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Study of wood pitch emulsions – interactions with Nile red and influence of pH

Master of Science Thesis in Chemistry and Bioscience

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This work was carried out at Eka Chemicals in Bohus (Sweden).

Cover:
Left: A density plot from a measurement of Nile red in model pitch emulsion with flow cytometry, showing the red fluorescence intensity versus the forward scattering intensity. Right: The fluorescent probe Nile red.

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Anna Palme, Gothenburg, June 2011

Abstract

Deposition of wood pitch causes detrimental effects both on the paper and on the paper machine. In this project, it was investigated how a model pitch emulsion is affected by changing the pH from 4 to 8 and back to 4. This method was used to mimic the pH changes in a paper machine. The system was studied using a model pitch emulsion, prepared from the three most significant substances in wood pitch; triglycerides, fatty acids and resin acids. The main techniques, used to investigate the changes during the pH-study, were flow cytometry and gas chromatography. During the project the fluorescent probe, Nile red, used in the flow cytometry experiments showed interesting fluorescence properties and therefore some effort was put into investigating this. The fluorescence from Nile red in model emulsions based on selected components were analysed with flow cytometry and fluorescence spectroscopy. The effect of the ethanol, the solvent used for Nile red, on Nile red and the model pitch emulsion was also investigated with flow cytometry.

The conclusion from the investigation of the pH-effect on model pitch emulsion is that the resin acids might form small droplets alone when varying the pH up and down, instead of returning to the bigger droplets. This was indicated by the distribution of resin acids between the droplets and the water phase by results obtained from the gas chromatography. Some results suggest that the charge of the droplets may affect the fluorescence from Nile red to a larger extent than earlier believed. The earlier theory of droplets with a shell containing the resin and fatty acids and a core with triglycerides was questioned, since the three components proved to be miscible.

Nile red was dissolved in ethanol and it was found that ethanol affects both Nile red and the model pitch emulsion. Investigations showed that methanol does not affect the emulsion in the same way and thus methanol should be used instead of ethanol as the solvent for Nile red. Addition of Nile red also proved to change the apparent size of the droplets.

Keywords: Pitch deposition, colloidal wood pitch, Nile red, Flow cytometry, fluorescence, gas chromatography.

Contents

Introduction

Sense the feeling of holding a glossy magazine in your hand and then opening it only to discover that on one of the pages the ink is unevenly distributed. This could be a problem caused by pitch deposition, where chemical substances change the surface of the paper leading to inking problems. Paper where deposition has occurred during production will of course never become a glossy magazine and will probably end up as recycled paper. Pitch deposition is a huge problem when considering that every year more than 2 billion books, 350 million magazines, and 24 billion newspapers are published only in the US. (TAPPI, 2001) In order to reduce both costs and waste, a better understanding of the chemical mechanisms behind pitch deposition is needed. Problems from pitch deposition has been a problem for a long time, but has increased with higher environmental standards leading to less water usage and more recirculation. (Mosbye, 2003) (McLean, Stack, & Richardson, 2009) (Mosbye, Richardson, & Parsons, 2008) The deposition also causes runnability problems, apart from printing problems, since the paper machines need more cleaning, which causes more down time.

Eka Chemicals is a large player on the market for pitch control products and it is important for the company to gain more knowledge of the mechanisms behind pitch deposition. The objective of this project was to investigate how the composition of wood pitch emulsion changes when increasing and decreasing the pH-value. The main techniques used were flow cytometry and gas chromatography. The probe used in the flow cytometry experiments, Nile red, showed interesting fluorescence properties and this was also investigated in the project.

Background

Pulping and wood components

The purpose of wood pulping is to separate the fibres in the wood in order to make them suitable for papermaking. This can be done either by mechanical or by chemical means. In chemical pulping, the lignin is removed and long fibres are maintained whereas in mechanical pulping the final paper contains a mixture of very long fibres down to fragments of the fibre wall. The yield in mechanical pulping is 95-98%, which is much higher compared to chemical pulping. (Sjöström, 1993) (Rundlöf M. , 1996) In this project, the focus is on thermomechanical pulp, TMP, which is made in a two step process; first the wood chips are ground at high temperature and pressure (110–130°C) in order to soften the lignin and then they are refined at atmospheric pressure. (Biermann, 2008) When the mechanical pulp is suspended in water different components are released and these are generally referred to as extractives. When process water is being recirculated the extractives are concentrated in the white water system (recirculated water) and may lead to pitch deposition. These problems are larger in TMP compared to chemical pulping since most of the tree is used in the pulp. In this report a model pitch based on TMP from the softwood Norwegian Spruce (*Picea abies*) is used.

The main constituents of Norwegian Spruce are cellulose, hemicellulose and lignin (Table 1). Cellulose fibres are built up from a homopolysaccharide of β-D-glucopyranose which is ordered into larger bundles, forming microfibrils. The microfibrils form fibrils and the fibrils build up the fibres of cellulose. (Sjöström, 1993)

Table 1: Chemical composition of Norwegian Spruce (Sjöström, 1993)

Component	Percentage
Cellulose	41.7
Hemicellulose	28.3
Lignin	24.7
Extractives	1.7
Residual constituents	0.9

Hemicelluloses

Hemicelluloses belong to a group of heterogeneous polysaccharides and were originally believed to be intermediates in the biosynthesis of cellulose, but now it is known that the biosynthetic routes of hemicelluloses are different from that of cellulose. Hemicellulose is the second most abundant component of wood. Most types of hemicellulose have a degree of polymerization between 70 and 200, which is small relative to cellulose, which has a degree of polymerization of 2500-3000. Hydrolysis with acid of hemicellulose gives the monomeric building blocks consisting mainly of D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose. (Sjöström, 1993)

Extractives

Extractives are the least abundant component in wood and are closely connected to pitch deposition and will therefore be in focus of this report. Extractives can be regarded as non-structural wood components and the group contains almost only extracellular, low-molecular weight compounds. Lipophilic extractives, commonly called wood resin or pitch consists of fatty acids, resin acids, sterols, steryl ester and triglycerides (Figure 1).
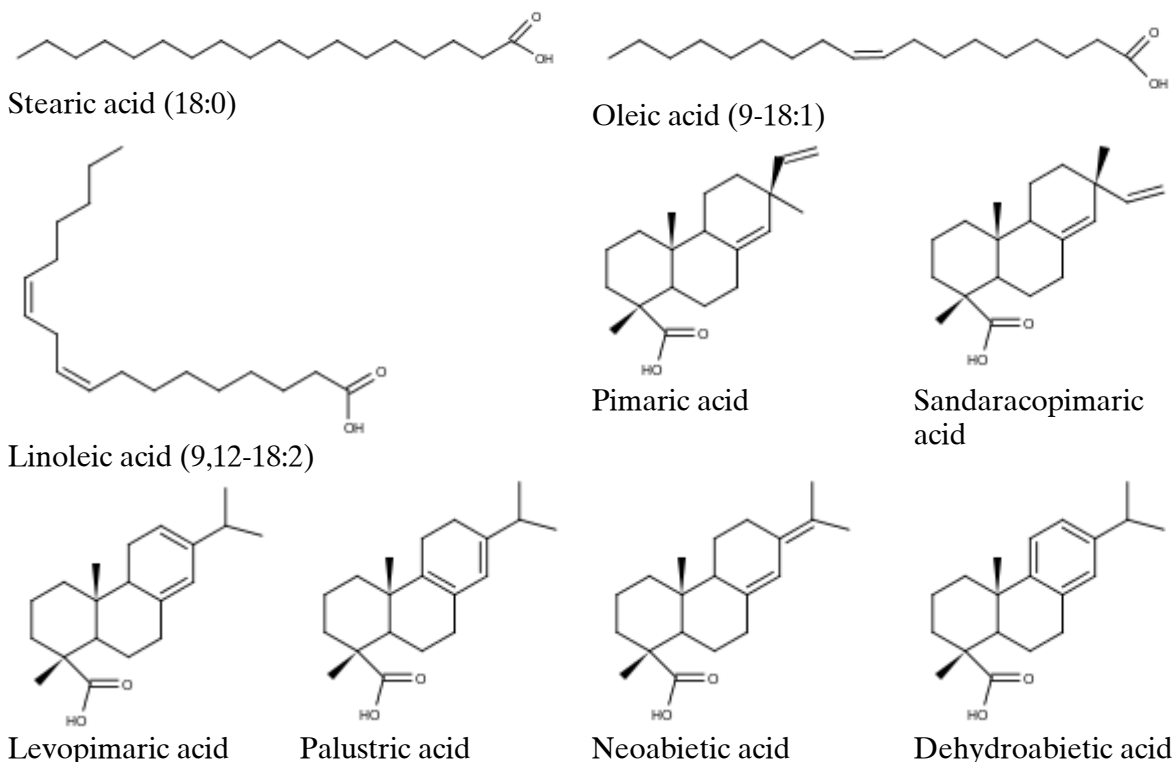

Stearic acid (18:0)

Oleic acid (9-18:1)

Linoleic acid (9,12-18:2)

Pimaric acid

Sandaracopimaric acid

Levopimaric acid   Palustric acid   Neoabietic acid   Dehydroabietic acid

Figure 1: Structures of major fatty acids and resin acids (Sundberg, Strand, Vähäsalo, & Holmbom, 2009)

Apart from these the wood also contains hydrophilic extractives such as lignans, which are phenolic compounds that consists of two phenylpropane units. (Qin & Holmbom, 2008)The different parts of a tree, such as the stem, branches, roots, bark and needles contain different amount and composition of extractives. (Sjöström, 1993) The ratio in the pulp between sapwood (outer part of stem) and heartwood (inner part) and thus the age of the tree to a large extent determines the composition and amount of extractives. Old trees usually contain a larger amount of heartwood and this results in less lipophilic extractives, such as triglycerides. (Örså, Holmbom, & Thornton, 1997) The resin acids have a relatively low solubility in water (Solubility of resin acids in waterTable 2). The dehydroabietic acid is aromatic and this leads to a higher solubility and possibility of pi-stacking interactions.

Table 2: Solubility of resin acids in water (Peng & Roberts, 2000)

Resin acid	Solubility in water (mg/l)
Isopimaric acid	1.70
Sandaracopimaric acid	1.82
Pimaric acid	2.17
Palustric acid	2.41
Neoabietic acid	2.31
Levopimaric acid	2.54
Abietic acid	2.75
Dehydroabietic	5.11

A study of accumulation of wood pitch in TMP showed that the amount of wood pitch in the recirculated water increased linearly with the number of accumulation cycles. This effect could be observed both at low and at high pH and the dissolution of resin acids above pH 6 did not prove to have any large impact on the amount accumulated. In the same study the temperature proved to be an important parameter in the accumulation of wood pitch. (Lehmonen, Houni, Raiskinmäki, Vähäsalo, & Grönroos, 2009)The deposition tendency at different pH was not reposted in this study, but Stack and co workers showed that the deposition tendency is highest around pH 6. (Stack, Stevens, Richardson, Parsons, & Jenkins, 2000)

Fines

Deposition of extractives onto fines may cause problems in paper production. (Rundlöf, Htun, & Höglund, 2000) Fines are defined according to Rundlöf as the fraction of pulp that passes though a screen of a given fractionator (Rundlöf M. , 1996). The most common is to use a wire with a pore size of 76 μm called a 200 mesh, and thus the fines that pass through such a screen are called P200. Using this definition, TMP consists of 25-35% fines (by weight) and the extractives are a part of the fines. Fines mainly come from the outer parts of the wood fibre and are created by the peeling action in the mechanical process. (Rundlöf M. , 1996)

Colloidal wood pitch

When extractives are released into water they form colloidal droplets with a size of 0.1-2 μm. (Sundberg, Strand, Vähäsalo, & Holmbom, 2009) These wood pitch droplets have been shown to have a severe detrimental effect on the sheet properties, as they reduce the sheet density which both leads to a reduced sheet strength and an increased light scattering. (Johnsen & Stenius, 2007) A better understanding of pitch droplets may lead to an increased understanding of the mechanisms behind pitch deposition and aid in solving the problems related to pitch deposition.

Results from several investigations on the structure of colloidal pitch have been published. Nylund and co-workers, who measured critical surface tensions of the different components in colloidal wood resin, proposed a two-layer model, with a hydrophilic outer shell and a hydrophobic core. (Nylund, Sundberg, Shen, & Rosenholm, 1998) Qin and co-workers came to the same conclusion when measuring the work of adhesion and showed that an increased ratio of fatty acids or resin acids increased the work of adhesion. (Qin, Hannuksela, & Holmbom, 2003) In this model it was proposed that the more hydrophilic resin acids and fatty acids interact at the surface and extend the hydrophilic parts out towards the aqueous phase and that the core contains the hydrophobic extractives, such as triglycerides. Based upon the

ratio of the different components the surface layer was calculated to be 100 Å thick, which corresponds to approximately 5 fatty acids. This model has been supported in studies using molecular modelling, where the resin acids and the fatty acids proved to have extensive interactions. (Vercoe, Stack, Blackman, & Richardson, 2005a) Questions are raised in this report on the correctness of this model.

The pK_a of hydrophobic molecules in aggregates in water is different from the pK_a of the monomer in water. The pK_a of resin acids is between six and seven and thus around pH 6 they will start to dissolve in the water phase and the distribution between the colloidal droplets and the water phase will change.

Shah and Kanicky investigated the pK_a of fatty acids and found that although electronic effects are not felt further away than 2-3 carbons, an increased chain length leads to a higher observed pK_a. Their explanation to this phenomenon is that already at low concentrations, far below the critical micelle concentration, CMC, there are pre-micellar aggregates present, leading to an increase of the apparent pK_a as the chain length increases. Their investigation also proves that addition of just a small amount of a short chain fatty acid decreases the pK_a of the mixture below the pK_a of both the individual components, due to more disorder in the system. (Kanicky & Shah, 2003) This decrease of pK_a is important to pay attention to in a pitch colloid solution, which is a mixture of different acids.

McLean and co-workers measured the colloidal pK_a, which is the pK_a of a substance above its CMC. Significant differences between the pK_a at 20°C and 50°C could be observed (Table 3). (McLean, Vercoe, Stack, & Richardson, 2005)

Table 3: Colloidal pK_a values of extractives (McLean, Vercoe, Stack, & Richardson, 2005)

Type of acid	pK_a at 20°C	pK_a at 50°C
Saturated fatty acids		
Palmitic (C16:0)	8.34	8.63
Stearic (C18:0)	9.89	9.28
Unsaturated fatty acids		
Elaidic (18:1;(trans)9)	8.31	7.65
Resin acids		
Abietic	7.26	6.18
Dehydroabietic	6.77	6.18
Isopimari	7.08	6.23
Neoabietic	7.07	6.23

Instead of using the "colloidal pK_a" Sundberg and co-workers introduced the term pK_{lw}, which is defined as the point where 50% of the fatty acids or resin acids are associated with the lipophilic, colloidal phase (l) and 50% is dissolved in the water phase (w). The concentrations in these measurements were below the CMC. The temperature proved to have the same effect on the pK_{lw} (Table 4) as on the colloidal pK_a. (Sundberg, Strand, Vähäsalo, & Holmbom, 2009)

Table 4: pK$_{lw}$ values of extractives (Sundberg, Strand, Vähäsalo, & Holmbom, 2009)

Type of acid	pK$_{lw}$ at 30°C	pK$_{lw}$ at 50°C	pK$_{lw}$ at 70°C
Resin acids			
Pimaric acid	7.8	6.8	6.1
Sandaracopimaric acid	7.4	6.7	6.9
Isopimaric acid	7.6	6.8	7.0
Abietic acid	7.5	6.7	7.2
Palustric acid	7.6	6.8	7.2
Neoabietic acid	7.3	7.3	7.3
Dehydroabietic acid	6.0	5.9	6.0
Fatty acids			
Palmitic acid (16:0)	7.7	7.4	7.8
Linoleic acid (9,12-18:2)	8.1	7.4	7.9
Oleic acid (9-18:1)	8.4	8.1	8.2
Stearic acid (18:0)	11.6	10.1	9.2
Arachidic acid (20:0)	13.5	12.7	12.1

Galactoglucomannans, one kind of hemicelluloses, also dissolve into the water phase in mechanical pulp and provide a steric stabilisation to the wood pitch colloids. This leads to a decreased tendency of deposition on fibres and fines. (Sundberg, Strand, Vähäsalo, & Holmbom, 2009) If the pulp is washed the hemicellulose might be washed out and can thus not help to stabilise the extractive colloids, possibly this could be a positive effect of recirculating white water. (Vercoe, Stack, Blackman, & Richardson, 2005a) The effect from hemicellulose was not investigated further in this project.

The colloidal stability has been shown to be dependent on both addition of calcium ions and polysaccharides, such as hemicellulose. Calcium ions are commonly studied, since the levels in the pulp are high. In a recent study, it was shown that the colloidal stability against calcium ions was very low in a dispersion of acetone extractives without polysaccharides. (Qin & Holmbom, 2008) The ions compress the electric double layer of the colloids and this may lead to aggregation. However, if there is hemicellulose present in the water, the colloids will not aggregate completely. Sundberg and co-workers also showed that the dissolution of resin and fatty acids after a pH change is very fast; a new equilibrium was reached already 1 minute after the pH change. (Sundberg, Strand, Vähäsalo, & Holmbom, 2009) Lee and co-workers came to the same conclusion and characterized the pitch colloids as "soft colloids" which reorganises upon changes to its environment. (Lee, Stack, Richardson, Lewis, & Garnier, 2009)

Deposition

Deposition is a big problem caused by a number of different factors. Investigations of adhesion of wood resin colloids on different surfaces in the paper machine suggested that the emulsion will accumulate on the hydrophobic surface materials and detach from wet paper web surfaces composed of carbohydrates or cellulose. (Kallio, Lindfors, Laine, & Stenius, 2008)

A solution to wood pitch problems may work for one paper mill but not for another, even though both have problems connected to extractives. This can be illustrated with an example from two paper mills in Australia and New Zeeland, where large problems with pitch deposition occurred. (Mosbye, Richardson, & Parsons, 2008) Through analysis of components in the pulp, the authors could determine that the amount of extractives bound to fibres and the calcium levels were important parameters to control. In one of the paper mills the pitch deposit problems could be avoided if the concentration of fibre bound extractives were below 55 mg /l. On the other mill it proved important to prevent formation of localised alkaline pH-conditions. Other strategies to solve the problem of pitch deposits include adsorption of extractives onto bentonite or talc, fixation of colloidal pitch to fibre surfaces and removal of extractives from the white water system. (Grubb, Wray, & Richardson, 2009)

Flow cytometry

Flow cytometry, FCM, is an analytical technique, which is routinely used in medicine for identification and counting of cells. Flow cytometers have been commercially available since the beginning of the 70's, but the use of FCM for white water analysis first started in the 90's by Vähäsalo and co-workers. (Vähäsalo, 2005)

The term cytometry refers to measurement of physical and/or chemical characteristics of single cells or particles. (Shapiro, 2003) The principle behind FCM is to measure a combination of light scattering and fluorescence of a particle system flowing by the measuring device (Figure 2). The sample is fed into a measurement cell by hydrodynamic focusing with the sheath fluid where the laser illuminates the sample. (Grubb, Wray, & Richardson, 2009)
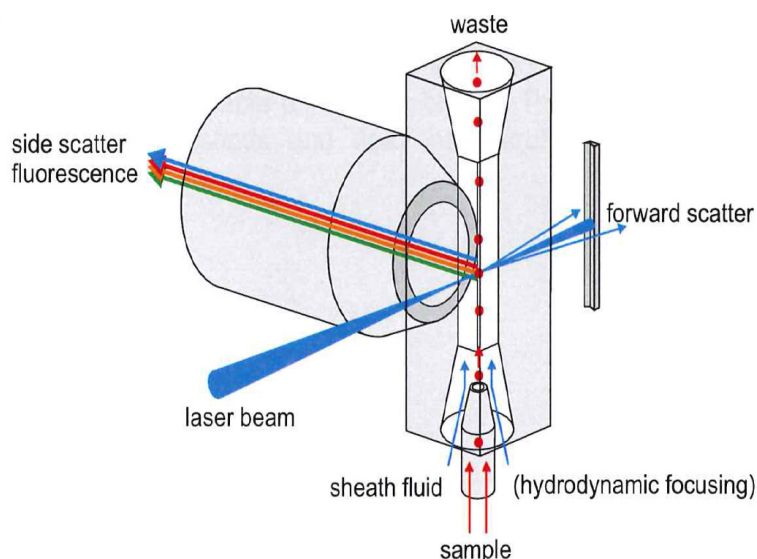

Figure 2: Schematic picture of the flow cuvette

The particles in the sample can range from 0.2 μm to 100 μm and commercial instruments available today can detect 100 000 particles per second and measure 10-20 different parameters for each particle. Since every particle is measured individually, the concentration of each particle type can be determined independently and rare occurrences can be measured without risk of disappearing in a mean value. (Vähäsalo, 2005) (Grubb, Wray, & Richardson, 2009)

It should be noted that almost all parameters that are measured with FCM, could also be measured using other techniques, such as confocal microscopy and scanning cytometry. The advantages of these techniques are that they have better spatial resolution compared to FCM and can examine the same sample repeatedly over time. The flowing sample in the flow cytometer has the advantage that one of the most common problems connected to fluorescence, photo bleaching, is avoided, since the probes only are subjected to the laser light during a very short time period. (Shapiro, 2003)

In this project a flow cytometer measuring five parameters were used, side scattering, forward scattering and red, green and orange fluorescence (Figure 3).

| FL1 Green: 512-542 nm | FL2 Orange: 575-605 nm | FL3 Red: 615-645 nm |

Figure 3: Wavelength intervals of the three fluorescence parameters in the flow cytometer used in this project

The size of the measured particles was estimated by a size calibration performed with latex particles. The particles were of four different sizes (0.431 μm, 1 μm, 2.87 μm and 3 μm) and based upon correlation between the side scattering and the known sizes, a calibration curve was calculated.
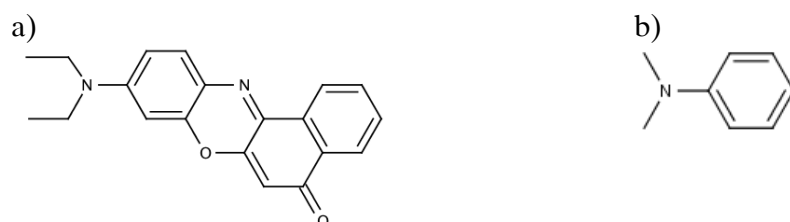
a)

b)

Figure 4: a) Structure of Nile red b) Dimethylaniline

In this project, the lipid probe Nile red, (Figure 4a) has been used as proposed by Vähäsalo. (Vähäsalo, 2005) Nile red was presented by Greenspan and co-workers in 1985 (Greenspan, Mayer, & Fowler, 1985)as a fluorescent stain excellent for detecting intracellular lipid droplets, with a very low fluorescence in water and a high fluorescence in hydrophobic environments. The same year they also published results indicative of a blue shift, proportional to the hydrophobicity of the environment. This change in fluorescence characteristics has been attributed to changes in the microenvironment and much effort has been put into explaining the excited state dynamics. In 1996, Dutta and co-workers reported results pointing towards a non-radiative, polarity dependent, twisted intramolecular charge transfer (TICT). (Dutta, Kamada, & Ohta, 1996) Yablon and co-workers investigated Nile red in nonpolar solvents with similar dielectric constants, but a wide range of viscosities and found an increase in quantum yield with viscosity and this was related to that the path to the TICT state was cut off. (Yablon & Schilowitz, 2004) The presence of a TICT-state was rejected by

Cser and co-workers in 2002, who found no sensitivity towards dielectric solvent – solute interactions but instead proof of hydrogen bonding in the excited state. (Cser, Nagy, & Biczók, 2002) In this study, the viscosity did not prove to have a large effect. In 2006, Dias and co-workers reported findings that supported Cser and co-workers in the statement that hydrogen bonding affects the fluorescence characteristics. (Dias, Custodio, & Pessine, 2006)

Comparing Nile red to dimethylaniline (Figure 4b), which has a pK_a of 5.1, it is reasonable to believe that the tertiary amine in Nile red have a similar value and thus will the protonation change in the investigated pH-interval. Wagner and co-workers propose that protonation of Nile red may lead to a decrease of the quantum yield. (Wagner, Boland, Lagona, & Isaacs, 2005) This protonation will also change the solubility of Nile red, but this effect has not been investigated further.

It can be concluded that the microenvironment of Nile red will affect the probe to a high extent. In all earlier published studies where pitch has been investigated with flow cytometry, Nile red dissolved in ethanol has been used. In order to gain increased understanding of how the fluorescence from Nile red can be interpreted, more detailed fluorescence measurements were made in this project. The main use of the fluorescence from Nile red today is as a rough probe of the hydrophobicity, but with an increased understanding of the fluorescence of Nile red in different environments, the interpretation of the results from flow cytometry may lead to an understanding of more parameters related to the colloidal droplets, e.g. composition and surface charge.

Experimental

Model resin preparation

Resin acids were Soxhlet extracted from solid oleoresin from Norwegian Spruce (*Picea abies*) for 8 hours with hexane. The oleoresin was kindly provided by Åbo Akademi. The hexane was then evaporated and the resin acids redissolved in acetone. The concentration and purity was determined with gas chromatography (GC). Equal amounts of a mixture of stearic and palmitic acid (50% stearic acid, 50% palmitic acid), oleic acid and linoleic acid was dissolved in acetone. All of the fatty acids were purchased from Sigma Aldrich. The triglyceride mixture (soybean oil) was directly dissolved in acetone.

The three components were mixed according to the weight ratios 5.1:1.5:1 of TG:RA:FA and the acetone was evaporated, with ratios according to Strand and co-workers. (Strand, Sundberg, Vähäsalo, & Holmbom, 2011) After evaporation, acetone was added and the three components were dissolved again. This solution was injected into water under stirring (250 rpm) to create an emulsion with a concentration of 500 mg/L. The acetone, corresponding to 2.5 % of the water volume, was removed by dialysis during 24 h where the water was changed approximately 8 times.

After the preparation, the emulsion was diluted to approximately 200 mg/L and stored cold. After dilution the emulsion had a pH of 5.5. All experiments were made on emulsion from the same batch.

Emulsions with single components and double components were also prepared for comparison according to the same procedure and using equivalent amounts.

pH-study

Model resin was added to a beaker, stirred with a paddle stirrer and the initial pH was measured. The pH of the emulsion was then sequentially set to 4, 6, 8, 6 and 4 with H_2SO_4 (0.05M) and NaOH (0.1 M). At each pH value, the solution was allowed to equilibrate for one hour during which necessary addition of acid and base was made to maintain the pH constant. After one hour, a sample was taken out and one fraction of the sample was filtered (0.2 µm, Whatman, blue ribbon "3"). After sampling, the remaining emulsion was set to the next pH. Measurements on the flow cytometer were made as soon as possible after sampling.

Extraction of model resin emulsion samples before GC-analysis

The pH of each 8 mL sample was first set to 3 and then 4 mL of methyl tert-butyl ether (MTBE) was added and shaken with the sample for 5 minutes. After shaking, the sample was centrifuged for 5 minutes (3000 g) and the organic phase pipetted off and dried. This procedure was repeated three times. The dry samples were then sent to GC-analysis performed according to Örså and Holmbom. (Örså & Holmbom, 1994)

Fluorescence spectroscopy

Emission spectra (excitation 488 nm, excitation slit 1.5, emission slit 10) were recorded on a Cary Eclipse Spectrophotometer (Varian) using 3 mL quartz cuvettes. The emulsion was diluted 30 times and 60 µl Nile red (EtOH, 20 ppm) was added to the 3 mL sample, if nothing else is noted.

Flow cytometry

Flow cytometry measurements were performed on a Partec CyFlow SL flow cytometer with 5 optical parameters, forward scattering, side scattering, FL1 green (512-542 nm), FL2 orange(575 -605 nm) and Fl3 Red (615-645 nm). The emulsion was diluted 100 times and 20 µl Nile red (EtOH, 20 ppm) was added to the 1 mL sample, if nothing else is noted. Each sample was mixed just before the measurement unless when noted otherwise.

Results and discussion

The main objective of this project was to investigate the changes in colloidal wood pitch after a pH change from 4 to 8 and back again to 4, a so-called pH-study. The changes were monitored with flow cytometry, gas chromatography and fluorescence spectrophotometry. Understanding of the effects of Nile red and solvent effects related to this is needed in order to understand these results and thus this will be presented first.

Effect of ethanol and methanol on Nile red

The effect on the apparent size and fluorescence, when adding ethanol was investigated since indications of that ethanol could affect the measurements was observed. This was not known in the beginning of the project and all published research on pitch and flow cytometry uses Nile red in ethanol. (Vähäsalo, 2005) (Grubb, Wray, & Richardson, 2009) In order to understand how this affected the results, the effects of ethanol were investigated further.
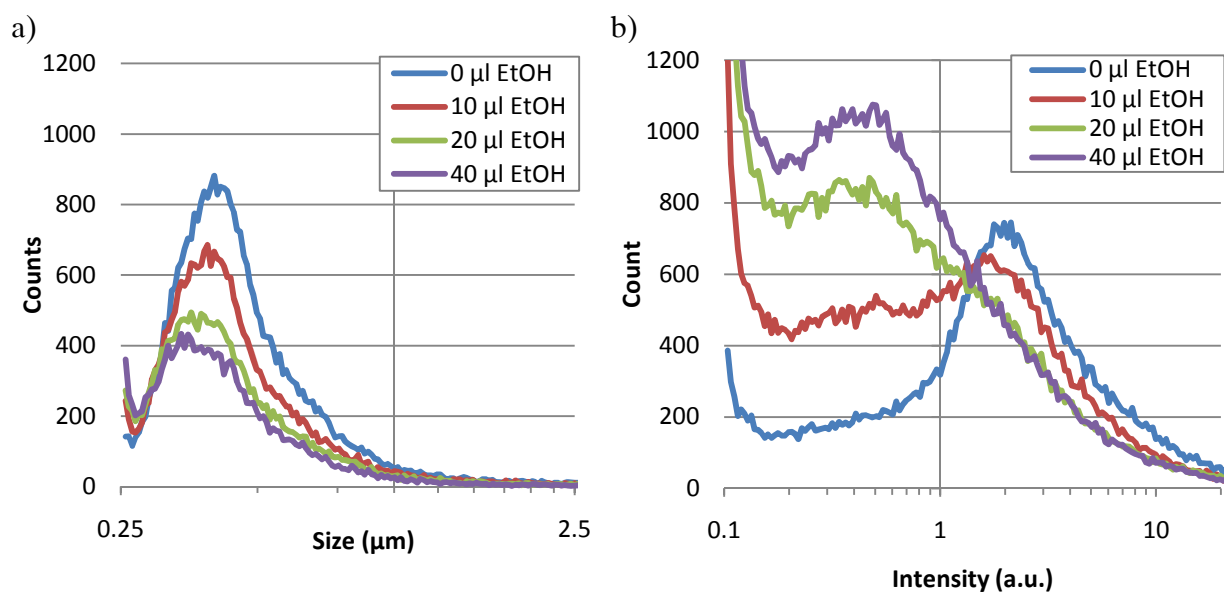

Figure 5: Flow cytometer measurements of a) size b) red fluorescence (FL3, 615-645 nm) on model pitch emulsion diluted 100 times (to 1 ml) and 20 µl Nile red (EtOH, 20ppm) added with subsequent additions of pure ethanol, mean value of two measurements.

Initially, when no extra ethanol has been added the emulsion gives rise to a peak with its maximum at 0.40 µm, but addition of increasing amounts of ethanol leads to an apparent decrease in the number of emulsion droplets (Figure 5). Simultaneously, the total number of particles in the diluted sample increases from 29000 to 88000 (not shown). This observed increase in particle count is due to an apparent increase of small particles. The observed decrease of the number of emulsion droplets could be an artefact caused by saturation of the flow cytometer. In the two last measurements the count rate was above the recommended level, but already before this point saturation could have occurred, due to the large increase of small particles.

Since the size is based on the side scattering, a change in refractive index could also disturb the measurements and lead to an apparent increase of small particles. When ethanol is dissolved in the sample fluid it leads to a different refractive index compared to the sheath fluid and this could lead to a disturbance on the side scattering measurements. This hypothesis could be tested though using a sheath fluid with the same ethanol concentration as the sample.

The increasing amount of ethanol leads to an apparent increased amount of particles with very low fluorescence intensity (Figure 5b). It is be reasonable to believe that the small particle fraction is the fraction with low fluorescence intensity. The fluorescence from Nile red in the emulsion droplets seems to decrease when adding more ethanol. If some of the added ethanol penetrates the droplets, this may lead to a quenching of Nile red inside the droplets. This could explain the decrease in fluorescence intensity at higher ethanol concentration. If this would happen the drops would swell, but measurements of the size indicate instead a size reduction. Ethanol could possibly also slightly dissolve the droplets and quench Nile red.

The effect of ethanol must be investigated further in order to understand if ethanol induces changes in the droplet size or if the change is only apparent and due to change in refractive index. Repeating the experiments using a sheath fluid with the same ethanol concentration as the sample could explain if the increase of small particles is due to a change in refractive index.

These effects from ethanol are not fully understood and have influenced the results in an undesired manner. As the droplet concentration fluctuates, so does the ethanol ratio per droplet and as a consequence the measurements of the model pitch emulsion will be affected. Therefore, ethanol is not a preferred solvent. This must be taken into account when analysing the results from the pH-study.
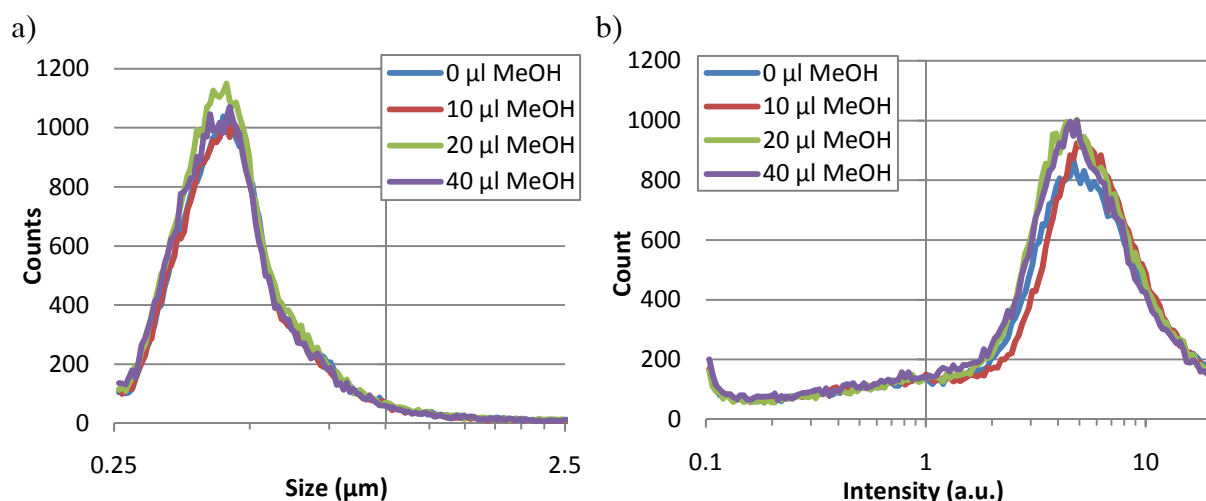

Figure 6: Flow cytometer measurements of a) size b) red fluorescence (FL3, 615-645 nm) on model pitch emulsion diluted 100 times and 20 μl Nile red (MeOH, 20ppm) with increasing additions of methanol, mean value of two or more measurements.

When Nile red is dissolved in methanol, a change in size of the emulsion droplets and fluorescence is not observed as in the case with ethanol. Since both ethanol and methanol are protic solvents, the major difference between them is their polarity. One explanation may be that methanol is too polar to diffuse into the droplets and will thus not be able to quench Nile red inside the droplets.
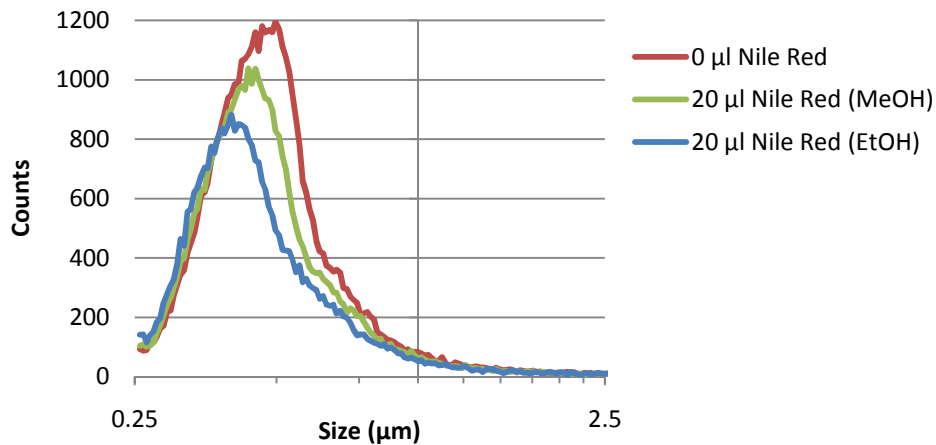
Figure 7: Flow cytometer measurements of the size of model pitch emulsion without Nile red and with Nile red dissolved in methanol and ethanol.

The side scattering from the model pitch emulsion is changed when adding Nile red in both methanol and ethanol. This leads to a change of measured size, but it is not known whether the real size distribution changes or if this is due to changes of refractive index. This could be studied though addition of very small amounts of Nile red in ethanol or methanol to the sample and study the size change.

The results from investigations of Nile red dissolved in ethanol and methanol indicate that more understanding of how both Nile red and the solvent interact with the pitch droplets in order to interpret the results. It is important to point out that an excess of undissolved Nile red is wanted in order to secure a constant concentration of Nile red in the droplets, but to investigate the effect from Nile red on the size a titration with small additions of Nile red could be performed. A more detailed discussion of this is found below. A sensitive probe, like Nile red, can be used to gain more detailed knowledge of the system, but on the other hand the results from a less sensitive probe could be easier to interpret and thus, this could be preferable in some cases.

14

Time effect on Nile red fluorescence

The emission spectrum of Nile red in water and model pitch emulsion reveals a change with time. According to literature Nile red, is quenched in water, but our measurements show a high peak at 660 nm. The intensity of this peak is only 30% of its original value after 120 min and no change in maximum wavelength is observed. This could be an effect of bad mixing on the molecular level between water and ethanol with Nile red, but results from experiments discussed below speak against this. If so, most of the fluorescence might come from Nile red inside ethanol droplets. After 120 min a better molecular mixing is achieved and the surrounding water quenches Nile red, and thus the intensity decreases.
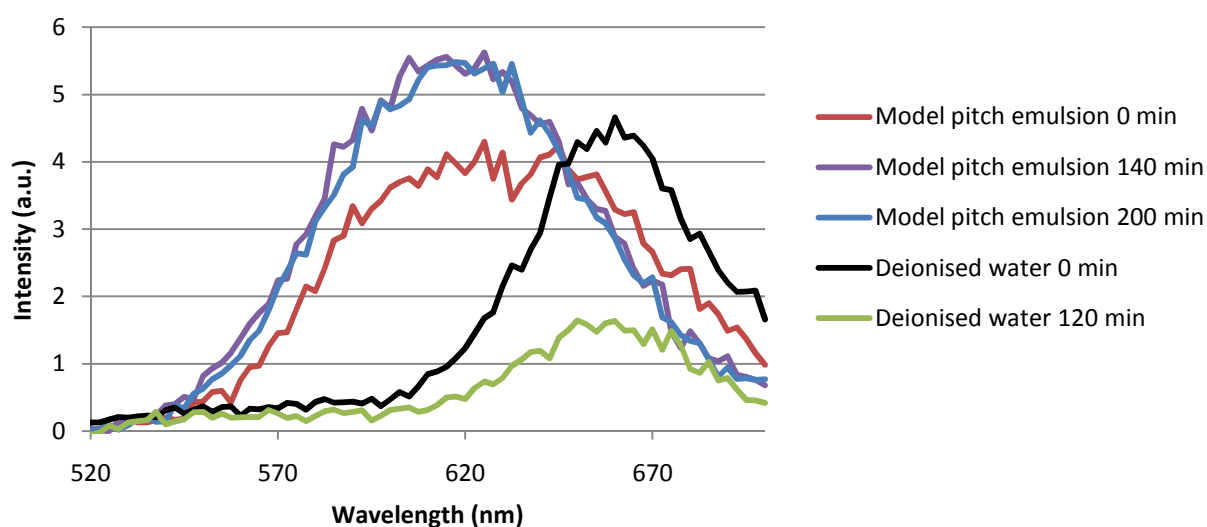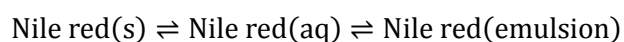

Figure 8: Emission spectra of Nile red in water and model pitch emulsion with time effect

The emission from Nile red in the model pitch emulsion both changes in intensity and in maximum wavelength when the sample is allowed to equilibrate. The initial overlap between the peak from Nile red in model pitch emulsion and the peak from Nile red in water decreases with time. Simultaneously the intensity at 615 nm increases and becomes dominating in the spectrum. The same observations of the time effect were made in measurements with flow cytometry (Appendix A).

These time effects are interesting to this project since it may give clues to the interaction between Nile red and the model pitch colloids. The time effect may be related to a slow equilibrium of Nile red between the emulsion and the surrounding water, see Equation 1.

Nile red(s) ⇌ Nile red(aq) ⇌ Nile red(emulsion)
Equation 1

Nile red has a very low solubility in water (< 1 µg/mL) (Greenspan & Fowler, 1985) and thus there will be a large fraction of undissolved Nile red in the water. In order for Nile red to fluoresce inside the emulsion droplets, the probe must first be dissolved in water in order to diffuse into the droplets and this may explain the time effect observed in Figure 8 and Appendix A. In all experiments where nothing else is noted, the samples were run

immediately after mixing. In future research it may be of interest to run the samples after equilibration for a certain time.

According to this model, the water is saturated with Nile red and the undissolved Nile red works as a reservoir, and thus the concentration, and also fluorescence in the droplets will be equal in all measurements, independently of the droplet concentration. This will be maintained as long as there is undissolved Nile red left in the reservoir.

Nile red in pure components and in component emulsions

In order to gain further understanding of the environment in the emulsion droplets, the fluorescence from Nile red in the pure components, not as emulsion, was analysed with fluorescence spectroscopy. The fatty acids, resin acids, and triglycerides mix well in a test tube and no tendency towards phase separation can be seen. I should be noted that only a small amount resin acids could be added since they were not available in a pure form. This suggests that the emulsion droplets may not be as well-structured, with the resin and fatty acids in the shell and the triglycerides inside, as earlier proposed. In the water-droplet interface it is reasonable to believe that resin and fatty acids are concentrated, stretching their hydrophilic parts out into the water. The proposed shell thickness of 100 Å corresponds to approximately 5 fatty acids but, it is more probable that inside of the monolayer there is a mixture of triglycerides, fatty acids and resin acids. Using 2D NOESY to analyse the model pitch emulsion, the distance between the fatty acids and the triglycerides could be determined. This could elucidate to what extent the fatty acids and the triglycerides are mixed in the droplets.
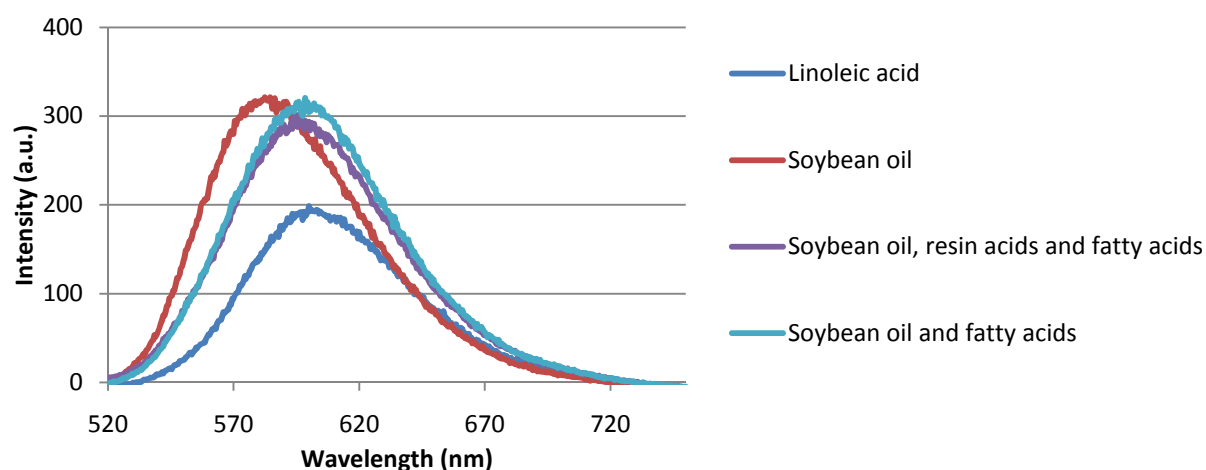

Figure 9: Emission spectra of Nile red (20 ppm, EtOH) in fatty acids, resin acids and triglycerides, ex 488 nm.

There are large differences between the emission spectra of Nile red in the different pure components that are present in the model pitch emulsion, both in terms of intensity and maximum wavelength (Figure 8). The differences between the pure linoleic acid and the mixture of linoleic acid and triglycerides are intriguing. The maximum wavelength of the emission spectrum from Nile red in linoleic acid is approximately 600 nm, whereas for triglycerides it is 580 nm. Another immediate observation is that Nile red in linoleic acid only has approximately 60 % of the intensity from Nile red in triglycerides.

When mixing the linoleic acid with the triglycerides the resulting spectrum has a higher intensity than linoleic acid and is slightly shifted towards shorter wavelengths. The addition of triglycerides to linoleic acid seems to protect the Nile red from quenching with only a small effect on the maximum wavelength. This indicates that there is more than one effect involved, not only solvent polarity or hydrogen bonding. Ethanol will possibly also affect the systems differently, since mixing between linoleic acid and ethanol will be better than between the triglycerides and ethanol and thus Nile red will be less protected from the ethanol in the linoleic acid.

Nile red in emulsions prepared from single and double components

In order to investigate the effect on the fluorescence of Nile red from the different components mixed in the model pitch emulsion, each of the components and mixtures of the different components were prepared as emulsions. Using NMR and acid-base titration a low concentration of fatty acids in the soy oil could be determined and thus this could also be prepared into an emulsion. The emission spectra of Nile red in these emulsions can be seen in Figure 10.
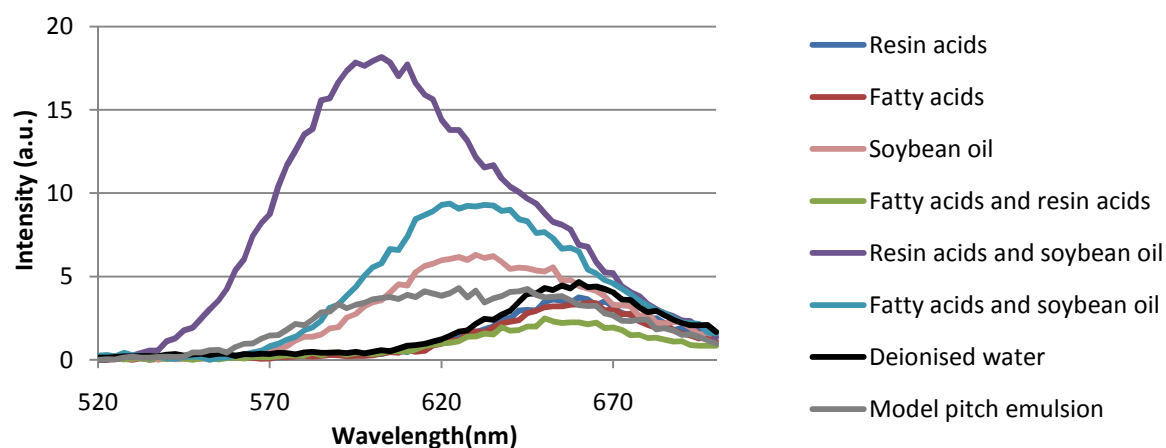

Figure 10: Emission spectra of Nile red (20 ppm, EtOH) in emulsions of fatty acids and triglycerides, ex 488 nm.

The emission spectra of Nile red in emulsions made from fatty acids, resin acids and a mixture of the two are interesting since they largely overlap with the peak from Nile red in pure water. This leads to the conclusion that Nile red has not entered these droplets and thus the fluorescence only comes from Nile red in the water phase. The spectra from the emulsion on pure soybean oil and the emulsion made from soybean oil and fatty acids have the same maximum intensity but Nile red in the mixed emulsion has a higher intensity. Using acid-base titration, a low acid concentration could be measured, probably from fatty acids originating from hydrolysed triglycerides. Using NMR, the fatty acids content was approximated to 10%. Thus, both of the emulsions contain a mixture of fatty acids and triglycerides but the composition and fractions are different. The emulsion made from soybean oil and fatty acids also contain more material and can therefore take up more Nile red and give rise to a higher fluorescence intensity.

The most diverging spectrum is that from the emulsion made of resin acids and soybean oil with a low maximum wavelength and high intensity, compared to the other emulsions. The reason could be an interaction between the aromatic ring in the dehydroabietic acid and the aromatic system in Nile red. Such an interaction could lead to a large change in the spectra since the non-radiative TICT would possibly be avoided and lead to a higher intensity. The broad peak from the model pitch emulsion could indicate a phase separation of the resin acids and the fatty acids.
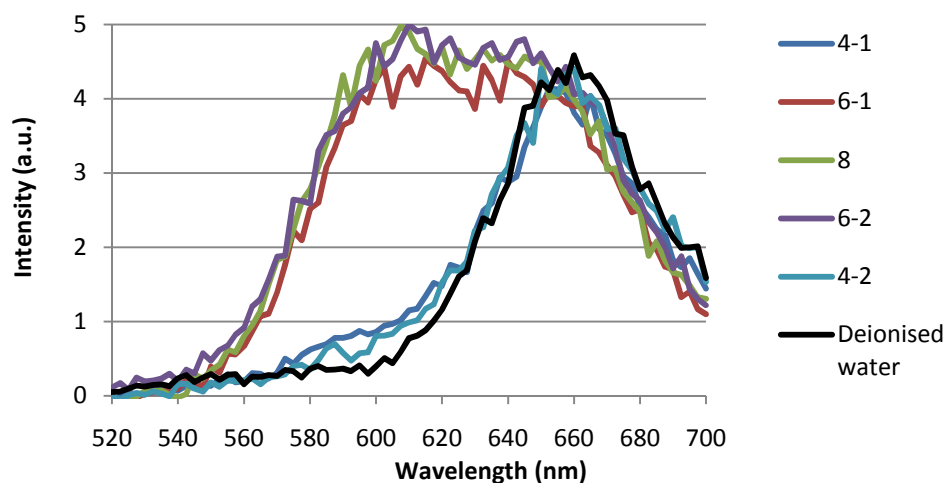
Figure 11: Emission spectra of Nile red in samples from pH-study (Ex 488 nm)

The spectrum from the emulsion made of resin acids and soybean oil also has large similarities with the spectra observed for pH 6 and 8 from the pH-study (Figure 11). Measurements of the emission spectrum of Nile red in samples from the pH-study reveal no difference between pH 4-1 and 4-2. The fluorescence from Nile red in the two pH 4 samples are largely overlapping with the emission spectrum of water, thus indicating that Nile red is mainly in the continuous phase. The emission spectra from Nile red in the samples 6-1, 8 and 6-2 are also similar, constituting a second group. The fluorescence has a large overlap with water, but is much broader and a second peak with maximum at around 600 nm can also be observed. The salt addition does not seem to affect the samples as the spectra from pH 4-1 and pH 4-2 is similar.

The composition of pH 6 and 8 lacks the resin acids, since they dissolve into the water phase, but in similarity with the emulsion made of resin acids and soybean oil, the emulsion at pH 6 and 8 has a surface charge. This leads to the conclusion that the surface charge may have a larger effect compared to the composition on the observed blueshift. Measurements of the surface charge on the component emulsions and the emulsions with different pH may elucidate this relationship.

pH-study on model pitch emulsion

Samples from each pH in the pH-study was extracted and analysed by gas chromatography. The results from the analysis by gas chromatography are shown as the distribution between the dispersed phase (the droplets) and the continuous phase in the model pitch emulsion (Figure 12). The content of the droplets was calculated as the difference between the total emulsion and the filtered phase.
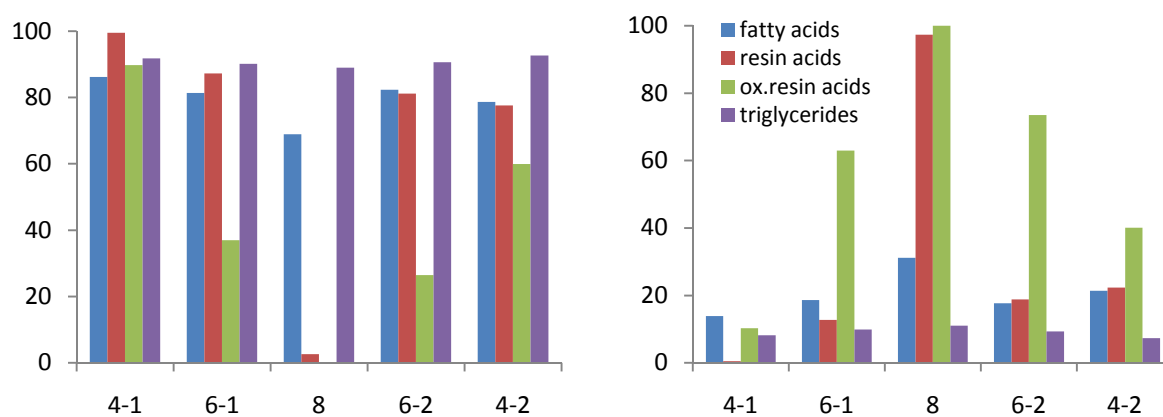

Figure 12: a) Percentage in droplets (dispersed phase) b) Percentage in filtrate (continuous phase)

The components in the model pitch have pK_a's ranging from 5 to 14 (Table 3), and thus the composition of the droplets will change during the pH-study. The resin acids, with pK_a's ranging from 6 to 8, are the components mostly affected in the studied pH-interval. At the first pH-value, 4, denoted 4-1, 99.4 % of the resin acids is in the droplets, whereas at the highest pH, 8, only 2.5 % are in the droplets. This leads to a change of both the composition and the surface charge of the droplets. When returning back to pH 4, the resin acids become uncharged and could either form aggregates of pure resin acids or go back into the droplets with the fatty acids and triglycerides.

Comparison of the levels of resin acids in the continuous and the dispersed phase at pH 4 in the beginning and in the end of the pH-study reveal some difference. At pH 4-1, 0.6 % of the resin acids are in the water phase, but at pH 4-2 22.83 % is in the water phase. This may indicate that some of the resin acids formed small colloids instead of returning to the bigger droplets. However, proof of such colloids could not be found in the flow cytometer measurements, (Figure 14), but only particles with a diameter above 0.25 μm are observed and thus these aggregates may be out of range. The critical micelle concentration, CMC, is 1 mM for a mixture of sodium abietate and sodium oleate, without any salt addition Appendix B. At pH 8, where the highest levels of fatty and resin acids are present in the continuous phase, the maximum concentration only reaches 0.07 mM and is thus far below the CMC.

The levels of fatty acids in the droplets are maintained between pH 4-1 and 6-1, but a decrease is observed at pH 8, which is due to the somewhat higher pK_a's of the fatty acids. When making the same comparison as in the case with the resin acids, a small change before and after the pH-study may be observed, but not as big as with the resin acids. It is thus reasonable to believe that when the fatty acids are protonated they return to the droplets.

The small amount of triglycerides in the filtrate indicates that some droplets may have slipped through the filter and thus some of the resin acids and fatty acids in the filtered sample may also come from droplets containing all of the three components. (Figure 12)

Samples from the pH-study was also analysed by flow cytometry. In Appendix C, the size histograms from the pH-study are shown. As no clear tendency could be seen, the number average volume and the weight average volume were calculated (Figure 13). There is a decreasing tendency in both the number average and the weight average and this tendency can also be seen in the total particle concentration (Appendix D). This could be an indication of deposition of droplets during the pH-study as large particles might deposit to a higher extent and thus leads to a change in average volume of the emulsion.
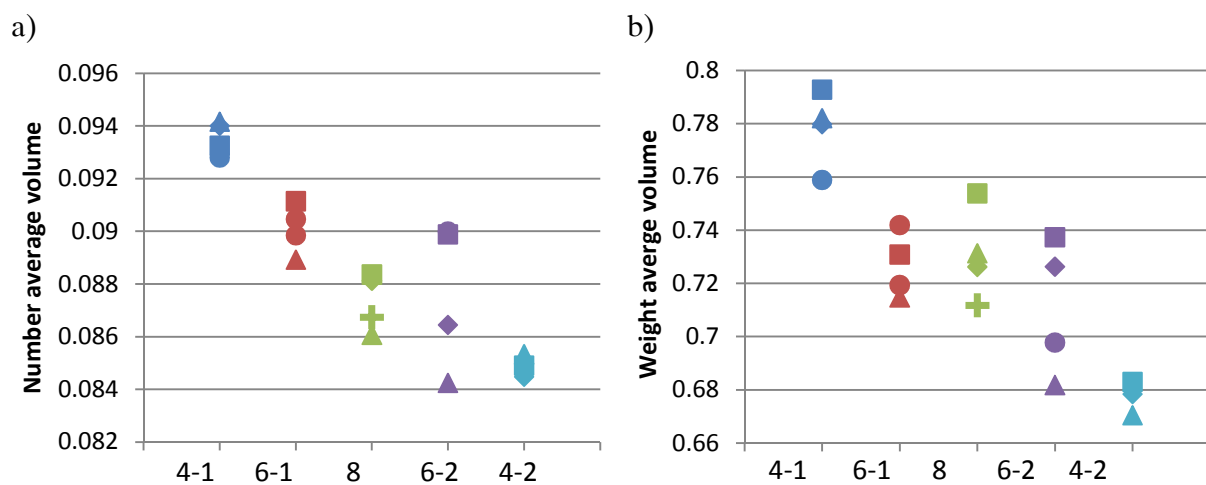
a)

b)

Figure 13: Size distribution from pH-study a) Number average volume b) Weight average volume

At pH 6-2, there is a large variation between the samples and the reason could be that pH 6 is close to the pKa of the resin acids and thus the droplets' composition and charge change fast. On the other hand, the samples at pH 6-1 do not have the same high degree of variation. The time between sampling and measurement also varied more for pH 6-2 than for the other samples and this could lead to more deposition and thus more variation between the samples. According to Stack and co-workers, the deposition is highest around pH 6. (Stack, Stevens, Richardson, Parsons, & Jenkins, 2000)

The density plots in Figure 14 from the pH-study reveal an increase in red fluorescence intensity at pH 8, compared to the other pH-values. From pH 4 to pH 8, both the composition and the charge of the droplets changed and the increase in fluorescence could be a sign of a more hydrophobic environment, caused by that the resin acids and some of the fatty acids have been dissolved. An increased understanding of the fluorescence from Nile red in the droplets may lead to more detailed statements.
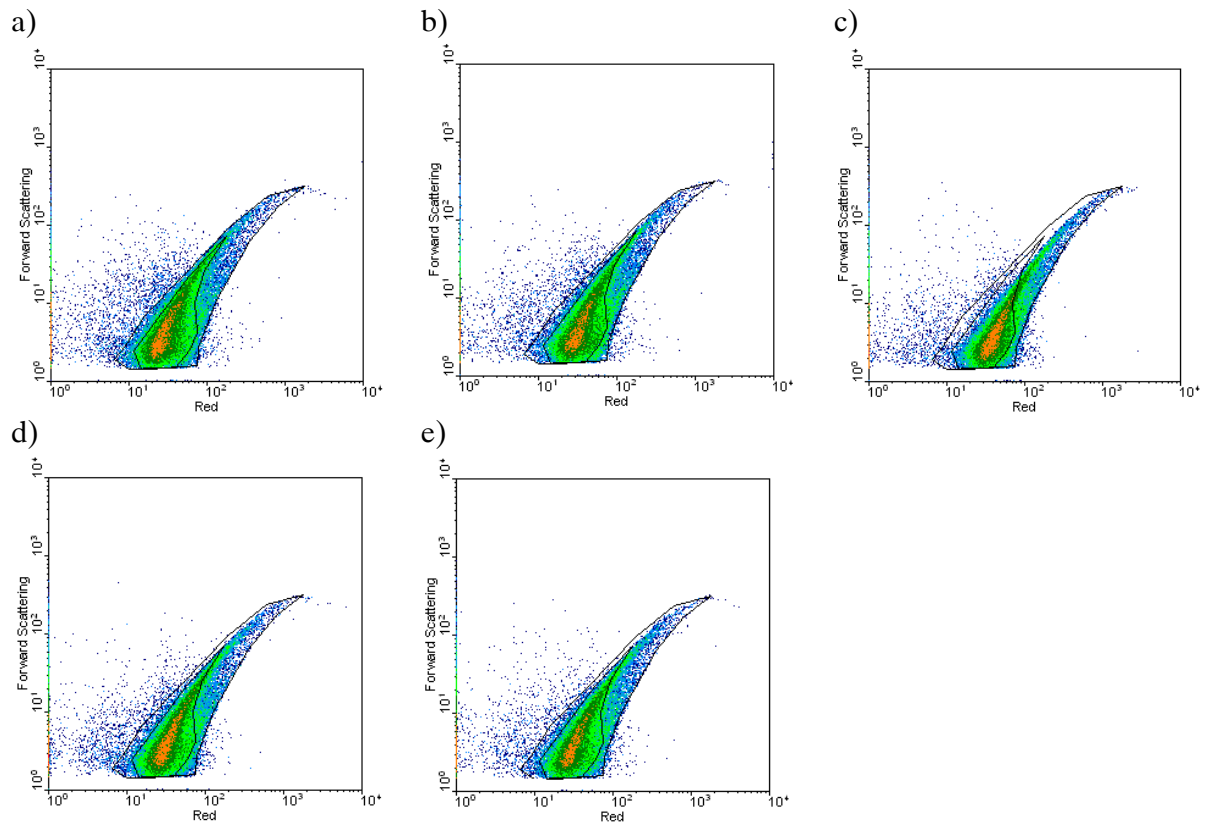
Figure 14: Density plots from pH-study, black lines according to pH4-1, a) pH 4-1 b) pH 6-1 c) pH 8 d) pH 6-2 e) pH 4-2

Conclusions

The conclusion from the investigation of the pH-effect on model pitch emulsion is that the resin acids might form small droplets alone when varying the pH. This was indicated by the distribution of resin acids between the droplets and the water phase.

Some evidence for that the charge of the droplets may affect the fluorescence from Nile to a larger extent than earlier believed was also found. This conclusion was based on fluorescence spectroscopy measurements on component emulsions and model pitch emulsion at different pH.

The earlier hypothesis of droplets with a shell containing the resin and fatty acids and a core with triglycerides was questioned since the three components proved to be miscible in a test tube. In the water-droplet interface it is reasonable to believe that resin and fatty acids are arranged in a monolayer, stretching their hydrophilic parts out into the water, inside this layer, the mixing between all components is probably better than in the earlier proposed hypothesis.

It was also found that ethanol, in which Nile red is dissolved, affects both Nile red and the model pitch emulsion. Investigations showed that when dissolving Nile red in methanol instead, the emulsion is not affected in the same way and thus methanol should be used instead of ethanol as the solvent for Nile red.

Future work

This work has elucidated the use of the probe Nile red to investigate pitch emulsions, but in order to find more evidence for the findings indicated by this work, more investigations are needed.

When using Nile red, the solvent effect must be investigated, since Nile red proved to be very sensitive to solvent effects. In order to measure the effect on the side scattering from the change in refractive index, caused by the solvent, a sheath fluid with the same solvent concentration as the sample could be used.

It must also be investigated how Nile red interacts with the droplets and if the side scattering is changed upon addition of Nile red. This could be studied through addition of very small amounts of Nile red in ethanol or methanol to the sample and measuring the size-change during the addition.

In further exploration of the pH-effect on the model pitch emulsion, the flow cytometer measurements could be combined with surface charge measurements. The size calibration based on the side scattering could also be supplemented with size measurements using dynamic light scattering, where the refractive index of the droplets does not influence the size estimate.

Using 2D-NOESY, it may be possible to determine whether the triglycerides are separated from the other components and reside in a core or if the fatty acids, resin acids and triglycerides are mixed in the droplets inside of a surface monolayer of fatty acids and resin acids.

Flow cytometry measurements of the pH-effect on the resin acid emulsion could lead to an increased understanding of how the pH affects the resin acids and if the resin acids form droplets without the other components after varying the pH. Using the flow cytometer, it could also be investigated how an addition of resin acid emulsion affects the model pitch emulsion.

The critical micelle concentration, CMC, for the mixed solution should also be measured in order to gain knowledge on how this concentration affects the droplets and the solubilisation.

Using a sensitive probe such as Nile red is more informative if more knowledge is gained of how the probe interacts with the system and what quenching and shift effects are the most important in the studied system. It could also be of interest to find and evaluate another probe, less sensitive to the environment. This probe could be used to examine samples where the analysis must be more straightforward and faster. Such a system would e.g. be of interest in paper mills.
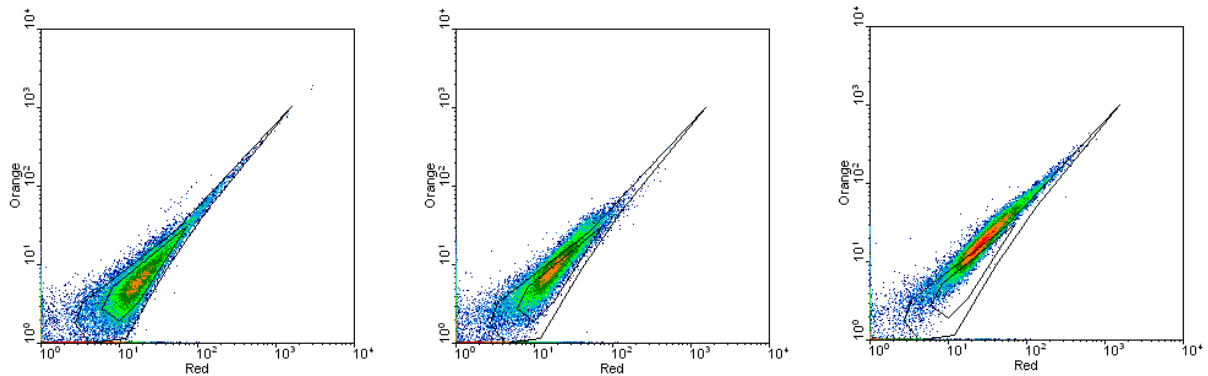
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Appendix A

Figure: Density plots of model pitch emulsion measured 0, 1 and 2 h after mixing
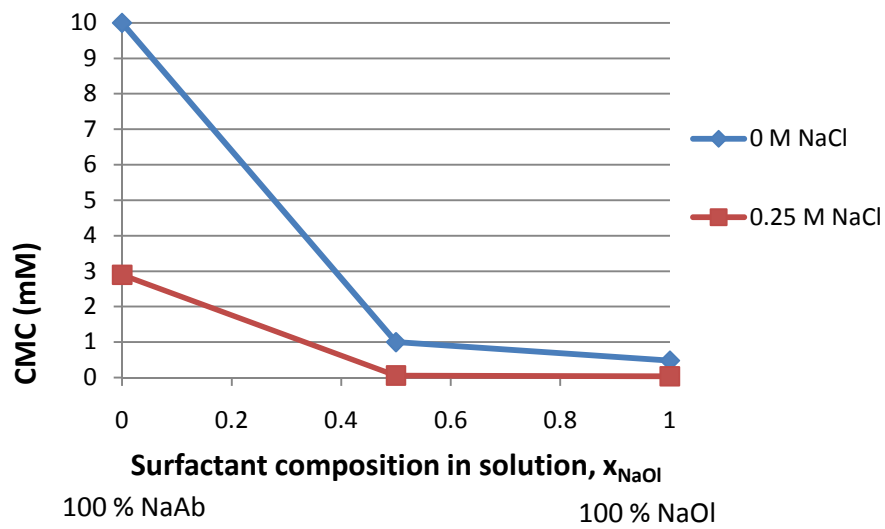
Appendix B

Figure: CMC of sodium abietate and sodium oleate. Adopted from Holmbom and co-workers. (Holmbom, Sundberg, & Strand, 2010)
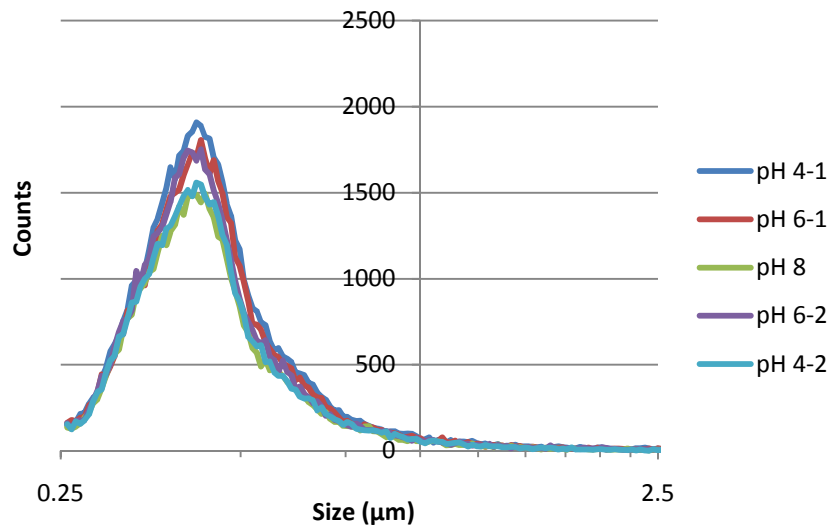
Appendix C

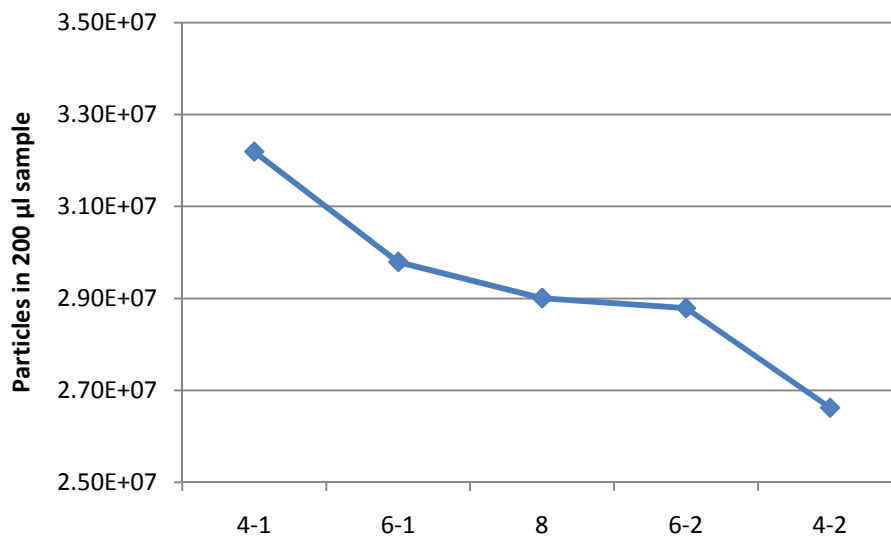
Figure: Size distribution from pH-study

Appendix D

Figure: Number of particles in samples from pH-study