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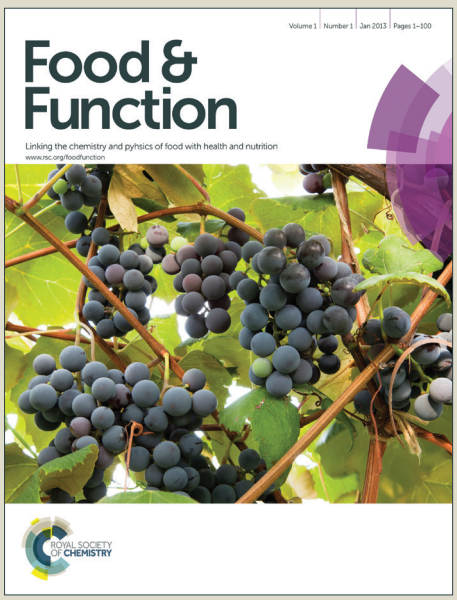

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Heat-induced aggregation of thylakoid membranes affect their interfacial properties

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Abstract

Many of our most popular lipid containing foods are in emulsion form. These foods are often highly palatable with high caloric density, that subsequently increases the risk of overconsumption and possibly lead to obesity. Regulating the lipid bioavailability of high-fat foods is one approach to prevent overconsumption. Thylakoids, the chloroplast membrane, creates a barrier around lipid droplets, which prolong lipolysis and increase satiety as demonstrated both in animal and human studies. However, a reduced lipase inhibiting capacity has been reported after heat treatment but the mechanism has not yet been fully established. The aim of this study was to investigate thylakoids' emulsifying properties post heat-treatment and possible links to alterations in lipase inhibiting capacity and chlorophyll degradation. Heat-treatment of thylakoids at either 60°C, 75°C or 90°C for time interval ranging from 15 sec to 4 min reduced ability to stabilise emulsions, having increased lipid droplets sizes, reduced emulsification capacity, and elevated surface load as consequence. Emulsifying properties were also found to display a linear relationship to both chlorophyll and lipase inhibiting capacity. The correlations support the hypothesis that heat-treatment induce chlorophyll degradation which promote aggregation within proteins inside the thylakoid membrane known to play a decisive role in interfacial processes. Therefore, heat-treatment of thylakoids affects both chlorophyll content, lipase inhibiting capacity and ability to stabilise the oil/water interface. Since the thylakoid's appetite reducing properties are a surface-related phenomenon, the results are useful to optimize the effect of thylakoids as an appetite reducing agent.

Keywords

Spinach, photosynthetic membranes, chlorophyll, heat stability, oil-in water emulsion, emulsion stability

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1. Introduction

Many of our most popular lipid containing foods are in emulsion form, such as culinary cream, ice cream, mayonnaise, beverages and sauces. Lipids are important from a sensory and nutritional perspective (omega 3 and 6 being essential)[1], also several vitamins i.e. A, D, E and K are lipid-soluble[2]. However, foods with high lipid content also have a high caloric density, which increases the risk of over-consumption[3]. It is widely accepted that high-fat diets leads to high-energy intake promoting obesity[4]. Obesity is increasing throughout the developed world and is becoming one of the major health problems of our time[5]. Obesity is associated with occurrence of type 2 diabetes as well as cardiovascular diseases and breast, colon, oesophagus and kidney cancers[6]. Regulating the lipid consumption and bioavailability of high-fat foods as emulsions is therefore crucial. Appetite can be suppressed by prolonged lipid digestion through the ileal brake[7]. A delayed lipid digestion leads to accumulation of undigested lipids or lipolysis products in ileum which in turn promotes secretion of satiety promoting hormones and peptides that slows down gastric emptying, reducing appetite, and in turn food intake[8]. Delay of lipid digestion could therefore potentially be a strategy to prevent the development of obesity and its associated health risks.

Lipid digestion is primarily an interfacial process. The triacylglycerols (TG) constitutes the lipid droplet and must be transported through the aqueous intestinal lumen to be absorbed by the epithelial cells. This requires a hydrolysis process from TG into monoglycerides (MG) and free fatty acids (FFA)[9]. Hydrolysis is catalysed by the enzymes lipase and its cofactor co-lipase excreted from the stomach and pancreas. Lipase and the hydrophobic lipid substrate must come in close proximity for the lipolytic reaction to take place. This unfavourable thermodynamical condition is facilitated by surrounding amphiphilic molecules (i.e. bile salts), which minimize the surface free energy of the emulsion droplets[10].

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A considerable amount of effort has been spend on developing methods and approaches to change the interfacial properties of emulsions to modulate digestion. The main approaches to prolong GI transit time for dietary lipids include increasing droplet size[11,12], varying the molecular structure of the lipids[13,14] and the interfacial composition[15,16]. Many obstacles have to be overcome and a substance nominated to modulate lipid digestion must meet a long list of criteria to be successful and approved by regulatory bodies. The candidate substance must survive enzymatic attacks and mechanical breakdown in the oral and gastric environment. When successfully reached the duodenal environment, it must be highly surface active, slowly digested by pancreatic enzymes, resistant to competing bile salt displacement and must be sterically inhibiting lipase activity at the interface[9,17].

One strategy to varying the interfacial properties of a lipid droplet is to create an impermeable barrier on the oil/water interface, which prolongs lipolysis in the intestinal environment by blocking lipase/co-lipase from its substrate. The barrier can be composed by a wide variety of components and can be constructed in different ways. For example a barrier can be created by layer-by-layer electrostatic deposition[17] or by partial gelatinization of starch granules to create a cohesive interfacial layer[18,19]. These approaches are similar in that the impermeable layers of biopolymers on the lipid droplet surface that cannot longer be displaced by other competing emulsifiers in the intestinal environment. The created barrier is also resistant to enzymatic degradation, which together reduces lipid bioavailability[17].

In this work a biological membrane, i.e. thylakoids, are used to create a barrier at the oil/water interface to modulate lipid bioavailability. Thylakoids, a membrane found in the chloroplast in green leaves (Fig 1a) has been found to inhibit lipolysis *in vitro*[20] under duodenal conditions as well as *in vivo* in animal models[21]. Supplementation of thylakoids to the diet in

human acute meal studies elevated the satiety hormones CCK, leptin and enterostatin while the hunger peptide ghrelin was supressed[22]. Supressed hunger sensations were also registered[23]. This has been attributed to prolonged lipid digestion, hence prolonged satiety via the ileal brake mechanism.

Thylakoid membranes are efficient emulsifiers with strong affinity for the oil/water interface[24] and can stabilise oil-in-water emulsions. Droplet size decreased with increasing thylakoid concentration reaching a plateau around 15 μm[25] although this could be a limitation of the homogenization device used. Electron micrographs showed thylakoids as bunched vesicles adsorbed on the surface of the lipid droplet (Fig 1b). The stabilisation mechanism was hypothesised to be a combined effect of mainly surface-active extrinsic membrane proteins but also membrane lipids, exposed on the surface of the thylakoid membrane[25].

Thylakoids are a new promising emulsion stabilising agent, which could be incorporated in functional foods as an appetite-reducing ingredient. Thylakoids are extracted from spinach leaves and must be processed to eliminate pathogens where the most common way in the food industry is by thermal treatment.

In a recent study it was reported that the thylakoids ability to inhibit lipolysis *in vitro* was reduced after thermal treatment[26]. The effect was more pronounced for higher temperatures and longer processing times. Also, chlorophyll was degraded simultaneously. Non-treated thylakoid displayed a bright green colour whereas hat-treated thylakoids were olive brown. The colour shift was also more pronounced for higher temperatures and longer processing times. The chlorophyll loss was strongly correlated to the thylakoids reduced ability to inhibit lipolysis post heat-treatment (R^2=0.95). A spectrophotometric absorption scanning revealed chlorophyll *a* to be a key factor for enzymatic inhibition. At the same time, the presence of

degradation products of chlorophyll were associated with decreased enzymatic inhibition. The components of thylakoids that provide its function as a lipolysis modulator are primarily photosystems I and II[20] (Fig 1a), which are structurally stabilised by chlorophyll. It was hypothesized that thermal treatment caused degradation of chlorophyll, which in turn induced aggregation of photosystem I and II, with reduced ability to inhibit lipolysis as consequence.

The question to be addressed in this study is: Could the reduced ability to inhibit lipolysis by thermal treated thylakoids be linked to the thylakoids' interfacial properties and ability to adsorb to the oil/water interface?

2. Material and methods

2.1 Preparation and analysis of thylakoid isolates

Thylakoid membranes were extracted essentially as previously described [27] modified as described in[26]). Spinach (*Spinacia oleracea*) were homogenized in a blender with water (1:1 wt/wt) and filtered through four layers of Monodur polyester mesh (20 μm). The filtrate was centrifuged at 5000 x g, 4°C, 30 min. The supernatant was discarded and the thylakoids in the pellet were collected and re-suspended with fresh water in a glass Potter Elvehjem homogeniser until a homogenous slurry was obtained. Dimethyl sulfoxide (DMSO, 99.5%, Sigma, St Louis, MO, USA) was added to a final concentration of 5 vol %, to avoid aggregation during freezing. The thylakoids were stored frozen (-18°C) and were thawed in a cold-water bath and stored on ice until start of thermal degradation experiments.

2.2 Dry matter analysis and determination of chlorophyll

Dry matter content in the thylakoid preparation (1.1±0.02%) was determined according to the official method of analysis (AOAC). Analysis was performed in triplicate. Chlorophyll

concentration was determined according to Porra et al[28] The total chlorophyll content in the non-treated thylakoid slurry was 0.767±0.002 mg/ml. Analysis was performed in quadruplicate.

2.3 Heat treatments

Heat treatments were carried out as previously described[26]. Thylakoid slurry was processed at three different temperatures (60, 75 and 90°C) for 15 sec – 4 min in a bent helical coil of stainless steel. A thermostatic oil bath (Julabo HC-8, Julabo, Seelbach, Germany) was used as heating device and the temperature was measured with ± 0.1 °C accuracy. The come-up time, determined by a thermocouple inserted into the annular centre of the metal tube, were 20.2 ± 3 sec at each processing temperature. After processing, the samples were immediately cooled in an iced water bath to avoid lag in cooling. The heat treatment was performed in triplicates at every time-temperature combination. The heat-treated thylakoid slurry was stored frozen (-18°C) and were thawed in a cold-water bath and stored on ice until use in emulsion trials.

2.4 Preparation of thylakoid-stabilised emulsions

Emulsions were prepared in four replicates in glass test tubes with 2 ml of the continuous phase (phosphate buffer), 1 ml of the lipid phase (Miglyol 812, Sassol AG, Germany) and varying amount of non-treated thylakoid slurry to conclude which concentration to use throughout the study. The concentration 350 µl slurry/ml oil corresponding to 3.85 mg dry matter was chosen as probe volume to avoid potential thylakoid saturation in the system yet still maximize the measurement sensitivity. Emulsions were prepared in four replicates in glass test tubes with 2 phosphate buffer, 1 ml miglyol (Miglyol 812, Sassol AG, Germany) and 350 µl thylakoid slurry (non-treated and heat-treated samples) by mixing (Ystral D-79282, Ballrechten-Dottingen, Germany) at 22 000 rpm for 60 sec. Two of the replicates were incubated dark and in 4°C for 60 min before photographs were taken and the particle size distribution were analysed. The remaining two replicates were incubated 7 days in dark at 4°C.

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The emulsions were photographed after 4 hours and after 7 days prior to particle size distribution analysis.

2.5 Particle size measurements of thylakoid-stabilised emulsions

The particle size distribution of the emulsions were analysed with a laser diffraction particle analyser (Mastersizer 2000 Ver 5.60, Malvern, Worcestershire, U.K.). The dispersing unit (Hydro 2000S) were filled with 100 ml MilliQ water and the pump was operated at 2000 rpm. A control experiment with phosphate buffer in the dispersing unit was performed to evaluate a possible dilution factor. No difference in droplet size was found and water was therefore used as background solution throughout the study. The glass test tubes were turned upside down three times before a small volume was added to the flow system and pumped through the optical chamber for measurements. Obscuration was between 10% and 20%. The RI of the sample was set to 1.45 (miglyol) and the RI of the continuous phase was set to 1.33 (water). Each emulsion replicate were measured three times.

2.6 Particle size measurements of thylakoid membranes

To examine whether heat treatment induced changes in the tertiary structure of the thylakoid membranes (i.e. aggregation), the particle size distribution of the thylakoid membranes themselves were analysed. The samples were prepared identical to the emulsions described above but without the lipid phase. 2 ml phosphate buffer and 350µl thylakoid slurry were mixed (Ystral D-79282, Ballrechten-Dottingen, Germany) at 22 000 rpm for 60 sec where after the particle size distribution were analysed with laser diffraction (Mastersizer 2000 Ver 5.60, Malvern, Worcestershire, U.K.). Obscuration was between 5% and 10%. The RI of the sample was set to 1.5 and the RI of the continuous phase was set to 1.33 (water). Each sample was measured three times.

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2.7 Light microscopy of thylakoid-stabilised emulsions

To ensure that the particle size distribution obtained from the laser diffraction analysis was detecting individual droplets and not aggregates, a manual size distribution was performed. Over 300 droplets from an emulsion stabilised by non-treated thylakoids were analysed with light microscopy and processed using the Java image-processing program ImageJ (version 1.42m) from which d_{43} and d_{32} were calculated as described by Timgren et al [29]. The manually measured d_{32} and d_{43} of the emulsions corresponded to these of the Malvern data within the accuracy of experimental framework (data not shown). The microstructure of emulsions stabilised with both non-treated and heat-treated thylakoids were also investigated.

2.8 Analysis of thylakoid-stabilised emulsions during storage

Test tubes with emulsified samples were photographed 1h, 4h and 7 days after emulsification, and the images were analysed with ImageJ. The emulsifying capacity of the thylakoid membranes and stability of the emulsions were expressed as volume of the emulsion compared to total volume of the sample, often referred to as the emulsification index (EI). The EI [30] was calculated as

$$EI = \frac{Volume\ of\ cream\ layer}{Total\ volume\ of\ emulsion} \quad \text{(Eq. 1)}$$

Specific surface area, S, which is the total droplet surface area covered by the adsorbed emulsifier was calculated as

$$S = \frac{6\phi}{d_{32}} \quad \text{(Eq. 2)}$$

where ϕ is the disperse phase volume fraction and d_{32} is the volume-surface mean droplet

diameter. Surface load, Γ_s, which corresponds to the minimum mass emulsifier required to cover a unit area of droplet surface (mg/m^2) was calculated as

$$\Gamma_s = \frac{C_a \cdot V_e}{S} = \frac{C_a \cdot d_{32}}{6\phi} \quad \text{(Eq. 3)}$$

where C_a is the initial concentration of emulsifying agent (assumed that all emulsifier is adsorbed at the oil/water interface), V_e is the emulsion volume, S is the specific surface area. Similarly, the maximum surface that can be created and stabilised by a unit emulsifier (m^2/mg) can be expressed as the Emulsifying Capacity, EC

$$EC = \frac{1}{\Gamma_s} = \frac{6\phi}{C_a \cdot d_{32}} \quad \text{(Eq. 4)}$$

2.9 Analysis of enzymatic inhibition capacity

The activity of lipase/co-lipase (Sigma-Aldrich, St Louis, MO, USA) was determined as described in Östbring et al[26] by pH-stat titration (Autotitrator Titralab TIM 854, Radiometer Analytical, France). Tributyrine was used as substrate and 0.1 M NaOH for titration. 15mL assay buffer (2 mM Tris maleate (pH 7), 0.15 M NaCl, 1 mM $CaCl_2$ and 4 mM NaTDC) was mixed with 0.5 ml tributyrine, 5 µl lipase, 5 µl co-lipase and 260 µl thylakoid slurry (non-treated or heat-treated). The incubation was performed at 25°C. The mean consumption rate of 0.1 M NaOH (ml/s) during 20 minutes was taken as activity of lipase/co-lipase. Six measurements were performed for each thermal processing condition.

2.10 Regression analysis between droplet diameter and enzyme inhibiting capacity

To investigate if the droplet size (or other surface activity-related variable) of lipid droplets

stabilised by thylakoids could be correlated to the thylakoids' enzyme inhibiting capacity a correlation analysis was performed using the curve-fitting tool in Matlab (version R2010b, Mathworks Inc., Natick, MA). Futhermore, correlations between the thylakoids' emulsifying properties and chlorophyll content (chlorophyll *a* and total chlorophyll) after heat-treatment were also investigated.

3. Results and discussion

3.1 Emulsifying capacity of non-treated thylakoids

Non-treated thylakoids stabilise oil-in-water emulsions, where mean droplet size decreased with increased thylakoid concentration levelling off at higher concentrations (Fig 2a), which is in accordance with previous studies[25,31]. The particle size distributions were centred around a single peak (non-treated thylakoids in Fig 3) with a relatively narrow span (Fig 2a). Also, the specific surface area of the emulsions was increased with increasing concentration thylakoids (Fig 2b).

3.2 Altered emulsifying capacity of heat-treated thylakoids

Heat treatment at either 60°C, 75°C or 90°C for time intervals ranging from 15 sec to 4 min affected the thylakoids emulsifying properties. The ability to stabilise the oil/water interface was reduced after heat-treatment. Higher temperatures (Fig 3) and/or longer processing times of thylakoids (Fig 4 and Table 1) resulted in progressively larger emulsion droplets. Heat-treatment at different temperatures affected the thylakoids ability to stabilise lipid droplets up to a certain limit but for the longest holding-times the droplets sizes were equal independent on processing temperature (Table 1). In Fig 4 the evolution of droplet diameter for the highest and lowest processing temperatures is plotted as a function of time for comparison. The

system had reached a plateau at approximately 2 min and extended processing time did not affect the droplet size further for any of the temperatures tested. The results were confirmed by light microscopy where the droplet diameter progressively increased with temperature but for 90°C the diameters were similar for 15 sec and 4 min (Fig 5). The droplet diameter of emulsions stabilised by thylakoids significantly increased when the thylakoid membranes were heat-treated prior to emulsification. Higher temperature and/or longer processing time resulted in larger droplets up to a certain limit, i.e. 90°C is such a high processing temperature that holding times longer than 15 sec did not result in significant larger drops and no further damage occurs with longer holding time. The micrographs also confirmed that the lipid droplets were dispersed in the continuous phase at all investigated processing conditions and that no aggregation or flocculation of droplets had occurred. After incubation the emulsions immediately creamed and the supernatant in the emulsion test tubes were transparent (Fig 5) suggesting that close to all emulsifier were associated to the cream layer. Both emulsions stabilised by non-treated and heat-treated thylakoids creamed due to gravitational separation and the emulsion droplets stayed intact during seven days storage and no oiling off was observed in any sample. The emulsification index (EI) did not differ significantly between four hours and seven days incubation for thylakoid-stabilised emulsions treated at 60°C, 75°C or 90°C (Table 1). The emulsions may therefore be regarded as stable against coalescence.

Why is the thylakoids' ability to stabilise an oil/water interface altered by heat-treatment prior to emulsification? We hypothesised that the structure within the thylakoid membrane is important when it comes to its surface-active properties. The components that provide its surface-active function both as a lipolysis modulator and, in this particular study, as lipid droplet stabiliser are primarily specific structures found inside the photosystems I and II within the thylakoid membrane[20]. The structures are called alpha helices and are proteins

formed in helices. In native thylakoids, the hydrophobic alpha helices are separated by chlorophyll molecules preventing the monomer structure from collapsing[32]. The chlorophyll molecule has a polar head group and a hydrophobic tail, which facilitates steric stabilization of hydrophobic alpha helices within the monomers in the photosystem I and II [33]. When the chlorophyll stays intact (i.e. non-treated thylakoids) both the emulsifying capacity (non-treated thylakoids in Fig 6a) and the lipolysis inhibiting capacity (non-treated thylakoids in Fig 7) of the thylakoids remains high.

When the thylakoid membranes are heat-treated, chlorophyll molecules are chemically degraded to pheophytin and/or enzymatically degraded to chlorophyllide[34,35]. For both degradation products the polarity of the molecule is altered and the hydrophobic parts cannot remain separated causing collapse inside the photosystem I and II of the thylakoid[36]. Since the hydrophobic parts are turned towards the inside of the aggregated thylakoid membrane, the ability to stabilise an oil/water interface is reduced. This hypothesis is supported by our results showing reduced emulsifying capacity when exposed to heat-treatment (Fig 6a). After heat-treatment, the stabilised lipid surface area per unit thylakoids are significantly reduced with temperature and processing time. The altered emulsification capacity also leads to larger lipid droplets as observed in particle size distributions (Fig 3). This phenomenon were more pronounced for higher temperatures and/or longer processing times, which is in accordance with the degradation kinetics of chlorophyll, following a first-order kinetic model[34]. Heat treatment also affected the surface load of thylakoids on the oil/water interface (Fig 6b). A higher temperature and/or longer holding time resulted in a thicker thylakoid layer covering the lipid droplet surface. These results suggest aggregation of thylakoid membrane induced by heat treatment where larger thylakoid aggregates creates a thicker absorption layer at the oil/water interface. To verify if the thylakoid membrane did aggregate to larger structures, the

particle size of the heat-treated membranes was analysed. Non-treated and heat-treated thylakoids membranes did not differ in particle size when dispersed in phosphate buffer (data not shown), which was unexpected. Also, the thylakoid membrane structures are known to form micelles with hydrophobic parts oriented towards the centre[37]. At 55°C and above the micelles are inverted and the hydrophobic parts are turned outside which facilitate aggregation due to minimization of free energy. Therefore, we suggest that the thylakoid membranes ability to stabilise an oil/water interface after heat-treatment are altered due to both reorganization of hydrophilic/hydrophobic parts inside the thylakoid membranes and heat-induced aggregation of membranes to larger particle cluster caused by inversion of micelles.

Emulsifying properties are correlated to ability to inhibit in vitro *lipase/co-lipase activity*

In previous studies, it was shown that thylakoids' lipase inhibiting capacity was reduced after heat-treatment[26] (Fig 7). Higher temperatures and/or longer processing times strongly reduced the inhibiting capacity. Similar results are shown in the present study were surface-related properties as particle size of emulsion droplets stabilised by thylakoids, surface load and emulsification capacity are altered by heat-treatment of thylakoids. Therefore, a regression analysis was performed to conclude if surface-related parameters could be correlated to lipase inhibiting capacity. A linear relationship was found between lipase inhibiting capacity and $1/d_{32}$ (R^2=0.80), which is directly proportional to the specific surface area (Eq. 2). When the thylakoids are heat-treated, the surface properties are altered and the lipase inhibiting capacity (which is a surface-related process) reduced. Another consequence of the altered surface structure is that the ability to stabilise lipid droplets in an emulsions are reduced, hence larger lipid droplets.

Since thylakoids' lipase inhibiting capacity after heat-treatment has been reported to be closely linked to chlorophyll degradation[26], a regression analysis between thylakoids' chlorophyll content and emulsifying properties were performed and a linear relationship (R^2=0.81) was established. Thus, all three variables i) lipase/co-lipase inhibiting capacity, ii) emulsifying properties and iii) chlorophyll content are correlated. These correlations support the hypothesis that heat-treatment induce chlorophyll degradation which promote aggregation within the alpha helical structures in the light harvesting complexes of thylakoid membranes known to play a decisive role in surface-related processes. Therefore, heat-treatment of thylakoids affects both chlorophyll content, the lipase inhibiting capacity and ability to stabilise oil/water interface. The results suggest that the thylakoids' ability to inhibit lipase/co-lipase is mainly a surface-related phenomenon, and if the surface-active properties of the membranes are reduced, the inhibiting capacity will decrease accordingly. We suggest that the chlorophyll-stabilized internal membrane structure is important for the thylakoids ability to attach to the oil/water interface. Probably are not chlorophyll *per se* needed to retain the membrane structure, but can possible be replaced by an artificial stabilizer if needed.
To better understand and modulate the function of thylakoids in terms of appetite reducing agent, attention must be paid to analysis of surface-related phenomena. Also, it may be possible to quantify the function of thylakoids by means of emulsion parameters. Potentially, the fast and less costly laser diffraction method can replace parts of more complex *in vitro* experiments. When effects of heat-treatment on thylakoids emulsifying properties are known, it is possible to choose an appropriate degree of heat-treatment, adjust the dose accordingly and get a potent and microbiological safe appetite reducing agent.

4. Conclusions

This study has provided new insights into the impact of heat-treatment on thylakoids ability to stabilise the oil/water interface. Heat-treatment of thylakoid membrane prior to emulsification reduced the ability to stabilise an emulsion with increased lipid droplets, reduced emulsification capacity and elevated surface load as consequence. We suggest that heat treatment both induced aggregation of the hydrophobic alpha helices inside the thylakoid membrane known to be active parts in stabilising an oil/water interface, as well as induced conversion of micelles leading to aggregation of thylakoid membranes to larger particle clusters. These two phenomena alter the thylakoids' ability to attach to the lipid surface and stabilise emulsions. Furthermore, our results demonstrate that the thylakoids emulsifying capacity after heat-treatment can be correlated to lipase inhibiting capacity since both are surface-related processes. This relationship open possibilities to use fast and inexpensive laser diffraction methods in screening processes, as a complement to the costly and time-consuming *in vitro* model.

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Table 1. Droplet diameter mode, span and emulsifying index (EI) of emulsions stabilised by non-treated or heat-treated thylakoids. Data are given as mean ± standard deviation.

		Incubation time 1 h			Incubation time 7 days			Incubation time 4 h	Incubation time 7 d
		d_{43} (μm)	d_{32} (μm)	Span	d_{43} (μm)	d_{32} (μm)	Span	EI	EI
non-treated		71±7	28±3	1.4±0.2	74±3	31±1	1.3±0.1	0.57±0.008	0.55±0.02
60°C	15 sec	92±14	66±28	0.88±0.1	84±6	48±15	1.1±0.1	0.54±0.02	0.54±0.01
	1 min	129±24	118±19	0.76±0.1	115±6	104±6	0.82±0.0	0.50±0.02	0.50±0.01
	2 min	144±8	131±6	0.79±0.1	146±15	134±13	0.78±0.1	0.44±0.03	0.50±0.05
	4 min	163±6	145±2	0.88±0.1	154±5	141±3	0.79±0.1	0.43±0.005	0.43±0.002
75°C	15 sec	131±18	121±16	0.75±0.0	122±6	112±6	0.75±0.0	0.42±0.03	0.47±0.008
	1 min	119±6	110±6	0.72±0.0	121±4	112±3	0.77±0.0	0.46±0.002	0.47±0.001
	2 min	120±4	111±3	0.74±0.1	111±2	102±2	0.77±0.0	0.51±0.007	0.49±0.01
	4 min	160±3	135±6	0.99±0.0	152±2	134±3	0.90±0.1	0.42±0.02	0.41±0.02
90°C	15 sec	152±4	132±5	0.96±0.1	163±6	131±1	1.1±0.1	0.49±0.02	0.43±0.04
	1 min	156±4	127±5	1.1±0.1	168±6	136±6	1.1±0.1	0.45±0.002	0.46±0.03
	2 min	153±5	131±2	0.99±0.1	169±7	138±6	1.1±0.1	0.44±0.01	0.43±0.002
	4 min	160±2	131±2	1.1±0.0	174±4	142±3	1.0±0.0	0.43±0.01	0.39±0.001

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Table 2. Emulsifying capacity (EC), chlorophyll content and relative inhibition capacity of non-treated and heat-treated thylakoid membranes. Data are given as mean ± standard deviation.

		Emulsifying capacity (EC) (m^2/mg)	Total chlorophyll (mg/ml)	Chlorophyll *a* (mg/ml)	Relative inhibition capacity (-)
non-treated		0.055±0.01	0.54±0.03	0.43±0.02	1.0±0.2
60°C	15 sec	0.024±0.01	0.42±0.02	0.33±0.02	0.70±0.1
	1 min	0.013±0.00	0.36±0.01	0.28±0.01	0.48±0.1
	2 min	0.012±0.00	0.34±0.03	0.26±0.02	0.50±0.2
	4 min	0.011±0.00	0.35±0.01	0.27±0.01	0.44±0.1
75°C	15 sec	0.013±0.00	0.42±0.01	0.33±0.01	0.50±0.1
	1 min	0.014±0.00	0.41±0.01	0.32±0.01	0.49±0.1
	2 min	0.014±0.00	0.38±0.00	0.29±0.00	0.49±0.0
	4 min	0.012±0.00	0.34±0.01	0.26±0.00	0.32±0.1
90°C	15 sec	0.012±0.00	0.34±0.02	0.27±0.01	0.32±0.1
	1 min	0.012±0.00	0.32±0.02	0.25±0.01	0.45±0.1
	2 min	0.012±0.00	0.33±0.03	0.24±0.01	0.62±0.1
	4 min	0.012±0.00	0.27±0.02	0.20±0.01	0.18±0.0

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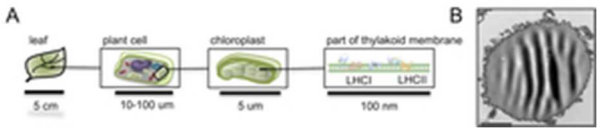


Overview of the natural location of thylakoids with sizes indicated. LHCI & LHCII = light harvesting complex I & II and are probably the active parts of the thylakoids in the enzyme inhibiting context. B. Electron micrograph of a lipid droplet covered with thylakoid membranes (published in Rayner et al, 2011).
25x5mm (300 x 300 DPI)

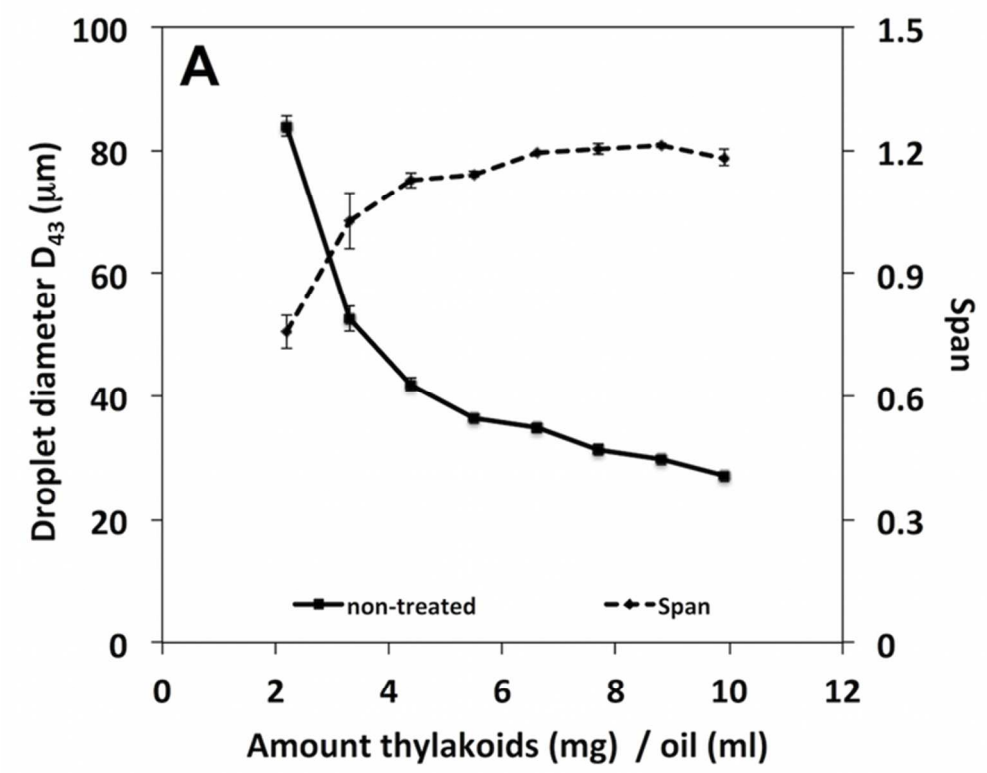


Fig 2. A. Droplet size and span as a function of amount added non-treated thylakoids (mg dry matter) per ml oil.
63x49mm (300 x 300 DPI)

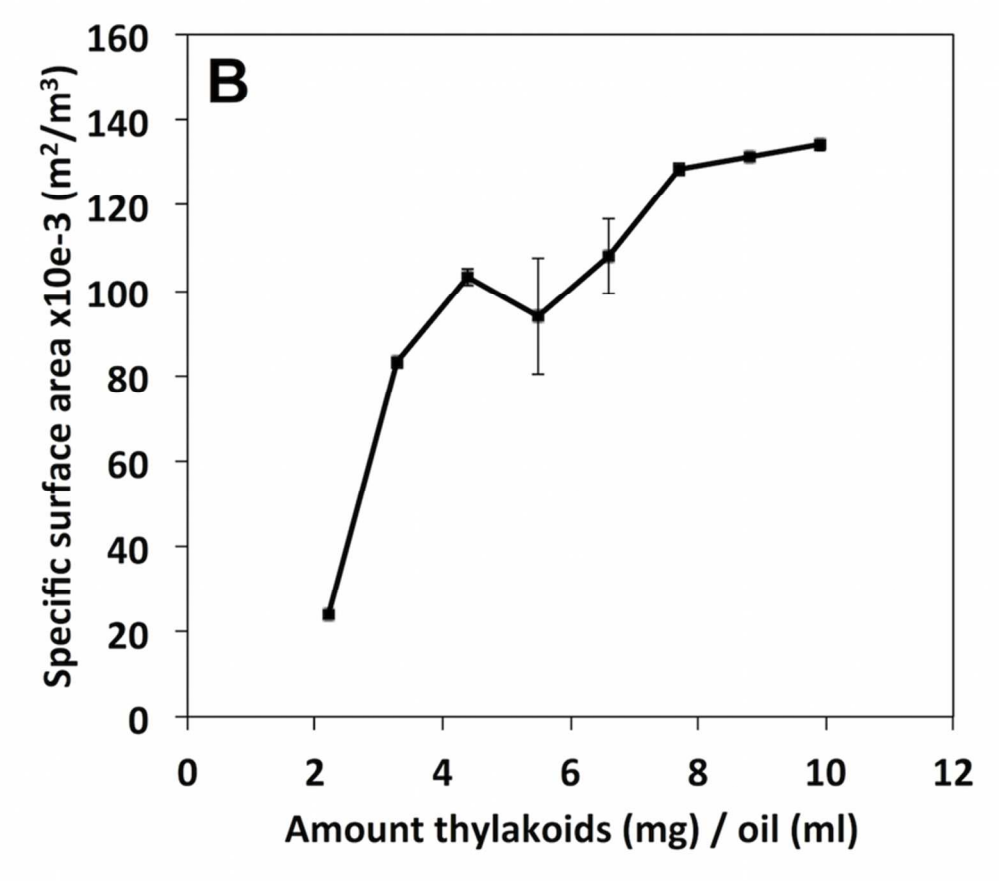
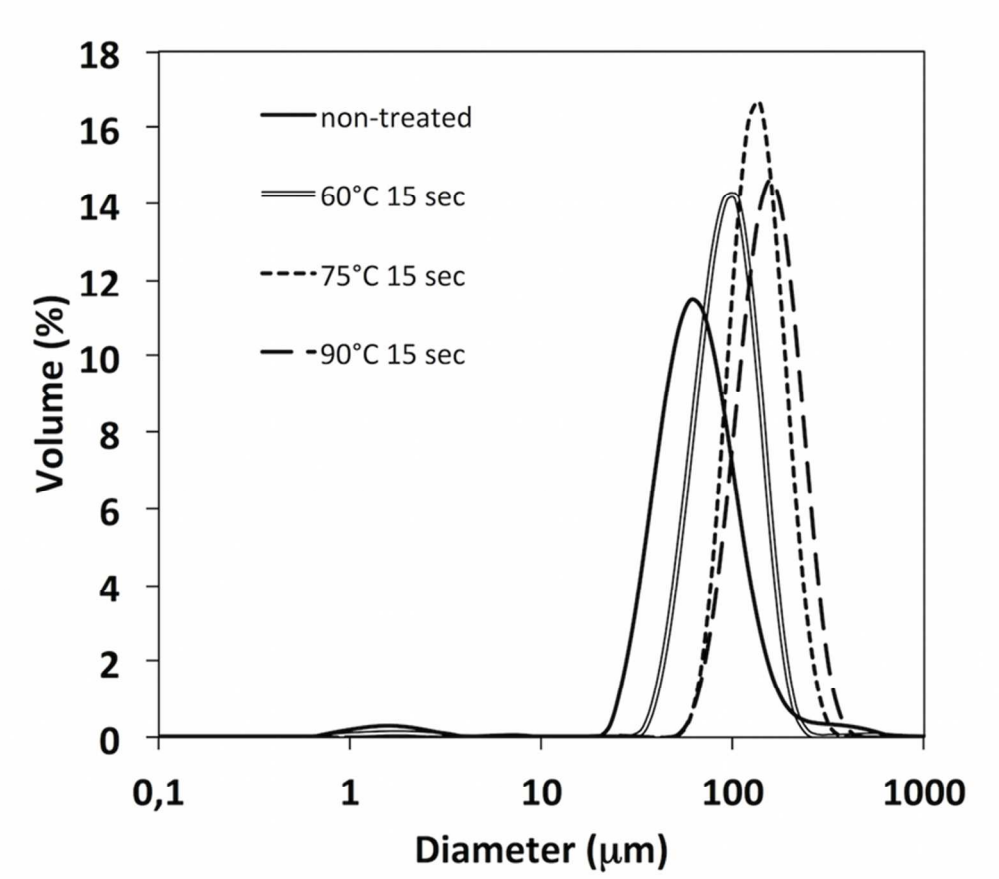
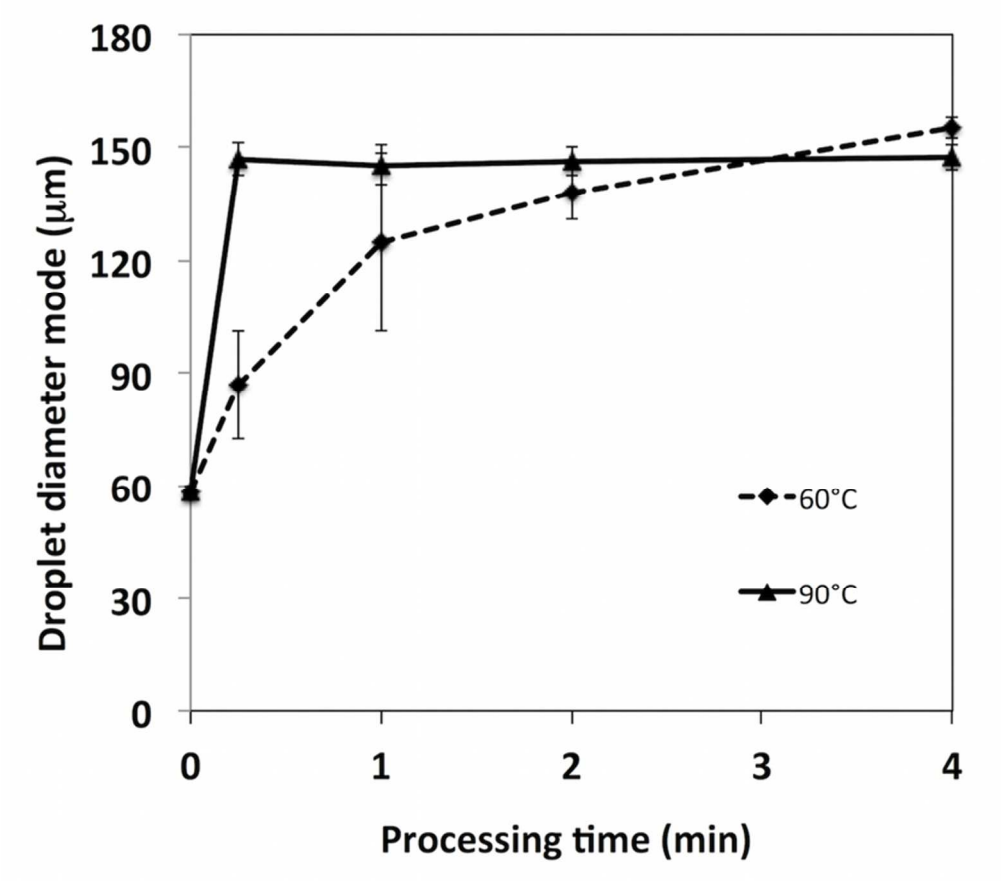


Fig 2. B. Specific surface area as a function of amount added non-treated thylakoids (mg dry matter) per ml oil.
70x62mm (300 x 300 DPI)



Size distribution of emulsion droplets stabilised by non-treated thylakoids and thylakoids heat treated at 60°C, 75°C and 90°C for 15 sec.
70x62mm (300 x 300 DPI)



. Droplet diameter as a function of processing time. Heat treatment affects the thylakoids ability to stabilize emulsion droplets up to a certain limit. After approximately 2 min the system has reached a plateau whereafter the droplet size are constant, independent on processing temperature.
70x62mm (300 x 300 DPI)

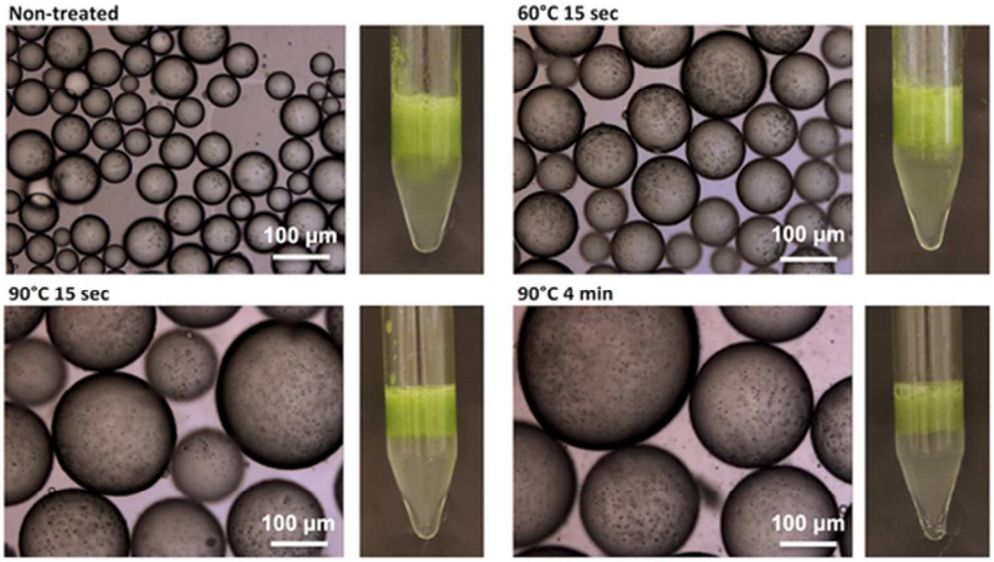


Fig 5. Micrographs (100X) of emulsion droplets stabilised by non-treated thylakoids (upper left), thylakoids treated at 60°C 15 sec (upper right), 90°C 15 sec (lower left) and 90°C 4 min sec (lower right).
48x29mm (300 x 300 DPI)

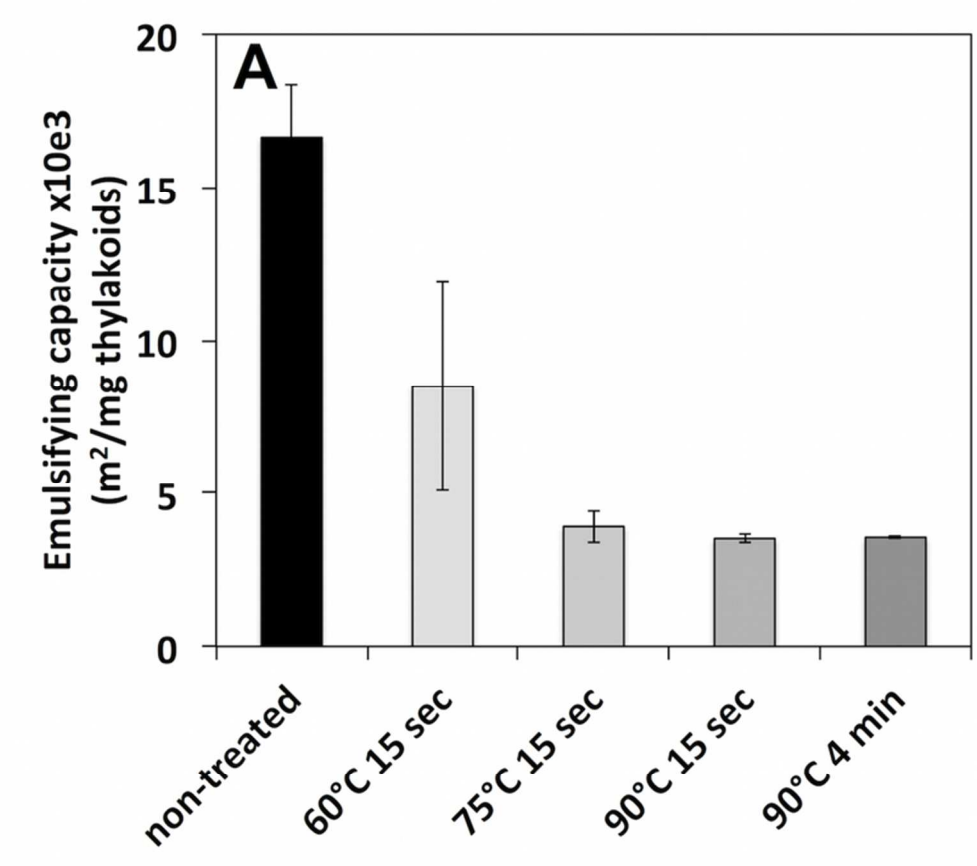


Fig 6. A. Emulsification capacity (EC) by non-treated and heat-treated thylakoids expressed as surface stabilized per mg dry matter.
69x60mm (300 x 300 DPI)

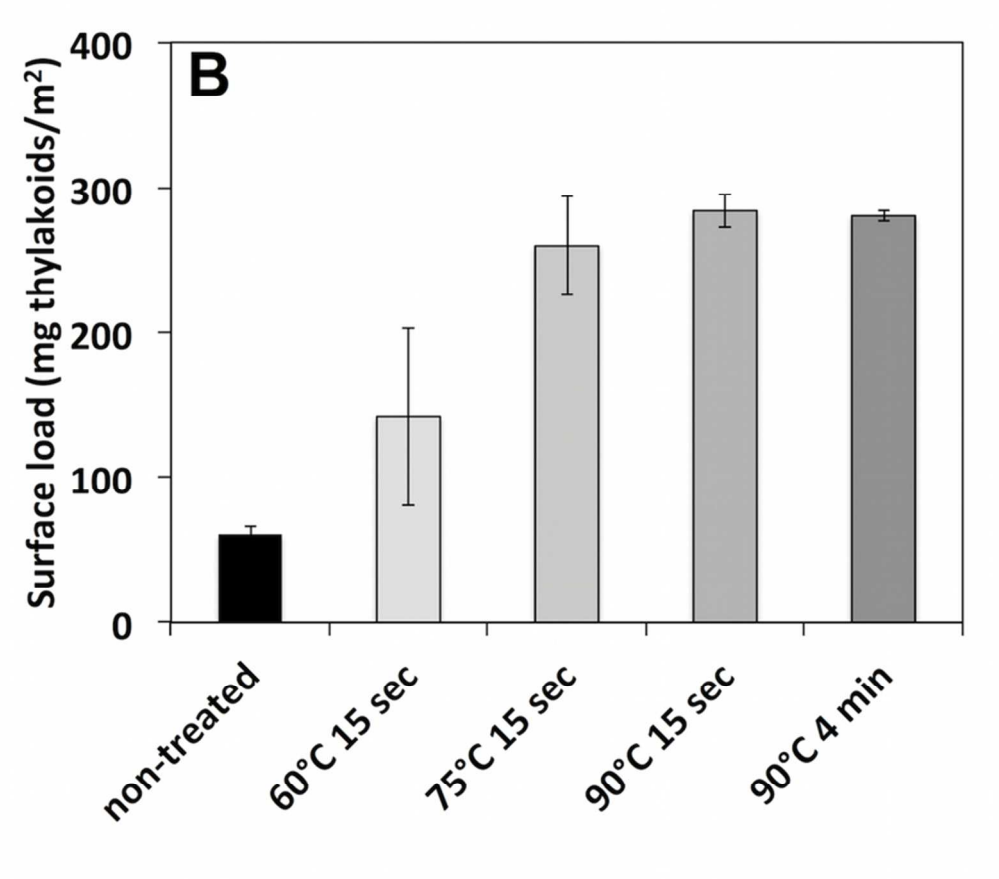


Fig 6. B. Surface load (Γs) by non-treated and heat-treated thylakoids expressed as mg dry matter adsorbed per unit created surface.
70x62mm (300 x 300 DPI)

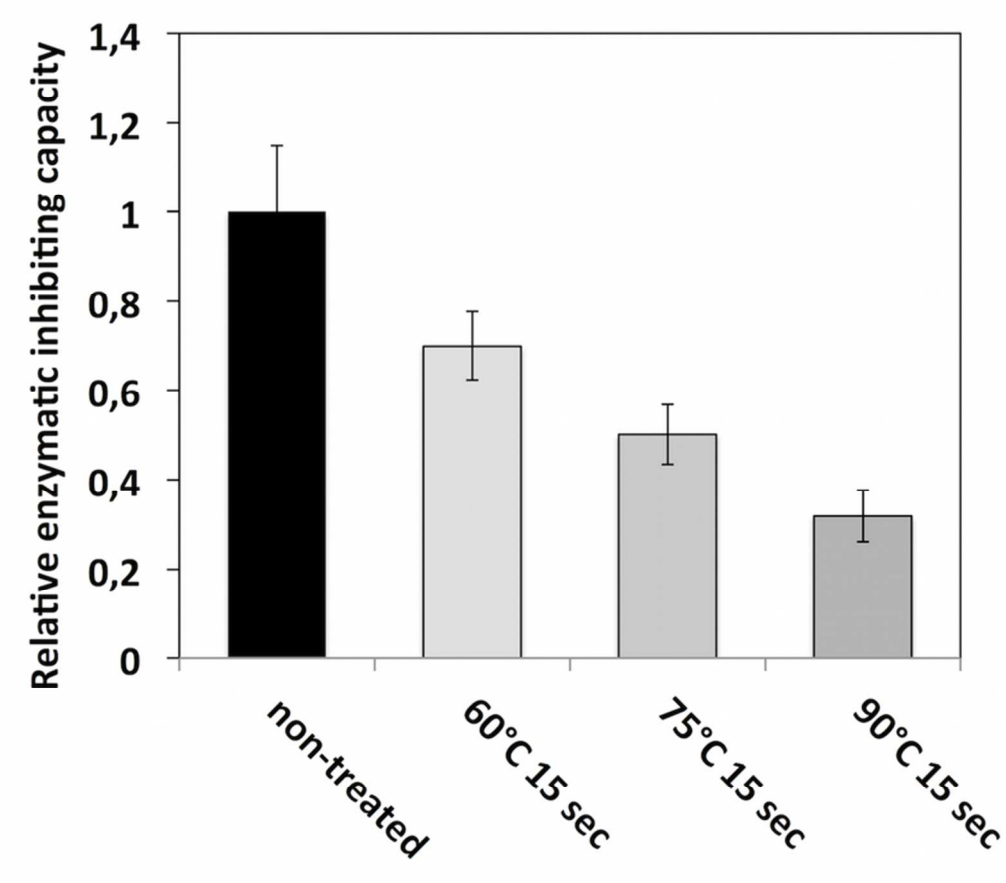
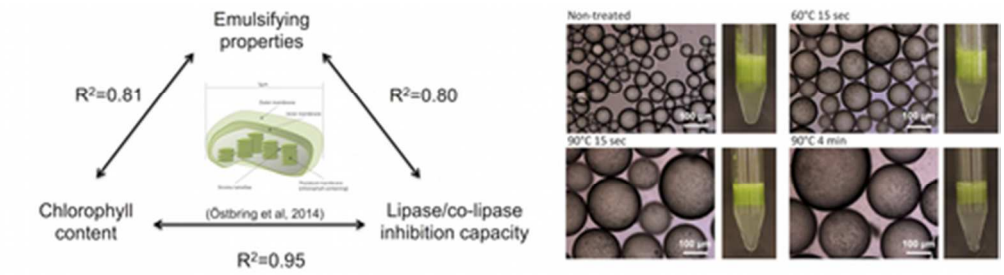


Fig 7. In vitro lipase/co-lipase inhibiting capacity by non-treated thylakoids and thylakoids treated at 60°C, 75°C and 90°C for 15 sec, data re-plotted from Östbring et al (2014).
70x62mm (300 x 300 DPI)



43x11mm (300 x 300 DPI)

Legends to figures

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