

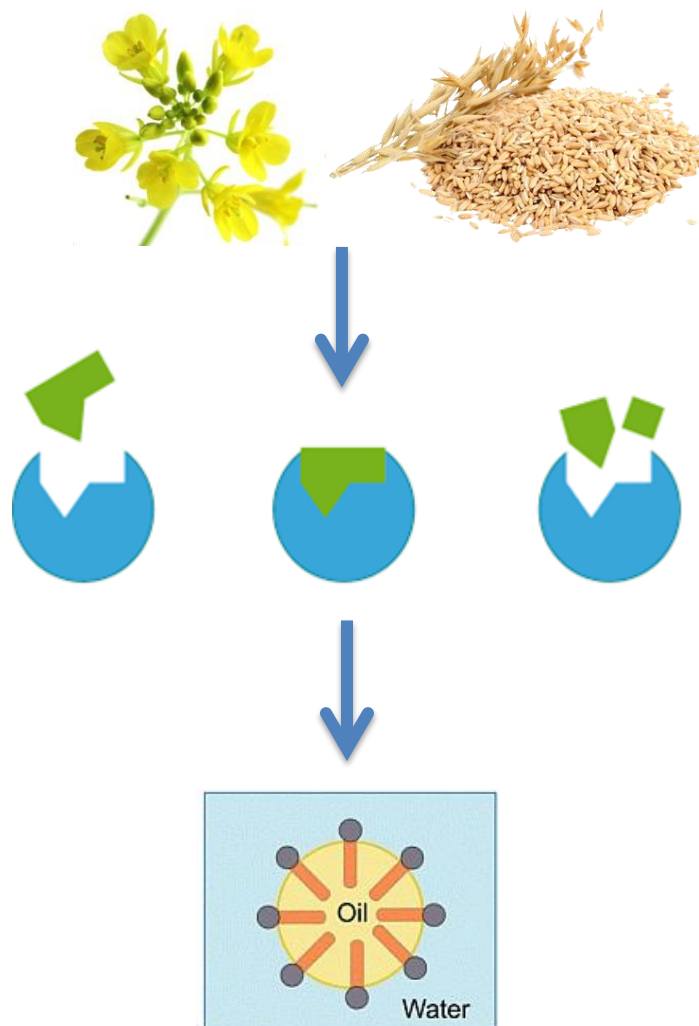
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Enzyme-aided production of lipid emulsifiers from side-streams of the food industry: rapeseed press cake and oat oil

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Abstract

The goal of this thesis project was to utilize side-streams of the food industry, namely rapeseed cake and oat oil, by enzyme-aided production of lipid emulsifiers.

The first objective was to extract lipids from the rapeseed press cake and increase protein yield, by adding an enzymatic step to the already existing protein extraction procedure developed by the food department of Lund University. A pre-treatment of the rapeseed press cake with Pectinex Ultra SP-L aimed to degrade the cell-wall matrix showed an increase in protein yield of approximately 10%.

Lipids were not extracted as no separate oil layer was seen during any of the treatments. It is hypothesised that the lipids were hydrolysed during the procedure leading to the bitter taste of the extract and solubilisation of the lipids. No starting material for modification of lipids was therefore obtained from rapeseed press cake.

The second objective was to determine the effect of enzymatic modification on the composition and emulsification properties of lipids. Two different oils were used: crude oat oil and polar lipid enriched oat oil (PL40). Firstly the unmodified oils were characterized. Column separation was performed, which successfully produced a neutral lipid, phospholipid and glycolipid fraction. Thin layer chromatography (TLC) showed that the neutral lipid fraction mainly contained di- and triglycerides and the phospholipid fraction contained phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and lyso-PC. Fatty acid profiles were determined by fatty acid methylation and gas chromatography. Main fatty acids in both oils were palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2). Minor fatty acids were palmitoleic acid (16:1), stearic acid (18:0) linolenic acid (18:3), arachidic acid (20:0), eicosenoic acid (20:1) and avenoleic acid (18:2 15OH), of which the latter was only found in the glycolipid fraction. Differences in fatty acid profiles of the two oils could be explained by the profiles of the different fractions. Emulsion stability was assessed by a fine-tuned spectrophotometric method supplemented by visual observations and light microscopy. Stability was significantly increased by the presence of unmodified oils, especially by PL40 oil. Surprisingly, contact time between oat oil and water before mixing also significantly affected stability, which was ascribed to the formation of liquid crystals.

Modification of the lipids was performed by a lipase from *Rhizopus arrhizus* and hydrolysed lipids were initially extracted by adding chloroform and methanol. However, this method was inconsistent and three other extraction methods were tested. Addition of water, chloroform and methanol proved most appropriate and was used for further experiments. Emulsion stabilizing ability increased as expected with incubation time for both crude oat oil and PL40 oil. Unexpectedly, the PL40 oil incubated without enzyme showed a higher stability than the hydrolysed oil. It is hypothesised that due to the hydrolysis more elaborate lipid structures are formed, which prevent coalescence but can cover less area than unmodified lipids, leading to bigger droplets and an increased creaming rate. From the results it is clear that the polar lipids in oat oil can be utilized as emulsifiers of which the properties can be changed by enzymatic hydrolysis.

In conclusion it can be said that both rapeseed press cake and oat oil can be utilized to a greater extent by aid of enzymes. The fine-tuned methods reported in this thesis for enzymatic hydrolysis and assessing change in molecular structure and emulsification properties can be used as a starting point for future research. This could eventually lead to implementation in industry and an increased utilisation of the side-streams of the food industry.

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1. Introduction

During the manufacture of food by-products are produced. These side-streams are not the focus of the production and are therefore often considered a waste. However, the streams frequently contain valuable components, which if utilized can increase the value of the crop while decreasing waste. Two by-products with potential for added value are investigated in this thesis, namely rapeseed press cake and oat oil.

Rapeseed press cake is the material remaining after oil extraction from rapeseed, *Brassica napus*. In the year 2011/2012 the worldwide production of rapeseed oil was approximately 24 million ton and as a by-product 33.6 million ton of rapeseed press cake was produced. Part of the press cake is used as a protein-rich feed, but despite the cake being more than 50% of the seed, it accounts for only 20-25% of the seed's value. (Carre & Pauzet 2014) The press cake shows potential for increased value as it is high in proteins, of which the amino acid composition is not only favourable for cattle, but also for human consumption (Rommi et al. 2014). Next to that, rapeseed cake can contain still quite high amounts of oil, depending on pressing method. Rapeseed oil is rich in unsaturated fatty acids (90%) and contains high levels of the essential fatty acids oleic acid (63%) and linoleic acid (20%) (Orsavova et al. 2015). Approximately 2% of the fat in rapeseed consists of phospholipids (Ambrosewicz-Walacik et al. 2015). This type of polar lipids is often used as emulsifiers in for example butter, sauces and mayonnaise (Kralova & Sjoblom 2009).

Oat oil is produced as a side-stream in the production of a fibre rich product by the company Swedish Oat Fiber AB. The fibres in oat, especially beta glucans, have recently gotten much attention for their health promoting abilities (Ekstrom et al. 2017; Patel 2015). The production of fibres is growing steadily (Swedish Oat Fiber AB n.d.) and so is also the amount of oat oil produced. Oat oil contains a high amount of polar lipids (Leonova et al. 2008) which gives the oil potential to stabilise emulsions.

Selection of the right emulsifier in the formulation of foodstuff is crucial for the stability and subsequently the quality of the product (McClements 2016). As many different foods with diverse stabilizing needs exist and also new foods are developed continuously, there is a constant demand in the food industry for emulsifiers with new combinations of properties. These new emulsifiers can be obtained by extracting polar lipids from previously overlooked sources, like oat. Furthermore, it is possible to modify the emulsifying properties of polar lipids by changing the molecular structure. Emulsifiers are molecules with a hydrophilic and lipophilic group. Changing the balance between the two groups affects what kind of emulsion can be made and its stability (Friberg 1997). The modification can be catalysed by enzymes. Enzymes are advantageous over other catalysts as they work under mild conditions, are considered safe and are specific for their substrate, meaning a complex material can be used as a starting material (Adlercreutz et al. 2016).

The goal of this thesis project is to utilize side-streams of the food industry, namely rapeseed cake and oat oil, by enzyme-aided production of lipid emulsifiers. The first objective is to extract lipids from the rapeseed press cake and increase protein yield, by adding an enzymatic step to the already existing protein extraction procedure developed by the food department of Lund University. The oat oil is already extracted and can be used directly to investigate the effect of enzymatic modification on the composition and emulsification properties of lipids. Due to this research insight will be gained in the possibilities to produce valuable functional ingredients from low-valued side-streams.

2. Background

The basics of emulsifying

Emulsions

An emulsion consists of two immiscible liquids, often oil and water, of which one is dispersed as small droplets in the other. Examples of emulsions that are encountered in everyday life are milk, an oil-in-water emulsion, and butter, a water-in-oil emulsion. Unexpected due its commonality, emulsions are actually unstable systems, destined to separation. Contact between oil and water is thermodynamically unfavourable, and as the interface between oil and water is very large in an emulsion, the system is thermodynamically unstable. This means that the system will move towards a minimal amount of interface, which leads to separation of the two liquids into two layers. This process can however be slowed down considerably by the presence of emulsifiers. An emulsifier is an amphiphilic molecule, meaning that it contains both a lipophilic and a hydrophilic group. Due to this, these molecules prefer to go to the interface between water and oil, arranging their lipophilic group towards the oil and their hydrophilic group towards the water. The emulsifier in this way lowers the interfacial tension and makes it harder for the dispersed droplets to come together and form one phase, leading to a kinetically stable emulsion. Depending on how effective the molecule is at stabilizing the system, such a metastable emulsion can take years to destabilize. (McClements 2016)

Destabilisation mechanisms

An emulsifier can delay destabilisation by affecting the destabilisation mechanisms. Important physical destabilisation mechanisms are gravitational separation and droplet aggregation.

Gravitational separation is caused by a density difference between the two phases. When the dispersed phase is denser than the continuous phase, the droplets will go down, called sedimentation. When the reverse is the case, the droplets will go up, called creaming. The rate of gravitational separation depends on the balance of forces acting upon it: the gravitational force versus the frictional force. Assuming no droplet interaction the gravitational separation rate can be approximated by Stokes law:

$$v_{Stokes} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}$$

where g is the gravitational acceleration, r is the radius of the droplet, ρ is density, η is the shear viscosity and the subscripts 1 and 2 refer to the continuous and dispersed phase. The sign of v_{Stokes} indicates whether creaming (+) or sedimentation (-) occurs (McClements 2016). It is clear from this formula that droplet size is an important factor determining separation rate. When droplets are under 1 μm , gravitational separation is of little importance. However, when droplet size increases above this, the rate increases exponentially (Friberg 1997).

Droplet aggregation leads to a larger effective droplet size, provoking an increased gravitational separation rate, and ultimately leads to the formation of large phases of the once-dispersed phase. Aggregation can occur in two different ways: flocculation and coagulation. When droplets flocculate, they are stuck together while still remaining several droplets. In contrast, when droplets coagulate one bigger droplet is formed. Both destabilisation mechanisms are driven by hydrophobic interactions, i.e. minimisation of the thermodynamically unfavourable oil-water interface. The rate of droplet aggregation depends on several factors. First of all, the droplets have to encounter each other before they can stick together. The likelihood of these encounters is determined by a range of conditions including temperature, which increases Brownian motion, turbulence and gravitational

separation rate. Secondly, whether droplets can come close enough to each other to aggregate depends on the interactions between the interfaces. Emulsifiers can prevent the droplets from coming close by steric repulsion, in the case of large molecules, or by electrostatic repulsion, in the case of charged molecules. Finally, the droplets can only coalesce when the film of materials on the interface ruptures or a hole is formed. When the interface is too strongly packed and the optimum curvature of the layer of emulsifiers does not favour hole formation, droplets can flocculate but not coalesce.

Lipids as emulsifiers

Emulsifying capabilities are found along a range of different molecules, both high-mass and low-mass (Kralova & Sjoblom 2009). Proteins are often used to stabilise emulsions as they are amphiphilic due to the variety of amino acids in their structure (Ozturk & McClements 2016). A more novel type of emulsifier is found in whole starch granules, which form Pickering emulsions (Timgren et al. 2013). However, in this research focus is laid on the emulsifying capabilities of polar lipids, which are low-mass surfactants. Lipids are defined as substances that are insoluble in water, but soluble in non-polar solvents (Coultate 1988). Although this definition includes an array of different substances, the term lipids commonly refers to a glycerol molecule esterified with one or more fatty acids. In the case of polar lipids, one of the alcohol groups of glycerol is esterified by a polar group. For instance, phospholipids are glycerol molecules of which the first alcohol is esterified by a phosphate, which is again esterified by a simple organic group. The exact class of the phospholipid is determined by this last group (Coultate 1988). Phospholipids are commonly obtained by degumming of crude oils. The resulting mixture of phospholipids is called lecithin. The lecithin can be treated in several ways to obtain different mixtures with different purities and is often used as emulsifier, for example in butter, sauces and mayonnaise (Kralova & Sjoblom 2009).

Another type of polar lipid is glycolipids, where the first alcohol on the glycerol is esterified by a sugar group. Glycolipids are mainly located in bacterial membranes and hardly found in plant tissue. However, the chloroplast is an exception. The membranes of the compartments of the chloroplast where the light-sensitive reactions take place, the thylakoids, contain a high amount of galactolipids. About 50% of the thylakoid lipids consist of monogalactosyldiacylglycerol (MGDG) and 30% of digalactosyldiacylglycerol (DGDG). (Hölzl & Dörmann 2007) It is predicted that eventually, when extraction costs have decreased, galactolipids will be the most commonly used natural emulsifiers (Kralova & Sjoblom 2009).

Polar lipids can be characterized by the ratio of their hydrophilic and lipophilic groups. This ratio is called the hydrophilic-lipophilic balance, HLB (McClements 2016). The concept of HLB was introduced by Griffin and defined as follows:

$$HLB = \frac{1}{5} \left(\frac{M_H}{M_T} * 100 \right)$$

where M_H is the molecular mass of the hydrophilic group and M_T the molecular mass of the whole molecule.

Another way to describe the HLB was proposed by Davies:

$$HLB = 7 + \sum H_{h,i} - \sum H_{l,i}$$

where $H_{h,i}$ is the contribution of each hydrophilic part and $H_{l,i}$ is the contribution of each lipophilic part. This additive model distinguishes between weak and strong hydrophilic groups. However, the hydrophilic/lipophilic contributions of many groups are not known yet, as they need to be determined empirically. (Ontiveros et al. 2014) Examples of group contributions that are known can be seen in Table 1.

Table 1 group contributions for several common chemical structures (Friberg 1997)

Group	<i>H</i>
-COO- (ester)	2.4
-COOH (acid)	2.1
-OH (hydroxy)	1.9
-O- (ether)	1.3
-CH / -CH ₃ / -CH ₂ - (carbon chain)	-0.5

The HLB number can be used to make predictions about the possible applications of the molecule. A low HLB number (3-6) indicates that the molecule can stabilize water-in-oil emulsions, a moderate HLB number (7-9) implies the molecule is a good wetting agent and a high HLB number (8-18) indicates that the molecule can stabilize oil-in-water emulsions (Friberg 1997).

A related method characterizes polar lipids by molecular geometry. The size of the hydrophilic head group and lipophilic tail are compared in the packing parameter p , which is defined as follows:

$$p = \frac{v}{l a_0}$$

where v and l are the volume and length of the lipophilic tail and a_0 is the cross sectional area of the hydrophilic head group. The packing parameter got its name due to the fact that molecular geometry determines the most efficient packing of the particles. This in turn determines the optimum curvature of a monolayer formed by the molecules, see Figure 1. (McClements 2016)

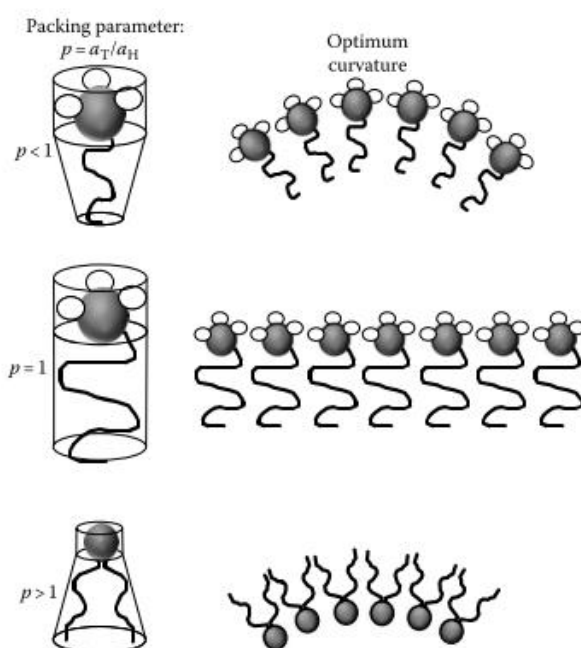


Figure 1 the molecular geometry (i.e. packing parameter p) of a polar lipid determines the optimum curvature of a monolayer formed by these lipids (McClements 2016)

The packing parameter and HLB number are related. A low p implies a small lipophilic group and thus a high HLB value. As mentioned before a high HLB value indicates the ability of the molecule to stabilise an oil-in-water emulsion. Such an emulsion contains oil droplets, with emulsifier molecules on the surface with their lipophilic tails sticking inwards and the hydrophilic heads pointing outwards. It can be seen in Figure 1 that a molecule with a $p < 1$ prefers exactly such curvature.

Characterisation of emulsion stability

The ability of an emulsifier to stabilize an emulsion can be investigated in several ways. The simplest method to determine emulsion stability is the bottle test. An emulsion is made and pictures are taken once in a while. Usually emulsions go through three stages during destabilisation, see Figure 2. At first droplets are dispersed homogeneously through the continuous phase and only one layer is seen. Due to gravitational separation droplets move up leading to a three-layer system: a depleted serum layer, an unaffected middle layer and an enriched cream layer. In the end all droplets will have moved up and only a serum and cream layer remain. Destabilisation can be quantified by measuring the height of the serum layer over time.

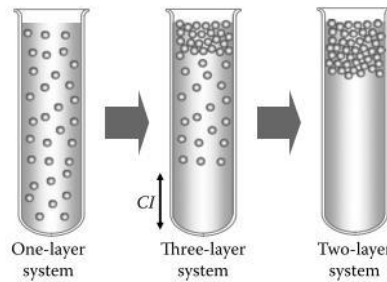


Figure 2 the three stages of emulsion destabilisation (McClements 2016)

Another way to determine emulsion stability is by assessing the droplet size distribution, for example by microscopy or a particle size analysis instrument using light scattering (Ozturk & McClements 2016). As discussed before, small droplets imply a lower rate of gravitational separation. Next to that, change in droplet sizes over time indicates whether droplet aggregation occurs. Droplet size information can be used to calculate the emulsion stability index (ESI), which is defined as follows:

$$ESI = \frac{d_0 * t}{d_t - d_0}$$

where d_0 is the initial mean droplet diameter and d_t is the mean droplet diameter at time t . The ESI is the time in which the mean droplet diameter doubles and is often used to estimate the life-time of an emulsion. (McClements 2016)

A relative novel method to assess emulsion stability is measuring light absorbance by the emulsion over time (Cabezas et al. 2012; Aizawa 2014). Cabezas and coworkers (2012) measured the backscattering of light ($\lambda=850$ nm) over the entire height of the emulsion by a vertical scan analyser. The change of the backscattering in the different layers over a period of 90 minutes was used to judge the stabilising properties of sunflower lecithin. Aizawa (2014) measured absorbance at 500nm at one fixed height over the course of half an hour in a spectrophotometer. A method was developed to determine the rate of destabilisation of the emulsion from the change of absorbance over time. It was seen that most of the absorbance curves showed a pattern resembling a first-order reaction. Therefore, the rate of destabilisation could be derived by plotting the natural logarithm of the absorbance against the time and calculating the slope of a linear trend line, see Figure 3.

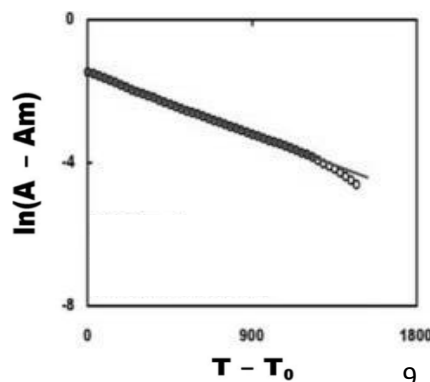


Figure 3 example of determination of destabilisation rate from absorbance data (Aizawa 2014)

Rapeseed press cake

Rapeseed press cake (RSC) is the material remaining after oil extraction from rapeseed, *Brassica napus*. Rapeseeds contain approximately 40% oil; the remaining 60% is obtained as by-product from the extraction (Rommi et al. 2014). The production of rapeseed oil has increased more than twofold over a period of 16 years (1996-2012), partly due to the development of biofuels. It has become the second most produced oilseed, behind soybeans. In 2011/2012 the worldwide production of rapeseed oil was approximately 24 million ton and as a by-product 33.6 million ton of rapeseed cake was produced. Part of the press cake is used as a protein-rich feed, but despite the cake being more than 50% of the seed, it accounts for only 20-25% of the seed's value. (Carre & Pauzet 2014)

The properties of RSC depend on the method of oil extraction. Commonly rapeseed oil is extracted using hexane and high heat pressing. A more gentle approach is cold pressing, in which no organic solvents are used and the temperature does not exceed 35°C (Gunnarshögs n.d.). The composition of cold-pressed RSC can be seen in Table 2 and is discussed in more detail below.

Table 2 composition of cold-pressed rapeseed cake provided by Gunnarshögs Jordbruks AB, analysed by Eurofins Food & Agro Testing Sweden AB. All values have an insecurity of +/- 10%.

Compound	%w/w
Water	10.3
Ash	6.04
Protein	27.3
Fat	15.5
Fibres	30.1
Carbohydrates (calculated)	10.8

The proteins in the rapeseeds are mainly storage proteins (napins <14 kDa and cruciferins 18-53 kDa), which are stored in protein bodies. These bodies are compartments bound to the cell membrane. The main functional proteins are oleosins (19-20 kDa). These proteins can be found in the membrane of oil bodies, also called oleosomes (Wanasundara 2011). As the name suggests, oil is stored in these organelles. The membrane of oleosomes consists of both phospholipids and oleosins, which together provide high stability both in vivo and in vitro (Deleu et al. 2010). Amino acid composition of RSC proteins is favourable for human consumption (Rommi et al. 2014) and as rapeseed is not a common allergenic, like soy, RSC has potential as a plant-based protein source for humans.

Due to the relative gentle extraction method, quite some fat remains in the RSC. Rapeseed lipids are rich in unsaturated fatty acids (90%) and contains high levels of the essential fatty acids oleic acid (63%) and linoleic acid (20%) (Orsavova et al. 2015). Approximately 2% of the fat in rapeseed consists of phospholipids, of which phosphatidylcholine is most abundant. During cold-pressing of rapeseed hardly any phospholipids are extracted (Ambrosewicz-Walacik et al. 2015), implying that RSC contains a relative high amount of phospholipid.

The fibres and carbohydrates present in the RSC are mainly cell-wall components, like cellulose, hemicellulose, lignin, pectin and arabinogalactan. The latter can covalently bind with proteins, forming proteoglycans (Rommi et al. 2014).

Current protein extraction procedure

A procedure to extract proteins from RSC has been developed at Lund University, Sweden. The first set-up can be seen in Figure 4. The pH of the macerate was varied by Nilsson et al (2015) in the range of 6-12.

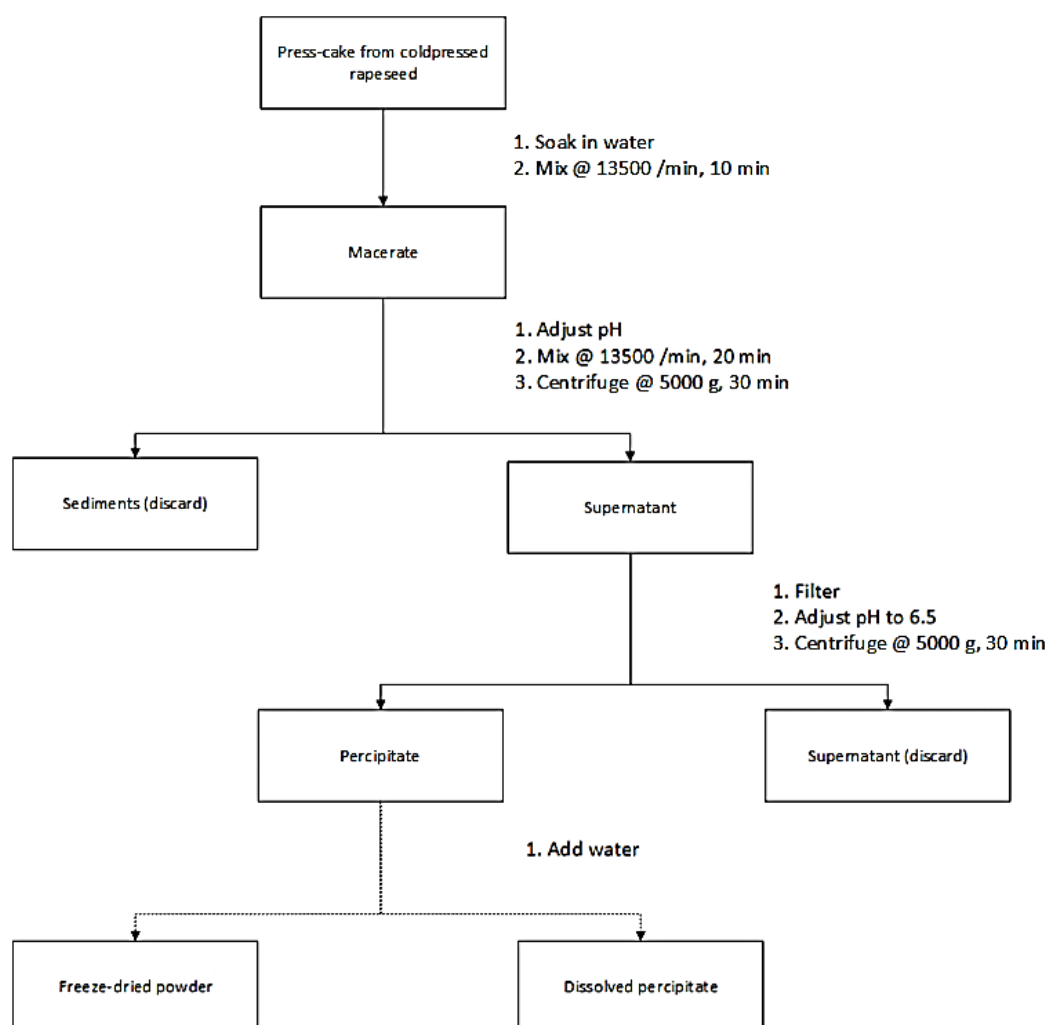


Figure 4 initial process scheme of protein extraction from cold-pressed rapeseed cake (Nilsson et al. 2015)

Protein content of several streams was determined by Eurofins Food & Agro Testing Sweden AB and can be seen in Table 3.

Table 3 protein content of streams in protein extraction procedure with macerate adjusted to pH 9 or 12, analysed by Eurofins Food & Agro Testing Sweden AB. All values have an insecurity of +/- 10%.

Stream	Protein content (%w/w)
Rapeseed press cake	28.9
Sediment 1 st centrifuge – pH 9	5.94
Sediment 1 st centrifuge – pH 12	3.38
Precipitate / wet protein extract – pH 12	12.6
Freeze-dried powder / dry protein extract – pH 9	42.6
Freeze-dried powder / dry protein extract – pH 12	58.0

From the table, it can be seen that some protein is lost to the sediment of the 1st centrifugation. A high pH resulted in less loss and a subsequent higher yield, but demanded more chemicals. The researchers at Lund University therefore decided to use a pH of 10.5 for the macerate in the current

extraction procedure. The filter step was discarded as the centrifugation was sufficient to separate the solids from the liquid. To promote protein precipitation the pH of the supernatant of the first centrifugation was adjusted to 6.0 and a heating step was added. The new process scheme can be seen in Figure 5. Protein yield and losses of this adjusted procedure are not as yet published.

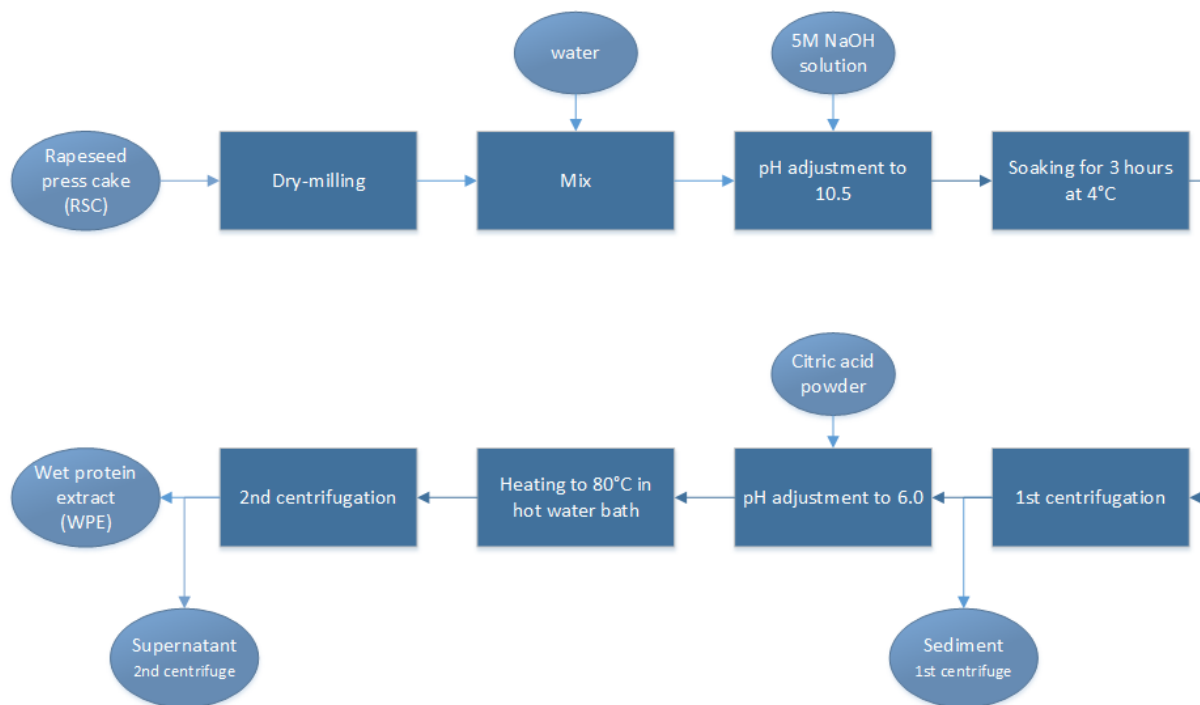


Figure 5 current process scheme of protein extraction from cold-pressed rapeseed cake, developed by Food department Lund University, Sweden

Oat oil

Oats are lipid-rich cereals containing up to 10% oil, and contain compared to oil seed crops a high amount of polar lipids (Leonova et al. 2008). Oat oil is produced as a side-stream in the production of beta glucan rich product by the Swedish company SweOat. The oats are defatted by an extraction and fractionation process using only ethanol and water. Next to the crude oat oil, containing 15% polar lipids, also polar lipid enriched oils are produced, containing 40% polar lipids and subsequently named PL40. Due to the high amount of polar lipids, oat oil has been used as emulsifier in chocolate, spreads and baking (Swedish Oat Fiber n.d.)

The polar lipids in oat oil include both phospholipids and glycolipids. The most abundant phospholipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Montealegre et al. 2012) and the most abundant glycolipid is digalactosyldiacylglycerol (DGDG) (Doehlert et al. 2010). Oat oil is rich in unsaturated fatty acids, with the essential fatty acid linoleic acid present in the highest amounts (Montealegre et al. 2012; Leonova et al. 2008). Next to the common fatty acids, oat contains unusual hydroxy and epoxy fatty acids. The most abundant unusual fatty acid is avenoleic acid, a fatty acid very similar to linoleic acid (18:2), but with a hydroxy group at the 15th carbon atom (Leonova et al. 2008). This fatty acid is found at galactolipids and can be esterified by another fatty acid, forming a mono-estolide. If this second fatty acid is also a hydroxy fatty acid, the polymerisation can continue and up to tetra-estolides can form (Moreau et al. 2008).

Enzymatic modification

Enzymes are proteins that catalyse specific reactions. This ability can be utilized in several instances, for example when a matrix needs to be broken down or when a molecule needs to be modified. Enzymes are advantageous over other catalysts as they are specific for their substrate, often very efficient, work under mild conditions and are considered safe. Disadvantages are however that enzymes are quite unstable and often expensive. Moreover, the enzyme with the required substrate specificity might not be available at all. (Adlercreutz et al. 2016) Fortunately, the enzymatic activity needed in this research has been found in the enzymes discussed below.

Pectinase for protein extraction

Pectinex Ultra SP-L mainly attack pectins, i.e. cell wall polysaccharides with a galacturonic backbone, but also other cell-wall components. Rommi et al (2014) assessed the activity profile of Pectinex Ultra SP-L, see Table 4, and determined the protein concentration of the enzyme solution to be 61 mg/ml.

Table 4 activity profile of Pectinex Ultra SP-L (Rommi et al. 2014) *nkat=10⁻⁹ mol/s

Type of activity	Main target	Activity (nkat*/mg protein)
Polygalacturonase	Pectin	2876
Endo-1,3(4)-β-glucanase	Arabinogalactan	135
Cellulase	Cellulose	22
Endo-1,4-β-xylanase	Hemicellulose	7.0
Protease	Protein	0.3
β-glucosidase	β-glucan	0.07

Lipase for lipid modification

The lipase produced by the mould *Rhizopus arrhizus* (also known as *Rhizopus oryzae*) is often used in lipid modification. The enzyme is highly sn-1,3 regioselective, meaning that it catalyses the hydrolysis of the fatty acid at the outer positions of glycerol molecule (Adlercreutz et al. 2001; Cao et al. 2016). The enzyme has been used successfully before for the hydrolysis of triglycerides (Li et al. 2015; Pérignon et al. 2012), glycolipids (Murakami et al. 1991) and phospholipids (Hara et al. 1997; Devos et al. 2006).

3.1. Study of rapeseed press cake

The aim of this research is to investigate whether the yield of the protein extraction procedure formulated by the food department of Lund University can be increased by addition of an enzymatic pre-treatment to the procedure, see Figure 6, and whether lipids can be extracted.

First of all, in order to get more insight in the losses that occur during the current protein extraction of rapeseed press cake (RSC), the protein composition of protein extract and one of the side streams (sediment of first centrifugation) are investigated by SDS-PAGE and compared to literature. It is hypothesised that loss could occur by entrapment of the proteins in the cell matrix. Breakdown of this matrix might therefore lead to release of the proteins to the supernatant and subsequently to an increased protein yield of the extraction procedure. The matrix, likely to consist mainly of cell wall materials is broken down in this research by a pectinase. The degree of breakdown is determined by DNS assay of reducing ends in the supernatant of the second centrifugation. Wet, dry and protein yields and protein losses are determined to assess the success of the protein extraction procedure.

Material & methods

Protein identification by SDS-PAGE

Two rapeseed protein extracts were produced using the current extraction procedure without enzymatic pre-treatment and a pH 5 or 6 in the denaturation step. Sediment was obtained after the first centrifuge step and only the upper yellow part was taken.

Approximately 45 mg of each material was re-dispersed in 1 ml of Milli-Q water. 16 µl of sample was mixed with 4 µl of loading buffer containing dye, heated to 100°C for 10 minutes and loaded on a premade gel. Electrophoresis was run at 150 V for approximately 40 minutes. The gel was stained with coomassie blue and de-stained with methanol/acetic acid mixture overnight.

Enzymatic pre-treatment

Cold pressed rapeseed cake (RSC) provided by Gunnarshögs Jordbruks AB was dry-milled using a coffee grinder. 10 g of RSC was weighed and deionized water was added up to 100 ml. When relevant, the pH was adjusted with citric acid powder. No, 0.1 (1%v/w RSC) or 0.5 ml (5%v/w RSC) of the enzyme Pectinex Ultra SP-L from Novozymes A/S was added. Samples were subsequently incubated in a shaking water bath at 30°C for 2 hours, 2 days or 3 days.

Protein extraction and wet yield

After incubation the pH of the samples was adjusted to pH 10.5 with 5 M NaOH solution in order to solubilize the proteins. After 10 minutes the pH was checked and readjusted to 10.5 when needed. The samples were stored for one hour at 4°C. Afterwards, the samples were poured in 50 ml centrifuge tubes, resulting in 3 tubes with approx. 33 ml per sample. The tubes were centrifuged at 4°C at 5000 rpm (3893g) for 30 minutes in Centrifuge 3-16PK from Sigma Laborzentrifugen GmbH. The supernatant was collected and the pH adjusted to pH 6.0 with citric acid powder in order to precipitate the proteins. Samples were placed in a hot water bath and stirred until they reached 80°C and then cooled down on ice. The samples were poured in 50 ml centrifuge tubes again, resulting per sample in 2 tubes with approx. 35 ml. The tubes were centrifuged at 4°C at 5000 rpm (3893g) for 30 minutes. Supernatant was poured off and assessed by DNS assay to determine the degree of breakdown by the enzyme. Sediment, i.e. wet protein extract (WPE), was weighed and stored. Wet yield was calculated as follows:

$$\text{wet yield (\%)} = \frac{\text{mass}_{WPE}}{\text{mass}_{RSC}} * 100\%.$$

Degree of breakdown

To an Eppendorf tube 600 µl of DNS reagent was added, containing per 100 ml: 1 g NaOH, 0.05 g sodium sulphite, 26.85 g sodium potassium tartrate tetrahydrate and 1 g DNS. Next, 400 µl supernatant was added to the Eppendorf tube. The sample was vortexed and put in boiling water for 5 minutes. After cooling down on ice, the samples were transferred to 1.5 ml cuvettes and the absorbance at 550 nm was measured with an UV-visible spectrophotometer UV-1650 PC from Shimadzu and displayed by the program UVProbe v2.01. A standard curve was made with glucose solutions with concentrations ranging from 0-10 mM, see appendix.

Dry matter content and yield

Oven disks were pre-dried in an oven at 105°C for half an hour, cooled down in a desiccator under vacuum and weighed empty. Approximately 2 g of each WPE and of RSC was added to the disks. Samples were left in the oven overnight at 105°C and weighed again after cooling down in a desiccator under vacuum. Dry yield was calculated as follows:

$$\text{dry yield (\%)} = \frac{\text{dry matter}_{WPE} * \text{mass}_{WPE}}{\text{dry matter}_{RSC} * \text{mass}_{RSC}} * 100\%.$$

Protein content and yield

Protein content was measured for rapeseed cake and all wet protein extracts at least in duplicate. Also, all layers of the sediment of the first centrifugation of two samples were measured. Protein content was determined by FlashEA1112 protein analyser from Thermo Scientific, using a conversion factor of 6.25. Protein yield was calculated as follows:

$$\text{protein yield (\%)} = \frac{\text{protein content}_{WPE} * \text{mass}_{WPE}}{\text{protein content}_{RSC} * \text{mass}_{RSC}} * 100\%.$$

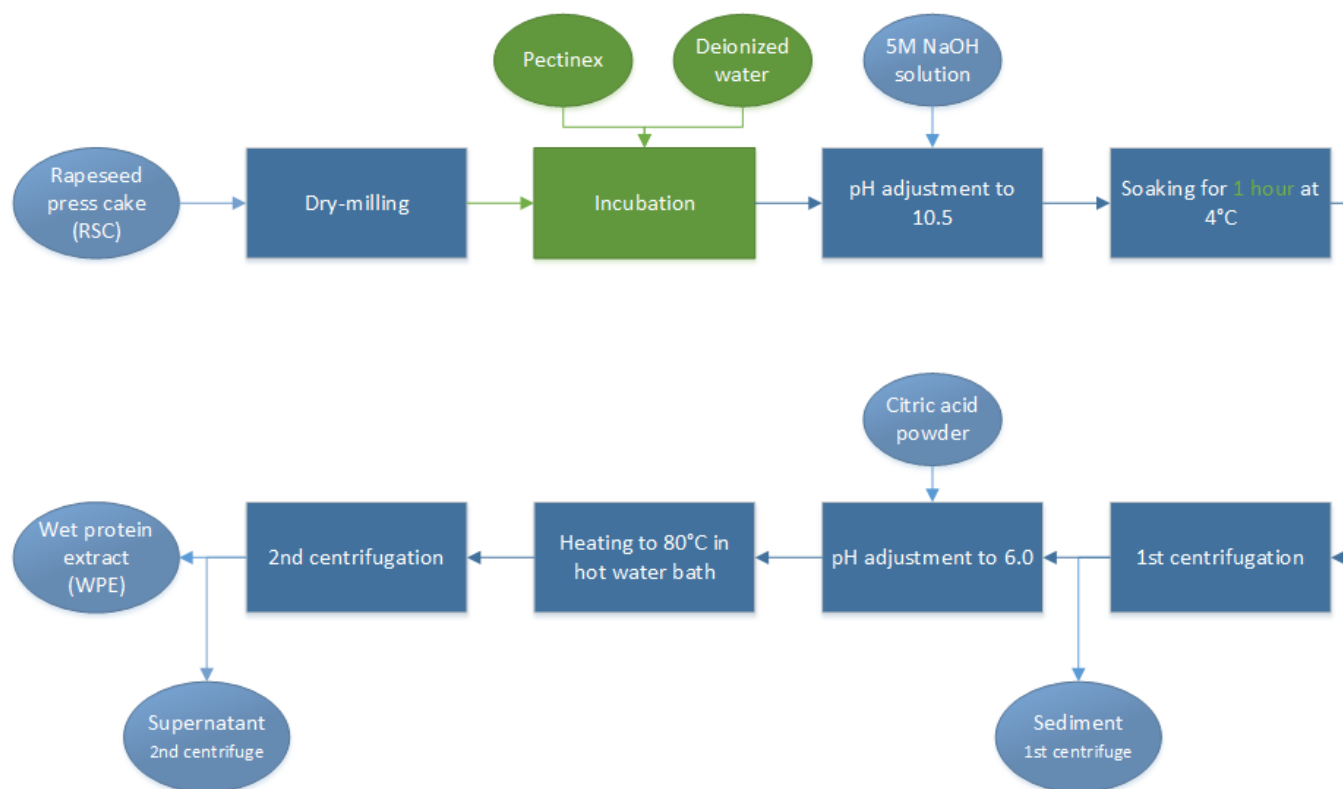


Figure 6 process scheme of enzyme-aided protein extraction from rapeseed cake

Results & discussion

Losses in original protein extraction

The protein composition of two protein extracts, extracted at different pH, and one of the side streams (sediment of first centrifugation) were investigated by SDS-PAGE and a picture of the gel can be seen in Figure 7. A ladder is included with protein sizes in kDa.

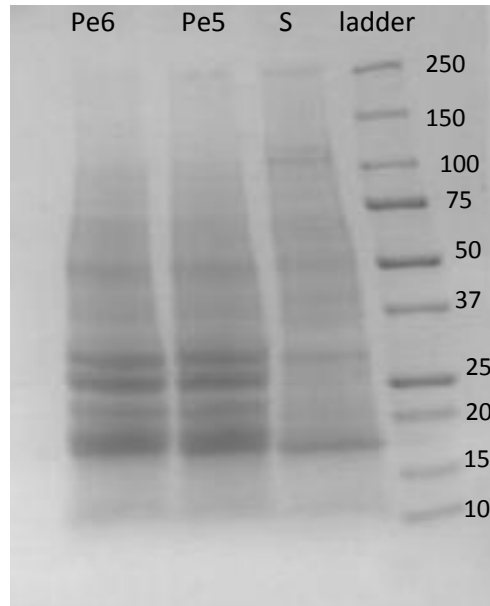


Figure 7 SDS-PAGE patterns of re-dispersed protein extract extracted by pH adjustment to pH 6 (Pe6), pH 5 (Pe5) and of sediment of the first centrifugation (S) with ladder as reference for protein size (kDa)

The protein extracts extracted at pH 5 and 6 do not show any significant differences, so the exact pH does not seem to matter for protein composition of the extract. This is not wholly unexpected as the heating to 80°C is a relatively harsh treatment, leading to denaturation of proteins also when the pH is not exactly equal to the isoelectric point.

From the figure it can be seen that the sediment contains proteins, which indicates that protein loss occurs during the first centrifugation step. The sediment has a different protein composition than the protein extract. Larger proteins are present, which is understandable as these are more easily centrifuged down. Interesting to see is that also quite some small proteins of ~18 kDa are present, which are the proteins of main interest (oleosin). This may be caused by the presence of other materials in the rapeseed cake. The cell wall matrix of the plant cells could have hampered the transfer of the proteins to the liquid phase by simply getting in the way. Next to that, arabinogalactan, a hemicellulose, is able to bind covalently with proteins, forming complexes called proteoglycans (Rommi et al. 2014).

To see whether all the different types of rapeseed proteins are obtained in the extract, the results of the extract can be compared to the initial protein composition of rapeseed. The protein composition of several different rapeseed cultivars has been studied by El-Beltagi and coworkers (El-Beltagi et al. 2011) and can be seen in Figure 8. From this gel it can be seen that different cultivars have similar protein composition. In contrast, there are large differences between the protein composition of the original rapeseed and the protein extract. As expected, the proteins found in the extract can also be found in the rapeseed. However, the reverse is not true. The rapeseed contains many small proteins, while the extract contains hardly anything under the size of 15 kDa. As the sediment also does not contain these small proteins, they might have been lost to the supernatant of the second centrifugation step.

Other clear lines on the rapeseed gel that are not seen for the extract are the ones between 35 and 45 kDa. It is not clear why these proteins are not seen. As the extract gel shows a light smear at this region, it might be that the proteins simply did not separate properly on the gel. It might also be that

these proteins have an isoelectric point far from pH 5 or 6, making them less prone to denaturation and sedimentation in the second centrifugation. Another possibility is that they are lost during oil pressing, meaning that they are not present in the rapeseed cake to start with.

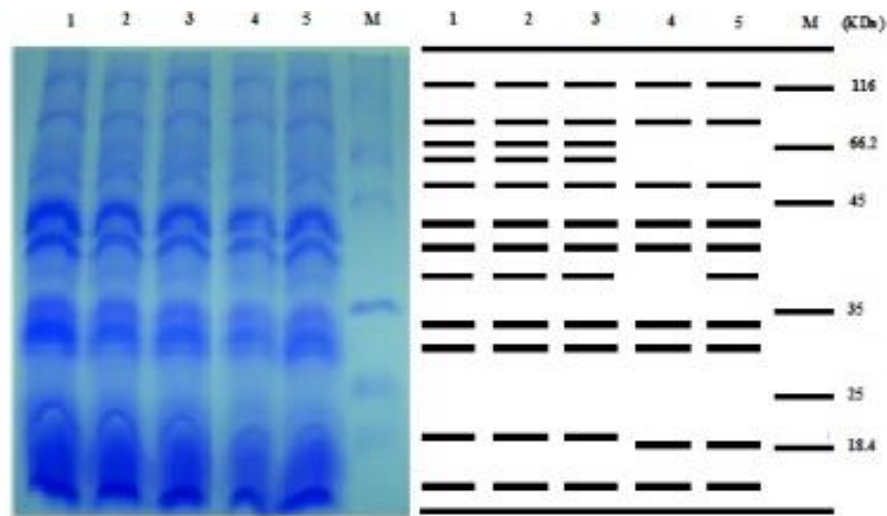


Figure 8 SDS-PAGE protein patterns of different rapeseed cultivars. 1: pactol, 2 silvo, 3 topas, 4 serw4, 5 serw6, M: marker. (El-Beltagi et al. 2011)

Another interesting article for comparison is by Rommi and coworkers (Rommi et al. 2014) in which rapeseed cake was enzymatically degraded. The protein composition of the liquid after degradation of the cake can be seen in Figure 9.

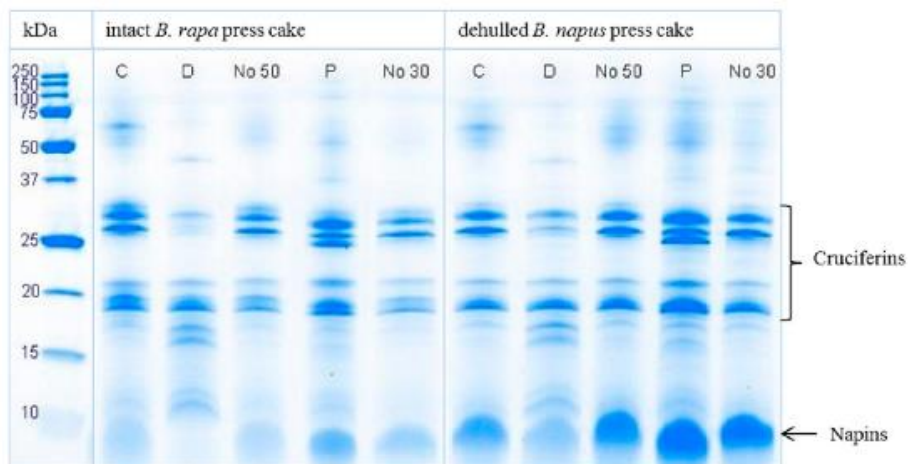


Figure 9 SDS-PAGE patterns of proteins released during enzymatic treatment of intact and dehulled RSC. The materials were hydrolysed for 48h with Celluclast (C), Depol (D) and Pectinex (P). No enzyme was added in the reference treatments carried out at 50°C (No 50) and 30°C (No 30). (Rommi et al. 2014)

The proteins slightly bigger than 25 kDa and slightly smaller than 20kDa (oleosins) seen in Figure 9 were also seen in the protein extract, Figure 7. As well similar is the absence of the proteins with a size between 35 and 45 kDa. This strengthens the hypothesis that these proteins are lost during the oil extraction and are not present in the raw material for the extraction. Different from the extract is that in the liquid proteins are present smaller than 15 kDa. This strengthens the hypothesis that the small proteins are lost to the supernatant in the second centrifugation step. To increase the protein yield it could therefore prove useful in future research to increase the heating time, in order to gel these proteins into larger complexes that will sediment during centrifugation.

Yields of enzyme-aided protein extraction

In order to study the effect of an enzymatic treatment, two experiments were done. In the first one short (2 hour, S) and long term (2 days, L) incubation was studied for incubation with no (X) or 1%v/w enzyme (Ez) at 30°C. Unfortunately the water bath could not keep a stable temperature and the sample heated up to 40°C in the second hour. Long term samples were subsequently kept at room temperature (approx. 20°C) for the remaining time. Results for this first experiment can be seen in Table 5. Yields were determined using the dry matter and protein content of the starting material, which were determined to be 91.4% and 27.7% respectively. These values are considered realistic as they are close to the values found previously by Eurofins Food & Agro Testing, which were 89.7% and 27.3% respectively.

Table 5 results for experiment 1, incubation of rapeseed cake with 1%v/w (Ez) and without (X) Pectinex Ultra SP-L for 2 hours (S) and 47 hours (L) incubation at inconstant temperature

Sample	Enzyme (%v/w)	Incubation time (h)	Reducing ends (mM)	Wet yield (%)	Dry matter (%)	Dry yield (%)	Protein content (%)	Protein yield (%)
X S	0	2	-	51.1	20.2	11.3	9.8	18.1
Ez S	1	2	-	56.0	18.3	11.2	7.8	15.7
X L	0	47	5.7	74.5	15.9	13.0	7.1	19.2
Ez L	1	47	10.9	75.0	16.4	13.4	7.8	21.1

Although the wet yield was higher for the sample Ez S than for the reference X S, the protein yield was lower. It is possible that due to the short incubation time, the enzyme did not have time to break down the cell matrix sufficiently to release protein. However, this can only explain why the protein yield is not higher than X S, and not why it is smaller. Curiously, the dry yield for both Ez S and X S is about the same, but the protein content is different. Apparently WPE of Ez S contains relatively much non-protein dry matter, which is able to hold water (as the wet yield is high). This could be pectin. It can be hypothesized that the enzyme has not yet broken down the pectin completely, but did facilitate release of pectin strands from the cell matrix. The pectin stays in solution during the first centrifugation, but might gel upon heating. Gelling of pectin normally occurs at very low pH (<3.5), but can also occur in the presence of calcium (Coultate 1988). Rapeseed cake contains approx. 0.65% calcium (Nwokolo & Bragg 1980). If a gel is formed, the pectin is likely to sediment during the second centrifugation. The pectin and the water it holds will consequently contribute to the wet yield, but not to the protein yield.

For the long term samples the wet yield was almost equal, but the dry yield and protein content are slightly higher. This is the reverse of what was seen for the short term samples. Using the pectin hypothesis, this could be explained as follows. After long soaking, pectin is solubilized even without the help of enzymes. Long strands of pectin are then present in the liquid of sample X L which subsequently gel and affect the protein content as described before. The pectin released in the sample Ez L is however broken down by the enzyme to small strands and monomers, which are unable to form a gel and sediment. This results in a higher protein content of the WPE. The protein yield of the enzyme containing sample Ez L is 1.9% higher than the reference X L, which is an increase of 10%. This indicates that when the enzyme is given the time to break down the cell matrix, more protein can be extracted than without this enzymatic step. For a relatively long incubation the increase in yield is lower than expected, but this could be caused by the low incubation temperature of approx. 20°C, at which the enzymes work only slowly. A higher temperature might increase the protein yield.

For the second experiment a working water bath was found and a new set of samples was incubated at 30°C. All samples were incubated over the weekend (67 hours). Samples contained no (X), 1%v/w enzyme (6-1 and 5-1) or 5%v/w enzyme (6-5). For most of the samples pH was unadjusted before incubation, and therefore had a pH of 6.1. Only the pH of sample 5-1 was adjusted to 5 before addition of the enzyme. Yields can be seen in Table 6.

Table 6 results for experiment 2, incubation of rapeseed cake with different amount of Pectinex Ultra SP-L (0, 1 and 5% v/w) and different starting pH (6.1 and 5.0) at 30°C for 67 hours

Sample	Enzyme (%v/w)	pH start incubation	Reducing ends (mM)	Wet yield (%)	Dry matter (%)	Dry yield (%)	Protein content (%)	Protein yield (%)
X	0	6.1	1.0	66.5	17.5	12.8	7.6	18.3
6-1	1	6.1	8.8	81.6	16.5	14.8	6.7	19.8
5-1	1	5.0	3.9	55.3	21.8	13.2	8.9	17.8
6-5	5	6.1	7.3	69.2	17.0	12.9	7.0	17.5

It was expected that for all enzyme containing samples the protein yield would be higher than the reference X. This was especially expected for sample 6-5, which contained the most enzyme. However, only sample 6-1 gave a higher protein yield, while sample 5-1 and 6-5 both gave a lower protein yield than the reference X. The unexpected low yield is probably caused by bacterial contamination. Both samples smelled oddly after incubation and when the bottle of sample 5-1 was opened a hissing sound could be heard, indicating gas production. The pH after incubation was usually the same for both reference and enzyme containing sample (5.8 for X S and Ez S, 5.5 for X L and Ez L and 4.3/4.4 for X and 6-1), but was lower for samples 5-1 (pH 3.8) and 6-5 (pH 4.0), indicating acid production. The supernatant of the 2nd centrifugation was clear for almost all samples, but not for 5-1 and 6-5. Especially 5-1 was quite opaque, indicating presence of bacterial cells or products from their metabolism. Microbial growth is likely to have hampered the protein extraction as bacteria release proteases, which can degrade both the enzyme and the product of interest. In previous research, microbial growth was prevented by addition of 0.02% sodium azide (Rommi et al. 2014). However, this substance is very toxic (Aldrich 2015). As the protein extract is intended for human consumption, such a substance cannot be used.

Sample X and 6-1 did not show signs of bacterial contamination and can thus be compared. Wet yield was 23% higher for the enzyme containing sample than for the reference. Dry matter and protein content were however somewhat lower for sample 6-1. This is the opposite of what was seen in the first experiment. The pectin hypothesis is unable to explain this result. It could be that the differences in protein content of WPEs are merely by chance. As no duplicates were run, the variance of the procedure in itself is unknown.

The increase in protein yield due to enzymatic pre-treatment was 8% in this experiment. This is lower than expected, as the increase in protein yield was higher (10%) for the long term samples incubated at a lower temperature (20°C) for most of the time. It is possible that the relatively short time at 40°C in the beginning of the first experiment had a significant effect on the enzymatic breakdown of the cell matrix. Both sample X L and Ez L contain a relatively high concentration of reducing ends (5.7 and 10.9) compared to sample X and 6-1 (1.0 and 8.8), indicating a higher degree of breakdown despite the lower temperature and shorter incubation time. The breakdown in the reference can be explained by the fact that rapeseed cells use enzymes for their metabolism. These enzymes might still be present in native conformation at the beginning of the extraction procedure due to the gentle cold pressing. When the temperature is high enough (e.g. 40°C) they might be able to significantly aid the breakdown of the cell matrix. This would also explain the relatively high protein yields of both samples X L and Ez L. In future research, an incubation temperature of 40°C might prove beneficial.

Losses in enzyme-aided protein extraction

Sediment after the first centrifugation was weighed and for both long term experiments the sediment of the enzyme containing sample (Ez L and 6-1) was approximately 3 grams less than the sediment of the control (X L and X). This indicates that the enzyme had effectively broken down some of the cell matrix. This is confirmed by the DNS assay which showed more reducing ends in the supernatant of the samples containing enzymes than for the reference, see Table 5 and Table 6. The sediment consisted of three layers, see Figure 10. The upper two yellow layers were less clear during larger-scale production and investigated together on SDS-PAGE, which showed that the sediment contained protein after the original extraction procedure. To investigate the effect of enzymatic pre-treatment on this loss, protein content was determined for all three layers for the sediment of sample X and sample 6-1. Results can be seen in Table 7.



Figure 10 sediment after 1st centrifugation

Table 7 protein content of the three layers of sediment after 1st centrifugation of the second experiment for sample X (no enzyme) and 6-1 (1% enzyme)

	Protein content (%)	
	Sample X	Sample 6-1
Upper layer	3.1	2.8
Middle layer	2.3	2.5
Bottom layer	4.2	4.2

As less sediment was obtained from sample 6-1 than from reference X and the protein content of the sediment appears also slightly less, it seems that less protein is lost to the sediment when an enzymatic pre-treatment is added. However, conclusions have to be drawn with caution as the protein content is based on only one measurement and enzymatic trials were only done once per set of conditions.

Whereabouts of the lipids

No starting material for further characterisation and modification of lipids was obtained from RSC. As the cake still contained about 15% oil, it was expected that some oil would be seen during the extraction procedure. Next to that, pectinase aided pressing of rapeseed has been seen to increase oil yield and pressing efficiency (Szydłowska-Czerniak et al. 2010). However, even with an enzymatic pre-treatment, no oil layer was visible in the sample after any of the centrifugations. It is possible that the lipids that remained after pressing are strongly attached to the cell matrix and are not released by the protein extraction procedure. Another possibility is that the oil is hydrolysed upon addition of 5M NaOH, a phenomenon called alkaline saponification. This reaction releases free fatty acids, which are amphiphilic and are soluble in water. This would explain why no separate layer is seen. Moreover, it explains why a bitter taste has been reported for the protein extract. Although the free fatty acids are solubilized, it is possible that they sediment together with the protein extract due to the formation of a gel during the heating step in the extraction procedure. This could lead to the reported off-taste, as unsaturated free fatty acids have a bitter taste (Belitz et al. 2009) and approximately 90% of the fatty acids in rapeseed oil are unsaturated (Orsavova et al. 2015). Oleic (18:1), linoleic acid (18:2) and linolenic acid (18:3) are the main culprits, as their bitter taste threshold levels are low, namely 119, 67 and 11.5 mg/100g respectively (Stephan & Steinhart 2000) and rapeseed oil contains significant levels of these fatty acids, namely 63.3%, 19.6% and 1.2% respectively (Orsavova et al. 2015). Subsequently the bitter taste might be decreased by minimizing the duration of the first soaking step, or - if taste is more important than yield - lowering the pH of this step.

3.2. Study of oat oil

The aim of this research is to investigate the effect of enzymatic modification on the composition and emulsification properties of lipids. As no rapeseed oil was obtained from the previous experiments, only oat oil is investigated.

Firstly, experiments are run which relatively simply and quickly assess the composition of oat oils. Lipid classes are determined by thin layer chromatography. As no mobile phase is known that can separate neutral lipids, phospholipids and glycolipids at the same time, a preparative separation step by column chromatography is performed. The fatty acid compositions of the oils and the separate fractions are determined by methylation and subsequent gas chromatography. Secondly, the emulsification properties of the oat oils are determined by a fine-tuned spectrophotometric method supplemented by visual observations and light microscopy. Finally, the oat oils are enzymatically hydrolysed and change in composition and emulsification properties are determined and compared to the unmodified oils.

Material & methods

Material

Crude oat oil and oat oil enriched in polar lipids (PL40) were received as gift from Swedish Oat Fiber AB. Solvents were obtained from Merck KGaA, VWR chemicals or Scharlau and of analytic grade.

Separation by column chromatography

The separation was performed as described by Leray (2016), except for an increased column size in order to obtain more product, an increased pore size of the silica gel in order to speed up the process, and an increased amount of lipid loaded on the gel in order to increase the amount of eluted glycolipid (as suggested by Pernet, Pelletier, & Milley (2006)). A column with a diameter of 3 cm equipped with a glass wool plug and stopcock was loaded with 2.7 g silica gel (300 Å) dispersed in 20 ml chloroform. After washing the column with chloroform and letting the liquid drop to the top of the gel, 135 mg lipid dissolved in 10 ml chloroform was loaded onto the gel. After complete drainage, 90 ml chloroform containing 1% acetic acid was added to the column, and the eluted liquid containing mainly neutral lipids was obtained in the first round bottomed flask. After complete elution, 90 ml of acetone/methanol (9/1) was added, and the eluted liquid containing mainly glycolipids was obtained in flask 2. After complete elution, 90 ml of methanol was added, and the eluted liquid containing mainly phospholipids was obtained in flask 3. All three fractions were evaporated to dryness on a rotary evaporator and weighed. The lipids were stored dissolved in a small volume of chloroform/methanol (2/1).

Thin layer chromatography (TLC)

Different mobile phases were used for the different lipid classes, as described by Price & Parsons (1975) and Christie (2003), namely:

- ❖ General separation: petroleum ether/diethyl ether/ acetic acid (70:20:4);
- ❖ Neutral lipids: petroleum ether/diethyl ether/acetic acid (90:10:1);
- ❖ Glycolipids: chloroform/methanol/water (75:25:4);
- ❖ Phospholipids: chloroform/methanol/water (65:35:4).

Standards that were included in the neutral lipid separation were palmitic acid and trilaurin as indicators for free fatty acids and triglycerides respectively. Phosphatidylcholine (PC) was included as a standard in the phospholipid separation.

About 30 ml of mobile phase was poured into a TLC chamber of 10x5x10 cm. The chamber was allowed to equilibrate for at least 20 minutes with a filter paper for increased surface area. Samples were applied one cm from the bottom and from each other with a micropipette on a TLC plate (silica gel 60Å) of 9 cm high and 7 cm wide. The plate was left in the chamber until the solvent front was approximately 2 cm from the top. After drying the plate was sprayed with a solution of 0.1% w/v 2,7-dichlorofluorescein in methanol, and a picture was taken under UV light. Spots were identified by

comparing retention factors (Rf), i.e. the distance the lipids travelled relative to the mobile phase, to TLC plates of cereal grain lipids previously published (Price & Parsons 1975).

Fatty acid analysis

The fatty acid content of the different oat oils and the three fractions obtained from crude oat oil were determined by the method described by Svensson (2011) in which fatty acids are methylated and subsequently separated by gas chromatography (GC).

Lipid fractions and modified lipids were evaporated to dryness under a stream of nitrogen and unmodified oils were used as is. In a dry glass test tube 5-10 mg of dry lipids were mixed with 0.7 ml dry cyclohexane, containing in later experiments trilaurin as internal standard. Next, 0.5 ml methylation reagent (dry methanol and sodium methoxide solution, 9/1) was added to each tube. After short shaking or vortexing to dissolve the lipids, the test tubes were incubated in a water bath of 50°C for 30 minutes. Next, 2 ml saturated NaCl solution was added to stop the reaction and the tubes were vortexed for three times 5 seconds. The tubes were centrifuged for 2 minutes at 3000 rpm. Samples were taken from the upper layer and added to a GC vial with insert. GC was performed with a Varian 430-GC equipped with a Supelcowax capillary column (length 51.6 m, film thickness 0.25 µm). 10 µl of sample was injected using an inlet pressure of 20 psi and an injector temperature of 230°C. Initial column temperature was 160°C and was increased steadily to 250°C over a time course of 30 minutes, after which the temperature was kept for another 10 minutes. Flow rate of helium, hydrogen and air was set to 25, 30 and 300 ml/min respectively. Detector temperature was set to 270°C. Peak areas were determined by the program Galaxy and identified by comparing the chromatogram to a previously obtained oat oil chromatogram with identified peaks by S. Leonova (unpublished).

In the first experiments, the weight of the lipids was not recorded properly and no standard was included, so fatty acid profiles were expressed in weight percentage. As peak areas can be assumed to be linearly proportional to the weight of material eluted (Christie 2003), the weight percentage can be calculated as follows:

$$\text{weight percentage (\%)} = \frac{A_{FA}}{\sum A} * 100$$

where A_{FA} is the area in UV*min of the peak related to the fatty acid of interest and $\sum A$ is the sum of the areas of all identified peaks. This calculation thus only takes into account the peaks that can be linked to a specific fatty acid and excludes noise (in contrast to the relative area given by the program).

Fatty acid profiles for the unmodified oils and the lipid fractions were compared to data from literature. When no specific information was reported a fatty acid profile was derived from the other data in literature, assuming:

$$X_{FA,total} * m_{total} = X_{FA,neutral} * m_{neutral} + X_{FA,polar} * m_{polar}$$

and

$$X_{FA,polar} * m_{polar} = X_{FA,glycol.} * m_{glycol.} + X_{FA,phosphol.} * m_{phosphol.}$$

where $X_{FA,y}$ is the weight percentage of a certain fatty acid in fraction y and m_y is the weight of fraction y. Weights of the fractions are taken from literature (Price & Parsons 1975) and are as follows: $m_{total} = 100$, $m_{neutral} = 72.9$, $m_{polar} = 27.1$, $m_{glycol.} = 17.0$ and $m_{phosphol.} = 10.1$. Also taken from literature are the weight percentages of the main fatty acids in polar lipids ($X_{FA,polar}$) (Leonova et al. 2008), phospholipids ($X_{FA,phosphol.}$) (Montealegre et al. 2012) and all lipids combined ($X_{FA,total}$) (Leonova et al. 2008).

In the hydrolysis experiments, the weight of the lipids was recorded and lauric acid was included (in the form of trilaurin) as internal standard in sufficient concentration for the peak to be distinguished

from the noise (5mM trilaurin, i.e. 15mM lauric acid). The peak areas in the GC chromatograms can be directly related to the weight of methylated fatty acids in the sample. The amount of attached fatty acids per kg of oil could therefore be calculated as follows:

$$\text{concentration (mol/kg)} = \frac{[is] * M_{w,mis}}{A_{is}} * \frac{A_{FA}}{M_{w,mFA}} * \frac{V}{m_{oil}}$$

where $[is]$ is the concentration of the internal standard in mM, A_{is} is the area of the peak related to the internal standard in $\mu V \cdot \text{min}$, V is the volume of the lipid containing layer after centrifugation (cyclohexane layer) expressed in ml and m_{oil} is the amount of oil used for the analysis, expressed in mg. $M_{w,mis}$ and $M_{w,mFA}$ are the molecular weights of the methylated internal standard and fatty acid under consideration. M_w was calculated by adding 14.0268 (weight CH_2) to the molecular weight of the unmethylated fatty acids. The total concentration of attached fatty acid concentration that was used to compare different treatments was calculated by adding up the concentrations of the quantified fatty acids.

Assessment of emulsification properties

Emulsions were made by mixing oat oil (emulsifier), sunflower oil and milli-Q water in a 4.5ml flask with an IKA Ultra-turrax T 25 equipped with the dispersion tool S 25 N – 8 G. Emulsion stability was assessed by visual observation, microscopy and spectrophotometry of the serum phase. Exact parameters of the procedure were chosen through iterative experimentation.

Oat oil and sunflower oil were mixed before addition of water, as this showed to create more homogenous emulsions regarding droplet size, see Figure 11.

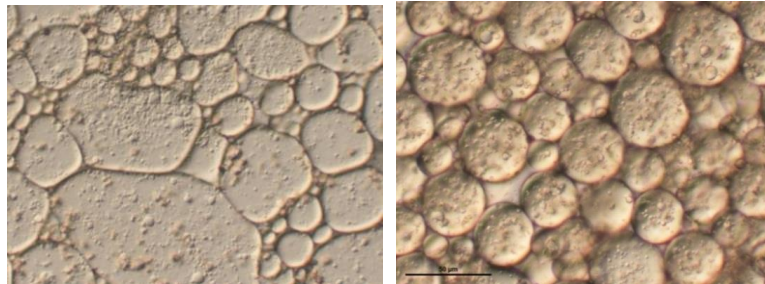


Figure 11 microscope pictures at $t=0$ of o/w emulsions without (left) and with (right) premixing of the oils. Emulsion made with ϕ : 30% sunflower oil, 1% (w/v emulsion) crude oat oil, mixed for 1 minute at 20.000 rpm.

A relative small dispersed-phase volume fraction (ϕ) of 15% was chosen in order to obtain a small cream layer which did not influence the spectrophotometry measurements; the interface between cream and serum had to be at a height of 2 cm or more in a 1.5 ml cuvette to evade the measurement area. The dispersed fraction was chosen to be sunflower oil, i.e. oil-in-water (o/w) emulsions were made, as the polar lipids have a high HLB and are thus suspected to prefer o/w emulsions. Also, a water-in-oil (w/o) emulsion was made with and without PL40 oil and the emulsion with PL40 oil showed a serum top layer before the control did, indicating that the polar lipids in oat oil prefer the o/w interface, see Figure 12.

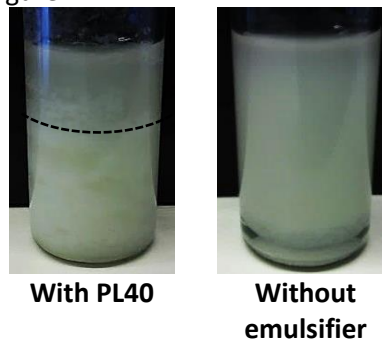


Figure 12 pictures taken after 90 minutes of water-in-oil emulsions, $\phi=15\%$ water, containing 0.5% (w/v emulsion) PL40 oil or no oat oil. Dotted line indicates approximate the interface between serum and cream layer

Emulsifier concentration was lowered from 1 to 0.5% (w/v emulsion) to create a relative unstable system of which the destabilisation rate could be measured in a time span of one hour. For the same reason the mixing speed of 10.000 rpm and mixing time of 30 seconds were chosen, as this combination showed visible destabilisation for emulsions without emulsifier within one hour, see appendix.

The duration of the contact between the three components of the emulsion before mixing turned out to be of importance, as will be discussed in the results. Soaking time in the standard method was therefore set to 10 minutes.

Absorbance by emulsions was measured in 1.5 ml cuvettes by the UV-visible spectrophotometer UV-1650 PC from Shimadzu and displayed by the program UVProbe v2.01. The kinetics mode was used to record absorbance every 10 seconds over the course of one hour at a wavelength of 517 nm. This wavelength was chosen by measuring the absorbance spectrum from 400 to 700nm for multiple samples; at a wavelength of 517nm all samples assessed showed stable absorbance, see appendix. The change in absorbance at 517 nm within an hour was used to evaluate rate of destabilisation. The destabilisation rate is defined as the negative slope of the natural logarithm of the absorbance over the time range 20-60 minutes. The first 20 minutes were omitted as the exact slope depended on the exact timing of the start of the measurement after mixing and the natural logarithm was not straight, resulting in large R^2 values. See for an example Figure 13.

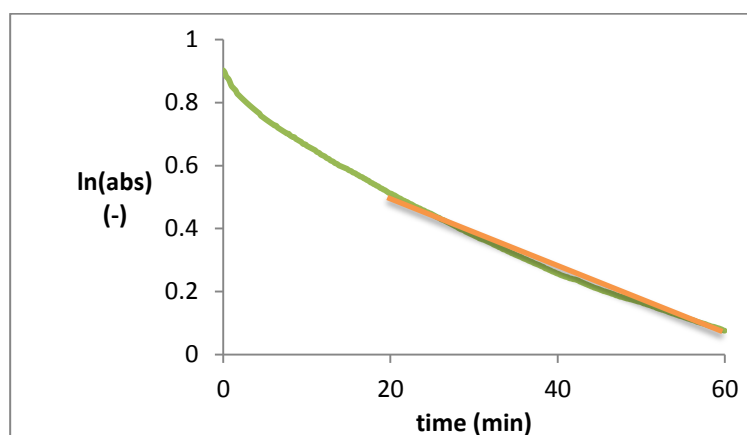


Figure 13 determination of destabilisation rate, which is defined as the negative slope of the natural logarithm of the absorbance over the time range 20-60 minutes

Microscope pictures were taken with a Nikon Optiphot-2 equipped with a DS Camera control unit DS-U2, comprising a DS-2Mv camera head (colour, 2.11 MP) and the imaging software Elements.

Enzymatic hydrolysis

Crude oat oil and PL40 oil were hydrolysed by a lipase from *Rhizopus oryzae* with an activity of ≥ 30 U/mg, obtained from Sigma Aldrich. The enzyme was added to the water to achieve a final enzyme loading ranging from 0.25 to 2 % (w/w oil). The enzyme containing water was subsequently mixed through approximately 2.5g oil. pH was adjusted to pH 7 for optimal enzymatic activity. At first, pH was controlled by adding drops of 5M NaOH to the reaction mixture, but this proved impractical, as the base did not mix in properly, see Figure 14. Instead, pH was controlled by using a 0.1 M phosphate buffer of pH 7 to mix the enzyme with before addition to the oil.



Figure 14 PL40 oil mixed with 20% (v/w oil) water and 50 μ L of 5M NaOH. Due to the high viscosity of PL40 oil, the base did not mix homogeneously, leading to a high pH at the top. The green colour is likely caused by phenolic acids present in oat oil, which are known to display a green colour when the pH is high (Doehlert et al. 2009).

Initially, water (i.e. buffer) content was varied from 5 to 3000% (v/w oil). Water content turned out to be deciding on the occurrence of gel-like structures. This formation of these structures was considered undesirable as it made the extraction of lipids more complex and likely shielded some of the lipids for the enzyme. Dilution of the system (to a water content of more than 3000%) did not prevent the gel from forming, instead a relatively low water content of 10% and below turned out to inhibit formation of the gels, see Figure 15. As water is the carrier of the enzyme, the highest possible water content of 10% was used in further experiments. Reaction mixtures were incubated in a shaking water bath of 40°C for 3, 24 or 48 hours.

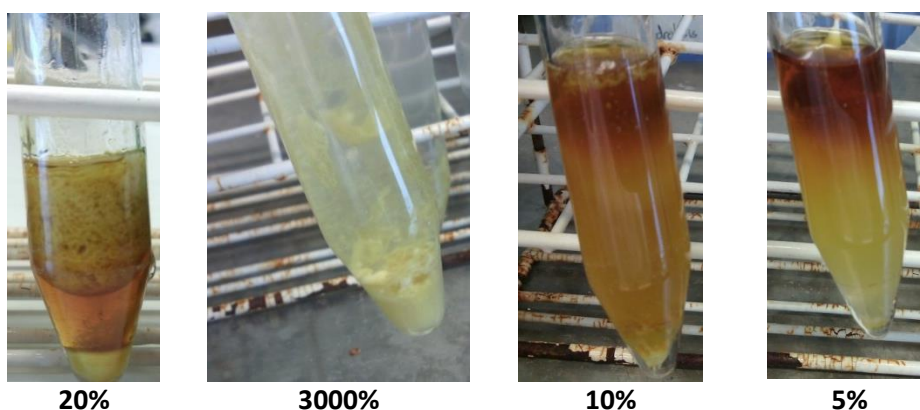


Figure 15 pictures of reaction mixtures containing PL40 oil, with differing water content (% v/w oil), after incubation, addition of chloroform/methanol and centrifugation.

Lipid extraction

After incubation lipids were initially extracted from the reaction mixture by addition of 3 ml chloroform/methanol (2/1), i.e. approximately the same volume as the volume of the reaction mixture. The tubes were subsequently vortexed and centrifuged for 2 minutes at 3000rpm. The top layer of the resulting liquid was transferred to Eppendorf tubes and stored for further analysis. The effect of deviations in this extraction procedure has been studied. Test tubes with 2.5 g of crude oat oil or PL40 oil with or without 0.5% (w/w oil) enzyme were incubated. After 3 hours and 24 hours four samples of 300 μ l were taken per test tube. Each sample was treated differently. The first was treated as before: 300 μ l of chloroform/methanol (2/1) was added. To the second 300 μ l diethyl ether was added instead of chloroform/methanol. The third was only subjected to centrifugation. To the fourth not only 300 μ l chloroform/methanol was added but also 300 μ l Milli-Q water. Success of extraction was determined by taking pictures and performing TLC for general lipid separation on the different layers. The most successful extraction method was used in subsequent hydrolysis experiments.

Statistical analysis

Whenever duplicates were available statistical analysis was done. T-tests (assuming equal variance) and balanced one- and two-factor ANOVAs were performed with the Analysis Toolpak in Excel. Unbalanced one- and two-factor ANOVAs and post-hoc tests were performed in R. All tests were performed with a 95% confidence level ($\alpha=0.05$).

Results & discussion

Unmodified oils

In this section detailed lipid composition and emulsifying properties of the unmodified oat oils are reported. The unmodified oils were separated in fractions by column chromatography and further separated with TLC into classes. Fatty acid profiles were determined for both the oils as is and the different fractions. Emulsification properties were determined for crude oat oil and PL40 oil.

Fractions

The weight of the fractions obtained for crude oat oil and PL40 oil can be seen in Table 8.

Table 8 Weight of fractions (after drying) obtained from column chromatography for crude oat oil and PL40 oil

Flask	Should contain	Weight (mg)	
		Crude oat oil	PL40
1	Neutral lipids	61.0	100.6
2	Glycolipids	54.4	69.0
3	Phospholipids	40.2	61.0
	Total eluted	155.6	230.6
	<i>Initial weight</i>	<i>134.2</i>	<i>133.7</i>

It can be seen that the weight of all three fractions combined exceeds the weight of the lipids loaded on the gel for both crude and PL40 oil. The excess of weight is likely to originate mainly from the neutral lipid fraction as this fraction was most liquid and contained small particles. The neutral lipid fraction of the crude oat oil was dried on the rotary evaporator several times in an attempt to fully dry the lipids. Even after extended evaporation the lipids looked wet and less viscous than the other fractions after drying. They did get dryer by the repeated drying, as is represented by the relatively low weight of this fraction compared to the neutral fraction of PL40, which was less extensively dried.

The glycolipid and phospholipid fraction did not look wet anymore after evaporation on the rotavapor, so the weight of these fractions can be compared. As expected, the separation of the polar lipid rich oil, PL40, resulted in a relatively high amount of both the polar lipid fractions. For both crude and PL40 oil, the glycolipid fraction was larger than the phospholipid fraction. This is as expected; Doehlert and coworkers (2010) and Price & Parsons (1975) reported phospholipid contents of 10% and 10.1% and glycolipid content of 11% and 17.0% respectively. Although exact numbers differ, there seems to be consensus on the superabundance of glycolipids in oat oil.

A general lipid separation was performed on the first fractions obtained and the oils in order to check if the separation method worked. The TLC plate of general lipid separation can be seen in Figure 16. Presumed identity of the spots is as follows: 1 polar lipids, 2-4 diglycerides, 5 triglycerides. From the plate it can be seen that PL40 contains more polar lipids than crude oat oil, as spot 1 is more clearly for PL40 than for the crude oat oil. It can also be seen that the column chromatography successfully fractionated the oat oil into a neutral lipid fraction and a polar lipid rich fraction. The neutral lipid fraction showed no stains at spot 1, not even at a high loading (Cn.6). The glycolipid fraction showed at high loading (Cg.6) only vague stains at spot 2 to 5, indicating that only a small amount of neutral lipids was present. Whether the fractionation is also successful for the separation of glycolipids and phospholipids cannot be seen from this plate.

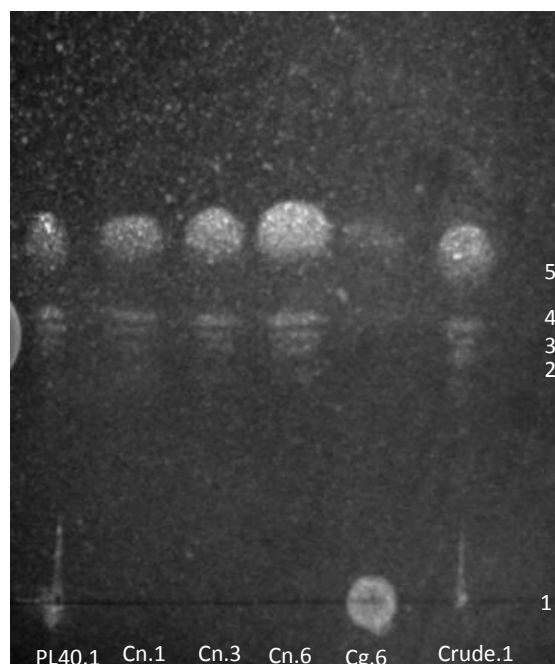


Figure 16 TLC plate of general lipid separation of PL40 oil applied 1 time (PL40.1), neutral lipid (n) fraction from crude oat oil (C) applied 1, 3 and 6 times (Cn.1, Cn.3 and Cn.6), glycolipid fraction (g) from crude oat oil applied 6 times (Cg.6) and crude oat oil applied 1 time (Crude.1)

Classes

The neutral lipid fractions were separated on the TLC plate seen in Figure 17. Clearly the mobile phase applied here was less capable of moving the lipids over the plate than the one in Figure 16, resulting in tightly packed bands which are difficult to analyse. Also, the standards applied did not give small, clear bands as would be ideal. In the future the mobile phase of the general lipid separation should be used for assessing the composition of the neutral lipids and a lower concentration of the standards should be applied.

Presumed identity of the spots is as follows: 1-2 diglycerides, 3 free fatty acids and 4 triglycerides. Spot 1 is also where polar lipids would be expected when present, but as the TLC plate in Figure 16 did not show any polar lipids in the neutral fraction, it is expected that spot 1 shows in this case 1,2 diglycerides.

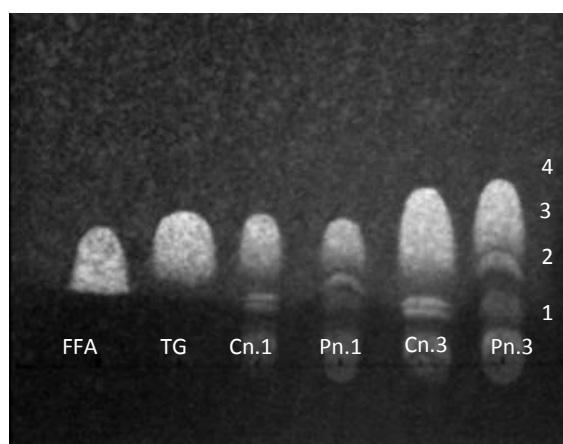


Figure 17 TLC plate of neutral lipid separation of palmitic acid and trilaurin as indicators for free fatty acid (FFA) and triglycerides (TG) respectively and of neutral lipid (n) fraction from crude oat oil (C) and PL40 (P), applied 1 and 3 times (Cn.1, Cn.3, Pn.1, Pn.3)

To investigate the types of glycolipids present in oat oil, a TLC was run with a mobile phase supposedly able to separate glycolipids, of which the result can be seen in Figure 19A. On first sight all samples show only one large spot at the top of the plate. These large streaks could indicate that too much sample has been used, smearing out individual bands. Another TLC was run with the same mobile phase but with 10 times diluted samples, see Figure 19B.

The TLC with diluted samples showed better resolution for the lipids that moved to the top of the gel, spot 5 and 6, showing bands that looked similar to the diglyceride bands in the general lipid TLC, Figure 16. The neutral lipid fraction (Pn.1) also moved completely to the top of the plate, making this identification more plausible. The phospholipid fraction (Pp.1) was separated as well into bands, indicating proper execution of the TLC. Next to this, the brightest stain for the phospholipid fraction, at spot 3, is not seen for any of the other samples. This indicates that the phospholipids were solely eluted during the third flushing of the column, implying that the fractionation process was successful.

According to Price & Parsons (1975) the most common glycolipids in oat, DGDG and MGDG, should show in this TLC just below halfway ($R_f=0.4$) and somewhat below the end ($R_f=0.8$) respectively. When looking very closely, spots can be seen at approximately these locations (named spot 1 and 4 in the figure) for Gc.D.3 and Pg.D.3. This indicates that these glycolipids are present, but more material is needed in order to get clear spots.

It is curious that the glycolipid spots did not show in the first TLC, when non-diluted samples were used. However, when looking more closely to Figure 19A, vague spots can be made out in the bottom part of the plate. By daylight these spots were vaguely red, instead of bright yellow as the other spots. It might be possible that these were glycolipids and that the dye acted differently with them, making it harder to spot them. It is also possible that the TLC was not performed properly in this instance. The mobile phase contained water and chloroform, which do not mix. Methanol is able to solve this problem, but only when the mobile phase is properly mixed. It is thus possible that the mobile phase was not homogenous in the first experiment, which would explain the low resolution and oddly reddish spots. For future TLCs the mobile phase should be prepared by first mixing water with methanol before adding chloroform and stirring the mobile phase in order to obtain a homogenous phase and a proper separation.

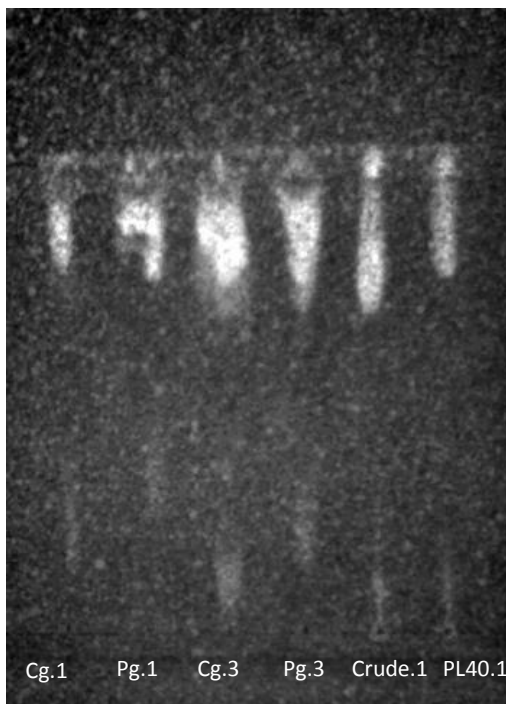


Figure 19A TLC plate of glycolipid separation of glycolipid (g) fraction from crude oat oil (C) and PL40 (P), applied 1 and 3 times (Cg.1, Cg.3, Pg.1 and Pg.3) and crude oat oil and PL40 applied 1 time (Crude.1 and PL40.1)

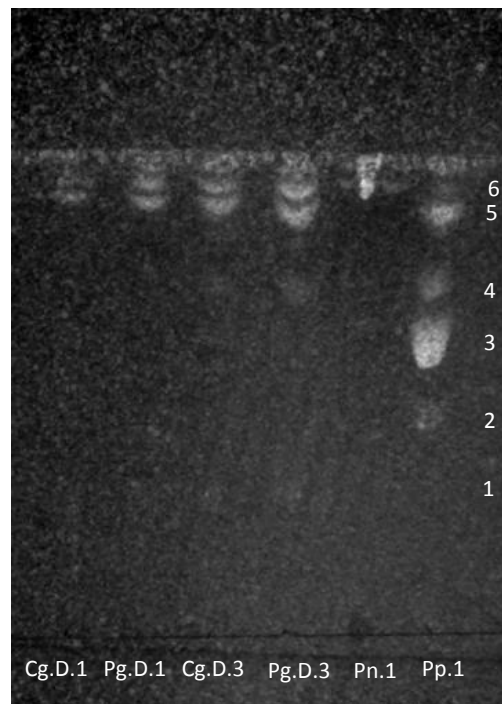


Figure 19B TLC plate of glycolipid separation of 10x diluted (D) glycolipid (g) fraction from crude oat oil (C) and PL40 (P), applied 1 and 3 times (Cg.D.1, Cg.D.3, Pg.D.1, Pg.D.3) and neutral lipid (n) and phospholipid (p) fractions from PL40 applied 1 time (Pn.1 and Pp.1)

The classes of phospholipids present in oat oil were investigated by TLC of the phospholipid fractions, seen in Figure 20. Spot 2 indicates the presence of phosphatidyl choline (PC), as is shown by the standard included. However, according to Price & Parsons (1975) the spot of PC overlaps with the spots of phosphatidyl serine (PS) and phosphatic acid (PA), which could explain the high intensity of the spot. Presumed identities of the other spots are as follows: 1 lyso-PC, 3 phosphatidyl inositol (PI) and 4 phosphatidyl ethanolamine (PE). According to Montealegre and coworkers (2012) PE is the most abundant phospholipid in oat oil, which strengthens the suspicion that spot 2 contains more classes of phospholipids than just PC.

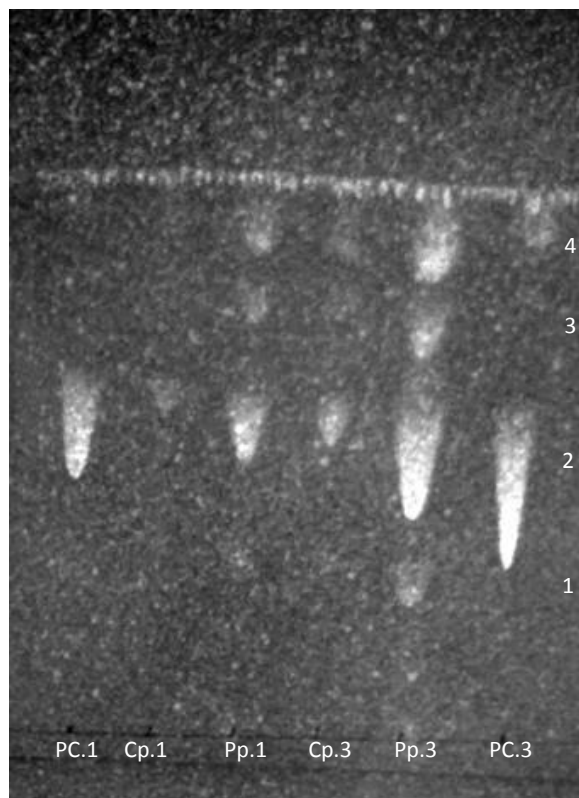


Figure 20 TLC plate of phospholipid separation of phospholipid (p) fraction from crude oat oil (C) and PL40 (P), applied 1 and 3 times (Cp.1, Cp.3, Pp.1, Pp.3) and phosphatidylcholine applied 1 and 3 times (PC.1 and PC.3) as standard

Fatty acid profile

The fatty acid profiles of crude oat oil and PL40 oil are visualized in Figure 21 and can be seen in more detail in Table 9.

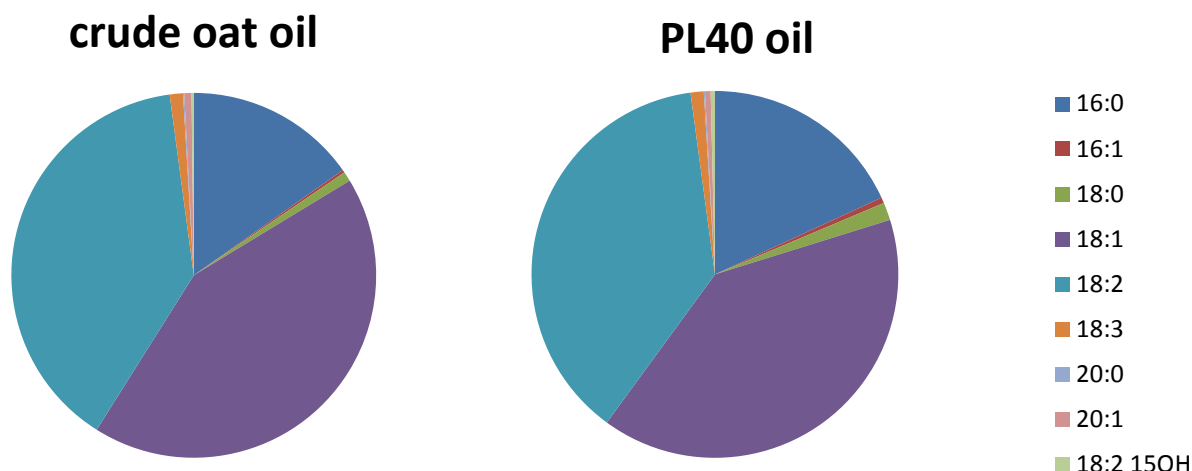


Figure 21 fatty acid profile (expressed in weight %) of crude oat oil and PL40 oil

From the figure it can be seen that the main fatty acids present in both oils are palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2). Minor fatty acids are palmitoleic acid (16:1), stearic acid (18:0), linolenic acid (18:3), arachidic acid (20:0), eicosenoic acid (20:1) and avenoleic acid (18:2 15OH). The abundances of the several fatty acids are compared with literature in Table 9.

Table 9 previously reported and measured (in duplicate) fatty acid profile oat oil. –: not reported. Stars indicate where significant differences exist between measured samples: *: 0.01<p<0.05, **: p<0.01

Fatty acid	Previously reported fatty acid profile			Measured fatty acid profile			Sign.
	Price & Parsons 1975	Leonova et al. 2008	Swedish Oat Fiber n.d.	Crude oat oil		PL40	
	Weight%	Number%	Weight%	Weight%		Weight%	
C16:0	18.0	15.9	16	15.2 ±0.12		18.1 ±0.06	**
C16:1	-	0.2	-	0.2 ±0.13		0.5 ±0.11	
C18:0	1.2	1.4	1	0.9 ±0.41		1.6 ±0.01	
C18:1	36.3	36.9	35	42.6 ±0.43		39.8 ±0.03	*
C18:2	42.0	40.4	44	39.0 ±0.05		37.8 ±0.12	*
C18:3	1.6	1.5	1	1.2 ±0.10		1.2 ±0.01	
C20:0	-	-	-	0.1 ±0.005		0.1 ±0.02	
C20:1	-	0.8	2	0.6 ±0.02		0.5 ±0.03	
C18:2 15OH	-	3.1	-	0.2 ±0.003		0.4 ±0.004	**

The measured amounts are in the same order of magnitude as the numbers found in literature. It was not expected that the exact same numbers would be found, as oat is a natural material and therefore known to vary in its exact characteristics. A curious discrepancy is that the abundance of linoleic acid (18:2) is lower than oleic acid (18:1) in all the samples, while in literature the reverse relationship is seen. Also, the amount of avenoleic acid (18:2 15OH) is lower than expected. Both these differences might be explained by an experiment-induced error. As polyunsaturated fatty acids

are relatively sensitive to oxidation, some might have been destroyed during the GC preparation, resulting in a false low estimate.

The company that provided the oils reported the same fatty acid profiles for crude oat oil as for PL40 oil. However, in this experiment significantly different fatty acid profiles were found, see Table 9.

The fatty acid profile could be affected by the fact that PL40 contains more polar lipids than crude oat oil. The fatty acid profiles of the three different fractions of crude oat oil have been investigated and are visualized in Figure 22 and can be seen in more detail in Table 10.

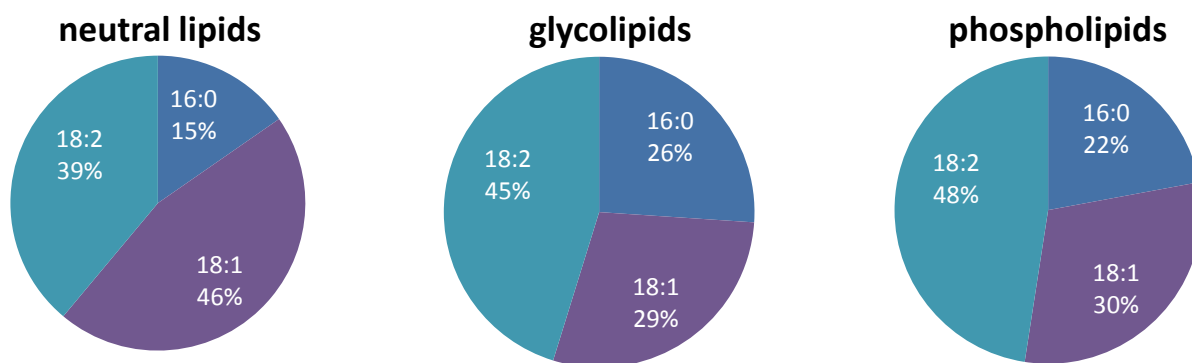


Figure 22 fatty acid profile (expressed in weight %) of all three lipid fractions of crude oat oil

The main differences between the two oils are the abundance of palmitic acid (16:0) and oleic acid (18:1). PL40 oil contains more palmitic acid (16:0), which corresponds to the high amount of this fatty acid present in glycolipids and phospholipids as opposed to the neutral lipid fraction. Crude oat oil is richer in oleic acid (18:1), which can again be explained by the fatty acid profile from the different fractions; neutral lipids have a significantly higher content of this fatty acid compared to the polar lipids.

The detailed fatty acid profiles of the fractions are compared to values found in and derived from literature in Table 10.

Table 10 previously reported, derived from literature and measured (in duplicate) fatty acid profile of fractions from crude oat oil. #: too low signal to quantify. Stars indicate where significant differences exist between measured samples: *: 0.01<p<0.05, **: p<0.01

Fatty acid	Neutral lipids		Polar lipids	Phospholipids		Glycolipids		Sign.
	Derived from literature Number%	Measured Weight%	Previously reported (Leonova et al. 2008) Number%	Previously reported (Montealegre et al. 2012) Weight%	Measured Weight%	Derived from literature Mix number% +weight%	Measured Weight%	
C16:0	14.6	14.8 ±0.23	19.3	24.2	22.1 ±0.28	16.4	25.3 ±1.64	**
C18:0	1.4	1.5 ±0.03	1.3	13.7	#	-6.1	#	
C18:1	43.3	44.0 ±0.19	19.8	18.9	30.4 ±0.07	20.3	27.9 ±0.73	**
C18:2	38.3	37.4 ±0.12	46.0	26.4	47.6 ±0.22	57.6	43.9 ±0.60	**
C18:3	1.3	1.0 ±0.02	2.1	1.7	#	2.3	3.0 ±0.31	*
C20:1	1.0	0.7 ±0.01	0.3	6.8	#	-3.6	#	

The measured and derived fatty acid profile of the neutral lipids are very similar, despite the different ways of expressing abundance. This is as expected as the fatty acids are close to each other in size, resulting in only a small systematic error when comparing the percentage based on amount of fatty acid molecules to the percentage based on the weight. The expected difference between the expressions is that the weight% would be slightly lower than the number% for relative small fatty acids and the reverse for the relatively heavy fatty acids. This is not seen in Table 10. It is likely that natural variation is cause for a large random error, obscuring the small systematic error. The measured and the previously reported fatty acid profiles of the polar lipids are not very similar. It is important to mention here that the measured abundances are relative peak areas. The abundances calculated thus depend highly on the amount of peaks found. As for both the polar lipid fractions the response was low in the GC and not all peaks could be quantified, the abundances displayed are relatively high. This could partly explain the unexpected high numbers found for the unsaturated fatty acids (18:1 and 18:2) in the phospholipid fraction. However, the amount of palmitic acid (16:0) is not higher than expected. It is likely that something else caused the unexpected high amount of unsaturated fatty acids. A possible explanation is that the separation was not perfect; some neutral lipids might have ended up in the phospholipid fraction, as might be concluded from Figure 19B. The neutral lipids contain high amounts of unsaturated fatty acids, and so their presence could higher the abundance of these fatty acids in the phospholipid fraction. The measured and derived fatty acid profiles for glycolipids are also not exactly the same. This is probably caused by the fact that the derived profile is calculated using data from three different sources, which used different raw material and different ways to express fatty acid abundance. However, the general profile is in agreement: the main fatty acids of the glycolipid fraction are linoleic acid, oleic acid and palmitic acid (in that order).

Next to the main fatty acids, the minor fatty acids can also be pinpointed to certain fractions. From Table 10 it is clear that eicosenoic acid (20:1) is mainly found in the neutral lipid fraction. This is also visible in the chromatograms in the appendix, as the corresponding peak ($t=21.4$ min) is only seen in the chromatogram of the neutral lipid fraction. Another interesting minor fatty acid is avenoleic acid. This hydroxy fatty acid has been reported to be specifically bound to galactolipids (Moreau et al. 2008; Doehlert et al. 2010). The hydroxy group allows for an extra fatty acid to bind, forming galactolipid estolides. Moreau and coworkers (2008) estimated that approximately 29% of the total glycolipid fraction is made up out of these estolides. In the current research, estolides are broken down during methylation and the individual fatty acids can be seen in the GC chromatogram ($t=31.5$ min). This peak was not seen for either the neutral lipid or phospholipid fraction, but is visible for the glycolipid fraction, see appendix. This confirms the specific placement of avenoleic acid at the glycolipids, as reported in literature.

Emulsification properties

The destabilisation rates of emulsions made with no oil (control), crude oat oil or PL40 oil are displayed in Figure 23.

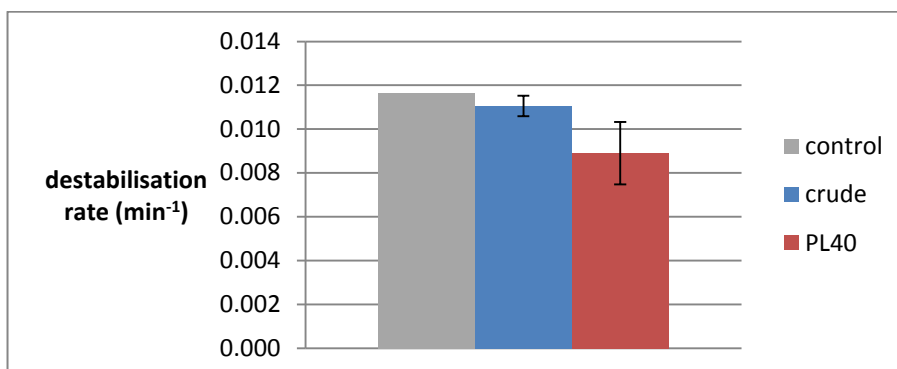


Figure 23 destabilisation rates for emulsions using no (control), crude or PL40 oil as emulsifier

No significant difference was found ($p=0.25$), but the results do indicate that PL40 oil creates the most stable emulsions. Microscope pictures of the emulsions after one hour, seen in Figure 24, indicate the same. The cream of the emulsion stabilized by the polar rich oil (PL40) consists of droplets of varying sizes, but droplets nonetheless, while the cream of the emulsions stabilized by no or crude oat oil show large oily phases which do not resemble droplets anymore. This indicates that in these emulsions coalescence has occurred at a relative high rate.



Figure 24 microscope pictures after one hour of cream of emulsions made with no (control), crude, or PL40 oil

Effect of soaking time

During the development of the emulsification procedure it was noticed that the interval between assembly of the ingredients in the bottle and mixing affected the destabilisation rate. This was unexpected and therefore explored further. The destabilisation rate for emulsions made with no (control), crude or PL40 oil was measured for different soaking times: short (less than 1 minute), 10 minutes (standard procedure) and long (approximately 70 minutes). The results can be seen in Figure 25.

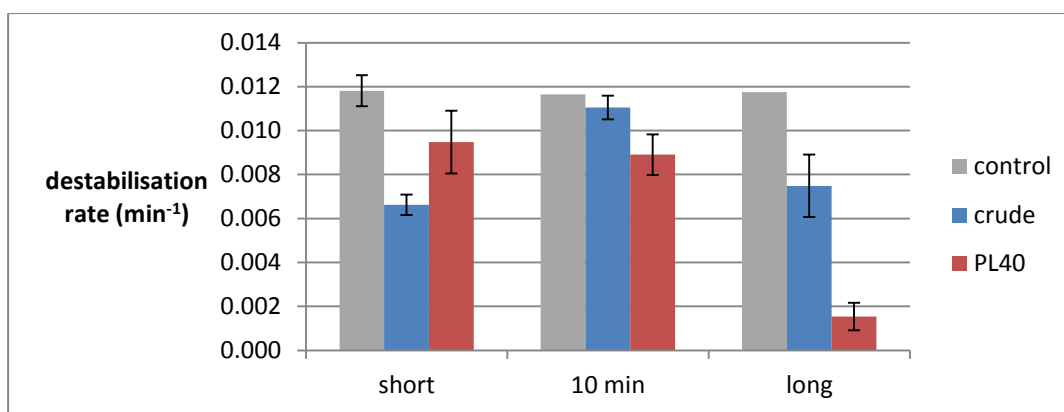


Figure 25 destabilisation rate for emulsions made with no (control), crude or PL40 oil and with different soaking times before mixing

Results from the statistical analysis can be seen in the appendix. The analysis showed that both time ($p=0.0006$) and emulsifier ($p=0.0002$) affected destabilisation rate. The control proved significantly different from both the crude oat oil ($p=0.042$) and PL40 ($p=0.011$). Another significant difference was found between long and 10 minutes of soaking ($p=0.035$). It is clear from both the analysis and the graph that the stability of emulsions without an emulsifier (control) was not affected by the contact time between oil and water before mixing ($p=1.00$). Emulsions stabilized by crude oat oil did show soaking time dependence; crude oat oil soaked for short time and 10 minutes gave significantly different destabilisation rates ($p=0.046$). Curiously, the long soaked crude oat did not follow in this trend of destabilisation, but shows a rate in between the two shorter soaking times. It is not clear why this is. The emulsions stabilized by PL40 show a decrease in destabilisation rate with an increase in soaking time; the long soaked PL40 oil gave a destabilisation rate significantly different from all the other samples, including PL40 soaked for 10 minutes ($p=0.003$) and for a short time ($p=0.0007$). This sharp increase in stability can be attributed to the formation of liquid crystals.

Liquid crystals

During the method development it was noticed that white gel-like structures would sometimes form when handling PL40 oil. The formation of this substance is shown in Figure 26. It is known that when polar lipids come in contact with oil and water, association structure can be formed called lyotropic liquid crystals. Due to weak intermolecular forces the polar heads align themselves in a crystalline fashion while the fatty acids remain flexible. It has been reported that liquid crystals can increase emulsion stability by forming a protective layer around the droplets (Larsson 1997). As is clear from Figure 26, it is only after 70 minutes that the structure has formed considerably, which is also the soaking time at which a considerable decrease in destabilisation rate has been seen.

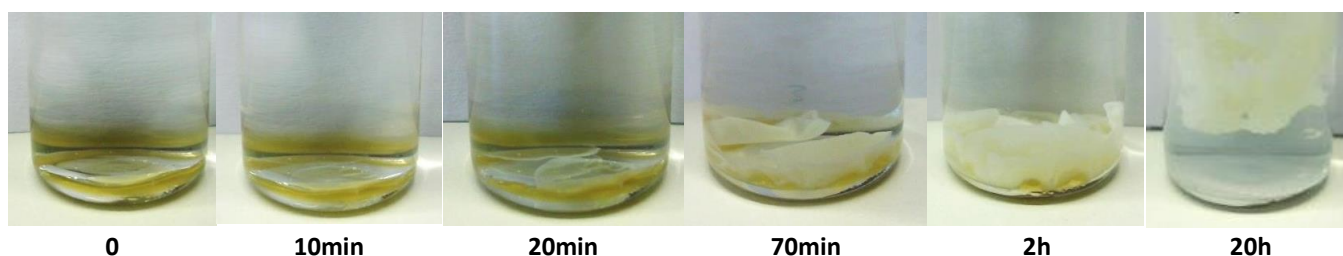


Figure 26 formation of white gel-like structure upon addition of water to polar rich oat oil (PL40)

Several types of liquid crystals exist, characterised by the repetition in the structure. If there is only repetition in one dimension, the liquid crystal is lamellar as seen in cell membranes. Repetition in two dimensions forms a hexagonal structure and three dimensions a cubic one (Larsson 1997). Which type of liquid crystal is formed depends, among other things, on the molecular geometry of

polar lipid present, see Figure 27. Polar lipids like monogalactosyldiacylglycerol (MGDG) and phosphatidylethanolamine (PE) that have a small headgroup with long and unsaturated fatty acids are prone to form hexagonal phases with their head groups pointing inwards (HII phase). Polar lipids like digalactosyldiacylglycerol (DGDG) and phosphatidylcholine (PC, or lecithin), that have a more even ratio of headgroup versus tail, are prone to form a bilayer (lamellar phase). Polar lipids with only one fatty acid (lysolipids) are prone to form hexagonal phases with their headgroup sticking outwards (HI phase) (Jouhet 2013).

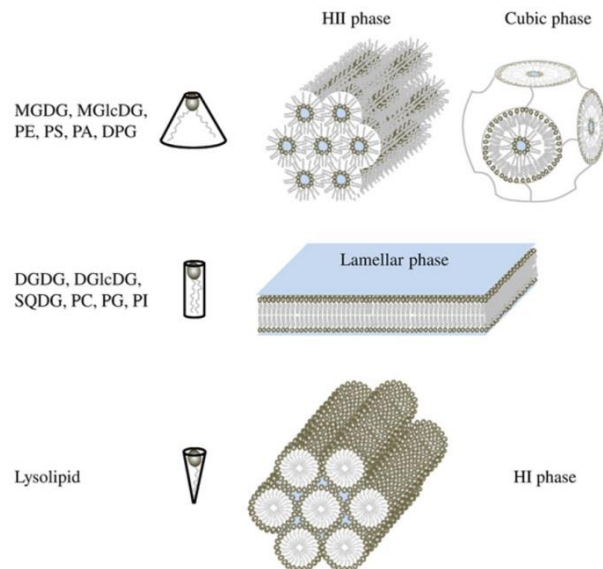


Figure 27 different types of liquid crystals and polar lipids favouring the structures (Jouhet 2013)

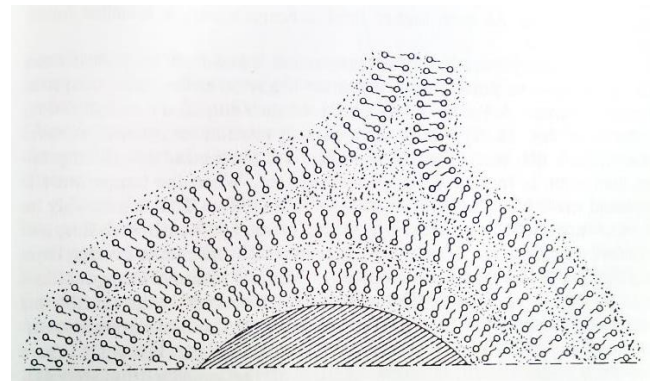


Figure 28 structure of a fragment of a particle of the lamellar liquid crystalline phase (a liposome) dispersed in water (Larsson 1997)

Oat oil contains a mixture of different polar lipids, meaning that it cannot be readily deduced which liquid crystal phase is formed. In the microscope pictures, Figure 29, it can be seen that the structure is thoroughly linked together and does not show any clear regularity. If hexagonal phases would be formed, worm like structures are expected. From these pictures a cubic phase could be suspected. However, the cubic phase is the most complex phase and transition to this phase is often facilitated by addition of other substances. It is therefore more likely that a lamellar phase is formed, considering as well the abundance of lamellar forming polar lipids, like DGDG and PC. It has been reported that when water is added to a lamellar liquid crystal to above its swelling limit, liposomes are formed, see Figure 28. The bilayers are stacked on top of each other with water layers in between and form normally a spherical particle, so to leave no loose ends. However, cylindrical threads can form, which can link several liposomes together. This structure appears as a gel-like homogeneous phase (Larsson 1997). This description fits very well with the white structure observed in this research.

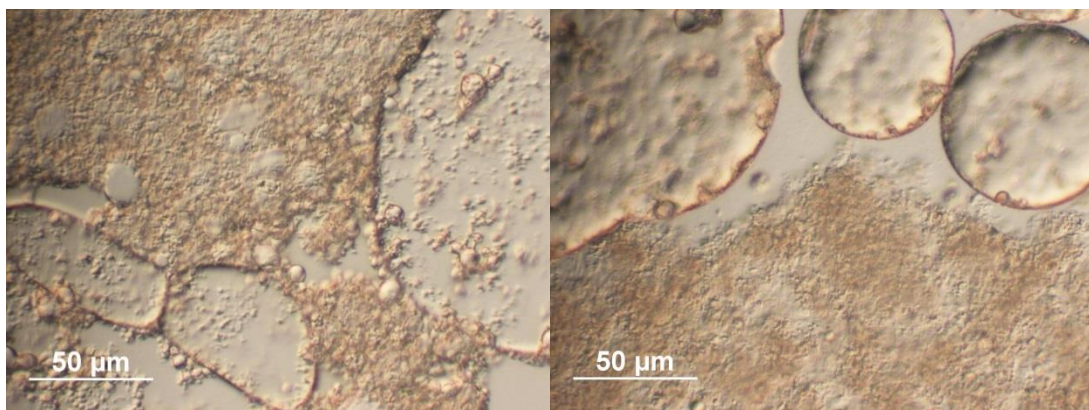


Figure 29 microscope pictures of white gel-like structure formed when PL40 oil is soaked in water

Hydrolysed oils, extracted with chloroform/methanol

The lipids obtained after short and long incubation with and without lipase, extracted by treating the reaction mixtures with chloroform and methanol were investigated for attached fatty acid content and emulsion stabilizing capabilities.

Short incubation

After 3 hours of incubation and extraction with chloroform and methanol, a viscous brown top layer was obtained from the PL40 sample. The enzyme containing crude oat oil sample however did not show a top layer after addition of chloroform/methanol and centrifugation and was therefore not analysed. The concentration of fatty acids attached to a glycerol backbone before and after incubation can be seen in Figure 30.

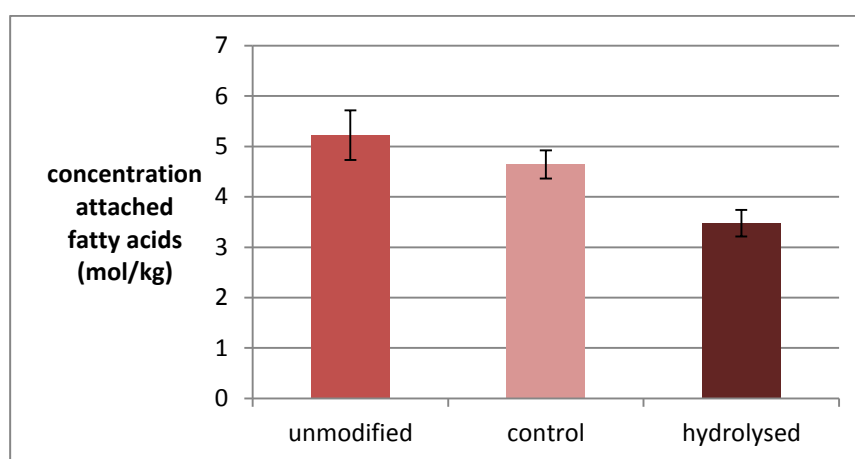


Figure 30 concentration of attached fatty acids in PL40 oil before (unmodified) and after incubation with (hydrolysed) and without (control) enzyme

Although the differences are not significant ($p=0.088$), the results do indicate that the enzyme hydrolysed some of the lipids. It also seems that the procedure itself is responsible for a lower concentration. This could be due to spontaneous hydrolysis of the lipids in the presence of water and a high temperature. However, it is also possible that the oil measured still contained some chloroform or methanol, diluting the sample.

The destabilisation rate of emulsions made with the different oils can be seen in Figure 31.

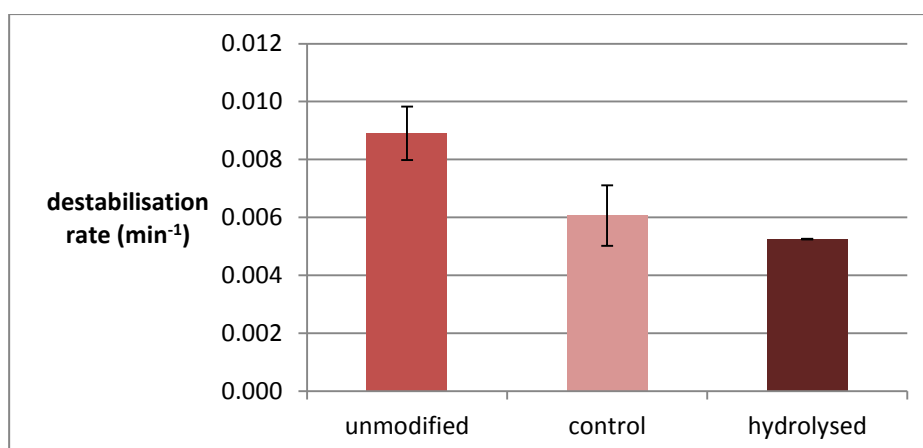


Figure 31 destabilisation rate of emulsions stabilised with PL40 oil before (unmodified) and after 3 hours incubation with (hydrolysed) and without (control) enzyme

No significant difference is found ($p=0.095$), but it does seem that where attached fatty acid content decreased the destabilisation rate decreases and the stability increases. The microscope pictures, seen in Figure 32, indicate the same. The incubated oils create emulsions with foams less packed

than the unmodified oil, indicating a higher stability against gravitational separation. A small difference between the control and hydrolysed oil is implied by the pictures of the serum layers; the serum layer of the emulsion stabilised by the hydrolysed oil contains more small droplets than the one stabilised by the control oil. This indicates that the hydrolysed oil creates the most stable emulsions.

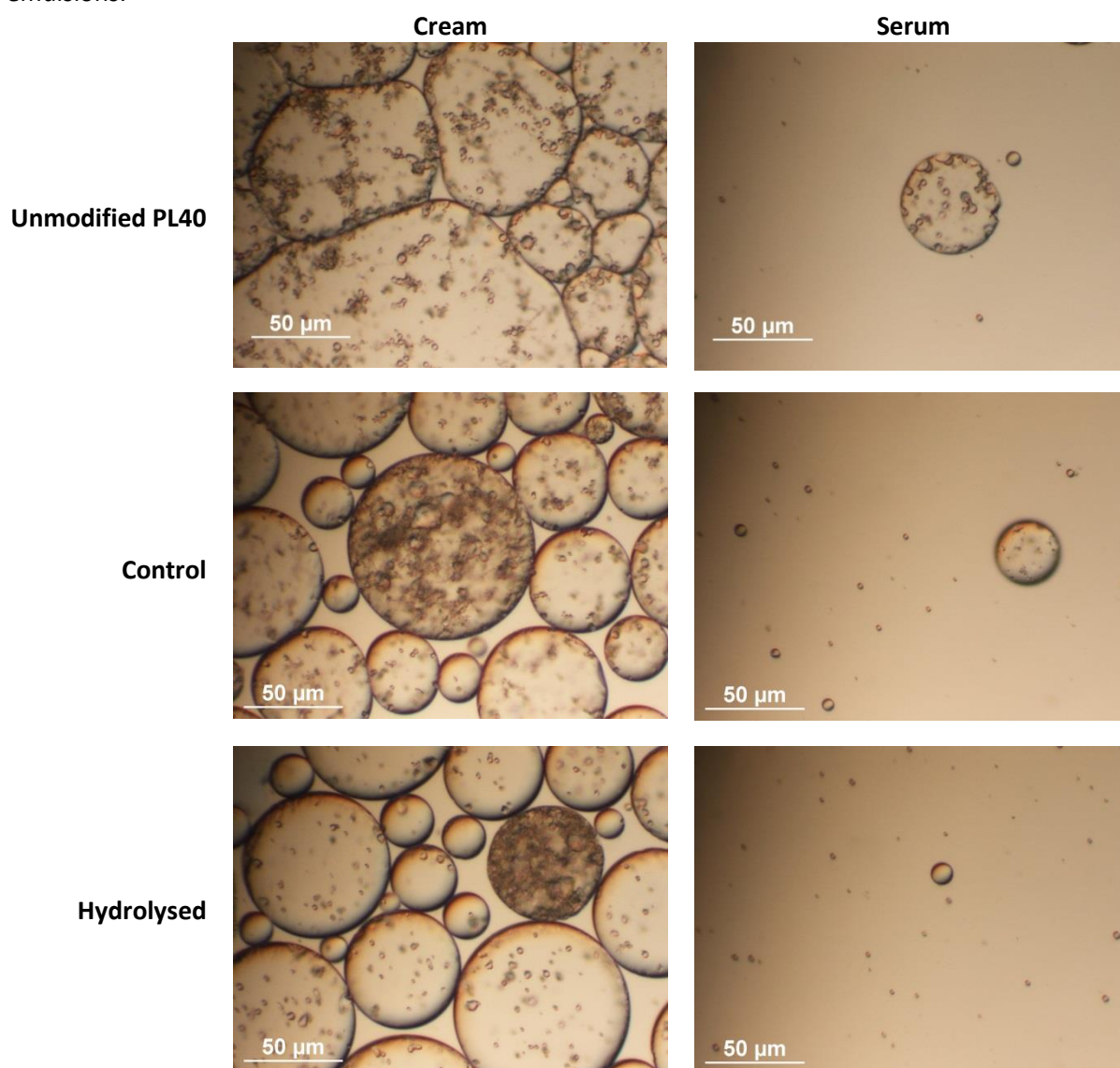


Figure 32 microscope pictures after one hour of cream and serum layer of emulsions made with unmodified PL40 oil, and incubated PL40 oil with (hydrolysed) and without (control) enzyme

The increased stability of emulsions containing hydrolysed oil is as expected. Due to the removal of fatty acids, neutral lipids become slightly amphiphilic. Because of their two-sided character the lipids will go to the interface between oil and water and lower the interfacial tension, just as the polar lipids already present. The increased amount of lipids able to stabilise the emulsion means that smaller droplets can be formed during mixing, which are more stable against gravitational separation (creaming). Furthermore, the polar lipids are affected by the hydrolysis; their HLB value increases, i.e. they become more polar. Because of this, the o/w droplets surface curvature will become more favourable. At the same time the curvature of a hole between two droplets, needed for coalescence, becomes less favourable, in this way stabilising the emulsion against coalescence (McClements 2016).

Long incubation

After one and two days of incubation, the crude oat oil samples did show a brown top layer that could be further analysed. For the PL40 samples a top layer also appeared, but this small layer was transparent and likely consisted of water, see Figure 33. Therefore this layer was not analysed.

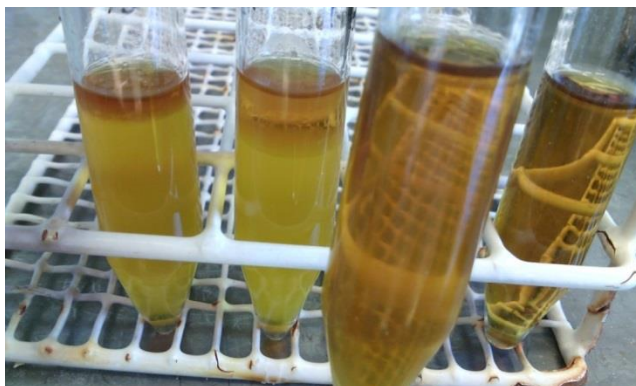


Figure 33 oat oils incubated for one day with and without enzyme, after mixing with chloroform/methanol and centrifugation. From left to right: crude oat with enzyme, crude oat oil without enzyme, PL40 oil with enzyme, PL40 oil without enzyme

The concentration of fatty acids still attached to a glycerol backbone before and after incubation can be seen in Figure 34.

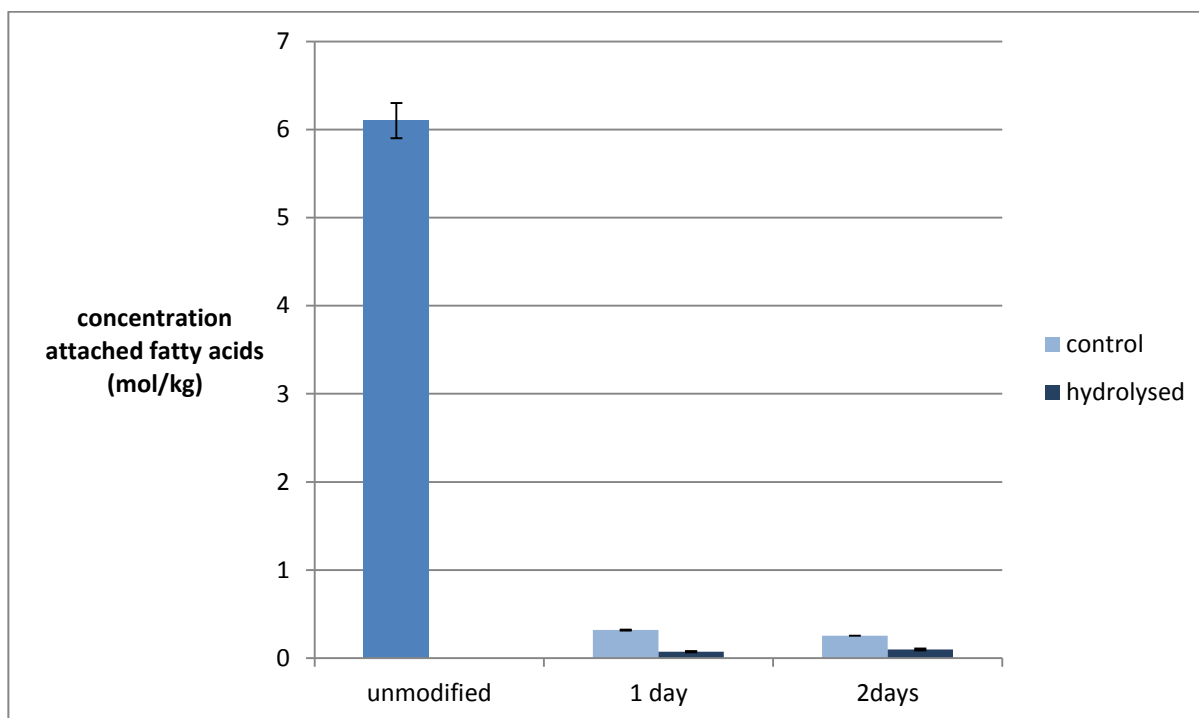


Figure 34 concentration of attached fatty acids in crude oat oil before (unmodified) and after incubation with (hydrolysed) and without (control) enzyme

The loss in attached fatty acids is enormous and unrealistic. The lipase is sn-1,3 specific, and can thus at most hydrolyse 2/3th of the fatty acids. It is likely that the most of the oil was not in the top layer, i.e. that the extraction procedure was inappropriate. In order to find an appropriate method, different methods of lipid extraction were investigated, as is discussed below.

Extraction methods

Different extraction methods are investigated on their ability to separate water from oil. First of all, the method used in the initial experiments of this thesis is investigated. This method was chosen based on previous reports of this method in literature (Christie 2003; Devos et al. 2006) and the fact that these solvents are also used in the separation and storage of lipids. Next, diethyl ether is tested. This organic solvent has been used before to extract (neutral) lipids from reaction mixtures (Pérignon et al. 2012; Cao et al. 2016). However, the usage of a solvent might not be necessary. Polar lipids have been separated from reaction mixtures before by only centrifugation (Li et al. 2015; Hama et al. 2015). Another possibility is to add next to chloroform/methanol also water, as this has been previously used to extract hydrolysed phosphatidylcholine (Hara et al. 1997). Success of extraction is determined by the presence of different layers and partitioning of the oil to one of these layers, which is determined by TLC.

Original method: chloroform/methanol (2/1)

The separate layers after addition of the solvents to the reaction mixture, vortexing and centrifugation can be seen in Figure 35.

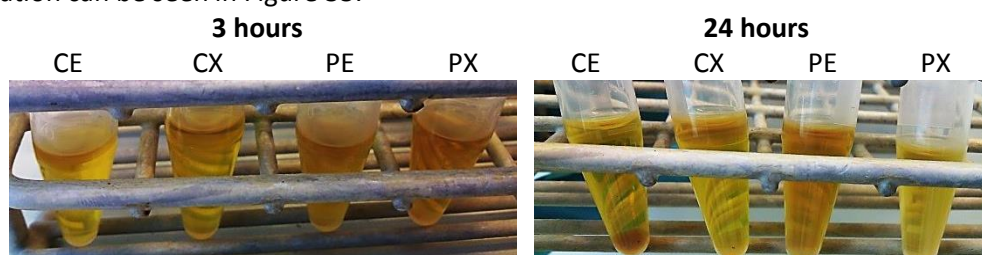


Figure 35 pictures of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme, after addition of chloroform/methanol and centrifugation

No distinct layers were seen for any of the PL40 samples, but the crude oat oil samples do show layers. After 3 hours a brown top layer is seen as was seen in previous experiments. After 24 hours of incubation however, one of the samples shows a brown sediment instead of a top layer. This was not seen before and it is not clear what caused the difference.

TLC was performed on the different layers of the crude oat oil samples, and on the PL40 samples, see Figure 36.

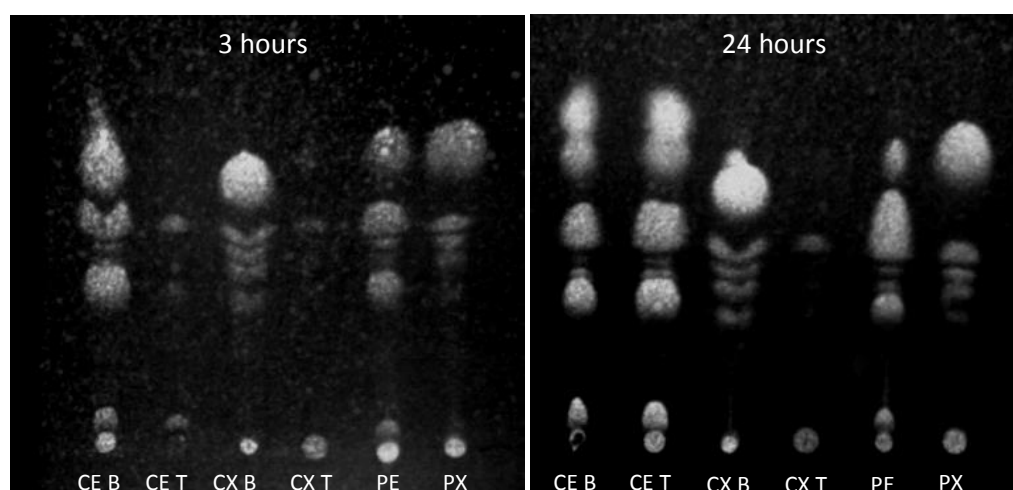


Figure 36 TLC plates of bottom (B) and top (T) layer after addition of chloroform/methanol and centrifugation of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme

As is clear from the TLC plates, the separation is not complete. Both layers contain some oil. Even more striking is that most of the lipids are found at the bottom layer. In the previously used procedure the top layer was taken for further analysis, assuming this the lipid rich fraction. In reality it is thus the opposite. Also considering that the PL40 sample did not show any separation, it can be concluded that this extraction method is far from ideal.

Diethyl ether

The separate layers after addition of diethyl ether to the reaction mixture, vortexing and centrifugation can be seen in Figure 37.

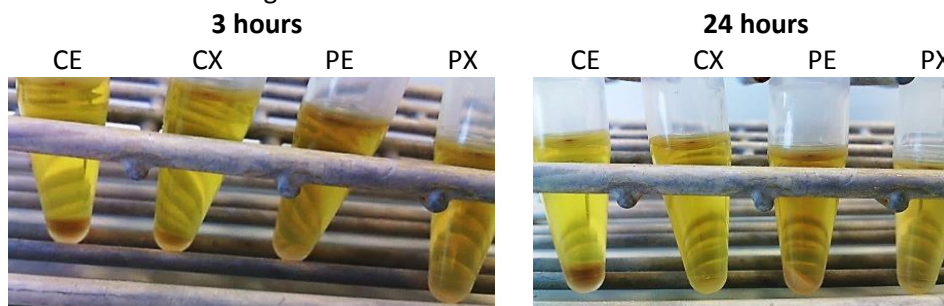


Figure 37 pictures of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme, after addition of diethyl ether and centrifugation

A separation is only seen for the crude oat oil samples after 3 hours of incubation and for the enzyme containing samples after 24 hours. The bottom layer of CE could be taken for further investigation, but for CX after 3 hours and PE after 24 hours the bottom layer was solid-like and it was difficult to take a sample without taking any liquid from the top layer.

The TLC plates of the samples and their different layers when relevant can be seen in Figure 38. Standards were included for triglycerides, free fatty acids and polar lipids.

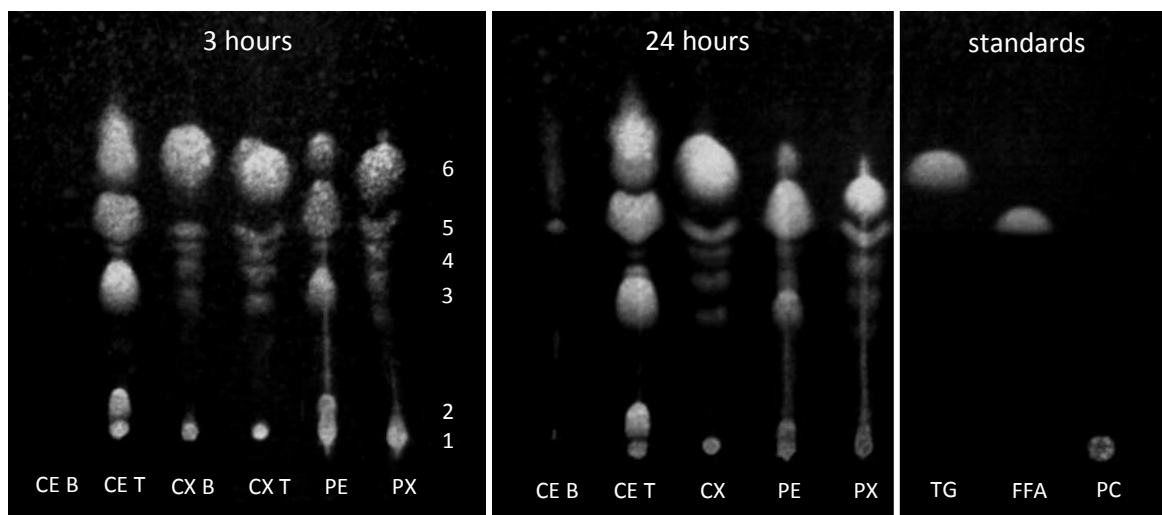


Figure 38 TLC plates of bottom (B) and top (T) layer after extraction with diethyl ether of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme, and of standards: trilaurin for triglycerides (TG), palmitic acid for free fatty acids (FFA) and phosphatidylcholine for polar lipids (PC)

The bottom layer of CE does not contain any oil. It is therefore likely that the the brown bottom layer is mostly water. However, the sucesfull separation only occurred in some samples while the other samples showed no separation at all. This extraction method is therefore also not optimal. Next to this, it can be seen from the TLC plates that hydrolysis occurred in the enzyme containing samples. Utilizing both literature (Price & Parsons 1975) and the included standards, the spots can be identified as follows: 1 polar lipids, 2 monoglycerides, 3 1,2-diglycerides, 4 1,3-diglycerides, 5 free fatty acids, 6 triglycerides. Both the enzyme containing samples (CE and PE) show bigger stains at

spot 2, 3 and 5, indicating that hydrolysis has occurred to such extent that for some lipids both the fatty acid at the sn1 and the sn3 position have been removed. No clear difference is seen between the 3 and 24 hours of incubation, indicating that in the first 3 hours most of the hydrolysis occurred.

Centrifugation only

The separate layers after centrifugation of the reaction mixture can be seen in Figure 39.

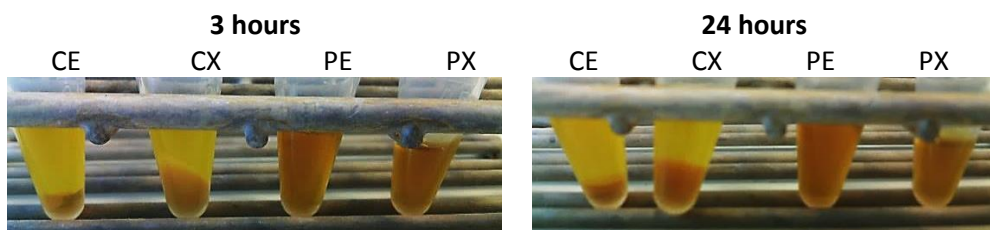


Figure 39 pictures of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme, after centrifugation

A separation is only seen for the crude oat oil samples. PL40 oil probably shows no separation due to the high viscosity of the oil, which prevents the water from traveling any significant distance during the two minutes of centrifugation.

The TLC plates of the samples and their different layers, when relevant, can be seen in Figure 40.

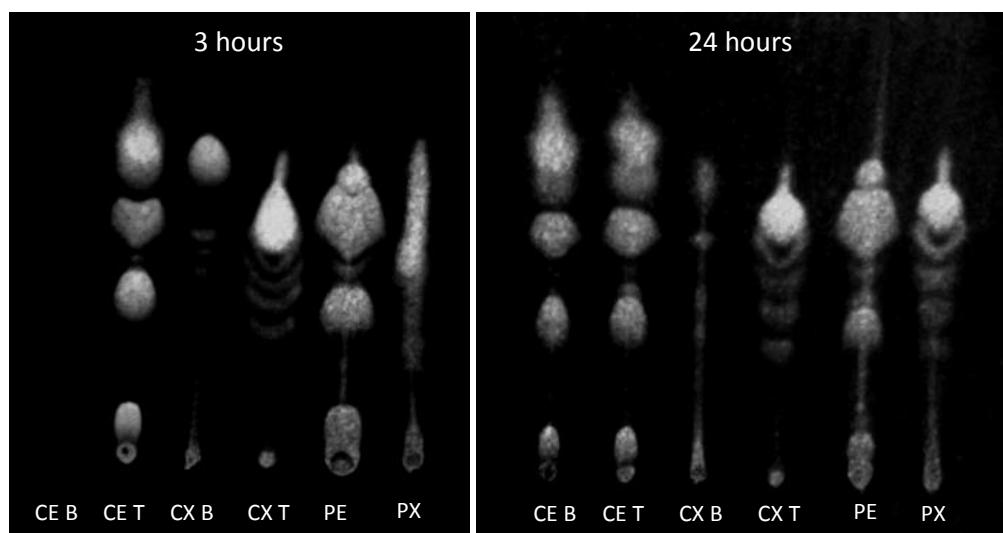


Figure 40 TLC plates of bottom (B) and top (T) layer after centrifugation of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme

The bottom layer of CE after 3 hours is completely empty, indicating that the water went to the bottom, leaving the oil on top and indicating a good separation. Unfortunately, the other bottom layers did show traces of oil. It is however possible that this is caused not by an unsuccessful separation, but by contamination of the bottom layer with the oil rich top layer when taking the sample. As water is denser than oil, it can be assumed that the water is in the bottom layer, making the top layer fit for further experiments. Also considering that the method is simple, cheap and safe as no organic solvents are used, the method is worthy of consideration. However, as PL40 did not show any separation, this extraction method is not appropriate in this instance.

Water/chloroform/methanol (3/2/1)

The separate layers after addition of the solvents to the reaction mixture, vortexing and centrifugation can be seen in Figure 41.

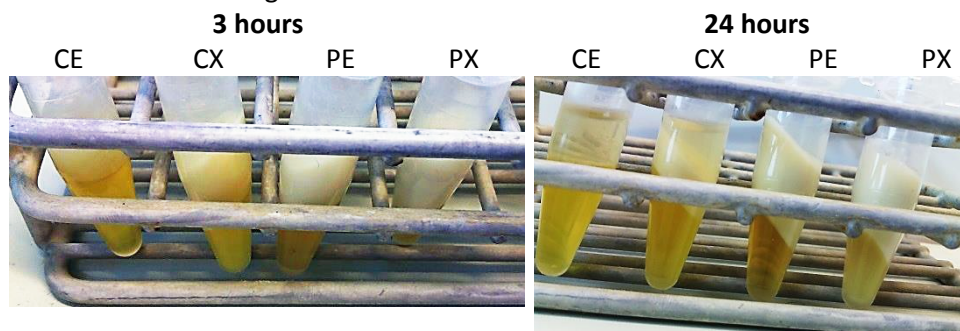


Figure 41 pictures of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme, after addition of water, chloroform and methanol and centrifugation

After 3 hours of incubation the samples showed in most cases three layers: a yellow bottom layer, a white gel-like middle layer and a transparent top layer, see left Eppendorf tube in Figure 42. Only sample CE only showed two layers, omitting the gel-like middle layer. After 24 hours, sample CE still only showed two layers and sample CX still showed three layers. The samples containing PL40 oil however showed two instead of three layers, omitting the transparent top layer. Instead, the white gel-like layer had increased in size, see right Eppendorf tube in Figure 42. It is likely that the white gel-like structures are liquid crystals, which are known to be able to take up water. In the case of the long incubated PL40 oil the lipids were able to take up all the water in the liquid crystal structure, in this way preventing the top transparent layer from forming.

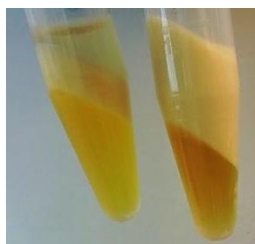


Figure 42 picture showing the different layers of oils mixed with water/chloroform/methanol and centrifuged.

The TLC plates of the samples and their different layers when relevant can be seen in Figure 43. No pure sample could be taken from the gel-like substance, except when it was the top layer (i.e. 24h PE and PX).

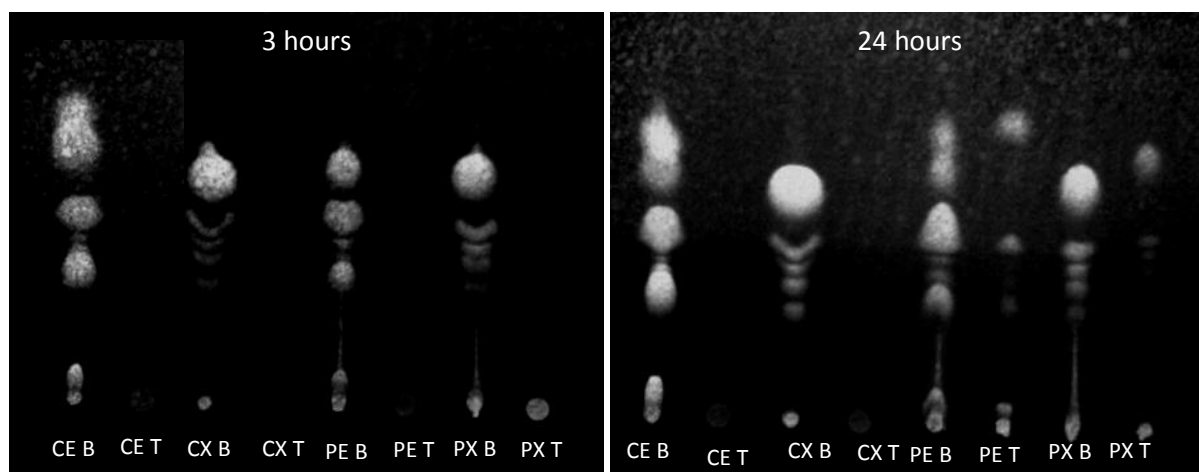


Figure 43 TLC plates of bottom (B) and top (T) layer after addition of water, chloroform and methanol and centrifugation of crude oat oil (C) and PL40 oil (P) incubated for 3 and 24 hours with (E) and without (X) enzyme

As is clear from the TLC plates, the transparent top layers hardly contain lipids. Only in sample PX some polar lipids are found in the transparent top layer. The two top layers of sample PX and PE after 24 hours correspondent to the white substance; the TLC shows that some lipids are found in this structure, namely some free fatty acids and triglycerides but mainly polar lipids. This strengthens the hypothesis that the gel-like structures are liquid crystals.

With this method, water is effectively separated from the oil in the form of a transparent top layer or captured in a liquid crystal. The oil rich bottom layer can therefore be used for further experiments.

Hydrolysed oils, extracted with water/chloroform/methanol

The lipids obtained by treating the reaction mixtures containing crude oat oil or PL40 oil with water, chloroform and methanol are investigated for attached fatty acid content and emulsion stabilizing capabilities.

Crude oat oil

The concentration of fatty acids attached to a glycerol backbone before and after incubation can be seen in Figure 44.

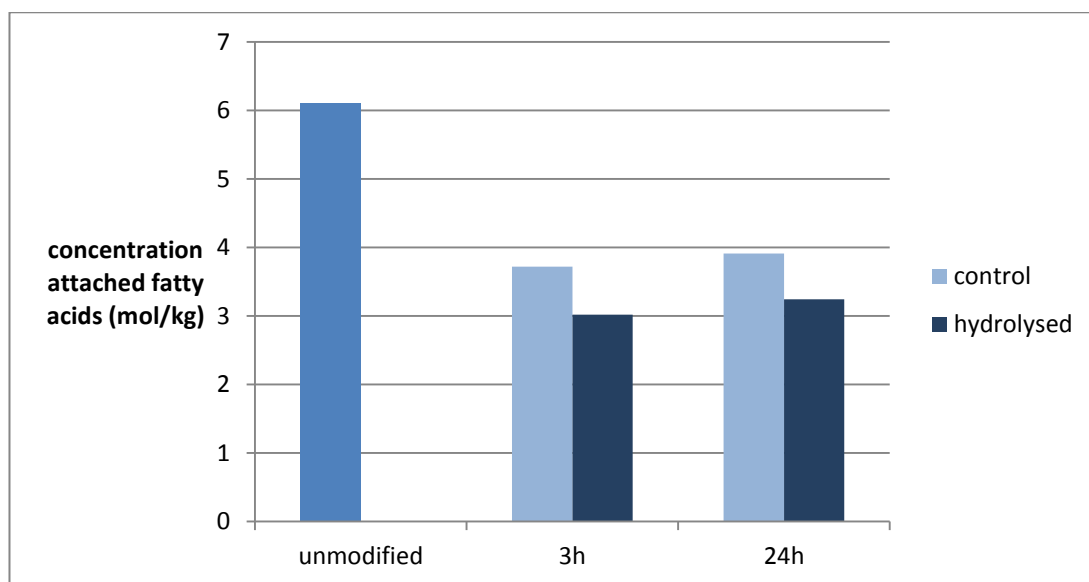


Figure 44 concentration of attached fatty acids in crude oat oil before (unmodified) and after incubation with (hydrolysed) and without (control) enzyme

It can be seen that the enzyme has effectively hydrolysed some lipids, as the attached fatty acid concentration is lower for the hydrolysed than for the control oil. After 3 hours the hydrolysis does not seem to continue as the fatty acid content is approximately the same after 24 hour as after 3 hours. However, it has to be considered that the measurements were only done once, meaning that small differences might not be visible due to mere chance.

The destabilisation rate of emulsions made with the different oils can be seen in Figure 45.

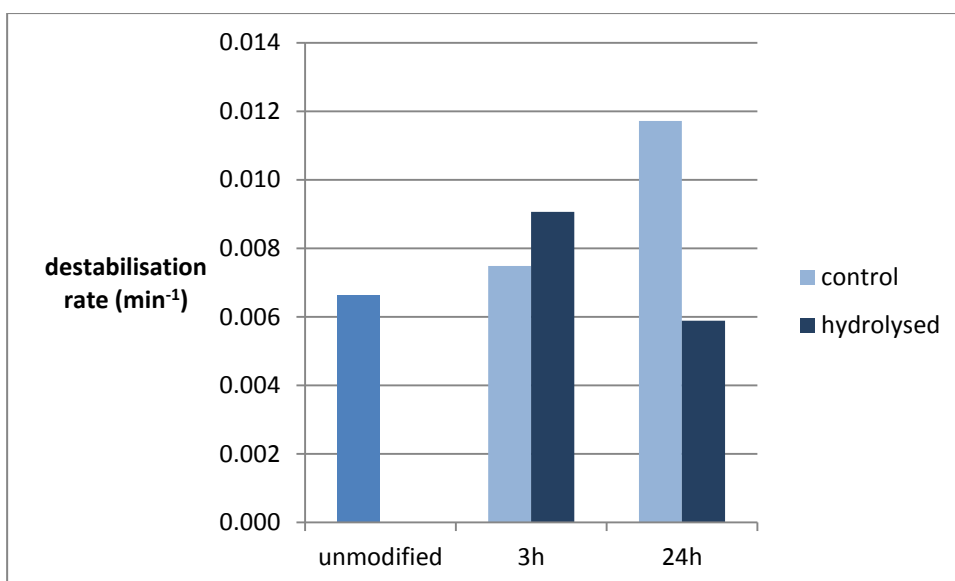


Figure 45 destabilisation rate of emulsions stabilised with crude oat oil before (unmodified) and after 3 hours incubation with (hydrolysed) and without (control) enzyme

Although fatty acid concentration did not visibly change over time, the emulsification properties did. The microscope pictures, seen in Figure 46, confirm the unexpected results. After 3 hours of incubation the emulsions are almost equally stable. After 24 hours a clear difference is seen between the emulsions stabilized by the different oils. The control oil creates unstable emulsions in which droplets coalesce within one hour to such a degree that a large oil phase is formed. The oil hydrolysed for one day is clearly more stable, as small droplets are seen, which are known to be more stable against gravitational separation (creaming). The observation that the hydrolysed oil is better able to stabilize the emulsion than the control was expected, as described before. It is however not clear why this effect was not yet seen after 3 hours of incubation.

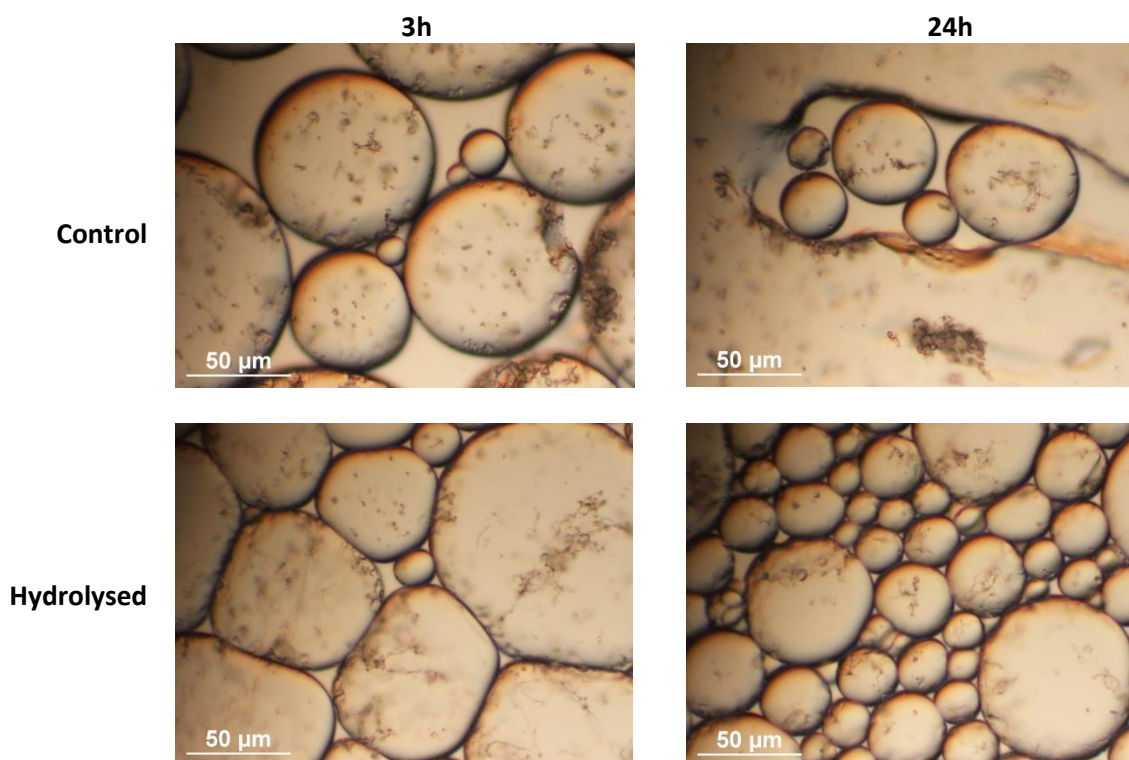


Figure 46 microscope pictures after one hour of cream layer of emulsions made with crude oat oil incubated with (hydrolysed) and without (control) enzyme for 3 and 24 hours

PL40

The concentration of fatty acids attached to a glycerol backbone before and after incubation can be seen in Figure 47.

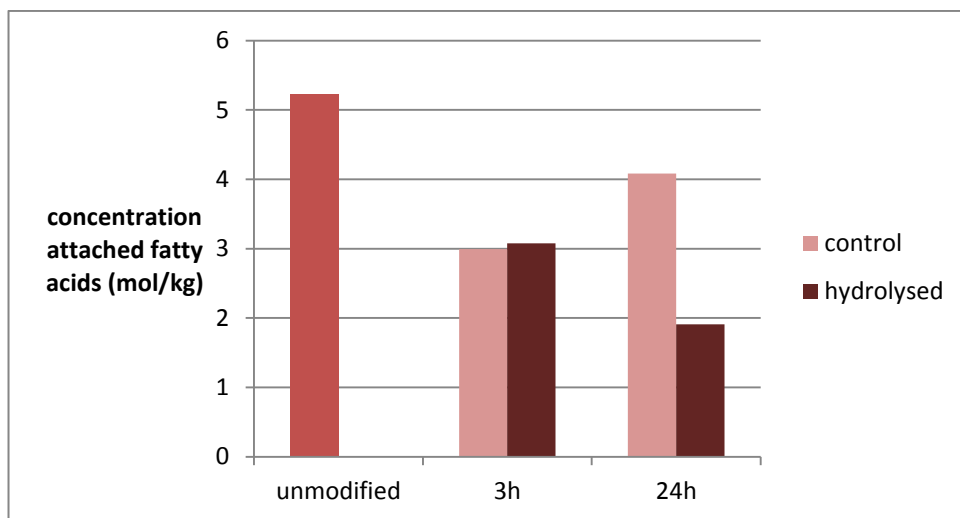


Figure 47 concentration of attached fatty acids in PL40 oil before (unmodified) and after incubation with (hydrolysed) and without (control) enzyme

The graph shows a decrease in attached fatty acids for the hydrolysed oil over time, as expected. However, the control shows the opposite. This is unexpected as the samples were taken from the same reaction mixture, implying that fatty acids were reattached after 3 hours of incubation. It is more likely that the concentration of the control after 3 hours was underestimated, possibly due to an improper drying of the sample before measurement.

The destabilisation rate of emulsions made with the different oils can be seen in Figure 48.

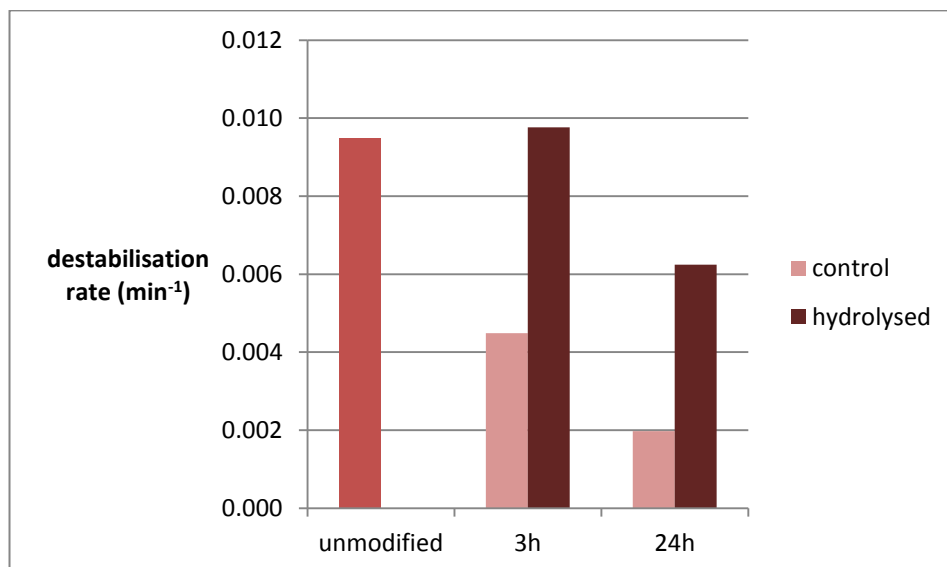


Figure 48 destabilisation rate of emulsions stabilised with PL40 oil before (unmodified) and after 3 hours incubation with (hydrolysed) and without (control) enzyme

A decrease in destabilisation rate is seen for the hydrolysed PL40 oil with increased incubation time, indicating an increased ability to stabilize emulsions when more fatty acids are hydrolysed and the HLB is increased, as was expected. It was however unforeseen that the destabilisation rate is higher for the hydrolysed oil than for the control. The microscope pictures, seen in Figure 49, do not show the same large stability difference as the spectrophotometer measurements; droplets sizes and packing density are approximately the same.

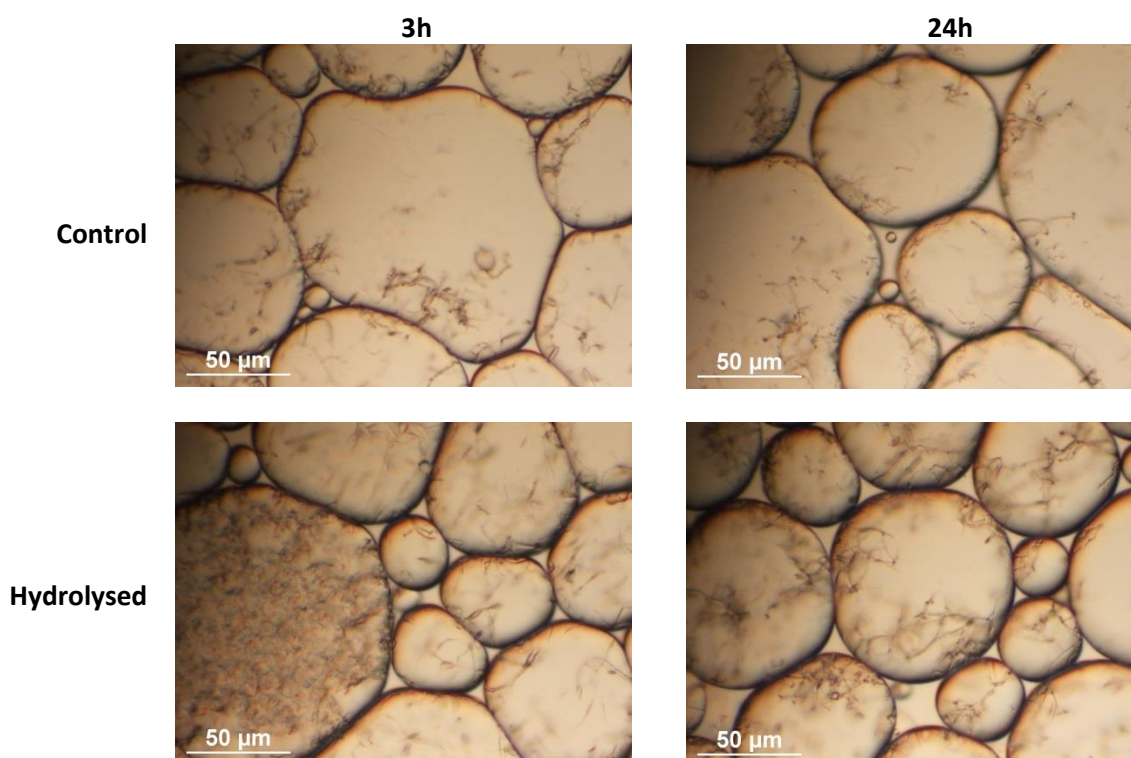


Figure 49 microscope pictures after one hour of cream layer of emulsions made with PL40 oil incubated with (hydrolysed) and without (control) enzyme for 3 and 24 hours

However, it was difficult to take pictures of the emulsion stabilized by the control oil. The droplets would quickly coalesce under the microscope, leading to an overestimation of the droplet sizes. The quick destabilisation under the microscope can be seen in the two pictures in Figure 50, taken only 5 seconds after each other.

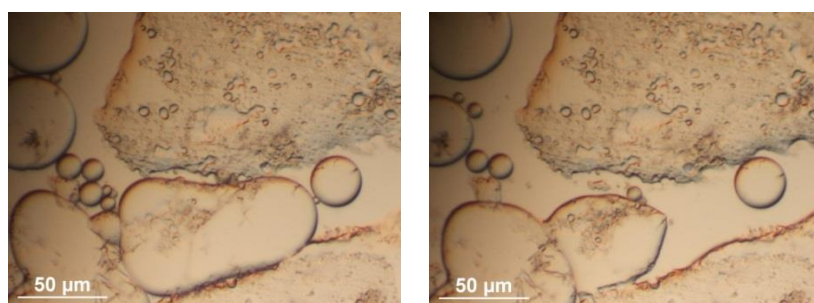


Figure 50 microscope pictures taken after one hour and 5 seconds apart of cream layer of emulsion made with PL40 oil incubated without enzyme for 24 hours.

It can be hypothesised that the type of lipid structure on the interface determines whether this fast coalescence occurs. It can be seen in the microscope pictures in Figure 49, that the droplets of the emulsion stabilised by the hydrolysed oil look rougher than the control. The quick destabilisation of the control under the microscope and its smooth surface imply that the lipids form a thin layer on the interface, which can easily be interrupted under stress. The hydrolysed lipids seem to form more elaborate structures, possibly a form of liquid crystals, leading to the visible roughness and a resistance to coalescence under stress due steric repulsion. However, because of the formation of these structures fewer lipids are available to cover the interface, leading to bigger droplets and a higher creaming rate, as seen in the spectrophotometry data, Figure 48.

If this is true, the larger destabilisation rate of hydrolysed PL40 does not mean that the hydrolysed lipids cannot be useful. Foods are complex systems which can have very different demands regarding an emulsifier. When a food system needs protection against coalescence, it is well possible that the hydrolysed PL40 oil is more useful than the original.

4. Conclusions & outlook

4.1. Rapeseed press cake

The objective was to increase the utilization of rapeseed press cake by increasing the yield of the existing protein extraction procedure and by extracting lipids.

In the original protein extraction procedure proteins of almost all sizes were lost with the rapeseed cell-wall matrix. A pre-treatment aimed to degrade this matrix showed an increase in protein yield of approximately 10%, but duplicates are needed for confirmation. Also, more research is needed to optimize the pre-treatment in order to increase efficiency and prevent microbial growth.

Lipids were not extracted as no separate oil layer was seen during any of the treatments. It is hypothesised that the lipids were hydrolysed during the procedure leading to the bitter taste of the extract and solubilisation of the lipids. No starting material for further extraction and modification of lipids was therefore obtained from rapeseed press cake.

4.2. Oat oil

The objective was to determine the effect of enzymatic modification on the composition and emulsification properties of lipids. As no rapeseed lipids were obtained, only oat lipids were investigated. Both crude oat oil and polar lipid enriched oat oil (PL40) were used.

First, detailed lipid composition and emulsifying properties of the unmodified oat oils were determined. Column chromatography showed that PL40 contained more polar lipids than crude oat oil and in both oils more glycolipids than phospholipids were found. Thin layer chromatography (TLC) showed that the neutral lipid fraction mainly contained di- and triglycerides and the phospholipid fraction contained phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and lyso-PC. Main fatty acids present in both oils were palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2). Minor fatty acids were palmitoleic acid (16:1), stearic acid (18:0) linolenic acid (18:3), arachidic acid (20:0), eicosenoic acid (20:1) and avenoleic acid (18:2 15OH). Differences in fatty acid profiles of the two oils could be explained by the fatty acid profiles of the different lipid fractions. Emulsion stability was significantly increased by the presence of unmodified oils, especially by PL40 oil. Surprisingly, soaking time also significantly affected stability. It is hypothesised that the polar lipids in (PL40) oil form lamellar liquid crystals when in contact with water, which then form a protective layer around the emulsion droplet, causing the observed sharp increase in stability.

Next, the oat oils were incubated with enzyme and hydrolysed lipids were investigated for attached fatty acid content and emulsion stabilizing capabilities. PL40 incubated with enzyme and extracted with chloroform/methanol showed a visible decrease in attached fatty acid concentration and an increase in emulsions stability, which is as expected. However, crude oat oil treated the same showed very low attached fatty acid concentration, implying an unrealistic high level of hydrolysis. This indicated that the extraction method did not work for both oils. Three other extraction methods were tested and addition of water/chloroform/methanol proofed most appropriate as TLC indicated that this method separated the lipids from the water for both crude oat oil and PL40 oil.

Crude oat oil incubated with enzyme and extracted with water/chloroform/methanol showed an increase in stabilizing ability with increasing incubation time, as expected. This could however not be related to a decrease in attached fatty acid concentration, as this remained constant. PL40 oil treated the same did show a decrease in attached fatty acid concentration with increasing incubation time, as well as an increase in stability. Unexpectedly, the PL40 oil incubated without enzyme showed a higher stability than the hydrolysed oil. It is hypothesised that due to the modification the lipids form more elaborate structures, which prevent coalescence but can cover less area than unmodified lipids, leading to bigger droplets and an increased creaming rate. It can thus be concluded that enzymatic modification altered the emulsification properties. Depending on food demands, the hydrolysed oil can be preferred over the original.

In conclusion it can be said that both rapeseed press cake and oat oil can be utilized to a greater extent by aid of enzymes. More of the rapeseed press cake's valuable proteins can be extracted when an enzymatic pre-treatment is added to the protein extraction procedure. The insights in this report can help to produce in the future protein extract with a higher yield as well as without a bitter taste, increasing the feasibility to produce for human consumption. The polar lipids in oat oil can be utilized as emulsifiers of which the properties can be changed by enzymatic hydrolysis. In this thesis tailored methods are reported for enzymatic hydrolysis and assessing change in molecular structure and emulsification properties, which can form a starting point for future research. This could lead eventually to commercialized production of a range of lipid emulsifiers with different properties enzymatically derived from oat oil, increasing the utilization and value of this side-stream from the food industry.

Acknowledgements

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Appendix

Rapeseed press cake

Standard curve DNS assay

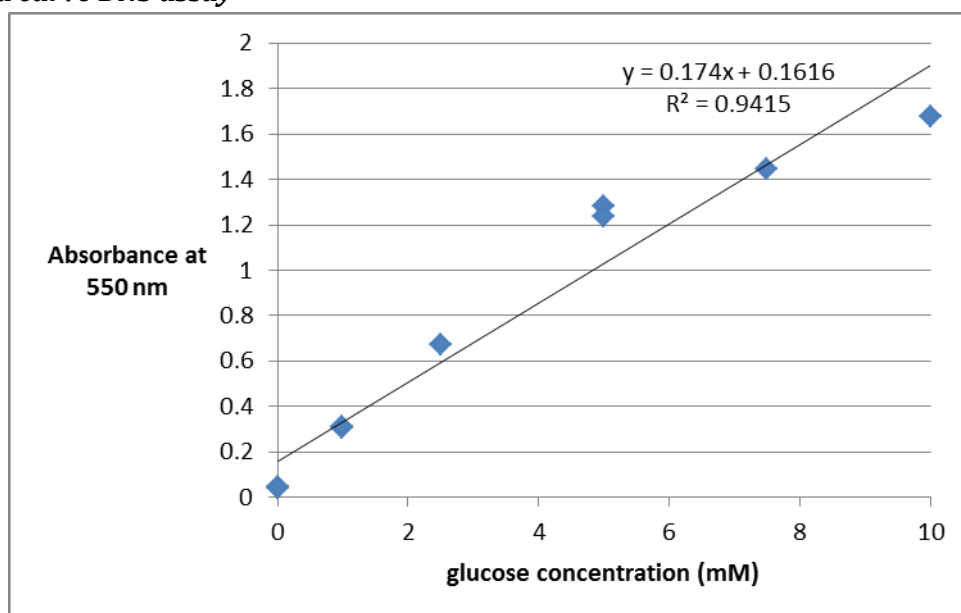


Figure S1 standard curve DNS assay, showing linear trend line with formula and R^2

Oat oil

Mixing parameters

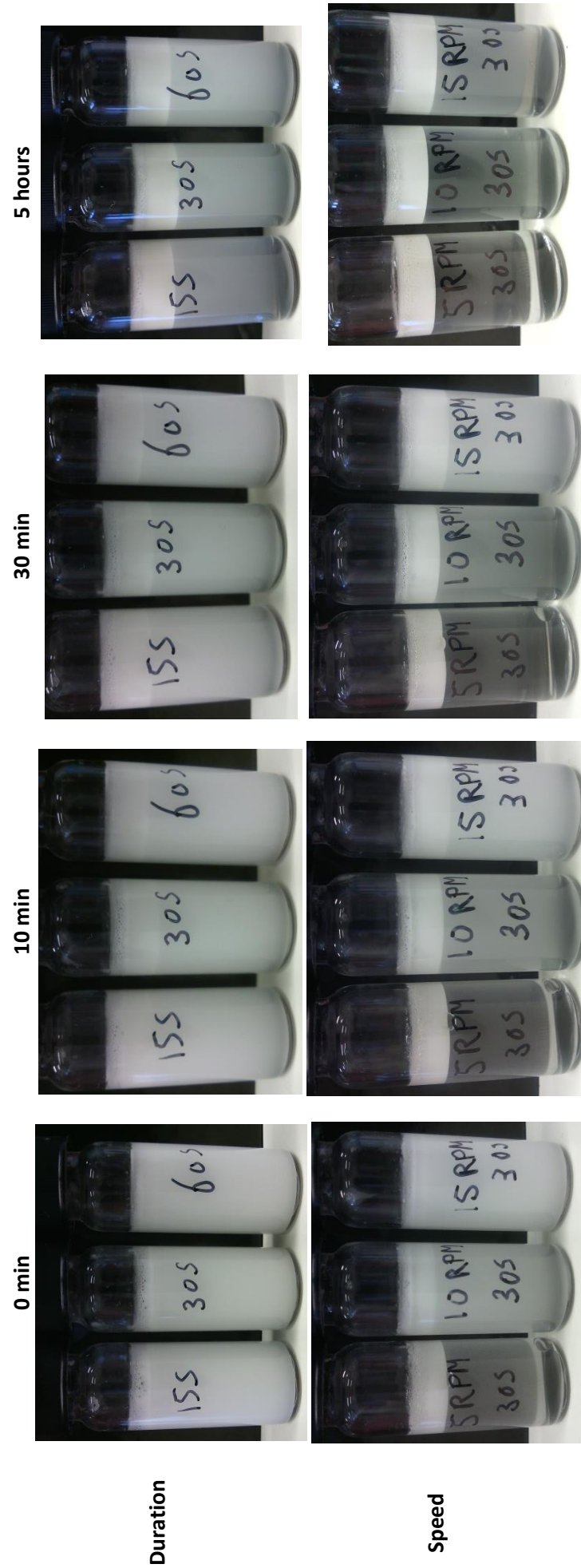


Figure 52 pictures taken at several time intervals of emulsion mixed for different times (15, 30 and 60 s) at 20.000 rpm and at different speeds (5.000, 10.000 and 15.000 rpm) for 30 s

Determining wavelength for spectrophotometry

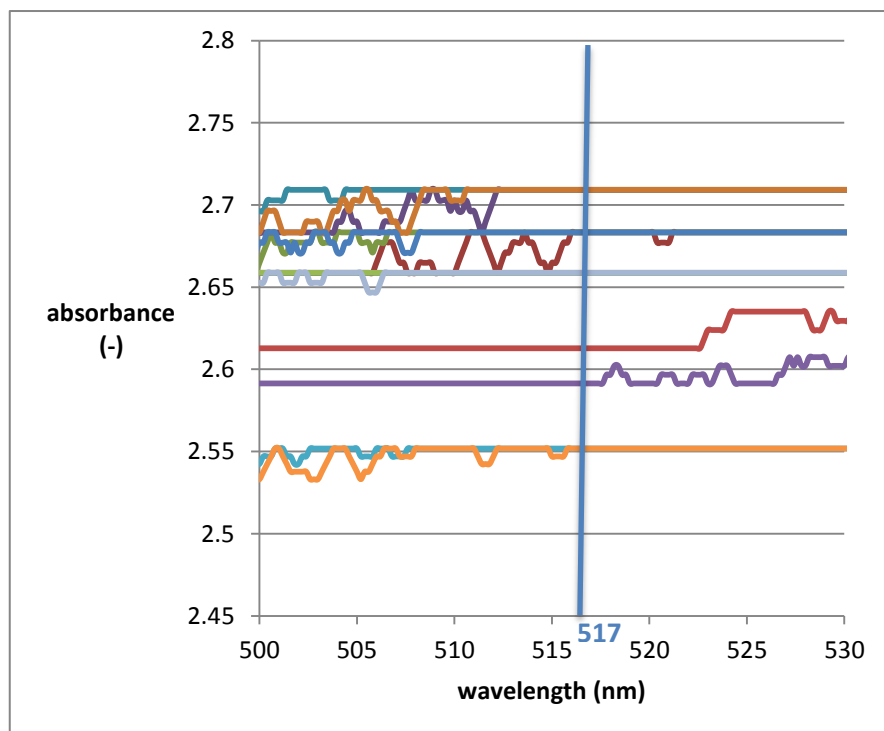
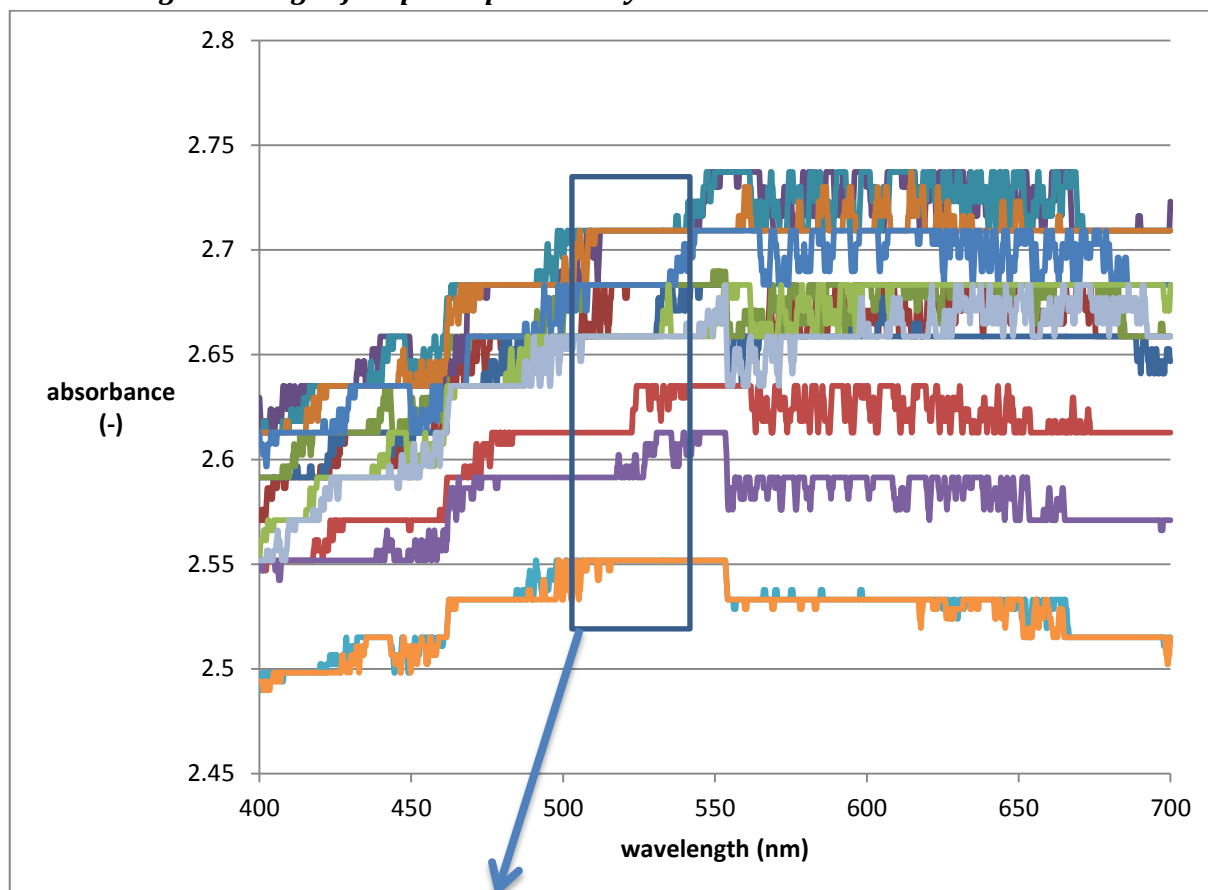


Figure 53 absorbance at wavelengths of visible light by several emulsions containing sunflower oil, water and in some cases oat oil as stabilizing agent. Enlargement shows stable absorbance for all samples at a wavelength of 517nm.

GC chromatograms of unmodified oils and fractions

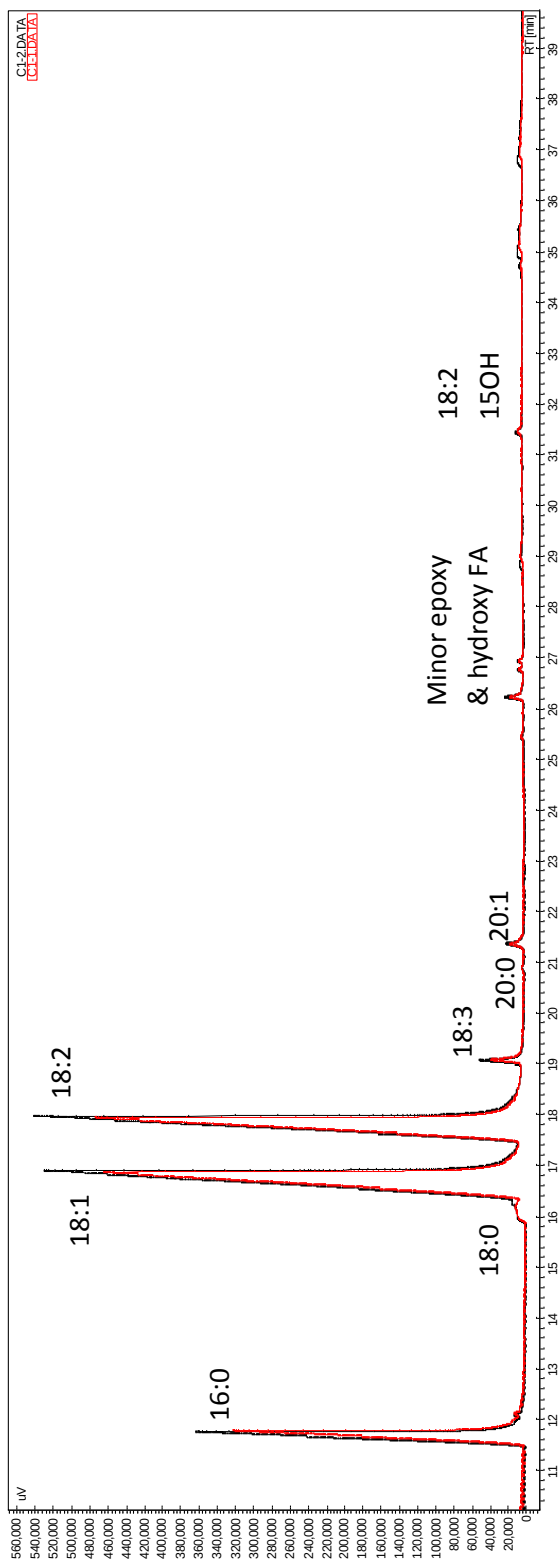


Figure 54 GC chromatogram of crude oat oil with presumed identity of the peaks (duplicates are shown in black and red).

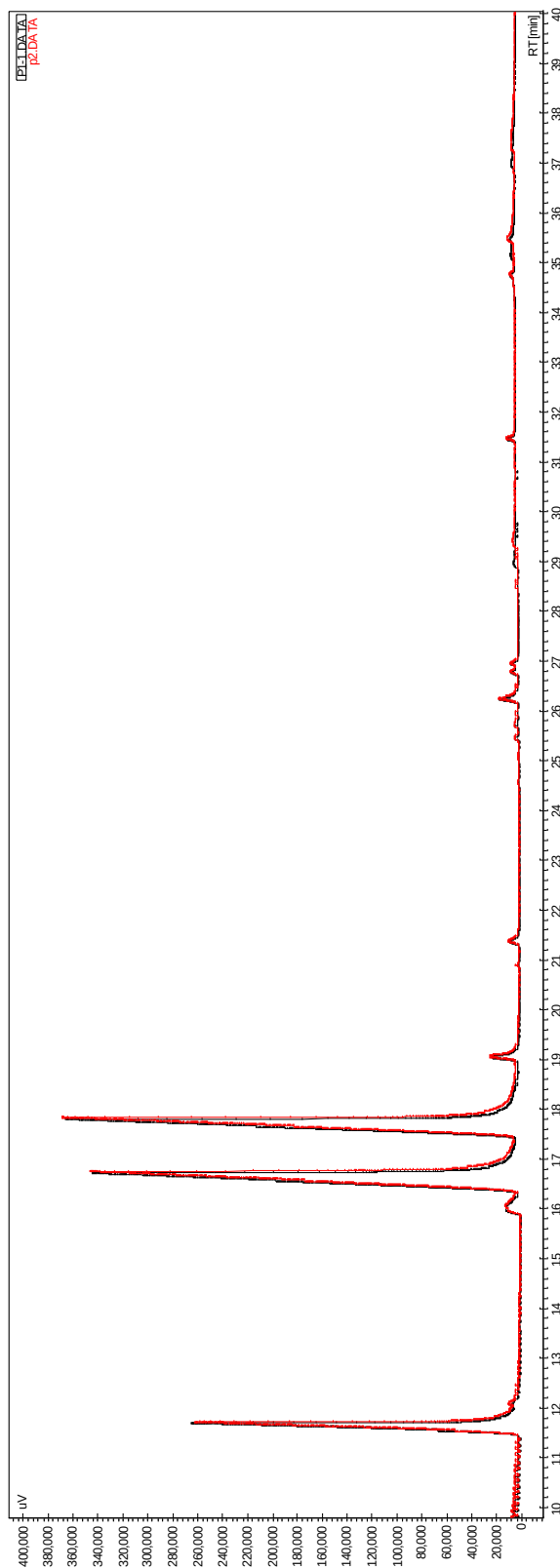


Figure 55 GC chromatogram of PL40 (duplicates are shown in black and red).

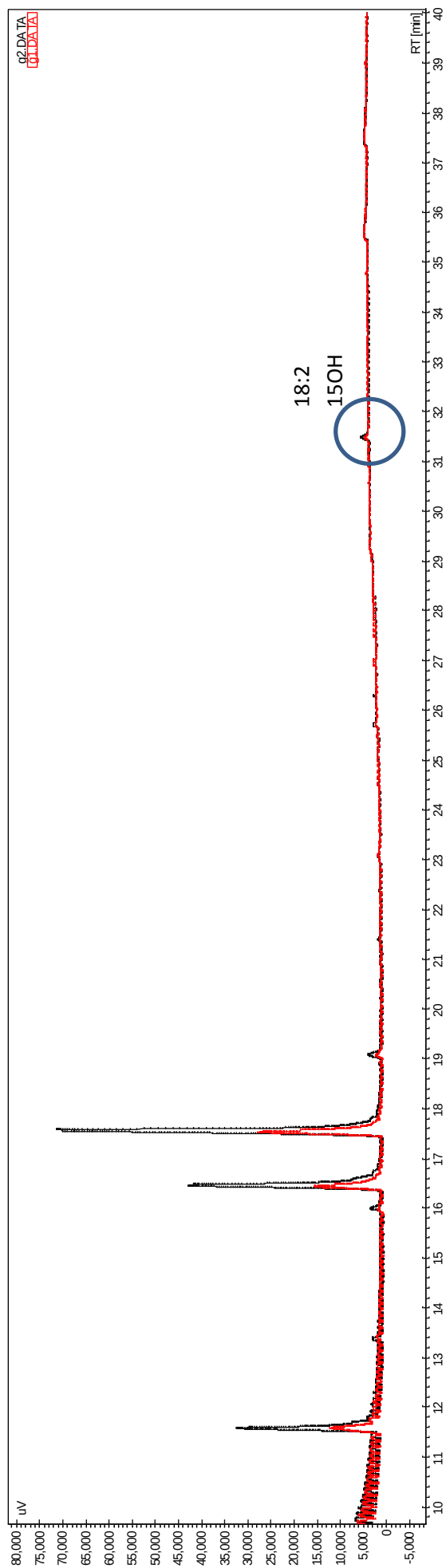


Figure 56 GC chromatogram of glycolipid fraction of crude oat oil (duplicates are shown in black and red). Circle indicates fatty acid only seen for this fraction.

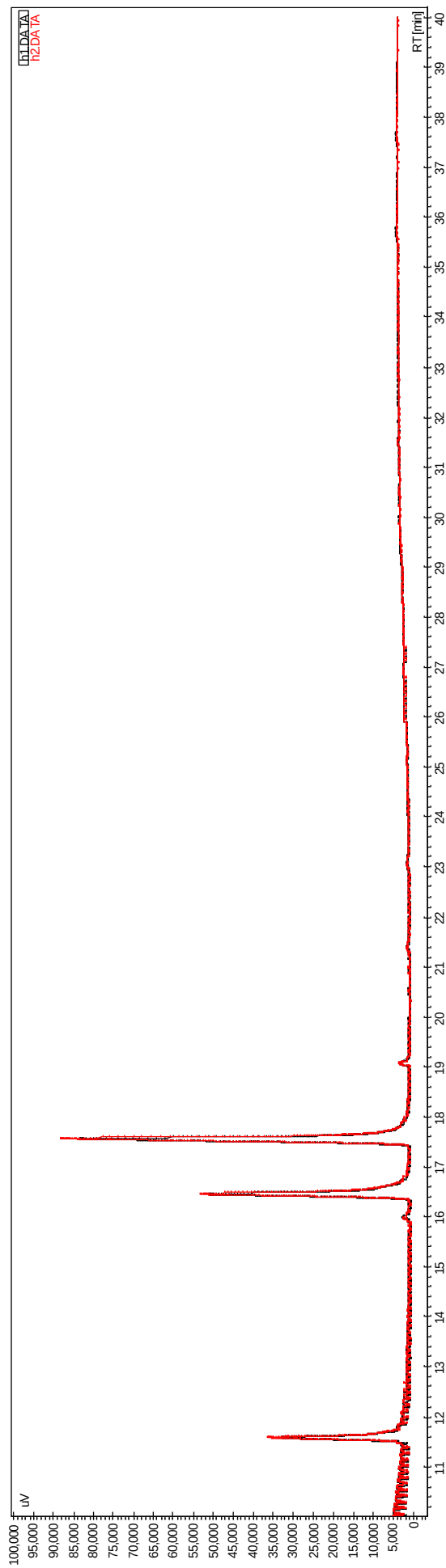


Figure 57 GC chromatogram of phospholipid fraction of crude oat oil (duplicates are shown in black and red).

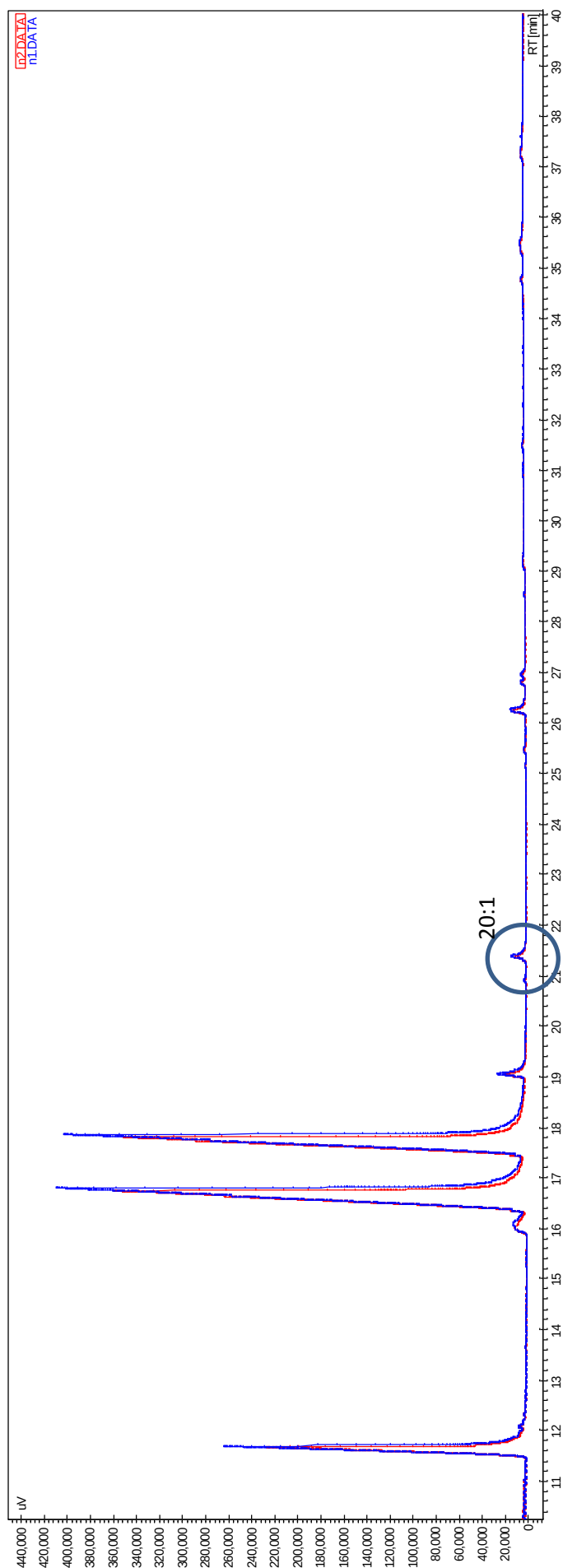


Figure 58 GC chromatogram of neutral lipid fraction of crude oat oil (duplicates are shown in black and red). Circle indicates fatty acid only seen for this fraction.

Statistical analysis for unmodified oils - output R

Post-hoc test of two factor Anova: pairwise t-test

```

      crude PL40
PL40 0.478 -
X    0.042 0.011

```

```

      10min long
long 0.035 -
short 0.557 0.061

```

Results pairwise t-test on destabilisation rates of samples grouped in both type of oil (crude oat oi (crude), PL40 oil (PL40) and no oil/control (X)) and soaking time. Bold numbers indicate significance ($\alpha=0.05$).

Post-hoc test of one factor Anova: Tukey test

	diff	lwr	upr	p adj
LP-LC	-5.950000e+01	-107.1921765	-11.8078235	0.0126943
LX-LC	4.300000e+01	-15.4107485	101.4107485	0.2125984
NC-LC	3.550000e+01	-12.1921765	83.1921765	0.2037230
NP-LC	1.400000e+01	-33.6921765	61.6921765	0.9545815
NX-LC	4.100000e+01	-17.4107485	99.4107485	0.2533278
SC-LC	-8.666667e+00	-52.2034681	34.8701348	0.9954671
SP-LC	2.000000e+01	-23.5368014	63.5368014	0.6969270
SX-LC	4.300000e+01	-0.5368014	86.5368014	0.0535812
LX-LP	1.025000e+02	44.0892515	160.9107485	0.0009920
NC-LP	9.500000e+01	47.3078235	142.6921765	0.0003467
NP-LP	7.350000e+01	25.8078235	121.1921765	0.0027611
NX-LP	1.005000e+02	42.0892515	158.9107485	0.0011631
SC-LP	5.083333e+01	7.2965319	94.3701348	0.0196497
SP-LP	7.950000e+01	35.9631986	123.0368014	0.0007169
SX-LP	1.025000e+02	58.9631986	146.0368014	0.0000813
NC-LX	-7.500000e+00	-65.9107485	50.9107485	0.9997867
NP-LX	-2.900000e+01	-87.4107485	29.4107485	0.6187872
NX-LX	-2.000000e+00	-69.4469228	65.4469228	1.0000000
SC-LX	-5.166667e+01	-106.7368485	3.4035152	0.0707039
SP-LX	-2.300000e+01	-78.0701818	32.0701818	0.7805441
SX-LX	-1.421085e-14	-55.0701818	55.0701818	1.0000000
NP-LC	-2.150000e+01	-69.1921765	26.1921765	0.7146422
NX-NC	5.500000e+00	-52.9107485	63.9107485	0.9999791
SC-NC	-4.416667e+01	-87.7034681	-0.6298652	0.0461025
SP-NC	-1.550000e+01	-59.0368014	28.0368014	0.8845131
SX-NC	7.500000e+00	-36.0368014	51.0368014	0.9982772
NX-NP	2.700000e+01	-31.4107485	85.4107485	0.6909704
SC-NP	-2.266667e+01	-66.2034681	20.8701348	0.5678732
SP-NP	6.000000e+00	-37.5368014	49.5368014	0.9996429
SX-NP	2.900000e+01	-14.5368014	72.5368014	0.3025028
SC-NX	-4.966667e+01	-104.7368485	5.4035152	0.0865984
SP-NX	-2.100000e+01	-76.0701818	34.0701818	0.8454515
SX-NX	2.000000e+00	-53.0701818	57.0701818	1.0000000
SP-SC	2.866667e+01	-10.2738324	67.6071657	0.2125984
SX-SC	5.166667e+01	12.7261676	90.6071657	0.0083026
SX-SP	2.300000e+01	-15.9404990	61.9404990	0.4284012

Results Tukey test on one factor Anova model comparing all samples, which vary in time (short (S), 10 minutes (N) and long (L)) and type of oil (no (X), crude oat oil (C) and PL40 oil (P)). Bold numbers indicate significance ($\alpha=0.05$).