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Method development and optimization for determination of lipid oxidation in emulsions

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Abstract

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This master thesis presents an analytical method used for analysis of oxidation products. The aim of this project was to come up with a method to separate the oil from the water-phase, to a sufficient yield and as pure as possible for analysis in a spectrophotometer called FoodLab fat. During the way experiments regarding stability of oils and finally a stability study on emulsions were done.

Starting the separation experiments with ethanol to break the emulsion, this turned out to be the best way to go. Further investigation through extractions with 2-propanol/isooctane and ethanol/heptane were tested. The method needed to be simple, easy to use and not too time-consuming but still be repeatable and reliable.

To optimize the separation, different centrifugation volumes, forces and times were tested. The results showed that in order to get the best separation the centrifugation volume and force cannot be too large or too small.

The final method proved to be successful for the use as research method and to be able to see trends of oxidation for the products. Further research and validation of the instrument and the method needs to be done before it can be used as a quality control method.

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Populärvetenskaplig sammanfattning

Vanligaste administreringsvägen för parenterala nutritioner (näringslösningar) är intravenöst, direkt i blodet. Med parenteral menas tillförsel av läkemedel eller vätska utan att det passerar mag-tarmkanalen. Parenterala nutritioner ges till patienter som inte har förmåga att få tillräckligt med näring genom oral eller enteral administrering, vid exempelvis sondmatning. I detta fall kan parenterala nutritioner vara livsavgörande.

Emulsioner existerar som en del i parenterala nutritioner men kan också användas såsom ensam byggsten. Emulsioner består av två icke-blandbara vätskor där den ena vätskan är dispenserad som droppar i den andra vätskan. Lipid oxidation är av stort intresse då det påverkar kvalitén hos emulsionerna som kan bestå av allt från en till fyra oljor med olika fettsyra mönster. De olika fettsyramönstren hos oljorna innebär också att de får olika benägenhet till att oxidera. Fettsyrorna kan klassificeras på olika sätt, antingen beroende av längden på kolkedjan, mättnadsgraden eller positionen av första dubbelbindningen. Desto fler dubbelbindningar desto snabbare sker oxidationen. Lipid oxidation består av en kedjereaktion där primära oxidationsprodukter som peroxider vidare oxiderar till sekundära oxidationsprodukter vilka kan mätas genom anisidinvärde.

Syftet med detta examensarbete har varit att ta fram en analysmetod för att separera olje-fasen från vatten-fasen i emulsionerna och vidare bestämma lipidoxidationen. Dessa parametrar bestäms med hjälp av ett FoodLab fat instrument, en spektrofotometer analysera för peroxider, anisidin och fria fettsyror (primära och sekundära oxidationsprodukter). Arbetet delades in i tre delar; i den första delen analyserades fyra oljor under åtta dagar utsatta för extrema förhållanden så som fri tillgång till syre och ljus under omrörning för att iaktta oxidationen. I denna del analyserades även dessa oljor under en fyra veckors period utan att bli utsatta för extrema förhållanden. Den största delen under arbetets gång var att ta fram en separationsmetod som generade ett utbyte av olja på ≥ 40 % samt så ren olja som möjligt för analys. Sista delen utfördes en stabilitetsstudie på emulsioner där den framtagna analysmetoden applicerades.

Målet är att hitta en metod som är simpel, enkel att använda inte för tidskrävande men samtidig repeterbar och pålitlig. Före bestämning av oxidationsstatus måste emulsionerna brytas. Enklast möjliga sätt att bryta emulsionen är tillsats av etanol, vilket senare kom att visa sig vara det bästa sättet att utföra separationen på. Extraktion med 2-propanol/isooktan samt etanol/heptan undersöktes också. I detta fall löser isooktan och heptan upp oljan i organfasen. Detta lösningsmedel måste i sin tur avdunstas innan analys vilket visade sig vara ett tidskrävande och svårt steg som behöver vidare arbete.

Metoden optimerades genom att olika volymer i centrifugeringsrören undersöktes. Förhållandet mellan emulsion och etanol var densamma för alla försök. Olika centrifugeringskrafter och tid var även det något som undersöktes för bästa separation. Det visade sig att volymen i provrören inte kan vara för stor då det medför svårigheter att bryta emulsionen. Det visade sig även att för hög centrifugeringskraft inte bidrog till den renhet på oljan som eftersträvades.

Den slutgiltiga metoden har visat sig fungera bra som forskningsmetod och för att se trender hos produkterna. Mer försök och validering behövs innan metoden skulle fungera som analysmetod för kvalitetskontroll.

List of abbreviation

EFA	Essential fatty acid
FA	Fatty acid
FFA	Free fatty acid
LE	Lipid emulsion
LCT	Long-chain triglycerides
MCFA	Medium chain fatty acid
MCT	Medium-chain triglycerides
MUFA	Monounsaturated fatty acid
NMT	No more than
O/W	Oil in water
PN	Parenteral nutrition
PUFA	Polyunsaturated fatty acid
SMOF	Soybean oil, MCT oil, Olive oil and Fish oil
W/O	Water in oil

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1. Introduction

When oral or enteral nutrition is impossible the parenteral nutrition's can be a lifesaving therapy [1]. Intravenous administration is the most common rout for parenteral nutrition's (PN) [2]. Lipid emulsions exists as a part in PN or as a single component. Emulsions consist of immiscible liquids. One liquid which is dispersed as droplets in the other liquid called the continuous phase [3]. Lipid oxidation is of great concern regarding the quality of emulsions in food as well as in parenteral nutrition's [4].

The first produced PN consisted of amino acids and glucose which led to a series of complications like the deficiency of essential fatty acids (EFAs) [1]. The first parenteral lipid emulsion (LE) was named Intralipid and is based on soybean oil (SO). LE can consist of a variety of fatty acids (FA) from different lipid sources. FAs can be classified in different ways, according to three structural characteristics which affect their physical and physiological properties. One classification is the chain-length, another the degree of saturation and the third classification depends on the position of the first double bond (ω). High number of double bond in e.g. polyunsaturated fatty acids (PUFAs) provides target for lipid peroxidation [5]. The presence of α-tocopherol, an antioxidant present in e.g. olive oil makes it less reactive to oxidize and can be added to PUFA LE. Another way to minimize the oxidation is to use structured LE by replacement of partial PUFAs with medium-chain fatty acids (MCFAs). SMOFlipid is a LE containing soybean oil which provides sufficient amounts of EFAs, medium-chain triglycerides (MCT) which offers rapidly available energy, olive oil which has an immunologically natural effect and fish oil which pursue immunomodulatory and anti-inflammatory effect [1]. When an emulsion contains of SMOF oils it comprises sufficient amounts of α-tocopherol to maintain an antioxidant status and minimize the oxidation and it leads to a well-balanced lipid emulsion.

When immiscible liquids as for emulsions are placed together the formation of two layers will appear with minimum contact with each other. Emulsions are thermodynamically unstable, this because of the need of free energy to increase the surface area between the oil and the water phase [3-4].The droplets will in contact coalesce in order to reduce the total interfacial area and the total surface area. Chemical substances like emulsifiers are added to form kinetically stable emulsions for a reasonable time period. The emulsifier is to prevent the reversion of the separated oil and water phases. They absorb to the surface of the droplets by being surface active and form a protective interfacial film to prevent the droplets to come close and aggregate. Emulsifiers used in parenteral nutrition are preferably phospholipids from egg-yolk, a fatty amphiphile that increase the surface charge of the droplets and increase the droplet-droplet repulsion. The interfacial film may have an important role for lipid oxidation and the orientation of the lipid molecules in relation to the interface will affect the accessibility for reactive oxygen species to attack. The interface is a region of each droplet consisting of oil, water and emulsifier molecules.

Lipid oxidation constitutes of chain reactions that yields primary oxidation products such as peroxides that can further react if extended to oxidation conditions and yield secondary oxidation products like aldehydes, ketones, oligomers and polymers [6]. Lipid oxidation will occur by different pathways; Auto-oxidation (the radical mechanism), photo-oxidation (the singlet oxygen-mediated mechanism) and enzymatic oxidation. The focus of this article will be on the non-enzymatic oxidation.

Auto-oxidation is a spontaneously oxidation which involves the unsaturated lipid and oxygen without the presence of light and catalysts [7]. Activation of either the unsaturated lipid or the oxygen is needed for the reaction to start [7-8]. The reaction involves three stages, initiation (formation of radicals), propagation (formation of hydro peroxides) and termination (formation of non-radical products). The photo-oxidation is started by highly reactive singlet oxygen species. These singlet oxygen species are formed by excitation of triplet molecular oxygen under exposure of light and presence of photosensitizers [6]. Triplet molecular oxygen species is barley reactive with unsaturated compounds, but once it reaches the reactive singlet state by excitation it will readily react with unsaturated compounds [9]. This reaction proceeds when the singlet oxygen reacts directly with the double bond at either carbon at the end of the double bond which yields an allylic hydroperoxide. The secondary oxidation products formed from the primary can be volatile and non-volatile and are formed by complicated sets of reaction paths. The amount of the oxidation depends on the number of double bonds. The π-bond is weaker than the σ-bond due to longer distances between the electrons and the nuclei and this makes the electrons more exposed [10]. Avoiding light, oxygen and heat will minimize the rate of oxidation in the emulsions [11]. The oxidation cannot be fully eliminated however by adding pro-oxidants and antioxidants it will prevent oxidation. Another important part is to know the composition of the fatty acids in the products to know the extent of possible oxidation.

Numerous techniques for determining these oxidation products have been described in literature. It includes spectroscopic methods as well as chromatographic methods [6]. The most common and used method for determining peroxides is iodometry where peroxides will react with iodide ions to generate iodine which is titrated using a solution of sodium thiosulfate and starch as indicator. Secondary oxidation products could be determined by spectroscopy methods, measuring the ρ-anisidine value when the ρ-anisidine reacts with the secondary oxidation products like non-volatile aldehydes. For determining free fatty acids (FFA) which is a mark of hydrolytic rancidity could titration methods be used [7]. By evaluation the oxidation status of the product only by one parameter it will not give a complete result over the oxidation, it requires evaluation of both primary and secondary oxidation products. The peroxide value, ρ-anisidine value and the FFA will give a good indication of the oxidation status of the product [6]. Sample preparation is sometimes needed to extract the lipids which can increase the oxidation and sometimes be tedious.

The methods used for analyzing oxidation products at Fresenius Kabi today are based on titration methods for free fatty acids (FFA) and peroxides [12-13]. The method for analyzing anisidine is based on the Chinese pharmacopeia, analyzed spectrophotometrically at 350 nm [14]. Both analysis for peroxides and anisidine requires freeze drying of samples. Another way of approaching the extraction is the use of different organic solvents in combination or by themselves, centrifugation for separation and drying of solvents under nitrogen gas [15-17].

The aim of this master thesis project was divided in three parts; the main part was to come up with a separation method for emulsions to be used when analyzing for oxidation products with a FoodLab fat instrument. Another part of this project was to look at the stability of oils used in emulsions. This was done in two ways, one by forcing the oxidation with the accessibility of free oxygen and light. The other one by looking at the oxidation when the oils were contained in closed bottles. The final part included a stability study of emulsions.

2. Theory

Theory and principles about the instrument used for the analysis can be read in this section.

2.1 FoodLab fat

The FoodLab fat instrument is an analysis instrument built as a spectrophotometer [18]. It is a simple and useful instrument for analysis of fats and oils to control the quality of the products. The instrument can carry out different test but the one of interest for this project are free fatty acid test, peroxide value test and ρ-anisidine value test.
The instrument consists of 12 cells for incubation of the cuvettes and three reading cells with beams at different wavelength. The incubation block is temperature controlled (37 °C ± 0.5 °C). It is calibrated and controlled automatically after a warm-up period. Pre-filled cuvettes consisting of a mix of alcohols and a cromogenous compound are used.

The principle of the free fatty acid test is that FFAs in the sample with pH < 7.0 will react with the cromogenous compound in the cuvette and decrease its color [19]. The decrease in color is proportional to the concentration of the FFAs in the sample. First a blank value is measured for all samples about to be analyzed. Then the sample is added to the pre-filled cuvette, mixed by shaking and put in the measuring cell for reading and the decease of color is measured. It is measured at 630 nm and the results are expressed as % of oleic acid.

The principle of the peroxide value test is that peroxides oxidize Fe^{2+}-ions to Fe^{3+}-ions that will be grouped and form a red complex [20]. This colorimetric intensity is measured at 505 nm and is directly proportional to the concentration of peroxides in the sample. First the sample is added to the pre-filled cuvettes and mixed by shaking, then a reagent (R2) containing a redox solution is added, mixed again and left to incubate for three minutes before put in the measuring cell for reading. The results are expressed as mekv/kg (milliequivalent of oxygen per kilogram).

The principle of the ρ-anisidine value test is that aldehydes, derived from primary oxidation products such as peroxides will react with ρ-anisidine [21]. A variation in absorbance is determined at 366 nm. The sample is added to the pre-filled cuvettes and put directly in the reading cell for measuring after it has been mixed by shaking. The results are expressed as AnV (anisidine value).

It is important to choose the right sample volume to fit in the right measuring range, since the instrument has different calibration curves depending on the sample volume and what kind of analysis wanted. The methods used for analysis with the FoodLab fat instrument are provided by a correlation with the AOCS (American oil chemists ‘society) official methods, Ca 5a-40 for FFA, Cd 8-53 for peroxides and Cd 18-90 for anisidine [19-21].

3. Experimental

3.1 Chemicals and materials

SmofKabiven, Intralipid 10% and Intralipid 20%, Omegaven and SMOFlipid were purchased from Fresenius Kabi, Sweden. Ethanol (99.9%) and Heptane (96%) were purchased from Fisher Scientific, Sweden. Isooctane (99.0%) and 2-propanol were purchased from Merck, Germany. Sodium sulfate was purchased from Acros organics, USA. Cumene hydroperoxide (89.5%) was purchased from Sigma Aldrich. Extrafluid®, Peroxide kit (peroxides), Anisidine kit (ρ-anisidine) and Free Fatty Acid kit (Acidity) were purchased from CDR, Italy. The oils for analysis were Soybean oil, MCT-oil, Olive oil and Fish oil produced at Fresenius Kabi, Sweden.

3.2 Instruments and apparatus

The analysis of the oxidation products were performed using a FoodLab fat instrument, a spectrophotometer with pre-filled cuvettes for analysis of FFA, peroxides and ρ-anisidine from CDR, Italy. The data were recorded on the instrument. The extraction procedures were mixed on a vortex-genie 2 (Scientific Industries, USA). It was centrifuged on eppendorf 5702, 5418 (Eppendorf, Denmark) and Heraeus megafuge 8230V (Thermo scientific, Sweden). An Excellence plus XP205DR scale (Mettler Toledo, Sweden) was used for weighting and a sample concentrator was used for evaporation (Techne, UK)

3.3 Analysis of oxidation products in oils

Stability analyses of oils were done to prove the theory about the ability of oxidation and for use in new products. It was performed using the FoodLab fat instrument on soybean oil, MCT-oil, olive oil and fish oil. At first to understand how fast the oxidation will increase an experiment with the four oils were performed with access to free oxygen, light and under rotation to force the oxidation. It was performed by analyzing for peroxides, FFA and ρ-anisidine at four points during eight days. After this experiment during a four week period analyses for peroxides, FFA and anisidine were done once a week on the same oils used for new products. These oils were kept in closed bottles and opened only for analyses once a week. After each analysis were the bottles put in a box with nitrogen before they were closed and kept in room-temperature and kept away from light.

3.4 Determination of optimal separation procedure of emulsions

Four separation procedures were performed, all based on liquid-liquid extraction/separation for the purpose to get a yield of oil equal to or more than 40% , but more important to get as pure oil as possible. A summary of the separation procedures can be seen in table 1 and description of the procedures below. The yields for the different methods were calculated by the amount of oil that was expected to be in the volume of emulsions used compared with the actual or weighted amount of oil after separation.
Taken into account were the densities of the oils in the different emulsion. After investigation, the densities for all the oils were nearly one and one was set as density used for calculations. A minimum of 40 % yield was required for the oils to get a good enough result over the oxidation due to experience.

Table 1 shows a summary of the separation procedures tested for the purpose of yielding as pure oil as possible and good enough yield for determination of oxidation products for the emulsions. Emulsions tested for the methods were Intralipid® 10%, Intralipid® 20%, SMOFlipid® and Omegaven®

Method	Separation fluid	Ratio (emulsion: solvent)
1	Ethanol	1:2 & 1:1
2	Extrafluid® (ionic solution)	1:5
3	Ethanol/Heptane (2:1)	1:2
4	2-propanol/Isooctane (2:3)	1:2

3.4.1 Method 1; Ethanol

Method 1 was a simple separation by addition of ethanol to break the emulsion and separate the phases, the oil ended up at the bottom of the vial. When adding ethanol to the emulsion it will change the solubility of the emulsifiers and disrupt the formation of an emulsion, coalescence will happen and the phases will be separated. This method was the one hoped for and therefor the most workup was done for this method. For the separation to appear the tubes were needed to be shaken, vortexed and then centrifuged at different forces and different times depending on the centrifugation force tried.
At lower g-forces the centrifugation time were needed to be longer. It was important that the oil ended up in a sufficient amount and that it was pure. Depending on the volumes used, the water-phase either needed to be removed to be able to reach the oil at the bottom. A pipette was carefully used to go through the upper layer and down to get the oil. To avoid getting water-phase in the pipette when it moved through the water-phase, air was gently pressed out of the pipette to get the oil. The collected oil was weight for yield determination. In table 2 the different volumes tried for the separation can be seen.

Table 2 shows the different tests done for method 1. For the separation with the 15 ml centrifugation tube and the 50 ml centrifugation tube with a total volume of 30 ml a double centrifugation was tried. In some cases the second centrifugation was done after the oil was collected. For the separation with the 50 ml centrifugation tubes with the total volume of 39ml, the difficulty was within the breaking of the emulsion.

Centrifugation tubes	Emulsion	Ethanol	Comments
1,5 ml centrifugation tube	0.4 ml	0.8 ml	
15 ml centrifugation tube	4 ml	8 ml	2 x centrifugation
50 ml centrifugation tube	10 ml	20 ml	2 x centrifugation
50 ml centrifugation tube	13 ml	26 ml	Difficult to break the emulsion with that large volume

3.4.2 Method 2; (Extrafluid®) ionic solution

Method 2 was based on separation with an ionic solution added to the emulsion to break it and separate it into phases. In emulsions there are interfacial components that will introduce charges in a double layer. When there is just the right amount of ions present it will shield the charges and the repulsive forces are dominant. If the ionic strength increases the shielding will be too large and the repulsive forces will decrease leading to dominant van der Waals forces and the emulsion breaks giving separated phases. For the separation to appear the centrifugation tube had to be shaken, vortexed and then centrifuged at 16 900 xg for 5 min. The fat ended up on the top of the vial as a white solid fat. The fat was transferred to a new vial with help of a spatula and then melted under a stream of hot water.

3.4.3 Method 3; Ethanol/Heptane

The extraction with method 3 is based on the separation of phases into water- and organic-phase with ethanol and heptane. The ethanol will change the solubility of the emulsifiers and disrupt the formation of an emulsion and the phases will be separated. The heptane will solve the oil in the organic phase. For this method, the oil was captured in the organic-phase (heptane) and ended up at the top of the vial. The centrifugation tube with the mixed liquids had to be shaken, vortexed and centrifuged at 3000xg at 10 min for the phases to separate properly. The organic-phase was then transferred to a newly weighted centrifugation tube with help of a Pasteur pipette through an open fiber matrix filter paper with sodium sulfate anhydrous to absorb potential water that followed the organic phase. To get rid of the solvent and to get the pure oil, the organic-phase was evaporated under a stream of nitrogen for two hours. After evaporation the centrifugation tubes were weighted again and the yield was calculated.

3.4.4 Method 4; 2-propanol/Isooctane

Method 4 uses the same procedure as method 3 with the difference in what solvent used and the solvent ratio. As for method 3 will the more polar solvent, the 2-propanol change the solubility of the emulsifiers and disrupt the formation of an emulsion and the phases will be separated. Isooctane as the more non-polar solvent will dissolve the oil in the organic-phase.

3.5 Detection of oxidation products

To evaluate for oxidation products, the FoodLab fat instrument was used, see section 2.1 for theory about the instrument.
Since the instrument uses fixed calibration curves a test sample was needed to be analyzed to make sure the correct curve was used. When the right measuring range was determined the analysis as described under section 2.1 could be done.
For FFA, peroxides and anisidine on fat matrixes there were six, seven and one measuring range that could be chosen depending on the oxidation of the sample, see table 3.

Table 3 shows the six intervals to choose of which two were for dilution when analyzing for free fatty acids (FFA), seven intervals of which two were for dilution for peroxide analysis and one for anisidine analysis. The volume depends on the measuring range, preferably a pretest will be needed to know in which measuring range the oxidation of the sample lies within [19-21].

Curve	Measuring range	Sample volume
Acid. 10µl	0.01-0.3 %	10 µl
Acid. 5µl	0.01-0.6 %	5 µl
Acid. 2,5 µl	0.03-1.1 %	2.5 µl
Acid. 1 µl	0.09-3.5 %	1 µl
Acid. dil-100 µl	1-12 %	2.5 µl diluted (100 µl)
Acid. dil-50 µl	5-24 %	2.5 µl diluted (50 µl)
Perox. 50 µl	0.01-3.4 mekv/kg	50 µl
Perox. 25 µl	0.1-5.5 mekv/kg	25 µl
Perox. 10 µl	0.5-11 mekv/kg	10 µl
Perox. 5 µl	0.3-25 mekv/kg	5 µl
Perox. 2,5 µl	1-50 mekv/kg	2.5 µl
Perox. dil-100 µl	4-275 mekv/kg	5 µl diluted (100 µl)
Perox. dil-50 µl	7-550 mekv/kg	5 µl diluted (50 µl)
ρ-anisidine	0.5-100 AnV	20 µl

3.6 Control/verification

The optimized method was verified based on accuracy for the different oxidation parameters and precision with peroxide analysis. The instrument was verified based on a linearity test, accuracy and precision with peroxide analysis. The peroxide results were given as mekv/kg, equation 1 shows the conversion of mM to mekv/kg.

Equation 1 Conversion of mM to mekv/kg, density of MCT-oil ≈ 0.945 [10]

$$C_{po,\frac{mmol}{L}(mM)} = \frac{C_{po,mekv/l}}{2} = \frac{C_{po,mekv/kg} \times \rho_{MCT-oil}}{2}$$

3.6.1Linearity test

MCT-oil used as zero-sample/standard solution due to the almost non-existing oxidation of the oil was spiked with cumene hydroperoxide 84.8%. The linearity test was done based on analysis method, Perox. 2.5 µl with a range between 1-50 mekv/kg.
The MCT-oil was spiked with cumene hydroperoxide to obtain different concentrations and single analysis of each concentration was analyzed.

3.6.2 Accuracy and precision

For the instrument, MCT-oil was spiked with cumene hydroperoxide 89.5% at different concentration. For within-day precision duplicate samples were analyzed and the coefficient of variation, CV (%) was calculated to be≤ 20%. For accuracy the samples were analyzed as they were unknown and the relative deviation was calculated with an acceptable limit of ≤ 20%.

For the method, multiple fat separations of emulsions were performed. The yields and the peroxide values from analysis were used to decide the within-day precision of the method. Single analysis of each separation experiment was done and the CV (%) needed to be≤ 20%. The accuracy of the method was determined by comparing values of oxidation parameters obtained from different extraction methods. The oxidation parameters were determined using the FoodLab fat and validated methods used for quality control. The deviation could not be more than ≤ 20%.

The acceptable thresholds are based on ICH guidelines for impurities and with modifications adjusted to Fresenius Kabi products [22-23].

3.7 Determination of lipid oxidation in parenteral nutrition's

A stability study of emulsions (Omegaven, Intralipid 20% and SMOFlipid) stored at 25°C, 40 % humidity, 30°C, 25% humidity and 40°, 25% humidity for determining of oxidation products was performed with method 1, 50 ml centrifugation tube. To a 50 ml centrifugation tube 10 ml emulsion and 20 ml ethanol were added. The emulsions were analyzed initially after one and three month. Duplicate samples were analyzed for all oxidation products (FFA, peroxides and anisidine). The analyses were performed as described in section 2.1 and 3.4.

New product emulsions containing of soybean oil, MCT-oil, olive oil and fish oil (see section 3.3) and new API were analyzed as for the stability study of emulsions above.

4. Results and discussion

This section will show the results connected to the methods and the aim. The best suitable separation method for analyzing of oxidation products with the FoodLab fat instrument along with important details regarding the analysis will be covered. It will cover the study over the stability of oils under different conditions and it will show the result for the stability study of emulsions.

4.1Trends over oxidation of oils

To prove the theory that the amount of double bonds in the fatty acids will have an effect on the oxidation an experiment with soybean oil, MCT-oil, olive oil and fish oil were done during eight days. The oils were kept in four bottles and samples from each bottle were analyzed during day one, three, five and eight. During the time the oils were continuously under stirring with free access to oxygen and light. The results are expressed as percentage of threshold values for the different oils according to internal specification. The threshold values are the highest acceptable oxidation that can be in oils used for production. If that value are exceeded the oil cannot be used. As seen in figure 1 over the results, the peroxide values of fish oil and soybean oil were exceeded. The anisidine threshold value for olive oil, soybean oil and MCT-oil was not reported, presumably because of the low primary oxidation in the oils when they are not stressed. Appendix 1 shows the analysis values and thresholds.
The results are consistent with the theory and there was no surprise that the peroxide values for fish oil will increase the most due to the great amount of double bonds. This was a good knowledge for further analysis of emulsions containing the different oils.

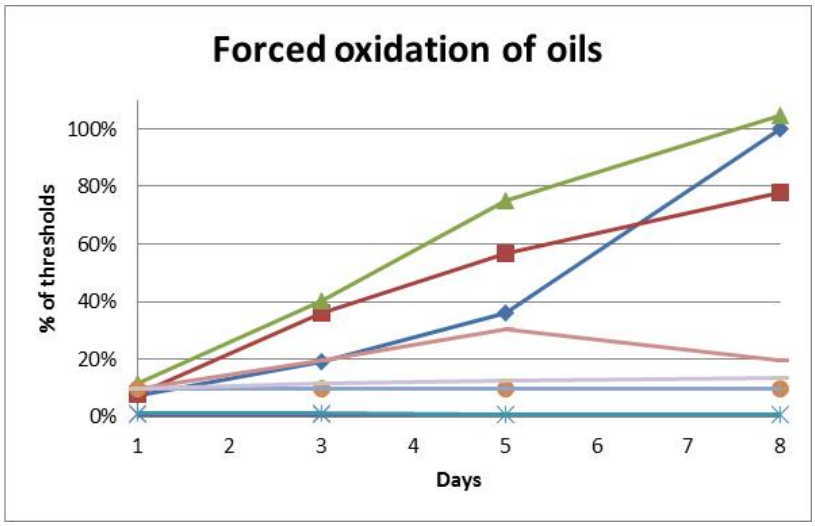

Figure 1, this graph shows the results of the forced oxidation of the SMOF oils regarding peroxide values, FFA values and anisidine values. The values are mean values of six or three replicate. Peroxide values of fish oil (dark blue, x7) with a distribution of 2-3%. Peroxide values of olive oil (dark red) with a distribution of 1-4%. Peroxide values of soybean oil (dark green, x3) with a distribution of 1-11%. Peroxide values of MCT oil (dark purple) with a distribution of 0%. FFA values of fish oil (light blue) with a distribution of 0-28%. FFA values of olive oil (orange) with a distribution of 0%. FFA values of soybean oil (light purple) with a distribution of 0%. FFA values of MCT oil (pink) with a distribution of 0-35%. Anisidine values of fish oil (light green) with a distribution of 0-6%. The results are expressed as the percentage of the threshold values. The thresholds are exceeded for fish oil and soybean oil for the peroxide analysis. The anisidine threshold for the olive oil, soybean oil and MCT-oil was not reported. Some distribution values became high due to very low values or values below the measuring range of the curve for the analysis. The smallest difference between values will result in a high distribution

The second part of analyzing these oils was done under different conditions than the forced ones and during a longer time-period. During a four week time period the oils were kept in

two closed bottles. One of the bottles was opened for analysis once a week and the second bottle were opened for the last analysis. This was to see how the opening and closing of the bottle during analysis will affect the oxidation and at the end production of new emulsions. Between the analyses time the bottles were kept closed from light and oxygen. The results are expressed as percentage of threshold values for the different oils. Figure 2 shows the results were the threshold for peroxide value of fish oil are close above limit. Appendix 2 shows the analysis values and thresholds, the only threshold exceeded was the peroxide value of fish oil at the last time point. There was no sufficient difference in the results when analyzing the two different bottles containing fish oil, the one only opened for the last time point and the one opened for every time point. The difference in analysis result was 0.04 mekv/kg. That resulted in a percentage difference between 101% and 102%. Both values exceeded the threshold limit, see results in appendix 2. Even if the bottles were kept under controlled conditions the oxidation of fish oil is relatively rapid compared to the other oils. Keeping the oils at four weeks will be a too long time-period to be able to keep the oxidation below threshold limits.

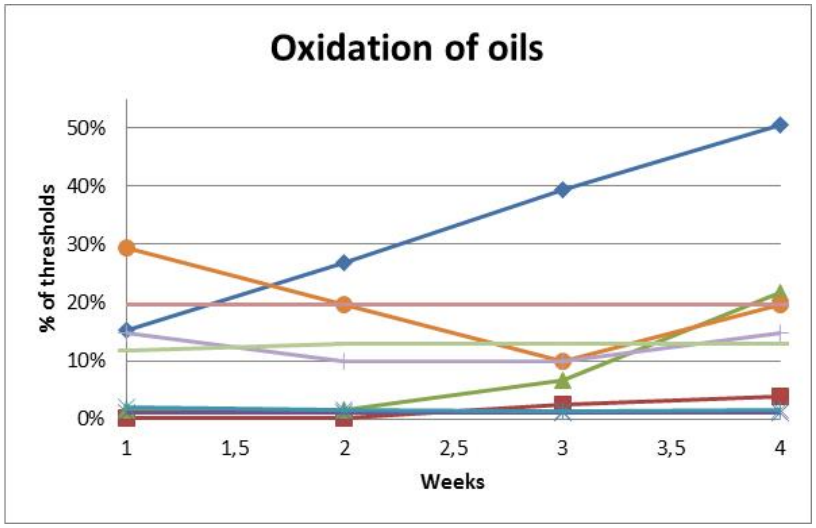

Figure 2, this graph shows the result of the oxidation of the SMOF oils regarding peroxide values, FFA values and anisidine values. The values are mean values of duplicate samples. Peroxide values of fish oil (dark blue, x2) with a distribution of 0-2%. Peroxide values of olive oil (dark red) with a distribution of 0-23%. Peroxide values of soybean oil (dark green) with a distribution of 0-43%. Peroxide values of MCT oil (dark purple) with a distribution of 0%. FFA values of fish oil (light blue) with a distribution of 0-20%. FFA values of olive oil (orange) with a distribution of 0%. FFA values of soybean oil (light purple) with a distribution of 0-33%. FFA values of MCT oil (pink) with a distribution of 0%. Anisidine values of fish oil (light green) with a distribution of 0-4%. The results are expressed as the percentage of the threshold values. The thresholds are exceeded for fish oil at the last time point for the peroxide analysis. The anisidine threshold for the olive oil, soybean oil and MCT-oil was not reported. Some distribution values became high due to very low values or values below the range of the curve for the analysis. The smallest difference between values will result in a high distribution

4.2 Optimization of conditions for separation methods

The optimization of separation methods were done to determine which separation method that will be suitable for analysis. A comparison of the methods optimized will be needed and an important point will be to keep the oil free from water and solvent.
The separation steps were optimized in order to get a minimum of 40 % yield of as pure oil as possible. A minimum of 40 % yield will be enough to give a fair picture over the oxidation according to experience. With a yield of oil below 40 % there is a risk of not getting the whole oil droplet for analysis and the results will be uncertain. There is a risk of only getting the outer layer containing the interface of the droplet. If any water or solvent remains in the oil it may interfere with the analysis due to the high sensitivity of the instrument. There is highly important that the oil does not contain any water or solvent. Different optimized conditions were studied for the methods, and the separation conditions tested were different

centrifugation volumes and forces. Filtration with sodium sulfate through an open fiber matrix filter paper compared with no filtration.

4.2.1 Conditions used for Method 1

For method 1, has centrifugation tubes with different volumes been tested, 1.5 ml, 15 ml and 50 ml. The same ratio was used regarded the volume of the centrifugation tube, emulsion: ethanol (1:2). Different centrifugation forces between 3000-9000 xg were investigated for the optimal separation. Since ethanol was used as the only separating agent there was no need of evaporation due to the fact that ethanol is a highly polar solvent and supposed to end up in the water-phase. The conditions tested resulted in different yields, shown in table 4. The reason of a variation in the yield can be the difficulty of getting the oil at the bottom of the centrifugation tubes without getting any of the water-phase. It is highly important that the oil does not contain any water or solvent and keep it as pure as possible. To be safe, some oil was left at the bottom and as long as it visually could be seen that the yield were more than 40 % this could be done and a variation of the yield could be seen.
When separating in 1.5 ml centrifugation tube the oils were pooled to get enough for analysis for all three oxidation parameters (FFA, peroxide and anisidine).

Table 4 shows the different conditions tried for method 1. Different volumes of centrifugation tubes and centrifugation forces resulted in different yields. The yields were based on multiple experiments at different time points and the range of mean values is seen in the table.

Centrifugation tube (ml)	**Centrifugation-force (g)**	**Yield (%)**	**Centrifugation-time (min)**
1.5	8600	52-76	10
15	3000	12-27	10
15	8000	61	10
50	8000	72-75	10
50	9000	*	10
50	7000	*	10
50	6000	*	15
50	5000	*	20
50	4000	*	17
50	3000	*	25

* = visually it could be seen that the yield were over 40% and that was the minimum goal, thereby there were no yield calculations done.

4.2.2 Conditions used for Method 2

For method 2 were 1.5 ml tubes used with a centrifugation force at 16900 xg and the ratio, emulsion: Extrafluid® (1:5). Only the Extrafluid® was used as extraction solution to break the emulsion and get separated phases. The solid white fat that ended up at the top was needed to be transferred to a new vial for melting. This step during the separation was difficult because of how easy the water-phase followed the transfer of the fat to the new vial. Therefore could no yield calculations be done and the analysis could not be performed
Centrifugation tubes of 1.5 ml were the only one tested and due to the poor result and difficulty of performing the separation no more centrifugation tubes with other volumes were tested.

4.2.3 Conditions used for Method 3

For method 3 were the centrifugation tubes of 15ml used throughout the separation. The same centrifugation force and ratio between emulsion and extractant solution has been used, emulsion: ethanol/heptane (1:2). This resulted in a two phase-separation were heptane will capture the oil in the organic-phase. The organic-phase were transferred to a newly weighted tube and kept under a stream of nitrogen for two hours to evaporate the solvent. The transfer of the organic-phase could be done either through filtration with anhydrous sodium sulfate or without filtration in which resulted in different yields, see table 5. A yield above 100 % indicates that solvent still remains and the oil will not be pure enough for analysis, the results will be uncertain due to potentially interfering solvents

Table 5 shows the difference in yield when extractions with ethanol/heptane were done with the use of filtration and no filtration of the organic-phase before evaporation. The yields were based on multiple extractions at several occasions and the ranges of the mean values are seen in the table.

Filtration	Centrifugation (g)	Yield (%)
Yes	3000	96-100
No	3000	101-117

4.2.4 Conditions used for Method 4

For method 4, see section 4.2.3 the only difference compared with method 3 was the extraction solution of 2-propanol: isooctane (2:3), the results are shown in table 6. As describe above, a yield above 100 % indicates that there is still solvent remaining. For this method a larger amount of organic solvent were used, which means that the evaporation may have had to be longer and that could be a reason that even with filtration the yield is above 100 %. A high yield above 100 % indicates on still remaining solvent and the oil will not be pure enough for analysis.

Table 6 shows the difference in yield when extractions with 2-propanol/isooctane were done with the use of filtration and no filtration of the organic-phase before evaporation. The yield was based on one single extraction.

Filtration	Centrifugation (g)	Yield (%)
Yes	3000	102
No	3000	100

4.3 Selection of separation method

To determine which separation method that will give a good enough yield above 40 % of pure oil and be as easy, environmentally friendly and effective as possible a comparison of methods were done. The most important thing when analyzing with the FoodLab fat instrument were the purity of the oils. Any solvent remaining in the oil can affect the results and give an inaccurate result. When this was taken into account and that the acceptable yield was more or equal to 40%, the separation procedure chosen were method 1 with 50 ml centrifugation tubes with a total volume of 30 ml, centrifuge at 7000 g for 10 min. No test for determining the purity of the oil was done. Based on the results for all methods was method 1 the most appropriate method to use.

4.4 Linearity test

A linearity test was done to investigate the possibility of doing a calibration curve for validation. From the results of the analysis of the standard solution sample with MCT-oil spiked with cumene hydroperoxide a linearity curve was constructed, see figure 3. It was constructed based on the obtained amount of peroxides from analysis in mekv/kg and the calculated amount of peroxides in mekv/kg. The choice of concentration interval was based on the measuring range, in this case was the interval 1-50mekv/kg. The linear regression with correlation coefficient at 0.9995 indicates on an acceptable linearity. Three standard solutions samples were prepared at concentrations of 3.13, 6.25 and 15.64 mM these concentrations were recalculated with equation1, to mekv/kg and that resulted in 6.07, 12.14 and 30.37 mekv/kg. After analysis the results were 8.91, 18.12 and 42.83 mekv/kg. There are a major difference between the calculated and the obtained values most likely due to interference from solvents further described in section 4.5.2, but still there was an adequate linearity for the test. With this experience it will be possible of doing a calibration curve for validation. More research of cumene hydroperoxide will be required before a full validation can be done.

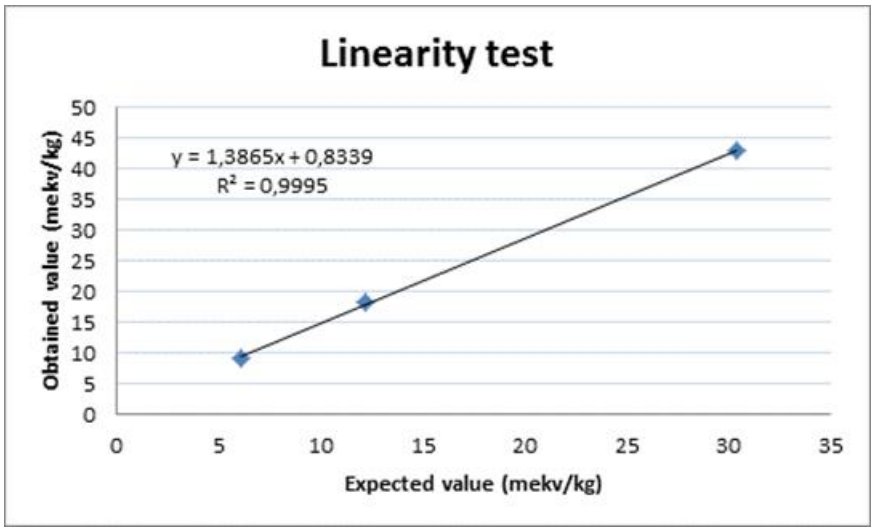

Figure 3 present the linearity test for analysis method: perox. 2.5 µl with measuring range between 1-50mekv/kg. The linearity test was based on MCT-oil spiked with different amount of cumene hydroperoxide yielding 30.37, 12.14, 6,07mekv/kg. When analyzed the results were 42.83, 18.12, 8.91 mekv/kg. Single analysis on each sample was done.

4.5 Accuracy and precision

Determination of accuracy and precision for the system and the method were done to assure that the results will be accurate and to investigate the possibility of doing a complete validation. The standard solution samples with MCT oil spiked with cumene hydroperoxide to obtain different concentrations at 3.30, 6.60 and 16.50 mM for system accuracy and precision. For method accuracy and precision multiple analysis with the selected separation method were done and comparison of oxidation parameters with different analysis techniques.

4.5.1 Method

The results when analyzing for method precision are shown in table 9. A total of six separations were done with the chosen separation method 1 and the yield was calculated based on oil that could be separated from the emulsion with the weighted mass of separated oil. The separated oils were analyzed for peroxide value and the coefficient of variation was calculated for both the yield and the peroxide value shown in table 9. The coefficient of

variation for both the yield and the analysis were satisfactory meaning that the separation method was stable along with the analysis.

Table 9 shows the within-day precision for method 1. The same emulsion was separated with method 1 and a total of six experiments were done. The yield was calculated and the oils were analyzed for peroxide values. The coefficient of variation is within acceptable limits of ≤ 20%

	Weighted mass (g)	Expected mass (g)	Yield (%)	Peroxide analysis (mekv/kg)
Average	1.6	2	**78%**	**0.19**
CV (%)			**5%**	**10%**

The results when analyzing for method accuracy are shown in table 10. The accuracy was calculated as the relative deviation (%) and the results are all based on average of duplicate samples. The FoodLab fat and the validated methods used for quality control were the methods compared. All values can be seen in appendix 3. The relative deviation was not what hoped for, usually when doing a comparison of methods more measurements than duplicate samples will be used. The values are either too high above the acceptable limit or right on the limit, see section 3.6.2. Looking at the accuracy for the method when analyzing for FFA when compared the oxidation values from the analysis with FoodLab fat and the validated method used for quality control the deviation is in acceptable limits. It was not optimal to compare the analysis results between two completely different types of methods. The analysis depends on different methods along with the sample preparations that may give different results.

Table 10 shows the accuracy for method 1. The same batches of samples were analyzed with the FoodLab fat instrument and the quality control methods. Duplicate samples were analyzed and the average calculated. One value belongs to one batch. A mean value from the average values was calculated and the relative deviations were based on the difference between the average value and the mean value. The relative deviation values are the same for both methods as seen in the table below. The acceptable limits were ≤ 20%

	Peroxide analysis	FFA analysis	Anisidine analysis
Relative deviation for the methods (%)	53	17	35
	51	19	27
	15	13	26
	80	19	28
	4	11	30
	-	7	14
	-	14	30
	-	20	31
	-	20	39

4.5.2 System

The results when analyzing for system accuracy and precision with the relative deviation between the obtained value and the expected value are shown in table 11 along with the coefficient of variation of the within-day precision. The accuracy of the system is not what expected but two results were close to the acceptable limit but in the upper region in which was not satisfactory. See the acceptable limit in section 3.6.2. One reason for the deviating results could be that the cumene hydroperoxide solution is around 80-90 % and contain solvents that might interfere with the analysis on the FoodLab fat, still it will make a linear curve with a correlation coefficient of 0,9916. No more investigations about the interference

with the analysis were done but will be needed before a complete validation can be set. The relative standard deviations for the three samples analyzed for within-day precision lies within acceptable limits, se section 3.6.2. Indicating on that the pipetting technique was repeatable.

Table 11 shows the accuracy and precision for the system. MCT-oil spiked with cumene hydroperoxide at different concentrations was analyzed and that results were compared with the expected giving a relative deviation. The coefficient of variation was calculated based on duplicate samples. Acceptable limits were ≤ 20%

Concentration, cumene hydroperoxide (mM)	Obtained value (mekv/kg)	Expected value (mekv/kg)	Relative deviation (%)	Within-day precision CV (%)
16.50	42.56	32.04	25	1.23
6.60	19.43	12.82	34	0.08
3.30	7.81	6.41	18	4.35

4.6 Analysis of lipid oxidation in parenteral nutrition's

The stability study was done to see if the different emulsions, containing different lipid sources will be affected by temperature and humidity. Intralipid 20%, Omegaven and SMOFlipid were analyzed with the selected separation method, see section 4.3. Figure 4 shows the results for emulsions stored at 25°C and 40 % humidity. Figure 5 shows the results for emulsions stored at 30°C and 25% humidity. Figure 6 shows the results for emulsions stored at 40 °C and no more than (NMT) 25% humidity. The results are expressed as the percentage of the threshold values for the different emulsions. The threshold values are the highest acceptable oxidation that can be in emulsions to guarantee the patients safety. If that value are exceeded there is no longer a guarantee for the patients.

The oxidation in the emulsions after three month presents to be below the threshold values. The analysis were done on duplicate samples were the mean value was calculated. The results are for a majority of the analysis at the lowest point in the linearity curve which makes the values very low and the smallest deviation between the values will give a large distribution value. Because the analysis results lie in the lowest part of the range it will be hard to get an accurate value. The method can still reveals trends for the oxidation over time. In appendix 4 can the mean values be seen as well as the threshold values for the emulsions.

An interesting observation was the peroxide curves for Omegaven. Omegaven contains fish oil which in section 4.1 were seen to be the oil with the most accelerated oxidation. Even when the oils are in emulsions the theory about the oxidation remains.

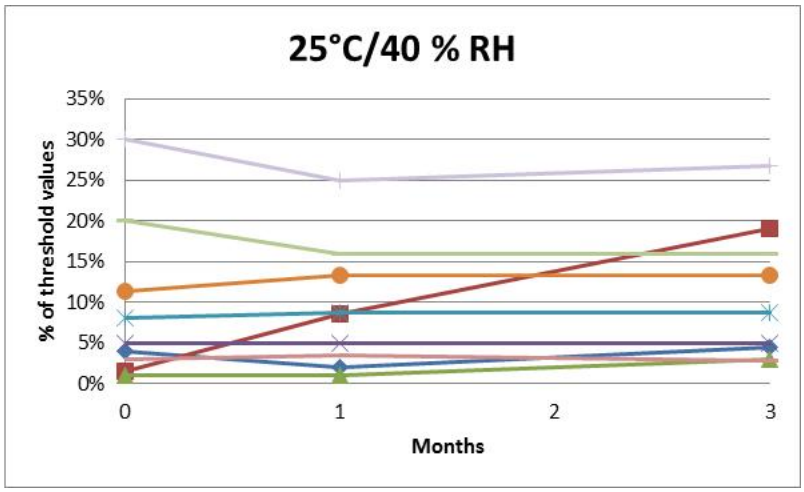

Figure 4, this graph shows the results over the oxidation of the stability study for the emulsions kept at 25 °C and 40 % humidity. The values are mean values of duplicate samples. Peroxide values of Intralipid 20 % (dark blue) with a distribution of 25-50%. Peroxide values of Omegaven (dark red) with a distribution of 5-33%. Peroxide values of SMOFlipid (dark green) with a distribution of 0-67%. FFA values of Intralipid 20% (purple) with a distribution of 0-33%. FFA values of Omegaven (light blue) with a distribution of 0-33%. FFA values of SMOFlipid (orange) with a distribution of 0%. Anisidine values of Intralipid 20% (light purple) with a distribution of 0-20%. Anisidine values of Omegaven (pink) with a distribution of 0-7%. Anisidine values of SMOFlipid (light green) with a distribution of 0-12%. The results are expressed as percentage of threshold values. Some distribution values became high due to very low values or values below the range of the curve for the analysis. The smallest difference between values will result in a high distribution.

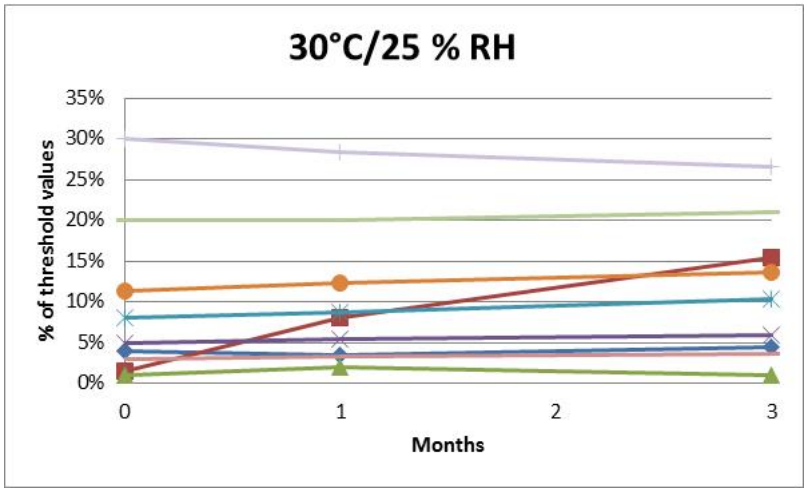

Figure 5, this graph shows the results over the oxidation of the stability study for the emulsions kept at 30 °C and 25 % humidity. The values are mean values of duplicate samples. Peroxide values of Intralipid 20 % (dark blue) with a distribution of 25-71%. Peroxide values of Omegaven (dark red) with a distribution of 0-33%. Peroxide values of SMOFlipid (dark green) with a distribution of 0-50%. FFA values of Intralipid 20% (purple) with a distribution of 0-9%. FFA values of Omegaven (light blue) with a distribution of 0-1%. FFA values of SMOFlipid (orange) with a distribution of 0-3%. Anisidine values of Intralipid 20% (light purple) with a distribution of 0-6%. Anisidine values of Omegaven (pink) with a distribution of 0-18%. Anisidine values of SMOFlipid (light green) with a distribution of 10-14%. The results are expressed as percentage of threshold values. Some distribution values became high due to very low values or values below the range of the curve for the analysis. The smallest difference between values will result in a high distribution.

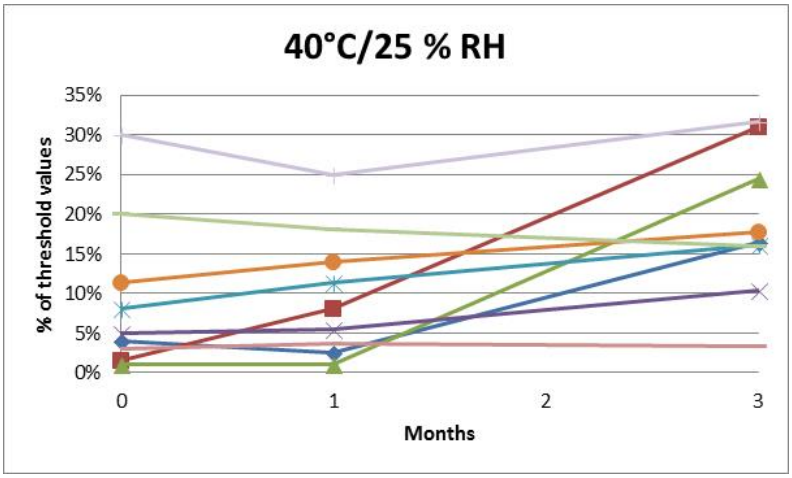

Figure 6, this graph shows the results over the oxidation of the stability study for the emulsions kept at 40 °C and NMT 25 % humidity. The values are mean values of duplicate samples. Peroxide values of Intralipid 20 % (dark blue) with a distribution of 21-60%. Peroxide values of Omegaven (dark red) with a distribution of 13-38%. Peroxide values of SMOFlipid (dark green) with a distribution of 0-50%. FFA values of Intralipid 20% (purple) with a distribution of 0-5%. FFA values of Omegaven (light blue) with a distribution of 0%. FFA values of SMOFlipid (orange) with a distribution of 0-2%. Anisidine values of Intralipid 20% (light purple) with a distribution of 0-7%. Anisidine values of Omegaven (pink) with a distribution of 0-7%. Anisidine values of SMOFlipid (light green) with a distribution of 10-12%. The results are expressed as percentage of threshold values. Some distribution values became high due to very low values or values below the range of the curve for the analysis. The smallest difference between values will result in a high distribution.

New product emulsion containing the oils from section 3.3 and 4.1 was analyzed with the selected separation method from section 4.3. The new product emulsions contained different composition of oils and new API, they were analyzed before and after sterilization in an autoclave. The values for the oils are based on the analysis form the first week, when they were first opened, see section 4.1. Table 12 shows the results over the oxidation.

The results showed that the peroxide values for the autoclaved emulsions were lower than the ones not autoclaved. The opposite result was shown for the anisidine and FFA analysis. When products are autoclaved they are exposed to high temperatures which will affect the oxidation. A reason can be that the oxidation has been accelerated and the primary oxidation products have been transferred to secondary oxidation products. That can be a reason that the FFA and anisidine value be higher for the autoclaved products.

Table 12 shows results of the oxidation in SMOF oils from section 4.1 and new product emulsion. For the new product emulsion there were one with different composition of oil and one with new API. They were analyzed before autoclaving and after. The values are based on mean values of duplicate samples and the values for the oils are from analysis week one.

Oil/Emulsion	Peroxide value (mekv/kg)	FFA value (mekv/kg)	Anisidine value (AnV)
Soybean oil	< 0.01	0.53	0.55
MCT-oil	< 0.01	0.71	0.60
Olive oil	< 0.01	1.06	2.10
Fish oil	1.54	1.06	2.35
SMOF 20 (autoclaved)	0.37	2.48	2.2
SMOF 20 (not autoclaved)	0.88	0.71	1.4
SMOF new API (autoclaved)	0.1	2.83	1.8
SMOF new API (not autoclaved)	0.54	0.53	1.3

5. Conclusion

An analytical method has been developed with the aim to separate the oil-phase from the water-phase in the emulsions and to be as sufficient, reliable and time effective as possible. For the method to be used as a quality control method a full validation of the method and system will be needed. As for now the method can be used as research method and for investigating trends for emulsions and oils regarding oxidation.

According to the tested verification parameters, the linearity test showed a linear correlation which makes the analysis reliable along with the within-day precision for the system indicating on a reliable pipetting technique. Looking at the accuracy for the system it was not as expected and another way of doing that verification will be needed or more research about the used cumene hydroperoxide. There is still some evaluation and optimization to do when looking at the method accuracy in which the values lies over the acceptable limits or right at the limit. The mean value of the analysis results between the methods are based on mean values of duplicate sample analysis within each method. It is not optimal to calculate a mean value of mean values and usually more than duplicate sample analyses are needed. Another important point is that the analysis methods used for quality control depend on different principles than the FoodLab fat instrument. They also have different sample preparation before analysis. All these aspects are needed to take into account when discussing the outcome of the results. Regarding the method within-day precision the results showed a reliable method indicating on a stable sample preparation.

The instrument turned out to be very sensitive when it comes to the purity of the oil and the volume of sample analyzed depending on the measuring range chosen. This was especially notable when doing the analysis of the system accuracy using 80 -90% cumene hydroperoxide. The values were far from the expected and one reason can be that the solvent in the cumene hydroperoxide, the remaining 10-20% interfere with the analysis.
Another way of confirming the sensitivity of the instrument due to pure oil can be seen when looking at the distribution. The distribution is higher for the majority of oils analyzed after separation from emulsion (stability study) compared to analysis of pure oil (stability test for oils). There is a possibility that the oils from sample preparation have some interfering component. Could potentially be residues from the water-phase.

The analysis of oxidation parameters in oils confirmed the theory about the pattern of fatty acids, the more double bonds present the easier the oxidation will happen. Even when the oils are kept in closed bottles away from light it will oxidize. It is the fish oil that needs the most control to avoid using it with exceeded thresholds.

The results from the three months stability study showed that the emulsions are still stable, and reliable due to that the oxidation level is below the thresholds. Results that exceed 20% of the threshold value for all analysis could be a guideline for further control and investigation. As can be seen from the graphs, the values below 20% are relatively stable. Notable is the peroxide curves for Omegaven containing fish oil that will keep increasing.
Because of the minimal oxidation in the emulsions, will the analysis result of the oxidation products end up at the bottom of the linearity curve which may cause results to fluctuate.
It is difficult to get a precise value when the results lie within the lowest point of the linearity curve.

The results from the analysis of newly produced emulsions before and after sterilization in an autoclave showed that the peroxide values were higher before sterilization and FFA and anisidine were higher after sterilization. A reason can be that the formation of secondary oxidation products from primary oxidation products will happen faster when the products were autoclaved and thereby exposed to high temperature.

The relevance of this work when all aspects are taken into account regarding analysis of oxidation products, the importance of pipetting technique, how to best do the analysis and how to separate the phases, will mean a more time efficient method and it will minimize the use of solvents compared to the methods used today. The instrument is small and thereby easy to transfer. It gives you all the analysis in short time which means lowering the costs and make it more health friendly. Due to the fast analysis it will help speed up the release of products that saves life and in the end saves time and money.

6. Future work

There are several interesting topics that can be tested and among them are the separation methods. When doing the centrifugation, centrifugation tubes containing filters could be tested in order to get a better separation or a safer separation between the phases. Another approach when trying the extraction with either 2-propanol/isooctane or ethanol/heptane is to figure out a way of drying the samples after evaporation of solvent, trying to get a 100% yield and pure oil.

Using simple methods, like Karl Fischer titration for investigation of the purity of the oil from the different separation methods are something that could be added to the optimization.

For the possibility of the method to become a quality control method the need of a full validation must be done. At this point the linearity test is only based on one range of the peroxide analysis and control over several ranges will be needed. It also needs to be analyzed for more concentrations points and triplicate analysis for each concentration. Doing the linearity test for FFA- and anisidine-analysis will also be required. Due to the sensitivity of the instrument the validation can be difficult in some cases.

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[20] http://www.cdrfoodlab.com/food-analysis/peroxide-value-oils-fats.html 2016-03-29

[21] http://www.cdrfoodlab.com/food-analysis/anisidine-value-fats.html 2016-03-29

[22] Corporate Guiding Document at Fresenius Kabi, cGD-PT-006, *Validation of analytical method,* 2011, *1,* 1-11

[23] ICH guidelines, *Impurities in new drug substances*, 2006, *4*, 1-15

Appendix 1

Result over analysis of soybean oil, MCT-oil, olive oil and fish oil during eight days with forced oxidation, table 1-4. The deviations can be seen in section 4.1

Table 1 Analysis results over forced oxidation during eight days with threshold value for soybean oil.

Soybean oil			
Days	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
1	0.21	0.35	1.1
3	0.72	0.35	1.2
5	1.35	0.35	1.2
8	1.88	0.35	1.3
Threshold	**≤ 0.6**	**≤ 3.6**	

Table 2 Analysis results over forced oxidation during eight days with threshold values for MCT-oil

MCT-oil			
Days	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
1	0.01	0.35	0.5
3	0.01	0.71	0.5
5	0.01	1.1	0.5
8	0.01	0.71	0.5
Threshold	**≤ 1**	**≤ 3.6**	

Table 3 Analysis results over forced oxidation during eight days with threshold values for olive oil

Olive oil			
Days	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
1	0.39	0.35	1.5
3	1.81	0.35	1.8
5	2.84	0.35	1.9
8	3.89	0.35	1.9
Threshold	**≤ 5**	**≤ 3.6**	

Table 4 Analysis results over forced oxidation during eight days with threshold values for fish oil

Fish oil			
Days	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
1	2.57	0.71	1.9
3	6.61	0.71	2.3
5	12.68	0.35	2.5
8	34.97	0.35	2.7
Threshold	**≤ 5**	**≤ 53**	**≤ 20**

Appendix 2

Result over analysis of soybean oil, MCT-oil, olive oil and fish oil during four weeks with analysis for use in new products, table 1-4. These oils were in closed bottles, avoiding light and only open for analysis. Samples of newly opened bottles were analyzed together with the other at the last time point. The deviations of the analysis can be seen in section 4.1

Table 1 Analysis results over oxidation during four weeks with threshold values for soybean oil

Soybean oil			
Weeks	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
0	0.01	0.53	0.55
1	0.01	0.35	0.7
2	0.04	0.35	0.7
3	0.13	0.53	0.7
3[1]	0.05	0.35	0.6
Thresholds	**≤0.6**	**≤3.6**	

3[1] = Results from the second bottle analyzed week four only

Table 2 Analysis results over oxidation during four weeks with threshold values for MCT oil

MCT-oil			
Weeks	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
0	0.01	0.71	0.6
1	0.01	0.71	0.55
2	0.01	0.71	0.55
3	0.01	0.71	0.55
3[1]	0.01	0.71	0.55
Thresholds	**≤1**	**≤3.6**	

3[1] = Results from the second bottle analyzed week four only

Table 3 Analysis results over oxidation during four weeks with threshold values for olive oil

Olive oil			
Weeks	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
0	0.01	1.06	2.1
1	0.01	0.71	2.35
2	0.13	0.35	2.45
3	0.20	0.71	2.5
3[1]	0.11	0.71	2.45
Thresholds	**≤5**	**≤3,6**	

3[1] = Results from the second bottle analyzed week four only

Table 4 Analysis results over oxidation during four weeks with threshold values for fish oil

Fish oil			
Weeks	**Peroxide (mekv/kg)**	**FFA (mekv/kg)**	**Anisidine (AnV)**
0	1.54	1.06	2.35
1	2.69	0.89	2.6
2	3.95	0.71	2.6
3	5.05	0.89	2.6
3[1]	5.09	1.06	2.7
Thresholds	**≤5**	**≤53**	**≤20**

3[1] = Results from the second bottle analyzed week four only

Appendix 3

Results for method accuracy analysis can be seen in table 1-3 below. The analyses were done on duplicate samples for each batch with the FoodLab fat and the validated method and the mean were calculated. The averages of the mean values from both methods were determined and the deviations are based on the mean values for each analysis and the average of the mean values.

Table 1 shows the results when analyzing for peroxide-values with the FoodLab fat and the validated method for quality control. The mean values were based on duplicate sample analysis. The relative deviation were needed to be ≤ 20%

Peroxide-analysis					
Batch:	**Mercury F2574 12m30°C25%**	**Mercury F2571 12m25°C60%**	**Mercury F2576 12m30°C25%**	**Mercury F2571 12m30°C25%**	**Mercury F2573 12m30°C25%**
QC	(mekv/kg)	(mekv/kg)	(mekv/kg)	(mekv/kg)	(mekv/kg)
Average	0,631	0,031	0,034	0,088	0,051
Relative deviation (%)	53%	51%	15%	80%	4%
R&D	(mekv/kg)	(mekv/kg)	(mekv/kg)	(mekv/kg)	(mekv/kg)
Average	2,04	0,01	0,025	0,01	0,055
Relative deviation (%)	53%	51%	15%	80%	4%
Average of average values	1,336	0,021	0,0295	0,049	0,053

Table 2, the two tables shows the results when analyzing for anisidine-values with the FoodLab fat and the validated method for quality control. The mean values were based on duplicate sample analysis. The relative deviation were needed to be ≤ 20%

Anisidine-analysis					
Batch:	**Mercury F2874 3m40°C25%**	**Mercury F2874 3m30°C25%**	**Mercury F2875 3m30°C25%**	**Mercury F2874 3m25°C40%**	**Mercury F2874 3m25°C60%**
QC	(AnV)	(AnV)	(AnV)	(AnV)	(AnV)
Mean	2.178	1.909	1.95	2.056	1.947
Relative deviation (%)	35%	27%	26%	28%	30%
R&D	(AnV)	(AnV)	(AnV)	(AnV)	(AnV)
Mean	1.05	1.1	1.15	1.15	1.05
Relative deviation (%)	35%	27%	26%	28%	30%
Average of mean values	1.614	1.505	1.55	1.603	1.499

Anisidine-analysis				
Batch:	**Intralipid 30% 10 IE1749**	**SMOF 1CB 10KC3281**	**SMOF 3CB 10IL7627**	**Mercury F2759 6m30°C25%**
QC	(AnV)	(AnV)	(AnV)	(AnV)
Mean	0.728	2.131	2.108	1.959
Relative deviation (%)	14%	30%	31%	39%
R&D	(AnV)	(AnV)	(AnV)	(AnV)
Mean	0.55	1.15	1.1	0.85
Relative deviation (%)	14%	30%	31%	39%
Average of mean values	0.639	1.641	1.604	1.405

Table 3, the two tables shows the results when analyzing for FFAs with the FoodLab fat and the validated method for quality control. The mean values were based on duplicate sample analysis. The relative deviation were needed to be ≤ 20%

FFA-analysis					
Batch:	**SmofKabiven 10KD4558**	**SmofKabiven 10KD4562**	**SmofKabiven 10KD4522**	**SmofKabiven 10KD4506**	**SmofKabiven 10KD4554**
QC	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)
Mean	1.763	1.688	1.631	1.671	0.5654
Relative deviation (%)	17%	19%	13%	19%	11%
R&D	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)
Mean	2.48	2.48	2.12	2.48	0.71
Relative deviation (%)	17%	19%	13%	19%	11%
Average of mean values	2.1215	2.084	1.8755	2.0755	0.638

FFA-analysis				
Batch:	**Intralipid 20% 10KC3273**	**SmofKabiven 10KC3152**	**SmofKabiven 10KC3156**	**SmofKabiven 10KC3196**
QC	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)
Mean	0.4	1.6	1.6	1.6
Relative deviation (%)	7%	14%	20%	20%
R&D	(mekv/L)	(mekv/L)	(mekv/L)	(mekv/L)
Mean	0.35	2.1	2.4	2.4
Relative deviation (%)	7%	14%	20%	20%
Average of mean values	0.375	1.850	2	2.000

Appendix 4

Results over stability study of Intralipid 20%, Omegaven and SMOFlipid, see table 1-3. The results were based on duplicate sample analysis and the deviations can be seen in section 4.7

Table 1 shows analysis results over stability study at 25 °C and humidity at 40% for Intralipid 20%, Omegaven and SMOFlipid. The results are mean values from duplicate sample analysis. The table contains the threshold values for the emulsions.

25°C/40%RH

Month	Perox. (mekv/kg) Intralipid	Perox. (mekv/kg) Omegaven	Perox. (mekv/kg) SMOF
0	0.04	0.02	0.01
1	0.02	0.09	0.01
3	0.05	0.19	0.03
Thresholds	**≤ 1**	**< 1**	**≤ 1**

Month	FFA (mekv/kg) Intralipid	FFA (mekv/kg) Omegaven	FFA (mekv/kg) SMOF	Anisidine (AnV) Intralipid	Anisidine (AnV) Omegaven	Anisidine (AnV) SMOF
0	1.77	4.25	6.02	0.90	0.75	1.00
1	1.77	4.60	7.08	0.75	0.85	0.80
3	1.77	4.60	7.08	0.80	0.70	0.80
Thresholds	**≤ 36**	**<53**	**≤ 53**	**≤ 3**	**≤ 25**	**≤ 5**

Table 2 shows analysis results over stability study at 30 °C and humidity at 25% for Intralipid 20%, Omegaven and SMOFlipid. The results are mean values from duplicate sample analysis. The table contains the threshold values for the emulsions.

30°C/25%RH

Month	Perox. (mekv/kg) Intralipid	Perox. (mekv/kg) Omegaven	Perox. (mekv/kg) SMOF
0	0.04	0.02	0.01
1	0.04	0.08	0.02
3	0.05	0.16	0.01
Thresholds	**≤ 1**	**< 1**	**≤ 1**

Month	FFA (mekv/kg) Intralipid	FFA (mekv/kg) Omegaven	FFA (mekv/kg) SMOF	Anisidine (AnV) Intralipid	Anisidine (AnV) Omegaven	Anisidine (AnV) SMOF
0	1.77	4.25	6.02	0.90	0.75	1.00
1	1.95	4.60	6.55	0.85	0.85	1.00
3	2.12	5.49	7.26	0.80	0.90	1.05
Thresholds	**≤ 36**	**<53**	**≤ 53**	**≤ 3**	**≤ 25**	**≤ 5**

Table 3 shows analysis results over stability study at 40 °C and not more than 25% humidity for Intralipid 20%, Omegaven and SMOFlipid. The results are mean values from duplicate sample analysis. The table contains the threshold values for the emulsions.

40°C/NMT 25%RH			
Month	**Perox. (mekv/kg) Intralipid**	**Perox. (mekv/kg) Omegaven**	**Perox. (mekv/kg) SMOF**
0	0.04	0.02	0.01
1	0.03	0.08	0.01
3	0.17	0.31	0.25
Thresholds	**≤ 1**	**< 1**	**≤ 1**

Month	**FFA (mekv/kg) Intralipid**	**FFA (mekv/kg) Omegaven**	**FFA (mekv/kg) SMOF**	**Anisidine (AnV) Intralipid**	**Anisidine (AnV) Omegaven**	**Anisidine (AnV) SMOF**
0	1.77	4.25	6.02	0.90	0.75	1.00
1	1.95	6.02	7.43	0.75	0.90	0.90
3	3.72	8.50	9.38	0.95	0.85	0.80
Thresholds	**≤ 36**	**<53**	**≤ 53**	**≤ 3**	**≤ 25**	**≤ 5**