alter the intestinal permeability and microbiota in a non-beneficial way, to be used in the food industry.

In the present thesis thylakoid membranes isolated from spinach has been shown to stabilize lipid droplets in oil-in-water emulsions. Due to their size and characteristics, thylakoid membranes fall under the category of high mass surfactants. Thylakoid membranes are rich in proteins and carries both positive and negative charges (see chapter “The structure and function of thylakoids membranes within plants” above for further details). Thylakoids have not shown any allergenic tendencies neither in toxic tests nor human long-term trials. Also, thylakoids have been demonstrated to enhance intestinal permeability (Paper I) and increase the diversity of the microbiota favouring beneficial bacteria as lactobacillus94. This makes thylakoids a suitable stabilizer in food emulsions.

In Paper III, IV and V emulsion models have been used as a tool to investigate the thylakoids’ interfacial properties post thermal and drying processing as well as during storage conditions. The results from Paper III and IV have been successfully correlated to the rate of lipolysis. When thylakoids are damaged by heat treatment or drying, the capacity to adsorb to the oil-water interface is reduced hence larger emulsion droplets. The same is true in the lipolysis system: damaged thylakoids cannot adsorb to interfaces (lipid droplets, lipase/co-lipase) as good as non-treated thylakoids, which facilitate access at the oil-water interface for lipase/co-lipase, which increase the digestion rate.

### Detoriation processes in dehydrated foods

Dehydrated foods are subjected to a wide range of biological, chemical and physical deterioration processes during storage (Fig 11). The processes are often initiated during processing and dehydration operations and continue during storage at a rate that is influenced by storage conditions95. The chemical, microbiological and nutritional quality during storage has been shown to be dependent upon storage temperature, oxygen, light, total moisture content and water activity of the food products96.

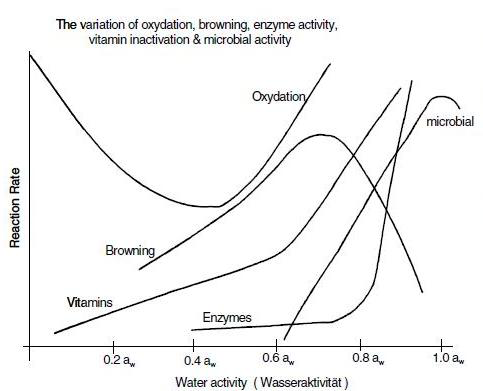


Figure 11. Deterioration processes in dehydrated foods are dependent on water activity. Illustration adapted from Neutec group.

Water activity (aw) is a key parameter that determines storage stability and is a measurement of the amount free water that can take part in biochemical reactions and allow for microbial growth. Water activity is defined as

where *p* is the partial vapour pressure of water in the material being measured and *p*0 is the vapour pressure of pure water at the same temperature. Per definition, the water activity of pure water is aw=1. The water activity of dried products should be relatively low to reduce microbial growth, enzymatic degradation processes and loss of nutrients. Growth of bacteria are generally inhibited below aw=0.9 and yeast and mould will not grow below aw=0.65. Most oxidative reactions and enzymatic reactions will be inhibited as aw is decreased. There is, however an important exception; at very low water activity, lipid oxidation is accelerated (Fig 11). At aw below 0.2, dehydrated products that contain even traces of fat may be subjected to auto-oxidation resulting in rancidity and off-flavours97. The relationship between total moisture content and the water activity of the food, over a range of values, and at a constant temperature yields a moisture sorption isotherm (Fig 12)98. Sorption isotherms of most foods are nonlinear, generally sigmoid in shape.

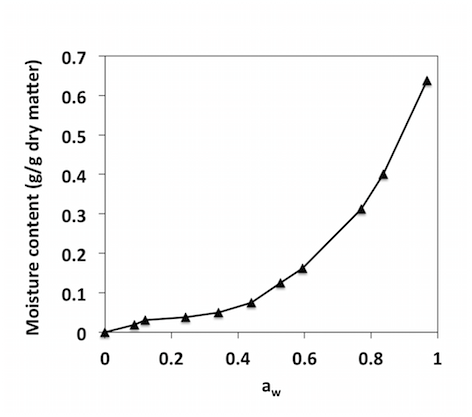


Figure 12. Moisture sorption isotherm at 25°C for freeze-dried spinach leaves. Adapted from King et al99.

Colour is a major quality attribute in dried fruits and vegetables. Usually during drying and storage, colour may change due to a number of chemical and biochemical reactions. Examples of chemical reactions that could affect colour during drying and storage are Maillard reactions, caramelization and ascorbic acid browning. Biochemical reactions affecting colour and other quality attributes during storage are often enzymatic degradation processes. Fruits and vegetables often contain phenolics, which are substrates to a naturally occurring enzyme in most plant tissues called polyphenoloxidase97. The enzymatic activity is initiated during drying and continues during storage. The enzymatic reaction may proceed to form oxidised forms of phenolics, which further polymerize to form brown pigments during storage. Chlorophyllase is known to catalyse degradation of chlorophyll100 and lipoxygenase is the major enzyme involved in carotene degradation101. A common way of preserving the colour attributes is blanching prior to dehydration and storage, which inactivates the enzymes101. Heat-induced inactivation of enzymes can thus minimize the loss of some colours and nutrients. However, there are enzymes that are heat stable. Peroxidase, the most heat stable enzyme in plants, produce phytotoxic free radicals that react with a wide range of food constituents including ascorbic acid, carotenoids and fatty acids. Some of these reactions cause undesirable changes in dehydrated foods affecting colour, aroma, and flavour as well as causing loss of nutrients95.

Unacceptable flavours in foods may develop through microbial and chemical action on food components during storage. Carotenoids are for example susceptible to oxidation when exposed to light, oxygen and enzymes during storage, and degradation of β-caroten is associated with the development of an off flavour95. The loss of quality attributes during storage can in some cases be dependent on specific drying technique applied prior storage. Loss of carotenoids has been reported to be greater in freeze-dried products compared to other drying techniques, due to the porous structure obtained from freeze drying. The porous structure facilitates oxygen transfer and promotes rapid oxidation, yielding dull colours and off flavours102.

A number of different deterioration processes reducing the nutrient content can take place during drying. In addition to destruction of nutrients during processing, a significant loss occurs during storage. This loss can be attributed to storage temperature, pH or exposure to oxygen, relative humidity and light. Ascorbic acid, vitamin C and vitamin A are sensitive to heat and oxidative degradation102. The levels are therefore greatly reduced during drying and subsequent storage when exposed for moisture accelerating the oxidative degradation96,103. Riboflavin, thiamine and niacin are moderately heat stable and riboflavin is sensitive for exposure to light during storage102.

The complexity of the deterioration processes makes it difficult to anticipate the quality of dehydrated foods during storage. On the other hand, the complexity opens possibilities to tailor storage conditions that minimize loss of quality attributes important for the specific food. For best retention of nutrients, colour and flavour in dried food, the food material should be stored cool and dark in airtight containers to avoid penetration of moisture. Generally, storage conditions allowing the food to keep aw=0.4-0.5 is optimal for dehydrated food. This range prevents most biochemical reactions and keeps the oxidation to a minimum.

# Methodology

The aim of this chapter is to give an overview over the methods and techniques used in the thesis and to provide a discussion and reflections over their strengths and weaknesses. Also, results from method optimisations are presented and discussed.

## Isolation of thylakoids

Thylakoids have been isolated in three different ways: the sucrose method, the pH method and the water method. The sucrose method was the first isolation method used in the project. The method yielded a very pure thylakoid extract and the isolation process was time consuming and the method was not applicable for larger quantities of raw material. Large amounts of thylakoids were required to perform animal and human studies and therefore a large-scale method (the pH method) was developed104. The water method is similar to the pH method and was developed by replacing protein precipitation with centrifugation as separation operation. The water method thereby reduces the time required for the isolation procedure.

Confounding factors for all isolation methods are choice of raw material. Spinach exposed to intense sunlight during cultivation has darker colour and more pigments compared to leaves grown under other circumstances. Spinach can be grown in different geographical areas and the cold chain during transport might not be properly maintained. If so, the leaves begin to deteriorate which alter the product quality in terms of microorganism growth. Also, it is harder to break the cell walls in thawed softened leaves, thus a reduced yield of thylakoid membranes in the isolation procedure.

### The sucrose method

The sucrose method aims to break plant cell walls via osmotic pressure in a series of dispersing and centrifugation steps, and several washing steps are also included. Due to the osmotic lysis and careful and repeated washing, the sucrose method yields the purest thylakoids of all investigated isolation methods. Fresh baby spinach leaves (*Spinacia oleracea*) were homogenized in a blender with homogenising buffer (50 mM phosphate buffer (pH 7.4), 5 mM MgCl2, 300 mM sucrose) (1:1 w/w) for 5 min until a homogenous slurry was obtained. A typical batch size was 100 g spinach and 100 g buffer. The slurry was filtered through four layers of Monodour polyester mesh (20 μm) and centrifuged (2000 g, 15 min, 4°C). The bottom sediment were re-suspended four times in different media: the first time in homogenising buffer, the second time in blast media (5 mM MgCl2) which induced cell breakage by osmotic lysis, and for the third and four times in washing media (10 mM tricine, 5 mM MgCl2, 300 mM sucrose, pH 7.4). After the final washing step, the bottom sediment was dispersed in a glass Potter Elvehjelm homogeniser until an homogenous slurry of thylakoid membranes were obtained. The isolated thylakoids were extremely concentrated in the Potter Elvehjelm step and it was impossible to transfer the slurry into test tubes without loosing some material, which affected the yield. Thylakoids isolated with the sucrose method were used in the intestinal permeability experiments (Paper I).

### The pH method

Thylakoids were isolated from spinach essentially as described by Emek et al104. Frozen spinach leaves (*Spinacia oleracea*) were homogenized in a blender with water (1:1 w/w) for 3-12 min. A longer mixing time resulted in a higher yield, around 25% for 3 min mixing time and up to 40% for 12 min mixing time. A typical batch size was 5 kg spinach and 5 kg water. The slurry was filtered through four layers of Monodour polyester mesh (20 μm) and the filtrate was diluted with water (1:10 v/v). The pH was adjusted to 4.7 with 1 M HCl and the suspension was incubated in dark, 4°C for 8h. The supernatant was discarded and the bottom sediment were re-suspended in fresh water (1:10 v/v) and incubated in dark, 4°C for 16h. The supernatant was discarded and the bottom sediment was collected. Confounding factors are the mixing time (as discussed above) and the individual handling technique in the elution of the supernatant. The slurry were incubated in 10 L buckets and it was difficult to separate the supernatant from the bottom sediment since the sediment were very loose with a flow profile close to laminar flow with no significant sharp interface between the supernatant and the sediment. Depending on how much of the sediment that was accidently discarded, the yield would differ. Thylakoids isolated with the pH method were used in spray drying experiments (Paper IV).

### The water method

Thylakoids were isolated from spinach essential as described by Albertsson et al2. Frozen spinach leaves (*Spinacia oleracea*) were homogenized in a blender with water (1:1 w/w) for 3-12 min. A typical batch size was 5 kg spinach and 5 kg water. The slurry was filtered through four layers of Monodour filter (20 μm) and was centrifuged at 5000 x g, 4°C, 30 min. The supernatant was discarded and the thylakoids in the bottom sediment were collected and re-suspended with fresh water in a glass Potter Elvehjem homogeniser until an homogenous slurry was obtained. Confounding factors were individual handling technique were the extremely concentrated thylakoids were impossible to transfer without losing some material which affected the yield. An advantage with the water method compared to the pH method is the higher chlorophyll content in the resulting powder. Thylakoids isolated by the water method had around 65 mg chlorophyll/ g powder compared to around 15 mg chlorophyll/g powder after isolation by the pH method. A possible reason may be a higher fraction of soluble proteins and fibres in the pH method compared to the water method. In the water isolation method, the soluble proteins and fibres were separated during centrifugation. Thylakoids isolated by the water method were used for thermal treatment experiments (Paper II and III), drying experiments (Paper IV) and storage experiments (Paper V).

## Intestinal permeability

Previous studies have demonstrated that thylakoid membranes prolonged absorption of lipids by inhibition of the lipolysis. To examine whether thylakoids inhibited uptake and absorption of other dietary substances as well (such as simple glucose, larger carbohydrates and proteins), an absorption study in Ussing diffusion chambers were performed. Ussing chambers are an *in vitro* model used for studying of diffusion of molecules over various tissues and has been widely used in physiological and pharmacological studies since the 1950s.

In this specific study, two half-cells were connected with a rat intestinal segment mounted between, with the mucosal side of the intestine facing one chamber and the serosal side facing the other105. The intestine was collected from the anesthetized rat, cut in pieces and was immediately mounted in the chambers. The connecting area between the chambers had an exposed intestinal area of 1.78 cm2. The chambers were filled with Krebs buffer, kept at 37°C and were connected to a carbogen supply. The mounted intestines were considered viable for a minimum of 2 hours. A test solution containing Krebs buffer, oleic acid, NaTDC, methyl-D-glucose (glucose marker), FITC-dextran (carbohydrate marker) and ovalbumin (protein marker) was added to the mucosal side of the intestine. Krebs buffer was added to the serosal side of the chamber system. Thylakoids isolated by the sucrose method were added in varying concentrations to the mucosal side together with the test solution. The passage of marker molecules from the mucosal side to the serosal side was quantified during 120 min. The Ussing chambers were mounted with several proximal and distal segments from 8 rats in total. Confounding factors are the individual handling technique when mounting the tissue in the chambers. The intestines must be stretched during the mounting in the chambers and can easily break with consequent leakage. The intestine *in vivo* is an elastic material. In the Ussing chamber, the tissue was stretched which may influence the size and elasticity of the intestinal cells and the transport of components through the tissue may therefore be affected.

## Pancreatic lipase activity

An estimation of the physiological function of the thylakoid membranes were desired to determine the effect of different treatments (heat treatment, drying methods etc.). The thylakoids’ ability to inhibit pancreatic lipase/co-lipase activity *in vitro* was chosen asfunction-related parameter. Lipase/co-lipase hydrolyses triacylglycerols to monoacylglycerols and free fatty acids, which results in a decreased pH. NaOH is titrated to neutralize and keep the pH stable at 7.0. The consumption of NaOH is taken as a measurement of the enzyme activity.

The enzyme activity was monitored essentially as described by Albertsson et al2. The substrate was prepared in a vial by adding 0.5 ml tributyrine to 15 ml buffer (2 mM Tris maleate, 0.15 M NaCl, 1 mM CaCl2 and 4 mM NaTDC, pH 6.9). Thylakoid membranes were dispersed in the solution and lipase (1 mg/ml, 3-15 μl) and co-lipase (1 mg/ml, 3-15 μl) were added. The incubation was performed at 25°C with stirring maintained by a magnetic stirrer under standardized conditions. The mean consumption during 10-20 min was taken as activity of lipase/co-lipase. The method have several confounding factors, one challenge are the varying quality of commercial enzymes. The purity and activity were different from batch to batch, which made it complicated to compare inhibition data between enzyme batches. The enzymes were often unstable and lost rapidly activity during storage in freezer between trials. In the present thesis, percent of control is used to somewhat overcome the problem with varying enzyme quality. Commercial enzymes are used in Paper II and III whereas enzymes manually purified from pancreas106,107 were used in Paper IV. The manually purified enzymes had higher activity and the activity was more stable over time. Therefore, the intra-variation coefficient was lower using these enzymes compared to the commercial, which reduced the numbers of replicates required to give sufficient reliable data. Typically six to nine replicates were used in experiments using commercial enzymes whereas three replicates were used using the purified enzymes. The temperature of the buffer was important for the results where room-tempered buffer gave results with less deviation. The incubation time varied between 10 and 20 min in the experiments. 20 min was used for experiments with commercial enzymes whereas 10 min was used for the purified enzymes. Control experiments were carried out to assure that 10 min incubation gave the same result as 20 min for the purified enzymes. The experiments were performed at 25°C, although 37°C would have simulated the *in vivo* environment better. The low incubation temperature was due to limitations in the equipment. Due to variation in enzyme quality, the enzyme activity at standardized conditions (0.5 ml tributyrine, 15 ml buffer, during 20 min at 25°C) were kept as constant factor. The enzyme concentration in the vial was therefore adjusted to give an activity of 0.0020-0.0025 mmol/s for the control. The added enzyme volume varied between 3 μl and 15 μl. A control (vial without addition of thylakoids) were run every fourth samples to assure that the enzyme activity were stable. Otherwise, the enzyme concentration was adjusted.

The pH stat method was designed to quantify enzymatic activity specifically. To achieve this, the enzyme activity should be the only limiting factor and all other parameters such as enzyme concentration and available lipid surface must be kept constant. Triacyglycerols with short-chained fatty acids were used as lipid substrate in the pH stat method. By adding constant amount substrate in the trials with stirring maintained on a constant rate, the available lipid surface could be kept constant. In the gastrointestinal tract the enzymes are released in excess and therefore the available lipid surface becomes the limiting factor. In the pH stat method on the other hand, the enzyme concentration and lipid surface were kept constant. By this arrangement, the enzymatic activity can be quantified.

In general, the pH stat method is an *in vitro* method, which cannot be directly extrapolated to the human body system due to several reasons:

* The route of the hydrolysed FFAs in the vial is not comparable to the *in vivo* digestion. In the GI tract, the FFAs are transported to the epithelial wall and are absorbed which affect the equilibrium and drives the lipid hydrolysis reaction further. In the *in vitro* model, all introduced molecules were present in the vial during the experiment. This may possibly lead to product inhibition of the lipolysis. In the present thesis, all inhibiting data were related to a control to minimize the problem.
* Tributyrate (4 carbon backbone) was used as substrate whereas dietary lipids are typically long chained fatty acids of 18-20 carbon backbone. Tributyrate is used due to high diffusion rate of hydrolysed FFAs. Since no active transport of hydrolysis products is available in the *in vitro* model, the hydrolysed FFAs must rapidly diffuse from the oil-water interface to not inhibit the hydrolysis reaction.
* When the food enters the intestine *in vivo*, it has already passed the oral cavity as well as the stomach. During these stages the food has been subjected to extensive chemical, physical and mechanical degradation. In the *in vitro* method, lipids are introduced directly to the enzyme system without any pre-treatment.
* The *in vivo* digestion process is much more complex with numerous components such as surface active proteins, emulsifiers and other bio-active substances present in the GI tract at the same time. These substances have affinity for the oil-water interface and are competing of the limited surface. The synergistic effect of these components is not fully known and cannot be neglected.
* The enzyme concentration *in vivo* differs from time to time and also between individuals. It is therefore difficult to mimic a true enzyme concentration *in vitro*.

However, the pH stat method can still be used in screening processes to evaluate enzyme inhibition candidates, which can be further evaluated with higher accuracy in animal and human models.

## Interfacial properties

The thylakoids’ interfacial properties are important for the ability to inhibit lipase/co-lipase. To inhibit the enzyme complex, thylakoid membranes must attach to the lipid surface and remain at the interface. Thus, ability to attach to and stabilize the oil-water interface was considered an important property and was quantified via the size of the stabilized emulsion droplets. An efficient emulsifier stabilizes small emulsion droplets at a given concentration compared to an inefficient emulsifier. An emulsion model system was set up to possibly complement the complex pH stat method.

Emulsions were prepared in glass test tubes with 2 ml phosphate buffer, 1 ml miglyol and varying amount of thylakoids by homogenizing with an UltraTurrax at 22 000 rpm for 60 sec. The emulsions were incubated dark and in 4°C for 60 min before the particle size distribution were analysed by laser diffraction (Malvern Mastersizer). In some experiments additional replicates were performed which were incubated 24 hours and 7 days to quantify the emulsion stability. The emulsion model were used in Paper III, IV and V.

The emulsion model system has been investigated and optimised:

* The oil fraction in the emulsions was varied between 7%, 14%, 20% and 33%. Oil fraction of 33% gave the smallest emulsion droplets and was therefore chosen throughout the thesis.
* The total emulsion volume in the glass test tube was varied between 3 ml and 6 ml. The smallest droplets were created in test tubes with total volume of 3 ml, probably due to limitations in the homogenizing equipment, and 3 ml were therefore chosen throughout the thesis.
* Air bubbles can be introduced during the mixing, which generates false large droplet size distributions in the particle size analysis, since air bubbles are typically larger than emulsion droplets. To avoid air entrainment the rotating knives in the mixing equipment were kept well below the liquid-air interface under the mixing to minimize bubble formation.
* The pump rate (1000 rpm, 2000 rpm and 3000 rpm) of the particle analyser was also evaluated. Higher pump rates resulted in smaller emulsion droplets. To investigate which pump rate that reflected the true droplet size distribution, a manual size distribution were performed. Over 300 emulsion droplets were measured in light microscope and were binned in a histogram comparable to the size distribution generated by the droplet size analyser. Pump rate of 2000 rpm gave an equivalent distribution with the same maximum as the manual size distribution and were therefore used throughout the thesis.
* The emulsion system is less complex compared to the pH stat method. For example no competing substances as bile salts were present in the system, the buffer was less sophisticated and the actual effect of enzyme inhibition were not monitored. Though the emulsion results demonstrated a strong correlation to the enzyme inhibiting results and the emulsion model were considered useful. Since the model is mainly used for comparison and evaluation of different treatments of thylakoids, interesting information regarding the thylakoids ability to stabilize the oil-water interface can still be provided.

## Determination of chlorophyll content

Chlorophyll content is used as a quantitative measurement of non-treated thylakoids throughout the thesis since there is a constant ratio between the two parameters. Since chlorophyll molecules are attached directly to LHCI and LHCII known to be the structures responsible for enzymatic inhibition, the quantitative method were considered overall useful. Chlorophyll was determined spectrophotometrically.

Thylakoids (10-50 μl slurry or 0.2-0.8 g powder) was added to 2 ml ice-cold acetone (80 vol%). The samples were vortexed and incubated dark and on ice for 20 min and were thereafter centrifuged at 12100 g for 4 min at 25°C, before spectrophotometric measurements were carried out. Spectra were obtained over the wavelength range 200 – 1000 nm at intervals of 0.5 nm against a blank of acetone (80 vol%). Samples were analyzed in triplicate. Chlorophyll content was calculated as described by Porra et al108.

Confounding factors are the challenge of pipetting acetone correctly. If the acetone were kept ice cold the variation between the replicates could be kept to a minimum. Determination of chlorophyll has been conducted in Paper I, II, III, IV and V.

## Powder production

Production of thylakoid powders was evaluated in Paper IV, by varying the isolation method (pH method and water method, see above for details), formulation (with or without maltodextrin) and drying method (drum drying, spray drying and freeze drying).

The drum-dried powder was produced under industrial conditions. Typical drum drying conditions have a drum surface temperature of 105°C with drying completed in approximately 30 sec.

The spray-dried powder was produced using a lab scale spray drier. Drying conditions were inlet air temperature 120°C, outlet temperature 72-75°C and feed flow rate of 0.6 L/h. The pre-heated air had a flow rate of 540 L/h. Optimization trials had been performed prior to the trials presented in the thesis, varying the flow rate (0.3 to 1.5 L/h) and the inlet air temperature (90°C to 200°C). Feed flow rate and inlet air temperature together determine the outlet air temperature, which in turn was the exposed temperature for the final powder. It was shown that the outlet temperature was negatively correlated to the lipase-inhibiting capacity of the powder: a low outlet temperature yielded a powder with high lipase-inhibiting capacity. Furthermore, powders with low outlet temperature had a high water content and high water activity. Due to a compromise between water activity and temperature, a setting yielding a low outlet temperature with an acceptable water activity was chosen. An outlet air temperature of 70-72°C was used throughout the spray drying trials in Paper IV, which corresponds to the outlet temperatures used in industrial scale. Also, the outlet temperature affected the colour of the resulting spray-dried powders. The powders exposed for the lowest outlet temperature were dark green and the powders exposed for the highest outlet temperature were light green. This was probably due to heat-induced degradation of chlorophyll. Drying in the lab scale spray drier limited the variables to inlet temperature, flow rate and feed concentration. The inlet temperature and flow rate were varied but the feed concentration (solids) were kept constant during all trials.

Freeze drying was carried out using a pilot freeze dryer. Thylakoid paste was distributed into an aluminium tray to form a layer of approximately 10 mm. The sample were frozen in -18°C for 24 hours before freeze drying. The plate temperature was 20°C, the condenser -50°C and the vacuum pressure of the dryer was 0.02 mbar. The residence time for the sample in the freeze-dryer was seven days. The residence time could probably be shortened but was not investigated in the thesis.

## Powder characteristics and quality

Powder characteristics and quality is important in evaluation of powder production processes. A product with the desirable physiological effect cannot be promoted unless the safety of the product can be assured. Furthermore, to ensure a standardised production of a functional food it is crucial to know and control the characteristics.

Paper IV evaluated thylakoid powders produced in different ways through a series of parameters, both characteristics and functional properties. A dry matter analysis analysed according to AOAC was included to evaluate if the moisture content was varying between the different production methods. If so, the concentration of the active component in the powder would vary, and the dose must be adjusted accordingly when exchanging production methods. Water activity was analysed to investigate the possibilities for microbial growth in the product and also the risk for accelerated lipid oxidation. Confounding factor can be exposure of the powder to surrounding air with higher humidity prior to analysis. To avoid this, the sealed airtight packages of thylakoid powder were opened directly prior to the analysis and were thereafter closed again. It was known that the chlorophyll content (se above for analysis details) was important for the lipase-inhibiting effect of the thylakoids in aqueous phase. Therefore, chlorophyll in the powders were analysed to evaluate if the same was true for the powder formula. Colour analysis was performed to investigate if the green colour could be used as marker and possibly replace the chlorophyll extraction method. The colour attributes (L-, a- and b-values) were measured on dry bulk powders with a portable spectrophotometer. Advantages with the colour method over the chlorophyll extraction method are no handling of solvents and less time consumption. Particle size distribution were analysed by the standard method sieving. Confounding factors are that the choice of sieves (both numbers and mesh size) has a large impact on the particle distribution results. Many sieves of different mesh size will give a distribution closer to the true distribution while few sieves will give a rough distribution, which can be difficult to interpret. In Paper IV five sieves with mesh size of 45-250 μm were used. The particle size of a powder is important for the powder flowability, which is an important factor in handling of the powder during storage in silos and transportation109. Also the two functional parameters lipase-inhibiting capacity and emulsifying capacity were determined for all powders (see above for details).

## Powder storage and sorption isotherms

It is important to control the quality of a functional food ingredient over the entire production chain, both in terms of powder characteristics (i.e. colour, water content, water activity), microbiological safety as well as the intended physiological effect. Dehydrated foods are most often hygroscopic and absorb moisture from the surrounding air. Sorption isotherms, information concerning the relation between water content and water activity in the dehydrated food is therefore useful information when designing packages. If a food is sensitive to moisture, the package must be airtight, not allowing moisture to penetrate. The product must also be microbiological safe and have the same physiological effect over the promised shelf life. Therefore, it is important to study alterations of the active ingredient during storage.

In Paper V, the characteristics (water content, water activity, colour, chlorophyll content), and interfacial properties of thylakoid powders dried in three different ways (drum drying, spray drying, freeze drying) after 8 months storage at 20°C in varying relative humidity were evaluated. Four relative humidity levels were used in the experimental setup: 10 RH%, 32 RH%, 47 RH and 61 RH%. The levels were chosen to reflect the indoor climate in Scandinavia with extreme levels included. The recommended indoor relative humidity is 30-70 RH% which is optimal for the human respiratory organs110. However, common indoor relative humidity often range from 20 RH% to 40 RH% in Scandinavia111, although it can be much higher in other parts of the world. Relative humidity is strongly dependent on temperature and during winter in cold climates, the relative humidity indoor is generally reduced, sometimes below 15 RH%. The relative humidity levels 32 RH% and 47 RH% included in the experimental setup thereby reflects average indoor relative humidity. The lowest level included, 10 RH% reflect the low relative humidity indoor a winter day and the highest level, 61 RH% reflect the high relative humidity a warm summer day.

In Paper V, emulsifying capacity was used as indicator for the active ingredient in the thylakoid powders since emulsifying capacity previously has been demonstrated to be linked to lipase/co-lipase inhibiting effect (Paper IV). Water content, water activity, colour, chlorophyll content, and emulsifying capacity were analysed before the start of the storage experiments to allow a reference level.

In order to produce controlled atmospheres holding the specific relative humidity stated above, saturated salt solutions were prepared in the proportions given in Table 1. Salt and water were mixed, transferred to desiccators, and were allowed to stand for seven days to equilibrate. The solutions were stirred for a few minutes every day. To assure that the system had reached equilibrium, the relative humidity in the desiccators was determined by a portable hygrometer. When the system had reach equilibrium, three grams thylakoid powder from each drying method was transferred to open Petri dishes and placed in the desiccators. After 2 weeks and 4 months, a series of non-invasive analyses (colour measurements and water activity) were performed to follow changes in the powder. These methods allow representative sampling over a large area without disturbing the system. After 8 months storage in varying relative humidities, the experiment was terminated. Water content, water activity, colour and chlorophyll content were analysed as described above. Sorption isotherms, describing the relation between water content and water activity in each of the powders investigated were calculated. Emulsifying capacity of the thylakoid powders stored for 8 months was also determined. Emulsifying capacity (m2 surface/mg emulsifier) corresponds to the maximal surface that can be created and stabilised by a unit emulsifier and is used as a measurement of the emulsifiers’ efficiency. Emulsions were prepared as described above and the particle size distribution was analysed. A probe mass of 64 mg (dry basis) thylakoid powder was used to maximize the measurement sensitivity and ability to register changes in interfacial properties of the thylakoids during storage. Emulsifying capacity were thereafter calculated from the particle size distribution, see Paper V for equations.

Table 1. The quantities of salt and distilled water used in the preparations of the saturated salt solutions112.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Salt | Salt quantity (g) | Water quantity (ml) | Theoretical RH% | Experimental  RH% |
| LiCl | 150 | 85 | 11 | 10 |
| MgCl | 200 | 25 | 33 | 32 |
| Mg(NO3)2 | 200 | 30 | 52 | 47 |
| KI | 111 | 75 | 69 | 61 |

## The multiregression model

Partial Least Square 1 (PLS1) and multivariate analysis is a useful tool to examine correlations of a series of variables to a single response. The data is converted and expressed in principal components (PC) to describe the variation and co-variation of the variables in less number of dimensions. The PLS1 analyses were carried out using Unscrambler (Camo Software, Norway).

In Paper II, the PLS1 model was used to correlate absorption wavelengths in the spectrophotometer analysis to lipase-inhibiting capacity of the thylakoids. The analysis was used as a tool in a screening process for potential biological or biochemical structures that were degraded by thermal treatment, which could explain the reduced lipase-inhibiting capacity of the heat-treated thylakoid membranes. If a single wavelength or a span of wavelengths were found strongly correlated to lipase inhibiting capacity, the structure that absorbs light at those wavelengths could possibly be identified. All wavelengths from the absorbance spectra (200 nm – 1000 nm) were loaded as variables and lipase/co-lipase activity were loaded as the response variable.

In Paper IV, the PLS1 model was used to evaluate which of the physicochemical properties of the thylakoid powder that had the highest influence on lipase-inhibiting capacity. When one or several physicochemical properties were identified, it would open possibilities to optimise the inhibiting capacity of the thylakoid powders. Moisture content, water activity, L-value, a-value, b-value, total chlorophyll content, chlorophyll *a* content, and emulsifying capacity (AUC for *d*43 for emulsions stabilised by thylakoids) were loaded as variables and lipase/co-lipase activity (AUC) was loaded as response. Since the regression model require replicates, the powder particle size obtained by sieving could unfortunately not been investigated due to lack of replicates. A disadvantage with the PLS1 is that the model only handles linear relationships. Non-linear relationships are therefore not discovered by the PLS1 method.

Statistical correlations are not causal relationships. To fully understand a system, other possible connective factors must be examined to explore other (secondary) pathways between the variables. Also, a likely mechanism must be assessed to claim causality between variables. We found a correlation between lipase-inhibiting capacity and emulsifying capacity. We also found that the two variables were linked via chlorophyll (and green colour). Finally we suggested a mechanism: chlorophyll might act as steric barrier inside the protein complex known to be responsible for the thylakoids inhibiting capacity. When the chlorophyll molecules are degraded in one or another way, the steric function was altered with internal membrane structural collapse and reduced inhibiting capacity as consequence. The statistical multivariate models were therefore used rather as an exploratory tool, in addition to as a descriptive tool in the present thesis.

# Results and discussion

In this section the results and discussion from the individual papers will be presented.

## Paper I

*Chloroplast thylakoids reduce glucose uptake and decrease intestinal macromolecular permeability.*

The passage and permeability for three marker molecules of varying molecular weight decreased over the rat intestine in the presence of thylakoid membranes on the lumen side. The molecules investigated were methyl-glucose (190 Da), FITC-dextran (4 kDa) and ovalbumin (45 kDa). The decreased passage over the rat intestinal wall was proportional to thylakoid concentration. Significant differences were found between control and all thylakoid concentrations investigated, as well as between 1.2 and 2.9 mg chlorophyll/ml and 1.2 and 5.8 mg chlorophyll/ml. Permeability was calculated as apparent permeability coefficient (Papp) and indicated reduced trans-mucosal transport of the marker molecules in the presence of thylakoids at the mucosal side of the intestine. Thylakoids were treated with trypsin prior to incubation in Ussing chambers to simulate enzymatic degradation of the thylakoids *in vivo*. Trypsin-treated thylakoids further reduced the passage of marker molecules. After termination of the experiments, the intestinal segments were covered by a green layer on the mucosal side, whereas the serosal side appeared unaffected. Electron micrographs of the green mucosal side showed thylakoids covering the mucosal layer of the microvilli on the intestinal wall (Fig 13). Interactions between methyl glucose, FITC-dextran and ovalbumin and thylakoid membranes were investigated. All marker molecules displayed an affinity to thylakoid membranes. Methyl-glucose had an affinity of 22%, FITC-dextran 22% and ovalbumin 12% respectively. Even though a partition of the marker molecules was associated to the thylakoid membranes, the dominating part of the molecules was still present as free molecules in the mucosal compartment of the Ussing chambers. The hindering effect could therefore not be explained solely by affinity between marker molecule and thylakoids.

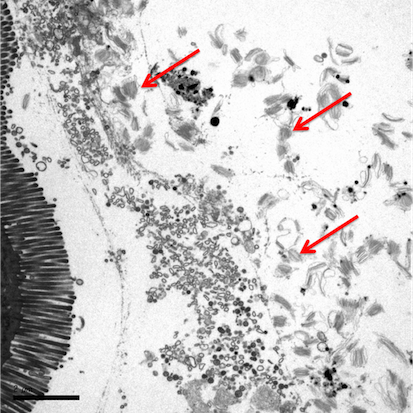


Figure 13. Electon micrograph of the mucosal side of the rat intestine. Microvilli are seen as a dark structure to the left and thylakoid membranes are indicated by the red arrows.

The suggested mechanism behind the decreased permeability of marker molecules was the thylakoids association to the mucosal layer on the intestinal wall. Thylakoid membranes have both hydrophilic and hydrophobic parts, and are displaying both positively and negatively charged surfaces. Even though the thylakoids net charge is negative in pH 7.4 (Krebs buffer used in the Ussing chambers) and the cells that lines the intestinal wall also is known to have a net negative charge, there are always regions with opposite charge. It is therefore suggested that the thylakoid membrane interact with the mucosal layer creating a hindering layer for passage of marker molecules. Thylakoids have been shown to swell in the presence of bile salts51, and treatment with trypsin results in an even larger swelling as demonstrated by Emek et al51. The elevated swelling results in an increased exposed thylakoid surface hence the decreased passage of marker molecules in the presence of trypsin-treated thylakoids. Affinity between the marker molecules and the thylakoids may contribute to the altered passage. Thus the present study presents a combined mechanism: i) thylakoids associated to the mucosal layer is creating a network which reduces the diffusion rate of the marker molecules investigated and ii) affinity between marker molecules and thylakoids physically hinders the marker molecules from passing over the intestinal wall.

The demonstrated results indicate that thylakoids can be useful both to control intestinal absorption of glucose as well as improve the barrier function of the intestine. A reinforced barrier has positive health effects as protection against harmful bacteria, avoidance of uptake of allergenic compounds and possibly avoidance or treatment for irritable bowel syndrome and disease.

## Paper II

*The effect of heat treatment of thylakoids on their ability to inhibit* in vitro *lipase/co-lipase activity*

Heat treatment at 60°C, 75°C or 90°C for time intervals ranging from 15 sec to 120 min reduced the thylakoids ability to inhibit lipase/co-lipase activity *in vitro*. Treatment at 90°C for 4 min reduced the lipase-inhibiting capacity to 20% of the capacity demonstrated for non-treated thylakoids. A colour shift from bright green to olive brown was also detected, attributed to chlorophyll degradation. The effects of heat treatment were both time and temperature dependent.

Thermal treatment induces degradation of chlorophyll to pheophytin and pheophorbide by replacement of Mg2+ with 2 H+. Replacement of Mg2+ affects both colour and polarity of the molecule, due to decreased polarity in the pyrrol part as is the case for pheophytin. The removal of the phytol chain, as is the case for pheophorbide, affects the amphiphilic properties drastically due to removal of the entire hydrophobic part. Chlorophyll is known to structurally stabilize hydrophobic alpha helices within the light harvesting complexes of the thylakoid membranes, known to be responsible for the lipase-inhibiting capacity. The degradation of chlorophyll to pheophytin and pheophorbide could lead to reduced stability inside the thylakoid membrane leading to aggregation of the hydrophobic parts. The aggregation in turn, affect the surface-active properties of the thylakoid membrane, hence the ability to adsorb to the oil-water interface and inhibit lipase/co-lipase activity.

A correlation between chlorophyll *a* and remaining lipase-inhibiting capacity after thermal treatment was established (R2=0.95) (Fig 14). Chlorophyll *a* can therefore be regarded as indicator of the structural status inside the light harvesting complexes of the thylakoid membrane. The relation between colour and lipase-inhibiting capacity opens possibilities to use spectrophotometric analysis as a complement to the pH stat method, to predict lipase-inhibiting capacity post heat treatment.

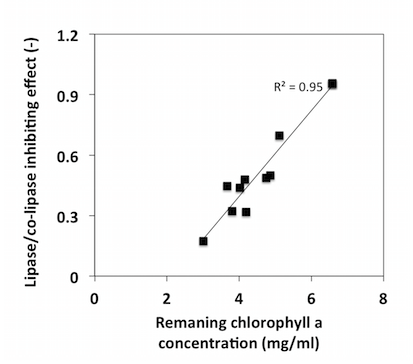


Figure 14. Chlorophyll *a* content and lipase/co-lipase inhibiting effect of thylakoids in aqueous solution after heat treatment (60°C-90°C for 15 sec-4 min) were closely correlated (R2=0.95).

Thylakoids were concluded to be heat sensitive. If heat treatment must be applied to assure microbiological safety, the dose must be adjusted accordingly. Knowledge about the degradation pattern of thylakoids post heat treatment makes it possible to design a thermal treatment process to ensure microbiological safety and at the same time minimize loss of function.

## Paper III

*Heat-induced aggregation of thylakoid membranes affect their interfacial properties*

Non-treated thylakoids stabilised oil-in-water emulsions. Droplet size decreased with increased thylakoids concentrations, levelling off at higher concentrations. Heat treatment at 60°C, 75°C and 90°C for 15 sec – 4 min prior to emulsification reduced the thylakoids ability to stabilise emulsions. Higher temperature and/or longer processing times resulted in progressively increased lipid droplets sizes, reduced emulsifying capacity and elevated surface load (Fig 15). The temperature/time effect was observed up to a certain limit. 90°C is such a high temperature that holding times longer than 15 sec did not result in significantly larger droplets and no further damage on the thylakoids occurred with longer times. All emulsions independent on heat-treatment creamed due to gravitational separation. Emulsion droplets stayed intact during 7 days storage with no visible oiling off. Emulsions were regarded as stable against coalescence.

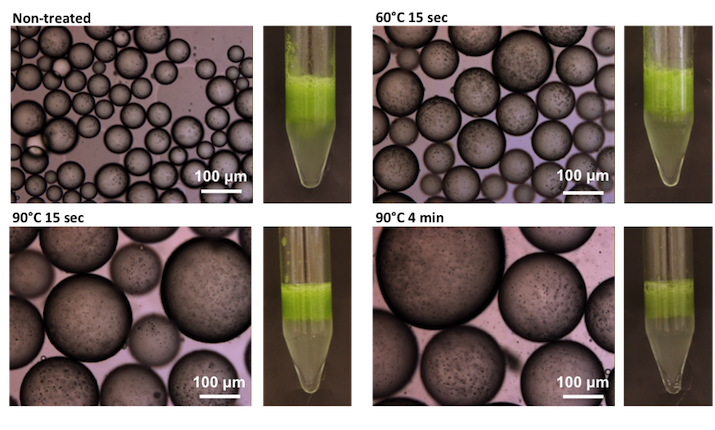


Figure 15. Micrographs (100X) of emulsion droplets stabilised by non-treated thylakoids and thylakoids treated at 60°C 15 sec, 90°C 15 sec and 90°C 4 min.

The surface-active function of thylakoids as lipid droplet stabilizers and lipolysis modulators are provided by alpha helices inside the light harvesting complexes LHC I and LHC II of the thylakoid membrane. In native thylakoids, helices are separated by chlorophyll to prevent the structure from collapse. Chlorophyll is an amphiphilic molecule with a polar head group and a hydrophobic tail which facilitate steric stabilization within LHC I and LHC II. Heat treatment induces degradation of chlorophyll with altered polarity as consequence. The hydrophobic helices cannot remain separated allowing aggregation. Hydrophobic parts are turned inside the aggregated LHC I and LHC II and the ability to stabilize the oil-water interface is thus reduced. Thylakoids are known to form inverted micelles at 55°C and above, with hydrophobic parts oriented towards the centre which facilitates aggregation of micelles. Thylakoids’ reduced ability to stabilize the oil-water interface post heat treatment is thus due to both i) reorganization of hydrophobic/hydrophilic parts inside the thylakoids and ii) heat-induced aggregation of membranes to larger clusters driven by inversion of micelles.

Lipolysis is mainly a surface-related process. To investigate if lipase-inhibiting capacity and surface-related emulsion parameters could be linked, a correlation analysis was performed. A linear relationship was established between the lipase-inhibiting capacity and the inverted surface-weighted mean droplet diameter 1/d32 (R2=0.80). Thylakoids’ lipase-inhibiting capacity after heat treatment has been reported to be closely linked to chlorophyll degradation (Paper II). A regression analysis was performed between 1/d32 and remaining chlorophyll *a* after heat treatment, and a correlation was established (R2=0.81). Thus all three variables i) lipase/co-lipase inhibiting capacity, ii) emulsifying properties and iii) chlorophyll content are linked.

The results suggest that thylakoids’ ability to inhibit lipase/co-lipase is mainly a surface-related process and if the surface-active properties of the thylakoid membrane are reduced, the inhibiting capacity will decrease accordingly.

To better understand and modulate the function of thylakoids in terms of appetite reducing abilities, attention must be paid to analysis and optimization of surface-related phenomena.

## Paper IV

*Characteristics and functionality of appetite reducing thylakoid powders produced by three different drying processes*

The effect of drum drying, spray drying and freeze drying on thylakoids physicochemical and functional properties was investigated. The different drying methods affected the powders physicochemical and functional properties.

Sieving experiment demonstrated drum-dried powders to have smaller powder particle sizes compared to freeze-dried and spray-dried powders. SEM-micrographs showed the size range for drum-dried powders to be large, ranging from very large structures (several hundreds μm) to smaller structures (around 50 μm). The drum-dried powders were dominated by large flake structures to some extent covered by very small spherical structures (1– 5 μm). The freeze-dried powder was also dominated by flake structures, but smaller compared to the drum-dried powders. The flakes were covered by spherical structures. The spray-dried powders were significantly different from the other powders, with exclusively very small spherical particles (1 – 5 μm). The particle size obtained from sieving and size information from the electron micrographs thus indicated different results. It is proposed that both are important. The sieving particle size is important for powder behaviour with respect to flowability. It is generally considered that powders with particles sizes above 200 μm is free flowing which is an advantage in handling processes such as packaging. The particle size information from SEM micrographs provided information regarding the powder morphology. It is suggested that thylakoid powders, when dispersed in aqueous solution are dissolved in smaller fractions and are thereafter not present as large powder particles but as much smaller structures. It is therefore proposed that the small aggregates (1 – 5 μm) detected in spray-dried powders, freeze-dried powders and to some extent in drum-dried powders are the functional size of the thylakoids with respect to enzyme inhibition and affinity for the oil-water interface. TEM-micrographs confirmed small aggregates in the size range 1 – 5 μm to be closely attached to the oil-water interface in oil-in-water emulsions.

Processing conditions affected the final colour of the produced powders. The colour of the powders was related to degree of heat treatment during drying which follows the degradation kinetics of chlorophyll. Powders dried at the highest temperature for longest time, i.e. drum-dried powders, had low chlorophyll content and less green colour compared to spray-dried and freeze-dried powders. Freeze-dried powders, not being subjected to heat during processing, had the highest chlorophyll content and most green colour of all powders investigated.

All powders stabilized oil-in-water emulsions. The lipid droplet diameter was reduced with increased amount of thylakoid powder. The spray-dried and freeze-dried powders stabilized smaller lipid droplets at lower concentrations compared to the drum-dried powders, indicating a higher affinity to the oil-water interface. Similarly, freeze-dried and spray-dried powders inhibited lipase/co-lipase *in vitro* to greater extent compared to drum-dried powders.

When optimizing and evaluating the effect of processing on a bioactive substance, it is crucial to identify a predominating (measurable) parameter determining the bio-functionality. In the present work, lipase-inhibiting capacity was chosen as predominating parameter. To evaluate which of the investigated physicochemical and functional parameters that had the highest impact on the lipase activity, a multiregression analysis was performed. L-value (lightness), a-value (greenness), chlorophyll content (both chlorophyll *a* and total chlorophyll) and lipid droplet diameter (d43) stabilized by thylakoid powders, was significantly correlated to lipase-inhibiting capacity. This confirms correlations found in previous work between chlorophyll, affinity to oil-water interface and lipase-inhibiting capacity for thylakoids in aqueous solution (Paper II and III). Of the significant parameters correlated to lipase-inhibiting capacity, the lipid droplet diameter (d43) stabilized by thylakoid powders had the highest correlation coefficient. It is therefore suggested that affinity to the oil-water interface is crucial for the thylakoids ability to inhibit lipase/co-lipase independent of formulation.

The active thylakoid structures interacting with lipid surfaces are known to be the hydrophobic alpha helices inside the light harvesting complexes. Chlorophyll acts as a steric barrier preventing aggregation of the hydrophobic helices. Furthermore, chlorophyll is known to be heat sensitive. When chlorophyll is degraded due to heat exposure, the polarity is altered and the ability to stabilize the alpha helices is reduced with aggregation as consequence. The aggregated protein complexes are less surface-active and thus a reduced emulsifying capacity compared to non heat-treated thylakoids. This might be the mechanism that explains why green colour, chlorophyll and emulsifying capacity is so closely linked to lipase-inhibiting capacity of thylakoids.

Thus, to optimise the thylakoids’ ability to inhibit lipase/co-lipase activity, the internal membrane structure displayed by the green colour should be preserved to as great extent as possible. The present study suggests the gentle freeze drying technique for production of thylakoid extracts with high lipase-inhibiting capacity. The results from the study can be applied towards production and optimization of a more standardised ingredient with appetite reducing ability.

## Paper V

*Effects of storage conditions on chlorophyll degradation in thylakoid powders produced by different drying methods*

The effect of storage in different relative humidities on thylakoid powders produced and dried in different ways was investigated. Powder characteristics and functionality, i.e. water content, water activity, chlorophyll content, colour and interfacial properties after 8 months storage were evaluated.

The water content of the powders increased during storage. The absorption of moisture was dependent on relative humidity of the incubation system with higher water content for powders incubated in higher relative humidity. The thylakoid powders were increasing in water activity and moisture content during incubation. Storage for 8 months affected the green colour and the lightness of the powders. All powders displayed an increased a-value (indicating loss of green colour) and decreased L-value (indicating darker colour) after storage. The changes were dependent on the specific relative humidity, with a greater loss of green colour when exposed to higher relative humidity.

Relative humidity showed a definite influence on the rate of chlorophyll degradation in thylakoid powders after storage (Fig 16). Chlorophyll content was dramatically decreased in freeze-dried and spray-dried powders after storage with a greater chlorophyll loss at exposure to higher relative humidity. Freeze-dried powder had 74 mg chlorophyll/g powder (dry basis) prior to incubation. After incubation in 10 RH% the chlorophyll content was decreased to 55 mg/g powder (-26% compared to prior storage) and after incubation in 61 RH% the chlorophyll content was decreased to 24 mg/g (-68% compared to prior storage). Spray-dried powder had 66 mg chlorophyll/g prior to incubation and 61 mg after incubation in 10 RH% (-8%) and 29 mg/g after incubation in 61 RH% respectively (-56 %). The spray-dried and freeze-dried powders displayed absorption spectra with a shift of maximum in the blue to shorter wavelengths after storage. This shift is associated with the chlorophyll degradation products pheophytin and pheophorbide. Freeze-dried powders displayed the overall largest chlorophyll degradation of the powders investigated. For spray-dried powders, chlorophyll was degraded to a low extent when the powders were exposed for low relative humidity, and the degradation was more pronounced for higher relative humidity. The loss of chlorophyll in the drum-dried powder was limited, which might be due to the much lower chlorophyll content at start of the incubation for the these powders. Drum-dried powder had 4.5 mg chlorophyll/g prior to incubation. After incubation in 10 RH% the chlorophyll content was decreased to 1.5 mg/g (-67%) and after incubation in 61 RH% the chlorophyll content was decreased to 1.04 mg/g (-77%). Drum-dried powders with maltodextrin had slightly lower initial levels, 2.9 mg chlorophyll/g. After incubation in 10 RH% the chlorophyll content was decreased to 0.96 mg/g   
(-67%) and after incubation in 61 RH% the chlorophyll content was decreased to 0.57 mg/g (-68%).

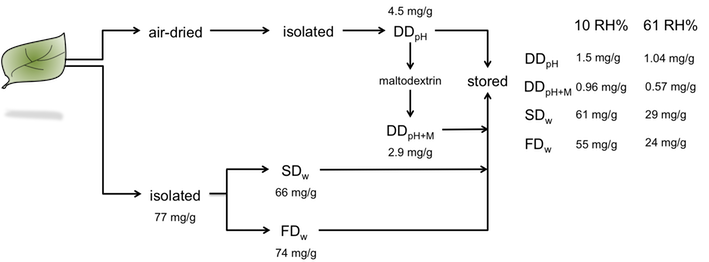


Figure 16. Schematic presentation of chlorophyll content in thylakoid powders in the different production steps and after storage in the lowest and the highest relative humidity (10 RH% and 61 RH%). Values indicated are chlorophyll content (mg chlorophyll/ g dry matter). DDpH= drum-dried powder produced by the pH method, DDpH+M= drum dried powder produced by the pH method with addition of maltodextrin, SDw= spray-dried powder produced by the water method, FDw= freeze-dried powder produced by the water method.

Chlorophyll is known to be degraded by two routes: heat- or acid induced degradation and enzymatic degradation by chlorophyllase. Furthermore, chlorophyllase is reported to be accelerated by higher relative humidity and inactivated by heat. It was therefore suggested that the chlorophyll degradation rate during storage was determined both by heat treatment applied in the drying processes prior incubation, and by enzymatic activity during storage.

It was suggested that drum drying (105°C for several seconds) induced chlorophyll degradation as well as inactivation of chlorophyllase. The subsequent absolute loss of chlorophyll during storage was therefore limited. This might be explained by low initial chlorophyll levels and limited enzymatic degradation. Drum-dried powders were therefore primarily restricted to one single degradation route (acid-induced degradation) during storage. Spray drying (72°C for a few seconds) induced limited chlorophyll degradation during dehydration and the heat treatment was severe enough to partly inactivating the chlorophyllase. Loss of chlorophyll during storage was therefore limited at low relative humidity due to low enzymatic activity. At higher relative humidity, the remaining chlorophyllase in the spray-dried powder was thought to be accelerated, -8% loss of chlorophyll during storage in 10 RH% and   
-56% during storage in 61 RH%. During incubation in high relative humidity, a significant loss of chlorophyll was observed due to two possible degradation routes (acid-induced degradation and enzymatic degradation). Freeze-dried powders were not subjected to any heat treatment at all during dehydration (20°C for 7 days). This gentle drying method preserved the chlorophyll content and the chlorophyllase in the produced powder. During storage, the loss of chlorophyll was significant due to high initial chlorophyll levels and intact chlorophyllase allowing chlorophyll to be degraded via two routes, as described for spray-dried powder above.

Thylakoids’ interfacial properties have previously been reported to be closely linked to lipase/co-lipase inhibiting effect (Paper III, IV). Prolongation of lipolysis is in turn correlated to prolonged satiety in animal and human models. Emulsifying capacity was therefore used as marker for the thylakoids’ functionality in the present study. Thylakoids’ emulsifying capacity was generally reduced after 8 months storage and the effect was dependent on the relative humidity during storage with a greater loss of emulsifying capacity at higher relative humidity. The reduction thereby followed the same pattern as for chlorophyll degradation. Freeze-dried powders were most subjected to loss of emulsifying capacity from their initial high level, followed by spray-dried powders. Both freeze-dried and spray-dried powders had an emulsifying capacity of 0.012 m2/mg before incubation. After incubation in 61 RH% the emulsifying capacity was decreased to 0.003 m2/mg (-75%) for freeze-dried powders and 0.007 m2/mg (-57%) for spray-dried powders. Drum-dried powders displayed a more modest reduction compared to the other powders. The initial emulsifying capacity was 0.006 m2/mg (drum-dried powder) and 0.005 (drum-dried powder with addition of maltodextrin). After incubation in 61 RH% the emulsifying capacity was decreased to 0.004 m2/mg for both powders. This was -34% decrease for drum-dried powders and -20% decrease for drum-dried powders with addition of maltodextrin.

The results indicated that the emulsifying capacity was linked to chlorophyll degradation. Chlorophyll is known to facilitate steric stabilization of the hydrophobic alpha helices inside the light-harvesting complex of the thylakoids. These alpha helices has been identified as the surface-active parts, enable thylakoids to associate to the oil-water interface. In the present study, chlorophyll in the powders was degraded not by heat (as in Paper IV) but by acid-induced degradation and enzymatic degradation. Though chlorophyll was degraded in another way, the emulsifying capacity was decreased. Thus the results indicate that chlorophyll is important for the internal structure of the thylakoid membrane. When chlorophyll is degraded, either by heat, acid or by enzymatic degradation, associations between the hydrophobic helices are promoted, causing reduced surface-activity thus reduced emulsifying capacity.

The results from the present study demonstrated that thylakoid powders are hygroscopic and absorb moisture from surrounding air during storage. Green colour, chlorophyll content and emulsifying capacity were reduced during storage and the effect was more pronounced for higher relative humidity. Freeze-dried and spray-dried powders had high initial levels and were more sensitive for deterioration processes compared to drum dried powders with low initial levels. Though sensitive for humidity, the spray-dried powders had the highest remaining levels of green colour, chlorophyll content and emulsifying capacity at all relative humidity levels investigated. Spray drying was therefore considered the most suitable drying method yielding a powder with best-maintained functionality after storage.

Packaging and storage are important factors for the shelf life of most foods. Based on the findings in Paper V, the thylakoid powders should be stored dark, in airtight packages, preferably single dose sachets, to control the powders’ total exposure to moisture during shelf life. The suggested packaging would ensure the thylakoids function as appetite-reducing ingredient after storage.

# General discussion

The work included in the thesis should be seen in the perspective of on-going work by the research group. In 2010, an attempt was made to incorporate thylakoids in meal components and investigate the effect on satiety and absorption of nutrients in humans. Thylakoids were incorporated in food items such as tomato sauce, granola müsli and focaccia bread which was subjected to heat treatment. The products were administrated to 20 overweight men in a three months crossover intervention study (data not published). This was the first time thylakoids were incorporated in heat-treated meal components and the thylakoid-enriched diet did not induce any further weight loss compared to control. The question of the effects of heat treatment and other standard processes on the thylakoids ability to prolong lipolysis and satiety were raised.

The present thesis investigates the effect of processing on thylakoids. Both non-treated thylakoids (Paper I, II and III) and treated thylakoids (Paper II, III, IV, V) have been evaluated. In Paper II and IV, the thylakoids’ effect on lipolysis *in vitro* investigated. However, the digestion and digestive tract is more than lipolysis. Therefore, Paper I investigates interactions between thylakoids and the intestinal wall.

Non-treated thylakoids displayed a high affinity against the rat intestinal wall (Paper I). It is hypothesized that the thylakoid membrane interacted electrostatically with the intestine. Although both the thylakoid membranes and the intestinal wall have a net negative charge at physiological pH, small positively charged areas are always present.

Due to the size of the thylakoid surface (1 mg chlorophyll corresponds to 2 m2 thylakoid surface) the number of exposed groups per thylakoid particle is large. Even if the individual group interactions are weak, the sum of them will be significant, hence a strong interaction with the intestinal surface. Thylakoids thereby act to enhance the barrier properties of the intestine, hindering passage of marker molecules of different size. Addition of thylakoids in various concentrations affected the passage of glucose molecules and larger macromolecules as FITC-dextran and ovalbumin *in vitro* in a dose-dependent way. This was the first time thylakoids was demonstrated to affect absorption of other dietary substances than lipids.

To address the question of heat treatments’ effect on the thylakoids, a study investigating the effect of heat treatment on thylakoids’ ability to inhibit lipase/co-lipase *in vitro* (Paper II) was performed.

The thylakoids’ interfacial properties post heat treatments, namely ability to stabilize the oil-water interface in oil-in-water emulsions, were also investigated (Paper III). Heat treatment (60°C, 75°C and 90°C for 15 sec - 120 min) affected both the thylakoids’ chlorophyll content, ability to inhibit lipase/co-lipase and ability to stabilize the oil-water interface.

The thylakoids were demonstrated to be sensitive to heat and the surface activity was reduced with both processing time and temperature. After 15 sec at 75°C, 50% of the enzymatic inhibition capacity was lost. Hence the results from the unpublished human intervention study may be explained by heat-induced inactivation of thylakoids. In later human studies, administration of thylakoids incorporated in non-heat treated meal components, has shown weight loss in human studies4.

Thylakoid powder dried by drum drying and freeze drying have been successfully used in several animal and human studies but the effect of processing had not been evaluated. Therefore, the effect of different drying techniques was evaluated (Paper IV). Thylakoids were dried by drum drying, spray drying and freeze drying. The effect of drying were proportional to degree of heat exposure during drying where freeze-dried thylakoid powder not subjected to heat at all had the highest chlorophyll content, the highest ability to stabilize oil-water interface and the highest lipase-inhibiting capacity. Multiple regression analysis revealed green colour, chlorophyll content and ability to stabilize oil-water interface to be significantly correlated to lipase-inhibiting capacity of the thylakoid powders.

A challenge for all dehydrated food products is to maintain their quality attributes during processing and storage. For functional foods, it is also important that the functionality is maintained. The food must have the promised physiological effect when it reaches the consumer, i.e. after storage. Therefore, the thylakoids’ powder characteristics and function was evaluated after 8 months storage in different relative humidities (Paper V). Both green colour, chlorophyll content and emulsifying capacity was decreased in all powders investigated, and the effect was moisture-dependent. Drum-dried powders displayed a limited loss during storage although low initial levels of green colour, chlorophyll content and emulsifying capacity. Freeze-dried powders displayed the greatest loss, due to high initial levels and maintained enzymatic activity, which could induce degradation of chlorophyll and green colour. Spray-dried powders displayed a limited loss at low relative humidity, which was attributed to partial inactivation of degradation enzymes during dehydration. The deterioration was accelerated with higher relative humidity and the effect was attributed to accelerated enzyme activity. Although the spray-dried powder was subjected to deterioration, this specific powder displayed the highest levels of green colour, chlorophyll content and emulsifying capacity compared to the other powders, at all relative humidity levels investigated.

Taken the results together, four variables (green colour, chlorophyll content, lipase-inhibiting capacity and ability to stabilize oil-water interface) were found to be highly statistically correlated. The findings are interpreted as chlorophyll degradation, induced either by heat- or acid induced degradation or enzymatic degradation, caused alterations in the polarity of the amphiphilic chlorophyll molecules. The altered polarity is due to decreased hydrophilicity in the pyrrol-part after heat-induced replacement of the central Mg2+. Enzymatic removal of the hydrophobic phytol chain also decreases the amphiphilic properties. The degraded chlorophyll molecules thereby lost their ability to stabilize protein complexes inside the thylakoid membrane, which promoted aggregation in structures known to play a decisive role in interfacial processes.

In Paper II, III and IV the degradation of chlorophyll was heat-induced and it could not be concluded that the observed decrease in interfacial properties was solely due to loss of chlorophyll. Heat-induced aggregation by inverted micelles could be another possible explanation. However, in Paper V, the loss of chlorophyll was induced not by heat, but by enzymatic degradation and/or acid-induced degradation, with similar decrease in interfacial properties as a result. It was therefore concluded that chlorophyll per se is important for the internal thylakoid structure, enabling absorption to the oil-water interface.

The close relations between green colour, chlorophyll content, lipase-inhibiting capacity and ability to stabilize the oil-water interface opens possibilities to replace the complex pH stat method *in vitro* with other less costly and less time consuming methods in screening processes for processing evaluation. Emulsion models and spectrophotometric methods can provide valuable knowledge of the thylakoids’ interfacial properties. However, human studies must be conducted to establish the thylakoids’ function in the gastrointestinal tract *in vivo*.

In summary, the present thesis investigates thylakoid membranes ability to interact with different surfaces such as the intestinal wall and oil-water interfaces of emulsions. The thesis provides a better understanding of how the thylakoid membranes’ different biophysical properties co-operates in different environments. The thylakoids’ interaction with the intestinal surface is mainly due to electrostatic interactions between the intestinal wall and extrinsic side groups of the thylakoid membranes. The thylakoids’ ability to interact with oil-water interfaces after different types of processing and subsequent storage has also been studied. Here, the interactions are mostly due to hydrophobic and hydrophilic properties of the thylakoid membrane. The knowledge provided by the thesis is useful in the formulation and quality control of a functional food ingredient with appetite suppressing abilities.

# Conclusions

When a functional food ingredient with appetite suppressing properties shall be created, it is necessary to ensure the microbiological safety and at the same time retain the ability to prolong lipolysis after standard food processing. So, how is the characteristics and subsequent physiological functions of thylakoids affected by processing such as heat treatment and drying and subsequent storage?

Non-treated thylakoids prolonged the passage of glucose and macronutrients as FITC-dextran an ovalbumin over the rat intestinal wall. This is explained by thylakoids attached to the mucosa layer enhance the barrier properties of the intestinal wall, hence a prolonged absorption of glucose and macronutrients.

Heat treatment induced degradation of chlorophyll, which reduced the thylakoids’ ability to inhibit lipase/co-lipase *in vitro* and reduced the ability to stabilize oil-in-water emulsions. The effect was both time and temperature dependent up to a certain limit where after no further damage occurred independent of processing time. A strong correlation was established between degradation of chlorophyll, ability to stabilize the oil-water interface and lipase-inhibiting capacity of the thylakoid membranes post heat treatment. It was concluded that chlorophyll was important for the internal thylakoid structure, enabling absorption to the oil-water interface, and hence ability to stabilize lipase/co-lipase.

Drying by different techniques (drum drying, spray drying, freeze drying) affected the thylakoids’ chlorophyll content, ability to inhibit lipase/co-lipase *in vitro* and the thylakoids’ ability to stabilize oil-in-water emulsions. Drying at higher temperatures reduced the emulsifying capacity, hence lower lipase-inhibiting effect. The subsequent thylakoid powders displayed decreased green colour, chlorophyll content and emulsifying capacity after 8 months storage. The effect was moisture dependent with a greater loss at higher relative humidity. It was concluded that deterioration processes were initiated during dehydration and continued during storage, with the extension depending on storage conditions. Drying at higher temperatures, such as drum drying, induced severe chlorophyll degradation as well as inhibition of enzymes catalysing chlorophyll degradation. Due to the low initial levels and low enzymatic activity, the loss of quality during storage was limited. Drying at low temperatures, as freeze drying, preserved the chlorophyll content and kept the degradation enzymes in the powder intact. These powders were subjected to substantial quality loss during storage. Drying at intermediate temperatures, as spray drying, induced limited chlorophyll degradation and partial inactivation of enzymes. The quality attributes for these powders were best preserved during storage compared to powders produced by other drying techniques. Spray drying was therefore considered the best production alternative for thylakoids when considering the entire production chain from raw material to consumer.

The relation between thylakoid powder characteristics and functionality after dehydration and storage was evaluated. Chlorophyll content, green colour, lightness and ability to stabilize oil-in-water emulsions were correlated to the thylakoids’ lipase-inhibiting capacity. The established correlations between characteristics and functionality of the thylakoids opens up possibilities to use the less time consuming and more cost efficient spectrophotometric analysis and emulsion models as a complement to the pH stat method in screening and optimization of processes.

In summary, it is possible to use thylakoids as appetite suppressor, with increased satiety between meals. The thesis contributes with knowledge concerning the effect of heat treatment, drying and storage on the thylakoids’ functional properties. When heat treatment and/or drying are used in production of thylakoids and when the powders are stored, the dose must be adjusted accordingly to compensate for the reduced functionality. Thus the results can be applied in production of a more standardised ingredient with optimized and predicted appetite reducing properties.

# Future perspective

The studies included in the present thesis describe effects of processing on thylakoids’ functionality *in vitro*, but there are still questions left to answer.

* Processing
  + Thylakoids are sensitive to heat but there are other strategies to eliminate potentially harmful bacteria such as irradiation. How do irradiation affect thylakoids’ functionality? Can it be a better alternative to heat treatment in production of thylakoids as appetite reducing agent?
  + Spray drying is a good dehydration alternative with high throughput, relatively low costs and the technique is yielding a thylakoid powder with best-maintained interfacial properties. But can addition of carriers such as maltodextrin affect the degree of heat-induced degradation on thylakoids during spray drying? Can even lower outlet temperatures be used to preserve the thylakoids’ functionality?
  + The studies included in the thesis are performed *in vitro*. Thylakoids are administrated in powder form in human studies in the research group. Do thylakoids in powder form interact with the intestinal wall *in vivo* as seen for thylakoids in aqueous solution in the present thesis?
  + Have heat-treated thylakoids reduced ability to inhibit lipolysis *in vivo* as well?
  + Are spray-dried powders after storage superior to drum-dried and freeze-dried thylakoids also *in vivo*?
* Formulation
  + Lipolysis is an interfacial process where a competition between various surface-active substances takes place. In which phase of the food should thylakoids be present to give the highest impact on lipolysis rate? It would be interesting to evaluate the effect of appetite of thylakoids pre-emulsified with dietary fat compared to thylakoids ingested separated from the fat phase.

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Figure 6, viewed 2015-02-01, illustration of the chlorophyll structure inside the light harvesting complex: http://commons.wikimedia.org/wiki/File:LHCII.jpg

Figure 7, viewed 2015-02-16, action of bile salts in emulsifying fats in the intestine: http://web.campbell.edu/faculty/nemecz/323\_lect/fatty\_acid/fa\_chapter.html

Figure 8, viewed 2015-02-16, overview of fat digestion and absorption: http://web.campbell.edu/faculty/nemecz/323\_lect/fatty\_acid/fa\_chapter.html

Figure 9, viewed 2015-04-06, drum dryer in industrial scale: <http://www.huaxiadrying.com/e028.htm>

Figure 10, viewed 2015-04-06, spray drier in industrial scale: http://www.mozoro.com/spray\_dryer.php

Figure 11, viewed 2015-03-28, the variation of oxidation, enzyme activity, vitamin inactivation and microbial activity in dehydrated foods: <http://www.neutecgroup.com/Water_activity_2.htm>