Development and Characterization of Omega-3 Tablets - a new administration form for omega-3

Dissertation for the Degree of Philosophiae Doctor

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Thanks to my children, Mathilda, William and Sigmund. You create the necessary work-free space every day.

I dedicate this work to Lars Olav Lien Vestland.

Oslo, September 2016
Tina Lien Vestland

Abbreviations

AUC_{0-24}	area under curve from 0 to 24 hours
AV	anisidine value
β-CD	β-cyclodextrin
CD	cyclodextrin
Cmax	maximum concentration
DC	direct compaction
DHA	docosahexaenoic acid (22:6 n-3)
EDTA	ethylenediaminetetraacetic acid
EE	ethyl ester
EFSA	European Food Safety Authority
EPA	eicosapentaenoic acid (20:5 n-3)
EU	European Union
FDA	Food and Drug Administration
GOED	Global organization of EPA and DHA
GRAS	generally regarded as safe
HPMC	hydroxypropyl methyl cellulose
MCC	microcrystalline cellulose
N.A.	not applicable
N.D.	not detected
Ph. Eur.	European Pharmacopoeia
PV	peroxide value
PVA	polyvinyl alcohol
RH	relative humidity
TG	triglyceride
Tmax	time for maximum concentration
Totox	total oxidation value
US	United States of America

Abstract

Omega-3 fatty acids are used in both nutraceuticals and pharmaceuticals in the form of triglycerides and ethyl esters. Administration forms available for omega-3 oil include bulk oil, soft gel capsules, emulsions and some powder compositions. However, the niche omega-3 tablets have so far remained unexplored.

In the present work direct compaction grade powders comprising omega-3 oil as triglycerides or ethyl esters were prepared utilizing β-cyclodextrin as encapsulating agent. Powders with omega-3 oil load ranging from 10-40% (w/w) have been prepared by vacuum drying, freeze drying or spray granulation of aqueous mixtures of omega-3 oil and β-cyclodextrin. Spray granulation proved to be the superior drying method for the preparation of compactible powders.

Powder X-ray diffractograms of the powders and crushed tablets show evidence of formation of new crystalline phases not present in pure β-cyclodextrin, indicating true complexation of ethyl ester and triglycerides. ^{1}H NMR data confirmed presence of ethyl ester:β-CD inclusion complexes.

The compactibility of the powders was explored by the preparation of tablets containing 20-40% (w/w) omega-3 oil as triglycerides or ethyl esters. It was found that powders with up to 35% (w/w) triglyceride oil and 30% (w/w) ethyl ester oil, respectively, could be directly compressed to tablets of good quality.

Recent years focus on the health benefits from omega-3 fatty acids has caused foundation for a diverse assortment of omega-3 supplements concerning quality. The discovery of a relatively extended sale of low quality products in several markets has resulted in an increased focus on essential properties of omega-3 products, like bioavailability of the omega-3 fatty acids from the formulation and oxidative stability through shelf-life.

The present work demonstrates that direct compaction grade powders based on spray granulated triglyceride oil and β-cyclodextrin, the corresponding tablet cores and coated tablets can be prepared with sufficiently low oxidation values to satisfy relevant monographs for omega-3 products, i.e. with initial totox values of the respective formulations < 10. Increasing levels of ascorbic acid in the formulation was correlated with lower totox values; however, the combination with EDTA as processing agent proved necessary to ensure sufficient oxidative stability of triglyceride powders. Spray granulating under nitrogen

atmosphere contributed to significantly decreased totox levels in powders after eight months of storage at accelerated temperature (37^{o}C), compared to spray granulation in air. In long-term stability studies, it was confirmed that coated triglyceride tablets remained at totox level < 10 after one year of storage at ambient temperature.

The bioavailability of EPA and DHA from the triglyceride tablets comprising 30% (w/w) triglyceride oil was established. It was found that the bioavailability, measured as relative levels of EPA and DHA in serum, was comparable to soft-gel capsules. It was further observed that time for maximum concentration of EPA and DHA in serum was significantly shorter administered as tablets compared to as soft-gels.

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1 Background

1.1 Omega-3 as health supplement and pharmaceutical

Long chain omega-3 fatty acids, in particular eicosapentaenoic acid (EPA; 20:5 n-3)) and docosahexaenoic acid (DHA; B22:6 n-3), have several documented health benefits that have caused great interest for omega-3 as a pharmaceutical ingredient and as a health supplement (Fig.1). A significant amount of scientific research has been conducted exploring the use of omega-3 and its impact on health. Largely, this research has reported positive effects. A few authors conclude that there are no health benefits gained by supplement of omega-3; for example, Rizos *et al* published a meta-analysis concluding that omega-3 had no effect on cardiovascular event rates [1].

The roles of omega-3 fatty acids in health and disease have been reviewed by several authors and have shown positive effects in infant development, cancer, cardiovascular diseases, mental illness including depression, attention-deficit hyperactivity disorder and dementia. Other health benefits are also suggested, like improved skin quality and general joint health [2-5].

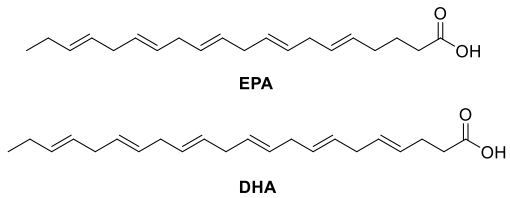

Figure 1. The molecular structure of EPA and DHA.

The larger part of the documentation is based on omega-3 fatty acids from marine sources, in particular fish oil. Natural and processed fish oils comprising omega-3 fatty acids are used as nutraceuticals in numerous commercial products and as pharmaceutical ingredients in a few drug products. Oils derived from fish are triglycerides (TG), implying that three fatty acids are connected to a glycerol backbone via three ester linkages. Processed oils can also be on the ethyl ester (EE) form, where the individual fatty acids are esterified with ethanol (Fig.2).

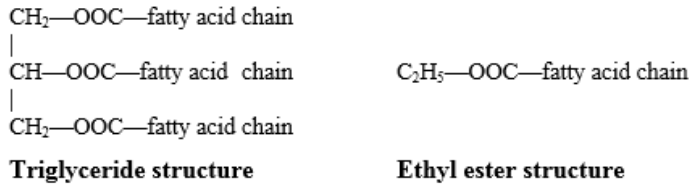

Figure 2. The structure of a triglyceride molecule compared to the structure of an ethyl ester.

Typically, a pharmaceutical omega-3 product comprise more than 85% omega-3 fatty acid esters and the prescribed dosage is above 2 grams per day. Omacor/Lovaza, developed by the Norwegian company Pronova Biopharma, and generic products dominates the global pharmaceutical market for omega-3 (1000 mg soft-gels containing >90% ethyl ester oil). In Norway, Omacor is prescribed for hypertriglyceridemia and as secondary prevention after myocardial infarction (Omacor, www.felleskatalogen.no (2016)).

The main share of fish oils available are derived from fish caught off the coast of Chile, Peru and Morocco, typically sardines and anchovies. Omega-3 fish oils are available in various concentrations, the most commonly used is 18/12. The designation "18/12" refers to the content of EPA and DHA, respectively, in area percentage of the total oil. This content, and ratio, of EPA and DHA is referred to as the "natural" composition, meaning that no attempts to further concentrate either of the individual omega-3 fatty acids have been made.

There are some differences reported between EPA and DHA when it comes to effect, causing DHA to become of more interest within the neurological field, while EPA is claimed to have a superior effect within the cardiovascular area [2]. For most products, the ratio between EPA and DHA is determined by the relative abundance of these fatty acids in fish oil. However, several products currently available on the market are characterized by high DHA/EPA ratios or has a high, specific EPA content. One such product is the pharmaceutical product Epadel® available on the market in Japan. The indications for this product are within the cardiovascular field.

The fish oils used in nutraceutical products are most often 18/12 or concentrates based on 18/12 oil. The omega-3 concentration can thus range from 30% to above 90%. The recommended daily intake of omega-3 fatty acids as health supplement is the cause of considerable debate and as of today, each country tends to issue their own individual recommendations. For Europe, Norway included, the European Food Safety Authority

(EFSA) has concluded that healthy adults can attain the health benefits offered by EPA and DHA by taking a daily supplement containing minimum 250 mg EPA+DHA [6].

In order to obtain powders comprising sufficiently high amounts of omega-3 it would have made sense to utilize a 90% fish oil concentrate. However, the prices for concentrates are increasing exponentially with the degree of concentration and higher price means less accessible supplements for the general population. For the intended tablet supplement to be available for the intended consumers and contribute to a general increase in the use of omega-3 supplements, the far more affordable 60% fish oil concentrate was used in this project.

Fish oils used in this work were triglyceride concentrates containing 600 mg/g of omega-3 as TG (EPA as TG 300 mg/g, DHA as TG 200 mg/g), ethyl ester concentrates containing 600 mg/g of omega-3 as EE (EPA as EE 300 mg/g, DHA as EE 200 mg/g), in addition to an ethyl ester concentrate containing >90% omega-3 as EE (800 mg/g EPA+DHA as EE).

Omega-3 as nutritional supplement is currently available in numerous administration forms, including bulk oil, soft-gels, emulsions and various semi-solid formulations typically added color and sweeteners. Of the available administration forms, the soft-gel capsules are still the most popular; as the taste and smell of the oil can be completely masked and the omega-3 dose comes in defined units.

1.2 Oxidative stability of omega-3 products

Omega-3 fatty acids are highly susceptible for oxidation due to the presence of multiple double bonds in the fatty acid chains. The mechanisms of the oxidation process involve various free radical reactions with hydroperoxide formation and hydroperoxide decomposition into complex mixtures of monomeric, polymeric and small molecular weight volatile compounds. Lipid oxidation requires the presence of oxygen, and can be catalyzed by among other metal ions, light and heat. The oxidation rate of fatty acids in omega-3 oil is influenced by the chemical composition of the oil, as well as the molecular structure of the fatty acids, the presence of impurities and antioxidants and the process conditions [7, 8].

The oxidative status of omega-3 oils is normally determined by the peroxide value (PV) and the anisidine value (AV) of the oils. The former value shows the primary oxidation products of omega-3 fatty acids, while the latter express the secondary. The PV value is measured in milli-eqvivalents (Meq) per mass, whilst the AV value has no designation. Normally the two

values are combined and presented as total oxidation (totox) values. The totox value combines the peroxide value and the anisidine value as follows [9]:

$$\boldsymbol{AV + 2PV = Total\ oxidation\ (totox)}$$

According to the European Pharmacopeia (Ph. Eur.) the maximum acceptable level for PV is 10 Meq/kg and AV is 30. The totox value has no defined limits; however, if the above mentioned values are taken into consideration the maximum totox level would reach 50. The recommended maximum levels are dependent on the type fish oil [10]. Values set by the Global Organization of EPA and DHA (GOED) are somewhat lower; PV is expected to be < 5 Meq/kg, AV < 20 and the combined totox value < 26 [9]. However, following these recommendations is voluntary for producers of health supplements.

The goal for various formulations containing omega-3 will naturally be to achieve long-term stability. Then a long shelf-life can be achieved, which is beneficial for all contributors in the supply chain, as well as for the end consumers. Furthermore, fish oil is a limited resource. A longer shelf-life of omega-3 products can therefore make an important contribution to optimal utilization of the resource.

1.3 Tablets as potential administration form for omega-3

Tablets, as soft-gels, represent an administration form that offer the active substance in convenient units. In addition, tablets are solids comprising dry powders and typically exhibit few compatibility issues when more than one active ingredient is included in the same unit. This is a potential advantage over soft-gels, as combining other actives with the oil inside the gelatin capsule requires the other actives to be compatible with the oil. Alternative methods for achieving a combination product inside a soft-gel exists, such as various capsule-in-capsule techniques; however, specialized equipment is needed. Tablets, on the other hand, can be mass-produced at low cost using conventional tablet machines. Being able to introduce omega-3 in a tablet may therefore represents easier and less costly production of both plain omega-3 supplements and combination products.

Most soft-gel capsules comprise of bovine gelatin and cannot be consumed by those requiring halal diet. Tablets as an alternative administration form, would eliminate the need for gelatin, while maintaining the form of an odor-free and tasteless supplement of omega-3.

In warm and/or humid areas, the soft-gel capsules become more permeable due to softening of the gelatin capsule. This represents a potential risk for oxidation of the content. Tablets are in general the most stabile administration form. If this property could be attainable for tablets containing omega-3 fish oil as well, it would be another benefit over soft-gels as an administration form.

In order to prepare tablets, the requested powder formulation has to have certain properties. It must flow evenly in the tablet machine and be compactible. Flow is important because conventional tablet machines rely only on gravity when powder is fed to the die table. Particle size influences flow and increasing particle size is associated with increasing flow rate up to a maximum at a particle size of 100-400 microns [11]. Moreover, particle shape is contributing to the flow properties of a powder and naturally, particles that are more spherical will improve flow. In this work compressibility index was used to describe flow properties. Good flow of a powder is typically indicated by a compressibility index of 12-16%, while 18-21% is indicating a fair to passable flow [12].

Compactibility is termed the ability of a powder to be compressed into a tablet with specified strength [13]. Compactibility is usually described by the crushing strength and friability measurements of tablet cores compressed from the powder in question.

Direct compaction (DC) is the method of choice preparing tablets with thermo-labile and moisture-sensitive compounds [14]. The whole compaction process happens in one single step. Potentially oxidative stressing extra process steps, such as wet granulation, can be avoided. The ideal preparation of omega-3 tablets would thus include compaction of a DC grade omega-3 powder. Then addition of other excipients than the omega-3 powder could be avoided to a larger extent, also securing the highest possible omega-3 oil load in the tablet.

In order to designate a powder DC grade, it must have sufficient flow and compactibility to be able to limit other necessary excipients in the tablet formulation to a minimum. In this work, all free-flowing powders resulting in tablets with crushing strength >90 N and friability less than 1% (w/w) are designated DC grade powders (applies when the powders comprise more than 95% (w/w) of the tablet formulation).

In addition to the technical properties necessary to form a tablet, a DC grade powder must ensure disintegration/dissolution of the tablet. Since the excipients should be minimized it is important that the powder itself holds certain properties that secure bioavailability of the active ingredient.

1.4 Cyclodextrins as carrier of lipophilic substances and potential direct compaction grade excipient

Cyclodextrins (CDs) are enzymatically prepared cyclic oligosaccharides comprising 6, 7 or 8 glucose units derived from starch, denoted α, β- and γ-cyclodextrins, respectively. CDs and chemical derivatives of the CDs can form complexes with lipophilic substances through non-covalent interactions between the hydrophobic cavity of the CD and the hydrophobic part of the guest molecule (Fig.3). As a result of this complex formation the properties of the guest molecule, e.g., its solubility and stability, can become modified. CDs are able to form complexes with solid, liquid and gaseous compounds [15-17].

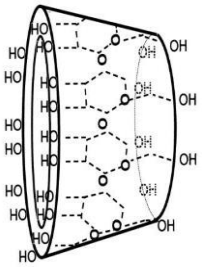

Figure 3. The cave-like supramolecular structure of β-cyclodextrin. A lipophilic substance may be included in the lipophilic interior under favorable conditions, whilst the more hydrophilic exterior can secure an improved solubility in hydrophilic environments (figure received from Omegatri AS).

The properties of cyclodextrins (CD) and their derivatives cause them to be used in several fields, including as components in pharmaceutical, food and cosmetic products. Worldwide there are around 30-40 pharmaceutical products on the market containing CDs, in addition to an unknown number of products within the nutraceutical and food area. Many of these products are tablets [17-19].

Of the three available CD forms, β-cyclodextrin (β-CD) is the most commonly used in pharmaceutical formulations and the best studied CD in humans [18, 20]. At the initiation of this project, the company Omegatri AS, had filed a patent application describing omega-3:β-CD complexes in various ratios. The main intention was to develop a powder possible to include in tablets.

According to literature, pure β-CD may function as a DC grade powder [20, 21]. Saleh showed that β-CD has similar compression-force profile as excipients like Emcompress and Starch 1500, which are commercially available excipients specifically designed for direct compaction [22]. Hence, utilizing β-CD as complexing agent could theoretically result in a DC grade powder. However, published work only describes compaction properties of pure

β-CD or β-CD encapsulating small amounts (less than 10% (w/w) of the formulation) of molecules with relatively high melting points (typically >100°C). The rationale for use of CDs described in published work is typically to improve bioavailability of lipophilic actives, not to solidify liquids for inclusion in tablets [18-20, 23, 24].

2 Aim of the project

Numerous published studies show that omega-3 has health benefits and the substance is extensively used in pharmaceuticals, food and health supplement products. There are currently no tablets containing omega-3 from fish oil on the commercial market. Tablets can be prepared without the use of gelatin, can be divided and typically exhibit superior stability compared to other administration forms. Therefore, the tablet as an administration form could prove advantageous relative to comparable administration forms, such as the soft-gel capsule.

By combining the abilities of β-CD to complex lipophilic substances and function as DC grade excipient, it was believed that omega-3 oil in doses necessary for a health supplement product could be included in tablets. A project outline was drawn, where the omega-3:β-CD complexes previously prepared by Omegatri should be formulated into a compactible powder possible to administer in tablets.

2.1 Overall aim for the project

Develop an omega-3 tablet based on omega-3:β-CD complexes for use as a health supplement product.

The following criteria should be met;

- The omega-3 source must be fish oil.
- The tablets should meet relevant requirements for the administration form as defined by e.g. the European Pharmacopeia.
- A daily dose of 250 mg EPA+DHA should be possible to administer with 2 tablets.
- The oxidative stability should enable a shelf-life of 18-24 months under conditions typically relevant for tablets (ambient conditions).
- The oral bioavailability of EPA and DHA from the tablets should be comparable to other relevant administration forms, e. g. soft-gel capsules.
- The production processes should be developed using standard equipment available in several sizes, in order to facilitate future upscaling.
- The powders prepared should not comprise any excipients not approved for use in tablets by relevant authorities.

2.2 Sub aims

In order to reach the overall aim, the following sub-aims were defined;

- Prepare compactible powders comprising omega-3:β-CD complexes, with the aim of preparing tablets with defined crushing strength (>90 N) and friability (<1% (w(w)) **(Chapter 4.1.1, Paper I and II)**
- Establish the maximum oil load in DC grade powders based on the impact of oil load on crushing strength and friability of tablet cores **(Chapter 4.1.2, Paper I and II)**
- Determine the superior molecular form of omega-3 fatty acid esters (EE vs. TG) to become included in tablets, based on the impact of the molecular form on crushing strength and friability of tablet cores **(Chapter 4.1.3, Paper I and II)**
- Investigate whether the developed powders could be characterized with XRD studies **(Chapter 4.1.4, Paper I and II)**
- Compare the abilities of developed powders to be compressed to tablets with commercially available omega-3 containing powders **(Chapter 4.1.5, Paper II)**
- Investigate the impact of necessary production process steps on oxidative stability of intermediate and final omega-3 products **(Chapter 4.2.1, Paper III)**
- Determine the oxidative stability of relevant omega-3 powder/tablet prototypes in long-term and accelerated stability studies **(Chapter 4.2.2, Paper III)**
- Establish the level of bioavailability of EPA and DHA from the tablets compared to soft-gel capsules **(Chapter 4.3.1, Paper IV)**
- Assess available regulatory information on the main components of the omega-3 powders **(Chapter 4.3.2)**

3 Overview of papers

Published papers or written manuscripts based on research performed in this project are essential attachments to the main text and are referred to in the text with their roman number as detailed below.

3.1 Paper I- *Compactible powders of omega-3 and β-cyclodextrin*

Vestland, T. L.; Jacobsen, Ø.; Sande, S. A.; Myrset, A. H.; Klaveness, J.
Food Chemistry, **2015**. 185: p. 151-158.

Powders with EE/TG oil loads ranging from 10-40% (w/w) were prepared by vacuum drying, freeze drying or spray granulation of aqueous mixtures of oil and β-cyclodextrin. The compactibility of the powders was explored, revealing that a dry and compactible powder can be prepared from various omega-3 oils and β-cyclodextrin. Spray granulation was found to be the superior drying method with respect to obtaining a compactible omega-3 containing powder. This was attributed to smaller amounts of surface oil in the spray granulated powders compared to the freeze dried powders; in addition to the achievable size, shape and size distribution of spray granulated particles.

Based on the properties of tablets made from freeze dried powders the maximum amount of oil in a compactible powder was up to 20% (w/w) for an EE powder and 30% (w/w) for a TG powder. The properties of tablets made from spray granulated powders showed that increased oil load was possible in compactible powders where the oil was an EE (up to 30% (w/w)) and indicated further that higher oil load could be achievable also for compactible TG powders (>30% (w/w)).

It was observed in XRD studies that the omega-3 powders contained other crystalline phases than pure β-cyclodextrin, indicating true complexation of at least parts of the omega-3 fatty acids present in the powders. Furthermore, it was observed that the oil load and drying method both significantly influenced the amount of radiation scattered by crystalline phases in the powders.

3.2 Paper II- *Characterization of omega-3 tablets*

Vestland, T. L.; Jacobsen, Ø.; Sande, S. A.; Myrset, A. H.; Klaveness, J.
Food Chemistry, **2016**. 197, Part A: p. 496-502.

Tablets containing 25-40% (w/w) EE/TG oil load were prepared utilizing spray granulated powders with β-cyclodextrin as encapsulating agent. It was found that powders with up to 35% (w/w) TG oil and 30% (w/w) EE oil, respectively, could be directly compressed to tablets with crushing strength >90 N and friability less than 1% (w/w). The results obtained suggested that the DC grade omega-3 powders comprised β-CD:fatty acid ester complexes with different stoichiometric compositions and that a smaller molar ratio of β-CD to fatty acid moiety than 3:1 was sufficient to prepare direct compaction grade powder suitable for tableting.

The properties of the prepared tablet cores were compared to the properties of tablet cores prepared from three commercially available omega-3 powders purchased from three different suppliers. It was observed that none of the commercial powders resulted in tablets of acceptable quality.

NMR spectroscopic studies of the prepared powders showed that the EE powders most likely contained inclusion complexes.

Furthermore, powder XRD studies confirmed that the spray granulated powders had different crystalline areas than pure β-CD, as observed in paper I. It was questioned whether the observed reduced crystallinity with increased oil load could be linked to the independent observation of decreasing crushing strength with increasing oil load.

3.3 Paper III- *Oxidative stability of omega-3 tablets*

Vestland, T. L.; Petersen, L. B.; Myrset, A. H.; Klaveness, J.
Journal of Lipid Science and Technology, **2016.** Technol. doi: 10.1002/ejlt.201500322.

Studies on oxidative stability of TG oil included in powders, tablet cores and coated tablets established that coated tablets and powder could be prepared satisfying relevant monographs for omega-3 containing products. The products were based on spray granulated, DC grade 30% (w/w) TG powders.

Addition of ascorbic acid, in combination with EDTA as processing agent, was correlated with lower totox in powders, tablet cores and coated tablets. Spray granulation performed under nitrogen atmosphere resulted in a significantly more oxidative stable powder than spray granulation in air.

The impact of necessary processing (spray granulation, compaction and coating) on oxidative stability of TG products was studied. It was observed that reduced access for oxygen triggers by compaction and coating of powders had a significantly stabilizing effect, despite the oxidative stress applied to the TGs during the production processes. In long-term stability studies, it was confirmed that coated TG tablets remained at totox < 5 after one year of storage at ambient temperature. The quantitative content of EPA and DHA was confirmed at 100 and 98% (w/w), respectively, in coated TG tablets after 1 year of storage in ambient temperature. The tablets were smell and odor-free.

3.4 Paper IV- *Bioavailability of EPA and DHA from omega-3 tablets*

Vestland, T. L.; Åsberg, A.; Klaveness, Aa. J.; Klaveness, J.
Journal of Lipid Science and Technology, **2016** (submitted article).

Coated TG tablets prepared from DC grade 30% (w/w) TG powder was included in a bioavailability study. The relative levels of EPA and DHA from the tablets in blood serum taken from healthy male volunteers was measured. The bioavailability from the tablets was compared to from soft-gel capsules, which was considered the most comparable administration form.

It was observed that the bioavailability from tablets was comparable to soft-gels when omega-3 oil type and dose was the same for both administration forms. It was further established that time to maximum concentration of EPA and DHA in serum was significantly shorter when the fatty acid esters were administered in tablets. A proposed explanation for the faster uptake was the more rapid passage through the gastrointestinal system due to altered solubility of the omega-3 triglycerides in complex with β-CD and less dependence of emulsifying bile during absorption.

4 Discussion on main observations

4.1 Preparation of omega-3 tablets

4.1.1 Preparation of compactible powders comprising omega-3: β-CD complexes, with the aim of preparing tablets with defined crushing strength and friability

As part of the patent application process, Omegatri AS had prepared omega-3:β-CD complexes and dried them utilizing vacuum or freeze drying. As detailed in Paper I these drying methods were not suitable in the preparation of DC grade powders intended for use in tablets, mainly because the morphology and particle size distribution of these powders caused hampered flow.

A defined goal going forward from freeze dried powders was to maintain, or preferably increase, the compactibility and improve the flow properties. More spherical powder particles, with smooth morphology and an easily defined particle size distribution were needed. De Castro *et al* describe spray granulation of β-CD complexes, where the main aim was to compress β-CD complexes without using a dry or wet granulation step as a part of the production process. The authors concluded that this technique allowed more possibilities for utilization of the inclusion properties of β-CD, also in tablets, by representing a less expensive and more convenient way of preparing granulated powders [25].

Spray granulation offers the combination of drying and granulation of powders in a fluid bed [25, 26]. This combination proved ideal for drying of the liquid mixture of omega-3 oil, β-CD and water in this project. The spray granulation process was normally initiated with a certain bed mass of powder already in the drying chamber (start material), but could also be initiated by simply spray drying some particles. The liquid mixture was then sprayed onto the fluidized particles in thin layers, finally resulting in a coarse granule. When the granules reached a certain size, it was possible to remove them from the process through an outlet equipped with counter pressure. The counter pressure in the outlet was adjustable, leaving the option to select desired size of the granules.

The variables in the process closely resembled the ones of a traditional wet granulation in a fluid bed [26]. The most important parameter for granule growth proved to be the balance between inlet and outlet air temperature. A narrow distance between inlet and

outlet air temperature caused over-sized agglomerates, while a wide gap between the temperatures resulted in powder particles that were unable to grow sufficiently large.

Unlike traditional spray drying, where water is typically evaporated at high temperatures (typically > 180^{o}C), it is possible to evaporate water at lower, adjustable temperatures during spray granulation [27]. This is an important feature for this particular product, as heat is a well-known trigger of oxidation. Experiments showed that the inlet air temperature could range from 25-50 ^{o}C, as long as the outlet air temperature was kept between 10 and 15 ^{o}C lower. This made the evaporation of water optimal for granule growth. Another important factor was the atomizing air pressure. This had to be just right, tearing the slurry into the correct sized droplets, supposed to hit the fluidized particles and dry them to the exact right moisture content before next droplet arrived, promoting adhesion of a new layer.

When spray granulation was utilized as the drying method, it was possible to control the particle size and particle size distribution. Visual observation of the powders showed that they consisted of off-white free-flowing granules, with each individual granule having a close to spherical shape (Pic.1).

Picture 1. Spray granulated 30:70 (w:w) triglyceride:β-CD powder. The oil contained 60% (w/w) triglycerides. (10x magnification)

For all the powders prepared for this work, the aim was a particle size distribution between 200 and 500 microns. It proved possible to attain the aimed for particle size for more than 92.8% (w/w) of the powder particles in spray granulated powders with TG oil (30, 35 and 40% (w/w) oil load). The corresponding figure for powders comprising EE oil was >77.8% (w/w) (25, 30 and 40% (w/w) oil load). Both EE and TG spray granulated powders were found to have compressibility indexes below 14%, indicating excellent flow properties (Paper II, Table 1).

Properties of the tablets from freeze dried powders indicated sufficient compactibility of these powders, however, the lack of flow represented a significant obstacle (Paper I). The spray granulated powders, on the other hand, expressed the properties of a DC grade powder. The flow was impeccable due to the size and shape of the particles and the resulting tablet cores had a high crushing strength and low friability (Paper II).

However, despite availability of a DC grade powder, the formulation of any powder into a powder blend to be compressed to tablets require some excipients to facilitate the process. A certain amount of lubricants is most often necessary to limit the friction between the movable part of the tableting machine and the powder blend or to avoid the powder blend sticking to the tablet punches [28]. For the powders prepared for this work, 0.5% (w/w) magnesium stearate proved necessary in the powder blend to limit friction, in addition to 1% (w/w) talc to avoid sticking to punches.

Many powders are sensitive to the addition of lubricants. Lubricants are most often fatty substances. Added in excessive amounts or mixed with the tablet formulation for too long, lubricants can generally cause decreased crushing strength in tablets due to coverage of binding sites. This applies specially to powders mainly expressing plastic deformation, due to the low number of new surfaces being formed during compaction [29, 30].

It was observed that the lubricant sensitivity of the prepared powders was not extreme, it was possible to use higher amounts of lubricants without decreasing crushing strength of the tablets. However, a goal was to limit added excipients to a minimum, to ensure highest possible oil load in the tablets. Therefore, the lowest effective amount of lubricants was used.

For tablets, and especially for tablet that shall be coated, a high crushing strength is a requirement. Normally, the higher the crushing strength of the tablets, the more resistant they are towards attrition applied in coating drums and through necessary handling [31]. Compared to many other tablets, the crushing strength of the tablets prepared was relatively low (Paper II, Fig.2).

Quite a few filler-binders were tested in the attempt to increase crushing strength of the tablets. The list includes among other microcrystalline cellulose (MCC), cellulose, lactose, sorbitol, di-calcium phosphate and tri-calcium phosphate in various grades and particle sizes, all advertised as superior filler-binders or binders.

One product was observed to contribute positively to the crushing strength. Avicel HFE-102 from FMC Biopolymer is a co-spray dried product of MCC and mannitol. MCC

probably is the most utilized filler-binder, popular due its combination of properties. It has good compactibility and solubility, can function as both filler, binder and disintegrant and is available in several grades [14]. Mannitol is often used in chewable tablets due to the negative heat of solution and sweetness, however, it is also reported as a filler-binder in tablets capable to compete with MCC [32]. The combination of the two is reported by the producer to have superior effect, especially when co-spray dried. It is possible that the spray drying secures a more amorphous material due to the instant cooling from high temperatures. This can contribute to increased compactibility. The Avicel HFE-102 added in the powder blend in a level of 2.5% (w/w) was sufficient to increase the crushing strength with up to 10 N.

A high crushing strength is, nevertheless, worth nothing if the friability is high. High friability is recognized as worn edges on tablets or, in worst case, broken tablets. High friability can be the result of insufficient compaction properties, however, in combination with high crushing strength, it is typically caused by an unsuitable tablet shape for the formulation. Change of tablet dies, hence, tablet shape, can is such cases resolve the issue.

The combination of crushing strength and friability was therefore used to describe the properties of the tablets prepared. These values represent the sum of the properties of the individual materials in the powder blend, the combination of the materials and the equipment used to prepare the tablet.

Tableting machines are often one of the oldest machines to be found in a modern pilot plant or production facility. The reason is that the technology has not changed significantly the last 50 years or more. This imposes high demand for the materials to be tableted. However, formulating the TG/EE powders with 0.5% magnesium stearate, 1% talc and 2.5% Avicel HFE-102 (w/w), resulted in tablets with acceptable crushing strength and low friability (Paper 1, Fig.4; Paper II, Fig.2).

The tablets were prepared on a standard tableting machine typical for lab/pilot environments, in other words, a machine of a certain age. This limited the possibility to monitor interesting parameters during the tableting process and leaves only the opportunity to visually observe the process, in addition to characterize the finished tablet. This is, however, not uncommon and the standard for tablets included in the final text for the International Pharmacopeia written by representatives from the World Health Organization includes visual observation as one of the important criteria for tablets in general (WHO, http://www.who.int/medicines/publications/pharmacopoeia/Tabs-GeneralMono-rev-FINAL_31032011.pdf (2011).

4.1.2 Establishment of maximum oil load in direct compaction grade powders

As established in Paper II, a powder with an oil load of about 10% (w/w) could be expected based on available literature on formation of β-CD complexes (22, 23). Utilizing a 60% fish oil concentrate in such powders would give a total omega-3 load of 6 % (w/w), including a specific EPA+DHA load of approximately 5% (w/w). In this work, a level of 4% (w/w) of facilitating excipients was deemed optimal. In case of 5 % (w/w) EPA+DHA load in powders, the EPA+DHA load in a tablet core would then decrease to 4.8% (w/w), hence providing 48 mg EPA+DHA per 1000 mg tablet. This would make the goal of 250 mg EPA+DHA in 2 tablets impossible to fulfill if the tablets were to have a reasonable size.

However, in tablet trials performed with the spray granulated powders prepared, significantly higher oil loads proved possible to include in tablets of acceptable quality, regardless of oil being in TG or EE form (Fig.4).

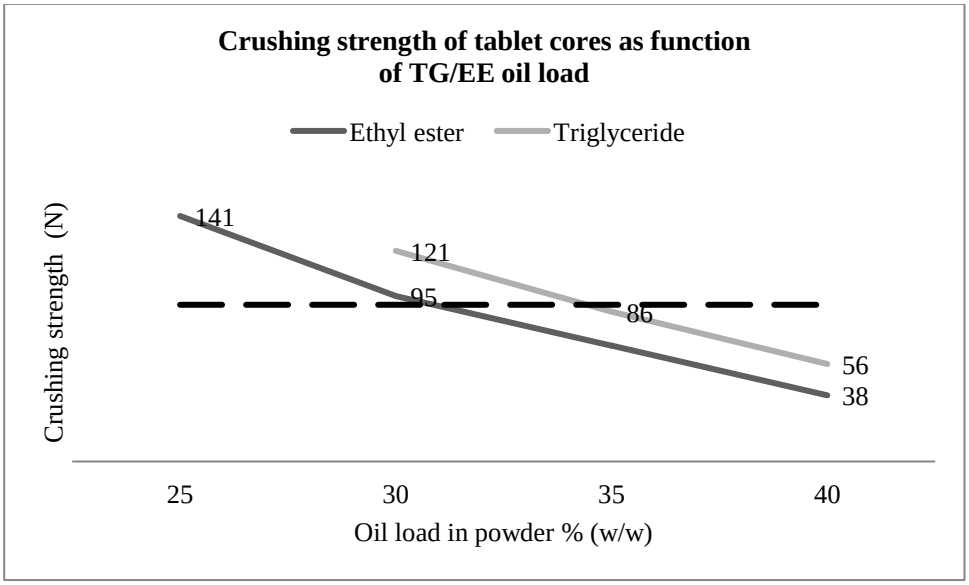

Figure 4. Crushing strength of tablet cores from spray granulated powders as function of oil load and oil type. The dotted line shows the limit of 90 N in crushing strength desired for tablets of acceptable quality. The tablets prepared were oblongs with a mean weight of 950 mg. The friability of the tablet cores varied between 0 to 0.59 % (w/w). The tablet formulation comprised; omega-3 powder (96%), Avicel HFE-102 (2.5%), talc (1%) and magnesium stearate (0.5%). N=10 tablets.

Freeze dried particles are generally porous with a high specific surface and this technique is used frequently for powders intended for immediate dissolution in contact with liquids, such as instant coffee. Anwar and Kunz showed that powders based on 25% (w/w) 33/22 EPA/DHA triglyceride oil embedded in various matrixes and dried by freeze drying comprised of very light, highly porous and irregular particles, similar to the freeze dried powders in this work

[33]. The authors further found that the freeze dried powders in their study had more free surface oil than similar powders prepared by spray drying or spray granulation. This was likely true also for the freeze dried powders compressed to tablets in this work. Surface oil will interrupt particle to particle binding within the tablet and it was observed that freeze dried powders resulted in weaker tablets overall compared to spray granulated (Fig.4; Paper I, Fig. 4).

Spray granulation can, if the correct parameters are achieved, build up a particle by adding layer upon layer, like an onion. This structure will give rise to a particle where the oil is less exposed on the surface. In addition, each particle will be close to spherical, a quality contributing to good flow properties.

As defined in this work, DC grade powders should be free-flowing and have the ability to be compressed to tablets with crushing strength >90 N and friability <1% (w/w) when the powder comprise more than 95% (w/w) of the formulation. Therefore, only spray granulated TG powders with oil loads ≤ 35% (w/w) and EE powders with oil loads ≤ 30% (w/w) could be designated as true DC grade powders.

4.1.3 Determination of the superior molecular form of omega-3 fatty acid esters

TG tablets prepared from spray granulated 35% TG oil powders comprised 168 mg EPA+DHA per gram tablet. EE tablets prepared from spray granulated 30% EE oil powder comprised 144 mg EPA+DHA per gram tablet. Hence, the EFSA claims could be fulfilled with both TG and EE tablets.

It was, nevertheless, consequently throughout all experiments presented in Paper I and II, observed differences between EE and TG powders and tablets that gave the respective products different properties overall. The observed variations indicated differences as to how the EE and TG molecules were complexed by β-CD.

The molecular structure of TGs and EEs makes several different stoichiometries in a complex conceivable. The molar ratio between β-CD and TG could range from 9:1 (corresponding to 3:1 for EE) to 1:1. The former implies that each fatty acid moiety in the TG molecule is complexed by three β-CD molecules. The latter implies that one β-CD molecule is associated with only one of the fatty acid moieties in the TG molecule. The stoichiometric composition of the β-CD:fatty acid complexes present in the powders prepared in this work is not known with certainty. Based on the wide variation of stoichiometries reported in the literature (1:1-

6:1), it is possible that complexes with several different stoichiometric compositions may be present in the powders prepared [34-39].

However, it is rather unlikely that a 3:1, or higher, ratio was the case in the complexes comprising the omega-3 powders. As detailed in Paper II, the achieved results in this work, with tablets of acceptable quality from spray granulated powders with an oil load of up to 35% (w/w), strongly indicate that most of the added oil was complexed by β-CD (Fig.4). This leaves no other option than a 1:1 or 2:1 stoichiometric relationship between β-CD and fatty acid ester molecule in the powders, due to the amounts of the respective ingredients added.

Further, results from tablet trials with freeze dried and spray granulated powders showed that more oil can be included in tablets when the oil was a TG (Fig. 4; Paper I, Fig.4). TG might require less β-CD because full encapsulation (3:1) of all three fatty acid esters in a TG molecule is unlikely, due to the steric hindrance caused by the glycerol backbone. In an EE, the fatty acid esters are singular. EE will presumably, due to absence of steric hindrance around the fatty acid esters, interact with an increased number of β-CD molecules per fatty acid moiety, thus requiring an increased number of β-CD molecules per mass oil (Fig.5). In the NMR study performed on both 30% (w/w) EE and TG powder it was indicated, by the observed presence of β-CD:EE complexes in D_2O, that EE associated closer to β-CD than TG (Paper II, section 3.2). This may be caused by the lack of steric hindrance around the EE molecules.

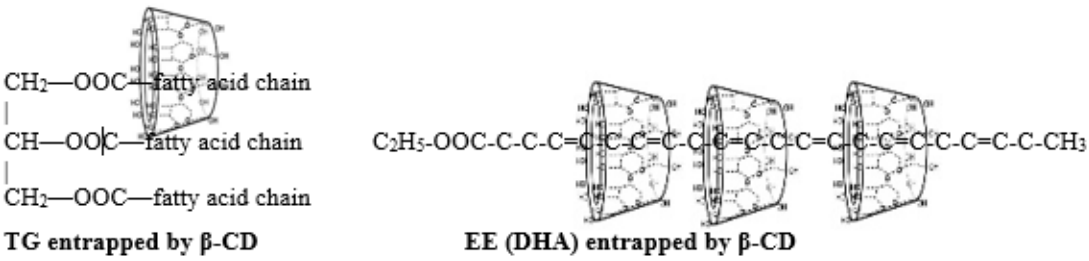

Figure 5. Theoretical display of a possible interaction between EE/TG and β-CD (scale not adjusted). Attempting to underline the EE, being less sterically hindered, can be able to interact with more β-CD molecules simultaneously. Also pointing at the two fatty acids in a TG molecule potentially not encapsulated by β-CD in dry form not being able to participate in an oil droplet in dry form.

For the tablets prepared there was a clear trend that increased oil loads caused decreased crushing strengths, strongly indicating increasing amounts of non-complexed oil components in the powders (Fig. 4; Paper I, Fig.4). Non-encapsulated oil in a powder will cover binding

sites and prohibit inter-particulate bond formation during compaction of a tablet, similarly to what can be observed when excessive lubrication is applied to a tablet formulation [30].

Similarly, in XRD studies it was established that increasing the oil load caused decreased fraction of radiation scattered by crystalline material. It was believed that this was caused by increasing amounts of free oil in the powders, since non-encapsulated liquid components in powders will appear as amorphous areas in XRD (Paper II, section 3.1).

Judging from the lower achievable crushing strengths for tablet cores produced from EE powders regardless of drying method, more non-complexed oil components were present in the EE powders than in the TG powders. This again points to the stoichiometric relationship between β-CD and TG molecule, compared to between β-CD and EE molecule. Based on the results from tablet trials and the XRD study, in case at least one of the three fatty acid esters in a TG molecule was complexed with β-CD, the remaining fatty acid ester(s) were not able to engage in the formation of an oil droplet, as opposed to the situation in an EE powder where any non-complexed fatty acid ester would be present as free oil.

Based on the observations made it was determined that TG was the superior molecular form for the omega-3 fatty acid esters for inclusion in tablets. Further research was hence focused on TG powder and tablets.

4.1.4 Characterization of the developed powders with XRD studies

The presumed presence of more free oil in EE powders was, however, not reflected as less crystallinity in XRD studies. On the contrary, for vacuum dried powders with 30% (w/w) EE/TG oil load, the EE powder clearly had more crystalline areas compared to TG powder (Paper I, Table 2, sample 2 and 6). Spray granulated 30% (w/w) TG powder was only slightly more crystalline than EE powder with the same oil load (Paper I, Table 2, samples 10 and 11).

Therefore, despite XRD showing that increasing oil loads corresponded with increasing amorphous area in powders, the method was not deemed suitable as an in-process test indicating suitability of powders as tablet excipients.

XRD was in this work used as a mean to characterize the prepared powder and it was in general found that the peaks originating from pure crystalline β-CD were absent in the diffractograms of the prepared powders, indicating that little, if any, crystalline β-CD was present in the final products, strongly indicating that the added β-CD participated in complexes.

New amorphous phases were established for all samples, as well as new crystalline areas compared to pure β-CD (Paper I, Fig.2 (example)). The latter was in contrast to the results reported by Choi *et al* and Choi *et al*, who did not observe any diffraction peaks corresponding to potential crystalline inclusion complexes of omega-3 fish oil (menhaden) and β-CD after use of a self-assembling aggregation method (20, 21).

A slightly larger fraction of the scattered radiation was scattered by crystalline material for powder made from the 90% (w/w) EE concentrate than for the corresponding powder made from 60% (w/w) EE concentrate (Paper II, Table 2, sample 2 and 3). This could indicate that EEs of omega-3 fatty acids are more easily included in the cavity of β-CD than the other fatty substances present in the oil. Research has shown that β-CD prefers unsaturated compounds as guest substances in a mixture of saturated and unsaturated molecules [40].

The exact nature of the crystalline and amorphous phases observed could not be determined with certainty from the powder diffraction data, most likely the omega-3 powders represent complex mixtures of several amorphous and crystalline phases comprising inclusion complexes, some free β-CD, free oil or a combination of these, formed during the production process.

4.1.5 Comparison of the abilities of developed powders to be compressed to tablets with commercially available omega-3 containing powders

There are several commercially available dry omega-3 powders on the market today. These powders are typically formulated as variations over oil droplets encapsulated in wall materials, most commonly used is animal-derived gelatin. Methods employed for encapsulation include spray drying, freeze drying or fluid bed drying of emulsions. More specialized methods like extrusion, melt injection and coacervation are also described. Alternatively, the oil is absorbed into porous materials or adsorbed to the surface of various materials or a combination of the two. Typical production methods include spray drying, freeze drying or vacuum drying of mixtures of oil and the porous/non-porous carriers, or oil being sprayed onto carriers in a fluidized bed process [33, 41-44]. Other means of solidifying omega-3 oil include complexation of the oil in suitable carriers, as used in this work.

To be able to study the abilities of a few selected commercial omega-3 powders to become compressed into tablets of acceptable quality, three different powders were purchased. With reference to Paper II, Commercial Powder 1 is Meg-3® 30% from DSM, Commercial Powder 2 is Dry N-3®12 Food from BASF and Commercial Powder 3 is Omega-Classic

Dry N 22 from Denomega. As could be established from the respective powder specifications, Meg-3® 30% from DSM represented a powder where omega-3 oil is encapsulated in a polymer (pork gelatin) while Dry N-3®12 Food from BASF and Omega-Classic Dry N 22 from Denomega are powders where omega-3 oil is adsorbed and/or absorbed to various materials (mainly carbohydrates). The powders are described in more detail in Paper II, page 501-502.

The powders were chosen partly due to the composition of the respective powders, partly due to the position of the brand manufacturers. Both BASF and DSM are large players in the omega-3 industry and it was deemed likely that they had the superior products within the field of omega-3 powders for dietary supplements.

The powders were compared on oil load and omega-3 load. The resulting tablets were compared on friability, crushing strength and mean weigh (Table 1). The mean weight was considered relevant because the tablet height was fixed at 6 mm. With a fixed tablet height, the mean weights of the resulting tablets indicate the filling of the tablet dies, and hence, speak of the flow properties of the tablet formulation. Insufficient flow will result in tablets with low crushing strength or no tablets at all. The problem arises when there is an insufficient mass of powder in the tablet die for the tablet punches to exercise pressure on. Even when the speed of the tableting machine is at minimum, or the machine is being manually operated, sufficient filling of tablet dies prerequisites a good flow of the tablet formulation.

However, mean weight must be considered in combination with other parameters to determine the ability of a powder to be compressed to tablets. A can be observed in the mean weight column in Table 1, Dry N-3®12 Food from BASF had sufficient flow. Nevertheless, the friability and crushing strengths measurements reveal that the powder lacked compactibility.

Upon comparison with spray granulated powders comprising 30% (w/w) EE and TG oil prepared for this work, the difference in suitability as tablet excipient becomes apparent (Table 1, columns 5, 6 and 7). Column 2 and 3 also show that despite the lowest oil load, the powders from this work had among the highest omega-3 loads.

Table 1. Properties of tablet cores prepared from commercially available omega-3 powders and selected powders from this work. Not applicable (n.a.) implies that there were no tablets to perform the test on due to lack of compactibility of the specified formulation. The tablet formulation comprised; omega-3 powder (96%), Avicel HFE-102 (2.5%), talc (1%) and magnesium stearate (0.5%). N=10 tablets.

Omega-3 powder	***Oil load in powder (w/w)***	***Omega-3 load in powder (w/w)***	***Omega-3 powder in tablet core (w/w)***	***Friability loss in tablet cores (w/w)***	***Crushing strength of tablet cores (N)***	***Mean weight tablet cores (mg)***
30% (w/w) TG powder	30%	18.0%	96%	0.11%	121	950
30% (w/w) EE powder	30%	18.0%	96%	0.25%	95	944
Meg-3® 30%	60%	18.0%	96%	n.a.	n.a.	n.a.
Dry N-3®12 Food	40%	12.0%	96%	10.00%	39	1010
Omega-Classic Dry N 22	40%	8.8%	96%	3.00%	55	813

Most probably, the outer shell of wall material(s) in powders based on encapsulated oil droplets, like Meg-3® 30% from DSM, will burst during compaction of the powders, causing large amounts of free oil in the formulation that will inhibit the formation of a dry tablet.

Oil adsorbed to the surface of particles, like in Dry N-3®12 Food from BASF and Omega-Classic Dry N 22 from Denomega, will prohibit inter-particulate bonding during compacting and result in tablets with very low crushing strength. It could be concluded from these tests that none of the selected omega-3 powders could be used as tablet excipients.

For this project, it was aimed at preparing omega-3 tablets with sufficient omega-3 oil per tablet to satisfy the EFSA claims with 2 units (minimum 250 mg EPA+DHA). The results from tablet trials showed that this can be achieved for tablets containing both EE and TG oil. No previous publications describe the use of CDs complexing EPA and DHA, or substances similar with regards to size and melting point, in a powder intended for use in tablets. In fact, in several publications it is concluded with β-CD not even being able to complex fatty acid esters from fish oils.

Choi *et al* and Choi *et al* describe the preparation of a dry powder of β-CD and oil comprising 18-30 % (w/w) EPA and DHA. This powder was intended as an omega-3 source in liquid food systems. The authors claimed that the technique for molecular inclusion of active substances in cyclodextrins cannot be applied with fish oils as core material due to the complicated composition of the oils [45, 46].

Anwar and Kunz prepared microcapsules with 25% (w/w) load of 33/22 EPA/DHA concentrates by spray drying, freeze drying and spray granulation utilizing various combinations of wall materials. One of the combinations of wall materials reported included a mixture of 15% 2-hydroxypropyl-β-CD, 10% soybean soluble polysaccharide and 50%

maltodextrin. The authors concluded that this specific combination was inferior to the other reported wall material combinations with regards to oxidative stabilization of the fish oil and attributed this to the inability of β-CD to include fatty acids in fish oil as guest molecules in the hydrophobic cavity [33].

Nicotine and nitroglycerine, with melting points of -79°C and 14°C, respectively, are among the few substances with lower melting points that previously have been complexed with CDs and included in hard tablets. Szejtli and Szente reported that a nicotine: β-CD complex was prepared by simply mixing nicotine with finely powdered crystalline β-CD and storing the mixture in a closed container at ambient temperature [23]. Less than 2 mg of the active substances were included in each tablet.

The finding that dry powders based on omega-3:β-CD complexes could be prepared represented therefore an inventive step. That the powder could be utilized as DC grade tablet excipient for the preparation of good quality tablets with oil load up to 35% (w/w) was very surprising based on what was known and published.

The next challenge was to ensure oxidative stability through all processes necessary going from bulk oil to finished tablet. As prototype, the 30% (w/w) TG powder and resulting tablets were chosen as that represented true DC grade powder and excellent quality tablet with regards to both crushing strength and friability.

4.2 Oxidative stability of triglyceride powder and tablets

4.2.1 Investigation of the impact of necessary production process steps on oxidative stability of intermediate and final omega-3 products

Oxygen is necessary for oxidation and all handling of omega-3 containing products should ideally be performed under inert atmosphere to reduce oxygen access [47, 48]. Inert atmosphere was, however, not possible to achieve under preparation of the powder and tablets in this work.

During the preparation of the products, the oil was initially introduced to an aqueous mixture of purified water, antioxidants and β-CD. The mechanical stirring caused the oil to divide into smaller droplets in the aqueous phase, closely resembling an O/W emulsion. The actual oil surface exposed to the ambient environment was, hence, small compared to the total volume of the liquid mixture. Oxygen has been reported to be up to three times more soluble in food oils than in water [49, 50]. Therefore, the risk of extensive initiation of oxidation at this stage was considered relatively small.

The spray granulation, on the other hand, introduced massive amounts of air through atomizing and fluidization gas, elevated temperature and applied mechanical stress. Following spray granulation, the TG powder was compressed to tablets. Even though direct compaction is considered the gentlest method for preparation of tablets, powders nevertheless undergo several unit operations when being transferred between containers, mixed into a tablet formulation and, finally, compressed into tablets. During these processes, the powder is exposed to atmospheric conditions, mechanical stress, light and heat.

It was expected that several successive production process steps would come with the risk of increased oxidation rates [51]. However, a powder has a high surface to volume ratio compared to a tablet, decreasing the surface of a powder by compressing it into a tablet may actually provide protection against oxidation due to reduced access for triggers of oxidation.

The assumption was made that the omega-3 tablets should be coated. Since the omega-3 fatty acid esters are believed to be homogeneously distributed in the powders prepared for this work, there will be fatty acid esters on the surface of the tablet cores that will reveal the distinct smell and taste of omega-3, unless masked.

A coating may in addition to taste- and odour-masking offer additional protection against oxidation by decreasing access for oxygen, moisture and light to the tablet core. On the other hand, application of a coating adds another process step that might induce additional oxidation.

The TG tablets were coated in a perforated drum coater in lab scale size (0.8 L drum). The position of the nozzle towards the moving tablet bed was angled towards the middle of the bed, to minimize loss of coating material. The spray rate and the drying rate were the two most important variables in the coating process. The balance between them decided the quality of the process. The objective was to achieve evaporation of liquid before it penetrated the tablet core, but not until sufficient coalescence of the polymer chains was achieved. The atomizing air pressure and the pattern air pressure were adjusted to suit the process, supporting desired droplet size and spread. During application of coating the balance between spray rate and drying rate was monitored with the outlet air temperature, which was fixed.

Fick's first law of diffusion states that the rate of change in concentration of a substance with time is directly proportional to the concentration difference between the two sides of a film;

$$Q = PA\Delta pt/d$$

where Q is the amount of substance that passes through the film, A is the available surface area, t is time, d is film thickness and Δp is the partial pressure difference of the migrating molecule between the two sides of the film. P is the product of diffusion coefficient and solubility coefficient for the molecule in the given film material.

The equation shows that film thickness, available surface area, time and differences in partial pressure are important parameters along with the physical and chemical properties of the substance. However, the law applies only under perfect conditions; any irregularity in the film structure like punctures and thinner parts will make the law invalid for the system [52]. In practice, it means that the law can be used only as a guide towards how to reduce diffusion.

To minimize Q the available surface area should be as small as possible. One way of achieving small surface area is to make the tablet core large, limited by how big a tablet the consumers would appreciate swallowing. The TG tablets were 950 mg and already a relatively large tablet.

The thicker the layer the molecule have to diffuse through, the longer before the permeant reaches the other side of the diffusion layer. The thickness of the coating layer can be measured in various ways, from hands on measurements to ultraviolet light measuring each and every tablet in a batch. An easy and inexpensive method is to record weight gain of the tablets. The drawback of this method is that it does not say anything about the quality of the coating layer, how evenly the gained weight from the polymer has spread out on the tablet surface. The method can be complemented by physical observation of the tablets.

The thickness of a coating film is limited by process considerations and economic factors. Coating is a time consuming and relatively expensive process. It was a goal to achieve sufficient stability with a minimized application of a coating. It was observed in introductory coating trials where HPMC film in layers of 3% (w/w) and 6% (w/w) were applied to TG tablets, that a 6% (w/w) was necessary to provide sufficient oxidative stability.

In addition to thickness of the coating film, physical and chemical properties of the film material are parameters that can easily be selected attempting to suit the tablet core. Looking at the diffusion processes related to migration of molecules through polymer films there are three main obstacles the diffusing molecule will have to overcome: It will have to adsorb to the surface of the film, diffuse through it and finally, desorb from the materials in the film and over to the materials of the tablet core [8]. The ideal coating for omega-3 containing tablets should therefore represent a barrier to oxygen in each step of the migration process.

Since oxygen is an apolar substance the coating should possess a certain polarity; making the adsorption process less facilitated and the solubility of the oxygen molecules in the polymer film low.

No commercially available coating materials are marketed specifically as oxygen protective per today; however, properties as “moisture protective” and “taste and odor masking” imply that the coating materials are less permeable to various gases. Aroma substances causing odor and taste belongs to a group of chemically and physically differing molecules, if a coating is impermeable for this wide range of molecules with regard to size and polarity, it might offer some protection against oxygen as well.

PVA is the polymer with the most references to possible protection against oxidation. It is a synthetic polymer, odourless, non-toxic and water soluble; hence, hydrophilic. The film forming properties are claimed to be very good, yet it dissolves fast in aqueous media. Since the coated tablet was intended for a marketed product, regulatory approval for use in health supplement products was required for the selected coating material. It should in addition be in the assortment of commercially available coatings from one of the well-known suppliers for convenient supply.

Nutraficient is a line of coating materials supplied by Colorcon, UK. Colorcon is a well-known supplier of coating materials, with a wide range of products and steady supply (Colorcon, http://www.colorcon.com/). Initially it was intended to use a coloured coating material. However, the tablet cores repelled any coating material containing titanium dioxide (TiO_2), which is the most commonly used opacifier in coloured coating materials. It seemed like the TiO_2 gathered in clusters on the surface of the tablets, causing thinner and thicker parts of the coating and even small holes. The reason is probably that the TiO_2 used in the coating material is of the water-dispersible kind and therefore repelled the fat on the surface of the TG tablets. There are other opacifiers available, however, it was chosen for the time being to move forward with the preparation of prototypes utilizing a clear version of Nutraficient. In all results presented on coated tablets in this work, the tablets were coated with a 6% (w/w) layer of Nutraficient Clear.

A short-time stability study (60 days) was conducted on TG 30% (w/w) powder, tablet cores and coated tablets. The intention was to get a quick glance at the impact of the production processes on oxidative stability of the fatty acid esters. It can be assumed that oxidation was initiated in all production processes necessary to prepare the samples tested. However, as can be observed in this study, both the compaction of the powder to tablets and the addition of a

coating layer delayed oxidation rates when compared to pure powder (Fig.6). It is highly likely that the effect observed was caused by decreased access for triggers of oxidation.

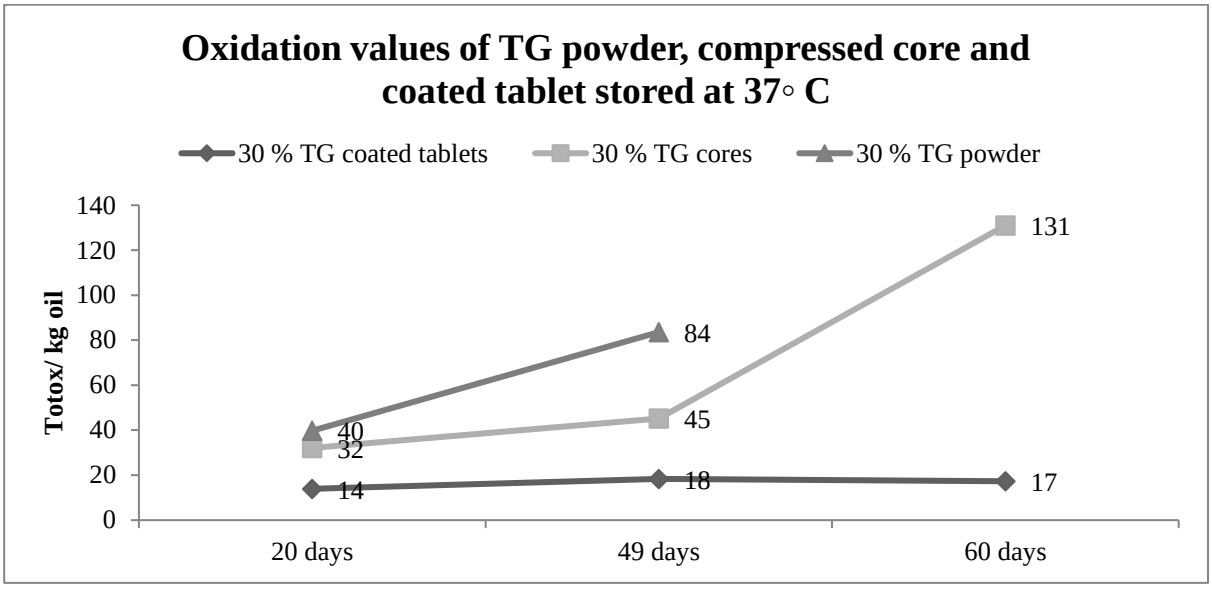

Figure 6. The triglyceride powder comprised 96% (w/w) of the tablet cores. The product samples were kept in small plastic containers permeable for gases and light. N=1, 3 analytical replicates, standard deviations for PV and AV analyses were less than 0.7 for all replicates.

This study was, as mentioned, intended as a quick glance. The TG samples were therefore prepared without the use of antioxidants and stored at 37ºC. For health supplement products it is possible to use stability study models based on pharmaceutical research; for tablets this would typically imply long-term and accelerated studies at various humidities and temperatures as detailed in the ICH guidelines [53]. However, it is known that for foods containing fats and oils, applying elevated temperatures can influence the oxidation process in an unpredictable manner. Heat can impact not only the rate of oxidation, but also the favored oxidation reaction patterns and the activity of the different pro- and antioxidants present [54]. Hence, results gained from extrapolation of accelerated studies may not necessarily be consistent with result from real-time stability studies at ambient temperatures.

Despite this fact it is common to use accelerated stability studies as an indicator for shelf-life among producers of omega-3 supplements, often because it is desirable to launch the product before a real-time stability study at ambient temperatures can be finalized.

Omegatri AS had prior to this project conducted a screening of antioxidant effect in combination with the omega-3:β-CD complexes. The effects of some selected antioxidants

in this particular system were screened in a stability study with freeze dried powders using multivariate analysis (Omegatri AS, unpublished results). The powder samples were stored for 1-10 weeks under ambient or accelerated conditions (37^{o}C). The multivariate setup included the antioxidants ascorbic acid, ascorbyl palmitate, cloudberry extract, rosemary extract and ethylenediaminetetraacetic acid **(**EDTA**)** in various concentrations.

The results from the screenings indicated that all antioxidants added value as protection against oxidation. It was demonstrated that ascorbic acid played an essential role, as decreasing totox levels were correlated with increasing concentrations of ascorbic acid. The data further suggested a superior effect of a combination of ascorbic acid and EDTA; the levels of the respective antioxidant and chelator were at 0.5% (w/w) and 0.11% (w/w).

Later it was discovered that EDTA is not allowed used in tablets in the European Union (EU) and efforts was made investigating whether EDTA could be excluded. The study of antioxidant effect in spray granulated powders presented in Paper III established that the powder containing ascorbic acid only exhibited oxidative stability until a certain time point. The importance of EDTA as processing agent during the aqueous phase of the production process was firmly emphasized (Paper III, Fig.1).

Upon a more extended study of the EU regulations, however, it proved possible to include EDTA in the formulation, see details in section 4.3.2. EDTA could therefore be re-introduced as a part of the standard formulation. The long-term stability study conducted to further observe the results indicated in Fig.6 was thus conducted on samples prepared with ascorbic acid and EDTA. The samples were in addition separated in two batches, to observe effect of storage at both 37^{o}C and at ambient temperatures.

4.2.2 Determination of the oxidative stability of relevant omega-3 powder/tablet prototypes in long-term and accelerated stability studies- Estimating shelf-life

The impact of reduced access for oxygen and light by compressing the powder to tablets was confirmed in the long-term stability study on TG powder, tablet cores and coated tablets presented in Paper III. This was demonstrated by the delayed oxidation of omega-3 fatty acid esters in the tablet cores compared to the oxidation rate in pure powder.

The totox values of the pure powder were above 30 at some point between 2nd and 3rd month of storage in samples kept at 37^{o}C and at around 11 months for samples stored in ambient conditions. The corresponding value for the tablet core samples was totox >30 after 5 months for samples stored in 37^{o}C. The tablet core samples kept in ambient temperature had just started to increase in totox 1 year after production (Paper III, Fig.3).

As detailed in Paper III, the stability studies suggested a preliminary shelf-life of the final coated TG tablet of approximately 22 months. The tablets were tasteless and odor-free. In the health supplement industry, accelerated stability studies are widely used to follow the stability of a product for 3-6 months. If sufficient stability is observed, the shelf-life is typically determined to 2 years. With the results observed, totox below 30 for 13 months for TG coated tablets stored at 37^{o}C and below 5 for samples kept at ambient conditions, there is little doubt that this product would receive a preliminary shelf-life of 2 years. Furthermore, the quantitative measurements of EPA and DHA in TG tablets performed after 1 year confirming the dosage of EPA and DHA contributes positively to the conclusion (Paper III, section 3.4).

The results from the pure powder samples indicated that the powder could be stored for a reasonable period of time under specified conditions. This promotes foundation for re-sale of the powder as raw material, given that the correct storage containers and facility.

The use of the tablet cores as a final product cannot without further precautions be recommended from the results observed in these studies. Coated and uncoated tablets are typically put in a box and stored at ambient conditions in stores and at the end consumer. However, if the tablet cores were blistered and stored cool, the tablet cores may have potential as a final product. As an intermediate product, the recommendation would be to coat the tablets as soon as possible, in order to slow down the oxidation initiated by the compaction process.

Looking at what has been accomplished previously on inclusion of fatty acid (esters) in CDs with the aim of protection against oxidation, the main theory states that lipophilic substances properly complexed with CDs can achieve protection against among other oxidation, light-induced decompositions and heat-induced changes. This can contribute to a longer shelf-life [16, 17]. Several publications describe the attempt to accomplish this protection with long-chained poly-unsaturated fatty acids.

Regiert *et al* performed a study where vegetable oils containing TGs were attempted complexed by α-, β- and γ-CD. They found that only when using γ-CD complete encapsulation was achieved. Light and access for oxygen was excluded during the complexation process. The following stability study revealed that only the powder prepared from γ-CD remained stable after two months of storage, and that increasing the amount of CD relative to oil increased the stabilizing effect [55].

Yoshii *et al* prepared inclusion complexes of EPA or DHA in α- and β-CD to investigate whether the oxidative stability of the fatty acids in the dry complexes was higher than for the corresponding pure oils. The complexes were prepared by mixing oils and CD and adding water up to 50% (w/w) of the weight of solids. The resulting powders were found to contain very small amounts of fatty acids included in β-CD, only about 0.01 per unit mass, the results were therefore inconclusive [56].

However, according to the results from tablet trials and XRD studies in this project, the amount of free oil in the powders prepared for this thesis was low enough to be able to prepare good quality tablets. It can therefore be assumed that the fatty acid esters from the 60% TG concentrate to a large extent was complexed by β-CD. The protective effect of the mere complexation by β-CD was confirmed in an unpublished study performed where a 20 g sample of 60% fish oil concentrate was stored in a closed box on a shelf, under otherwise ambient conditions. The sample reached totox 58 after one week (Omegatri AS, unpublished results), whilst the powder samples in the long term study stored at ambient temperature kept below totox 30 for 11 months (Paper III, Fig.3, top panel). These results demonstrate that the complexing of TG oil with β-CD results in longer shelf-life, unlike what was observed by among other Regiert *et al* and Yoshii *et al.*

Spray granulating under nitrogen atmosphere significantly added time to the shelf-life of TG 30% (w/w) powder stored at 37ºC. The spray granulation process introduces a massive amount of air, in addition to mechanical stress and elevated temperatures [26]. Typically, residence time in the fluid bed chamber was approximately 1 hour and the product temperature was 40-45ºC. Performing the spray granulation utilizing N_2 instead of air as fluidization and atomization gas reduced the totox in powder samples kept at 37ºC from 468 to 34 (Paper III, Fig.2).

Equal storage conditions for samples spray granulated in air and N_2 did not annihilate the initial differences observed in totox, despite permeable storage containers and the relatively large surface/volume ratio of all powder samples (8 g). Whether similar differences will be observed in powders stored in ambient temperatures is yet to be seen in the study. Nevertheless, the significant difference observed for powder samples kept at 37ºC does represent a firm indication of the positive impact of spray granulation in N_2 on oxidation rates. It can therefore be assumed that higher oxidative stability for coated TG tablets than seen in these studies can be achieved, if N_2 is utilized during spray granulation.

In sum, during the processes necessary to produce a dry powder, pressing powder to tablets and coat them, the usual protective surroundings applied to products containing omega-3 will typically not be available. It is normally necessary to perform production of powder and tablets in bright light and air, as it is challenging to achieve inert atmosphere within the required production equipment. Blending, tableting and coating applies massive mechanical stress and elevated temperatures.

Despite the quite extensive production processes applied to the TG oil described in this work, the overall results observed demonstrated that oxidative stable TG tablets can be produced utilizing standard production equipment. This shows that the protective measurements actually applied in the process from TG oil as raw material to finished tablet, had a strong enough impact on the initiation and rate of oxidation to withstand the influence of the non-ideal surroundings.

4.3 Bioavailability of EPA and DHA from triglyceride tablets

4.3.1 Establishment of the level of bioavailability of EPA and DHA from the triglyceride tablets compared to soft-gel capsules

The bioavailabilities of numerous omega-3 supplements have been reviewed recently and it was observed a pronounced variation in bioavailability [57, 58]. The health benefits obtainable from an omega-3 supplement requires that the omega-3 is bioavailable from the administration form. The final step in the development of a health supplement product was therefore to establish the bioavailability of the omega-3 fatty acid esters from the TG tablets.

In disintegration studies it was observed that the tablets disintegrated in hydrophilic environments after approximately 1 hour, ultimately releasing the oil. It was therefore assumed that the omega-3 fatty acids esters would be bioavailable. However, it was not easily predictable how the absorption would be influenced by the concurrent presence of β-CD. CDs are extensively used by the pharmaceutical and food industry, among other for increasing the bioavailability of lipophilic substances [16]. It could therefore be that the bioavailability of the omega-3 fatty acid esters proved higher from the tablets based on omega-3: β-CD complexes, than from certain other administration forms. On the other hand, it could be that the TG oil did not leave the complex *in vivo* or that it was released from the complex later than ideal for absorption.

The primary aim was to establish oral bioavailability of omega-3, represented by EPA and DHA in TG form, from the tablets. The oral bioavailability from the tablets was to be compared with soft-gel capsules, an administration form that is also a defined unit intended

for oral administration by immediate swallowing. The soft-gels used in this study contained the same TG oil, in comparable doses, as used in the prepared tablets.

The responsibilities included in this project were to organize the bioavailability study on behalf of Omegatri AS, interpret and publish the results. Omegatri funded the entire study and in clinical studies it is considered important that representatives for the funder is not involved in certain key activities. The study was therefore officially coordinated by a representative from Drug Discovery Laboratory AS. However, as typical is for small companies, the basis work is performed at the company, resulting in a draft which is quality assured by the hired consultant.

In this case, this basis work included among other the preparation of a draft for the clinical trial protocol, the case report form and invitation and information sheet to potential volunteers. In addition to the main bioavailability study presented in Paper IV, two smaller bioavailability study pilots were performed at the facility of Omegatri. Work with these included complete study design, recruiting of volunteers and a nurse to do the actual blood sampling on the test days, purchase of necessary blood sampling equipment and cooperation with the analytical laboratory. All analyses of blood samples were performed at an external laboratory (Vitas AS, www.vitas.no).

The main bioavailability study presented in Paper IV was performed at the Comprehensive Clinical Trials Unit, Oslo University Hospital (Rikshospitalet, Oslo, Norway). The study was approved by Regional Committees for Medicinal and Health Research Ethics.

The study was organized as a randomized crossover study to exclude bias. The participants were healthy male volunteers above the age of 18 years. The following inclusion/exclusion criteria were applied; sex (only males were included), age (only men >18 years) and body mass index (restricted to 20-28 kg/m^2). The reason behind the exclusion criteria was to secure a certain homogeneity in the population. Since the population was small (10 volunteers), this was thought of as an advantage for the comparison of bioavailability between the individuals.

The participants were asked to avoid certain types of food (fish, linseed, linseed oil, soy oil, walnuts, products containing grapefruit, vitamin supplements and supplements in general) 1 week prior to the first test-day and through the entire duration of the study (3 weeks). Moreover, they were asked to avoid caffeine and alcohol 72 hours prior to, and during, each test-day. This to exclude potential interference with results.

The diet on each test-day was standardized and the participants were asked to avoid eating or drinking from 8 p.m. the prior day. The standardized breakfast was a fat-rich meal served immediately after administration. Again, an attempt to exclude individual variations caused by the diet. The fat-rich meal was given to stimulate bile activity, attempting to enhance the uptake of omega-3.

On each test-day the participants were given a single dose TG 60% concentrate formulated in tablets *or* soft-gel capsules. The dosage chosen was high (3 g), in order to be ensure the fatty acid being easily detectable from the blood samples. A washout period of 14 days was applied between administrations of the individual dosage forms.

The baseline level of EPA and DHA was established prior to administration on each test-day and the relative increase in individual levels of EPA and DHA was followed for 24 hours after administration. Blood samples were taken before administration of omega-3 (baseline) and 2, 3, 5, 8 and 24 hours after administration.

As presented in Paper IV, the bioavailability study demonstrated that both EPA and DHA had a non-significant numerical higher systemic exposure following administration of omega-3 tablets, compared to soft-gel capsules (Paper IV, Fig.1, Table 1, Table 2). It could be concluded that the bioavailability of EPA and DHA from the TG tablets was comparable to from soft-gel capsules. Omegatri AS could, hence, prove that the omega-3 was bioavailable from the formulation form, which was the main intention behind the study.

However, the study design, with the first blood sample after administration scheduled 2 hours after ingestion, was not able to register the true maximum concentration (Cmax) for EPA and DHA after ingestion of the TG tablets. This can be assumed, as the peaks were registered at the first blood sample (t=2 h). This strongly indicates that the true peaks came earlier and were higher.

The foundation for the blood sample regime was taken from relevant literature on bioavailability studies, in addition to recommendations from the Comprehensive Clinical Trials Unit [59-61]. It was focused on 2-8 hours after administration, as it was believed that Cmax would be occurring during that time period. This was true for EPA and DHA from the soft-gel capsules, but not from the TG tablets.

Despite the study design not being able to register the true Cmax for EPA and DHA from the TG tablets, the differences observed in time for maximum concentration (Tmax) were still significantly different for both EPA and DHA from the administration forms (Paper IV,

Fig.1, Table 1, Table 2). These differences would most probably have been even greater, if the true Cmax for both fatty acids had been registered.

As discussed in Paper IV, it was believed that the difference was caused by the alternation of solubility of the fatty acid esters caused by the complexing with β-CD and less dependency of emulsifying bile during absorption caused by the delivery of the fatty acid esters as molecules rather than fat droplets.

The study could support that the administration form had an influence on the rate of absorption. However, one of the most exciting differences thought to exist between the administration forms could not be proved; the theory of the reflux-free omega-3 supplement.

Gastric reflux is defined as stomach content returning up the esophagus causing a burning sensation. It is estimated that as much as 40-60 % of the population suffers from mild to severe symptoms of reflux [62, 63]. It is not clinically proven that omega-3 fatty acids exacerbates the prevalence of reflux; however, when omega-3 oil has been ingested and reflux occurs, the oil can worsen the experience due to unpleasant taste and/or odor.

Soft-gels, pure oils or any administration form for omega-3 that releases the oil as bulk oil in the stomach, will increase the risk of fish-tasting reflux due to the oil being retained in the stomach as an oil layer on top of the hydrophilic stomach content. As detailed in Paper IV, individual omega-3 fatty acid esters complexed with β-CD in the stomach will pass over to the intestines faster due to the hydrophilic surface of the β-CD. The results from the bioavailability study, with the short time to Cmax for EPA and DHA from the TG tablets, supports this. Therefore, in theory, it will be no layer of oil on top of the stomach content being able to cause fish-tasting reflux after ingestion of the TG tablets.

The experience of reflux is, however, challenging to quantify. In the performed bioavailability study **t**he participants were asked to fill in a form after the study where they were asked whether they experienced any reflux during the 24 hours after administration of TG oil. No-one reported any incidents of reflux, either when administered as tablets or soft-gels. This is not so surprising, as the participants received only one single dose omega-3 as soft-gel or tablet per test-day. The participants were healthy adult males, typically not in the population mostly bothered by fish-tasting reflux after ingestion of omega-3.

A population that would benefit greatly from a guaranteed reflux-free omega-3 supplement, on the other hand, is pregnant women. It is considered crucial that this group receives their daily dose of the essential omega-3 fatty acids to ensure optimal development

of the foetus [4, 5]. Unfortunately, many pregnant women are especially bothered by reflux during pregnancy and are, moreover, extra sensitive to smell and taste. A reflux-free omega-3 supplement can therefore be considered essential.

As mentioned, the TG tablet being a reflux-free omega-3 supplement is currently just a theory based on the physical-chemical properties of the product, as well as biological conditions in the digestion system, and is only indirectly supported by the performed bioavailability study. Hopefully this can be further investigated in future studies.

4.3.2 Assessment of available regulatory information on the main components of the omega-3 powders

In order to fulfil the EFSA claim, the intended daily dose of EPA+DHA should be minimum 250 mg per day [6]. The goal was to be able to supply this amount with 2 tablets. A 900 mg tablet compressed from 30% TG powder, of which the powder comprises 96% (w/w) of the tablet formulation, will contain 129 mg EPA+DHA, when the triglyceride oil was a 60% concentrate.

The ingredients contributed by the TG powder in the 2 tablets would be as follows;

2 tablets	1800 mg
TG powder	1729 mg
β-cyclodextrin	1201 mg
Triglyceride oil, of which;	518 mg
EPA+DHA	259 mg
Other fatty substances	207 mg
Other omega-3 fatty acid esters	52 mg
Ascorbic acid	9 mg

Norwegian laws are coordinated with EU regulations, officially defining omega-3 as a medicinal product only when the recommended intake exceeds 3 grams per day (FOR-1999-12-27-1565, www.lovdata.no (2016)). Similarly, in 1997, the United States (US) Food and Drug Administration (FDA) issued a rule affirming omega-3 oil as generally recognized as safe (GRAS) for use as food ingredient, as long as the consumption does not exceed 3g/day (62 FR 30751, www.gpo.gov/fdsys/granule/FR-1997-06-05/97-14683/content-detail.html (2016). The intended daily dose of omega-3 oil from the tablets is well below this dose. The supplement is therefore considered a nutraceutical supplement, both in EU and the US. Omega-3 supplements in these doses are considered very safe and adverse effects, like upset stomach and loose stools, are not expected [5].

The legal status of a substance sold in the US is dependent on whether it is offered for use in a medicinal product, as conventional food or in a dietary supplement. There exists an own monograph for β-CD in the US Pharmacopeia for use of the substance in medicinal products. Furthermore, β-CD is included in the GRAS list, in defined amounts for defined uses in conventional food.

For use in dietary supplements, like the TG tablets, the regulatory status of a substance will depend on whether it is defined as the active ingredient or as an excipient in the relevant product. In the TG tablets, β-CD acts as a carrier for the active ingredient and will, hence, be categorized as an excipient. The approved use of an excipient in a product categorized as a dietary supplement in the US will finally rely on the safety profile accepted by the brand owner. The brand owner must take responsibility for the potential consequences for human health caused by the products that they supply to the US market. The safety standard is "reasonable certainty of no harm" under the conditions of intended use established by FDA (21 CFR 170.3(i), https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=170.3).

Dietary supplement is not an own category in the EU. All that is not medicine is categorized as food. The official EU regulation 1333/2008 states the use of β-CD (E 459) in food as *quantum satis* in coated and uncoated tablets and as maximum 1 g/kg final food in food other than uncoated/coated tablets. A separate monograph exists for β-CD in Ph. Eur. for use in medicinal products.

The restrictive allowed use of β-CD in food other than tablets in the EU is among other founded on a report from the European Medicines Agency (EMA). In 2014, EMA reviewed the use of CDs as excipients in various formulations. The report concluded that CDs in oral formulations are safe, mainly due to low absorption of the substances. Nevertheless, it was recommended a maximum level of 500 mg/day or a permitted level of exposure of 10 mg/kg/day for β-CD; probably as a precaution after observed renal toxicity with parenteral use of the substance (EMA/CHMP/333892/2013,http://www.ema.europa.eu/ema/index.jsp?curl=search.jsp&q=EMA%2FCHMP%2F333892%2F2013&btnG=Search&mid (2016)).

Marketing and sale of the TG tablets with regard to the level of β-CD included is hence allowed both in the US and the EU. However, taking into consideration the low levels of

exposure recommended by EMA, it is nevertheless advisable for a producer to assess the available safety profile of the ingredient, for internal documentation and information to the brand owners considering the product.

β-CD in systemic circulation can cause hemolysis of erythrocytes due do the extraction of cholesterol. However, the main concern regarding native CDs (α, β, γ) is nephrotoxicity caused by the formation of microcrystals in kidney. This may occur as native CDs are excreted unmetabolized in urine and have low water solubility. This effect has been observed as most significant with β-CD due to having the lowest water solubility (about 18.5 mg/ml) [15, 64, 65].

However, taken orally β-CD is considered practically non-toxic due to the low absorption. B-CD is composed of seven alpha- (1,4) linked D-glucopyranosyl units; the cyclic structure resists enzymatic hydrolysis by beta-amylases and saliva alpha-amylases. Therefore, β-CD is poorly hydrolyzed in the human small intestine. The relatively large molecule size of β-CD (1135 MW), as well as the general structure of the molecule with several hydrogen donors and acceptors, cause them to be poorly absorbed by biological membranes in intact form [18, 19, 64, 66].

2 tablets per day will give a daily exposure of 1201 mg β-CD. It is established that about 0.6% (w/w) of the β-CD taken orally reach systemic circulation by passive diffusion if the substance is taken in pure form. When taken in complex with a drug or nutrient, a reduction in bioavailability has been observed of up to 80% when compared administration in the pure form [67]. 1201 mg β-CD taken in complex with omega-3 fatty acid esters and other fatty components from the TG oil would then give less than 1.5 mg β-CD in systemic circulation. An adult has between 4 and 6 L of blood and will have no issues with precipitation of β-CD at this level.

Adverse effects caused by β-CD in the gastrointestinal system are not expected at this dose level. The substance is fermented by the colonic microflora and can, if consumed in excess, cause bloated stomach and reversible diarrhea. In animal studies, reversible diarrhea has been observed with ingested doses of >1000 mg/kg/day. This is a quite standard reaction to large amounts of indigestible carbohydrates [16, 65, 66].

B-CD can in theory reduce bioavailability of lipophilic nutrients, as vitamin A and D, by forming complexes. However, complexing with CDs is a dynamic, reversible process. It is not expected that any fixed complexes will be formed in the relatively diluted gastrointestinal system, especially if the CD was not administered in pure form.

Large food and pharmaceutical ingredient manufacturing companies (primarily Wacker and Roquette) have gathered extensive safety dossiers on the CDs, including numerous acute and subchronic toxicity studies, embryotoxicity/teratogenicity studies, human tolerance studies and other toxicity and pharmcodynamic studies. The observed levels of safe consumption are reported as significant. LD_{50} for rats ingesting β-CD orally has been estimated at >5000 mg/kg (del Valle, 2003=5). Non-toxic levels have been registered at up to 864 mg/kg/day for rats and 1967 mg/kg/day for dogs [68].

However, children below 2 years of age and persons with renal impairment should perhaps avoid this supplement due to the relatively high content of β-CD.

Other regulatory territories than the EU and the US have individual, various regulations describing the use of β-CD. It is worth mentioning that β-CD has a separate monograph in the Japanese Pharmacopeia and is otherwise categorized as modified starch approved for general food applications in this territory [16, 66].

According to EU regulation 1333/2008, EDTA (E385) is not allowed as an additive in tablets. It is, however, allowed in among other spreadable fats (like dressing, mayonnaise, margarines and so forth), canned and bottled fish and emulsified sauces. EDTA is hence not considered a dangerous substance, as reflected in the regulation.

However, EU regulation 1333/2008 also states that; *Substances not consumed as food itself but used intentionally in the processing of foods, which only remain as residues in the final food and do not have a technological effect in the final product (processing aids), should not be covered by this Regulation*. EDTA has its functions in the water phase in the intermediate aqueous mixture prepared prior to the drying of the powders in this work. Analyses for residues in the omega-3 powders showed an EDTA residue of about 0.01 mg/g powder. EDTA can therefore be categorized as a processing aid and is thus allowed according to EU regulations.

In the US, EDTA is recognized by the FDA as GRAS for use at levels of < 2% in foods.

Ascorbic acid (E300) is a food additive generally permitted in the EU. According to the list of additives, the maximum level for supplements is "*quantum satis*" (EU directive 98/72/EC and amending directive 95/2/EC). Ascorbic acid is included in the GRAS list without limitations or comments from the FDA for the US markets.

5 Conclusive summary

This project was initiated on the basis of a promising complex of omega-3 and β-CD developed by Omegatri AS. The complex was intended for use in tablets. The tablets were to fulfil certain criteria;

- The omega-3 source must be fish oil.
- The tablets should meet relevant requirements for the administration form as defined by e.g. the European Pharmacopeia.
- A daily dose of 250 mg EPA+DHA should be possible to administer with 2 tablets.
- The oxidative stability should enable a shelf-life of 18-24 months under conditions typically relevant for tablets (ambient conditions).
- The oral bioavailability of EPA and DHA from the tablets should be comparable to other relevant administration forms, e. g. soft-gel capsules.
- The production processes should be developed using equipment available in several sizes, in order to facilitate future upscaling.
- The powders prepared should not comprise any excipients not approved for use in tablets by relevant authorities.

The production processes developed in this work have caused foundation for the preparation of DC grade omega-3 powders and omega-3 tablets. Spray granulation as drying technique secured powder particles with shape, size and size distribution ideal for tableting. The compactibility of pure β-CD proved transferable to the omega-3:β-CD complexes. The search for the ideal tablet excipients and coating formulation resulted in coated tablets of good quality, utilizing regulatory approved excipients from well-known suppliers. All production processes, spray granulation, tableting and coating, were developed using standard production equipment.

The bioavailability of selected fatty acid esters from the administration form was found to be comparable to alternative products already marketed and a shelf-life of 2 years in ambient conditions could be preliminarily set for the TG tablets. The developed tablets fulfil the EFSA claims, requiring a daily dose of 250 mg EPA+DHA, with 2 tablets per day.

6 Further work

The production processes for preparation of TG powder and tablets have shown to be robust and up-scalable and production on ton scale is currently being performed on a regular basis. However, optimization of processes is continuously ongoing, with the aim of maximizing the output while minimizing process time. The water content of the intermediate liquid mixture is currently quite high, causing a relatively low output per hour of drying (spray granulating). Trials with lower water content of the initial hydrophilic mixture will be performed. The risk with reduced water content is not being able to suspend the complexes formed.

The methods for inclusion of high amounts of liquid oils in compactible powders will be further used to explore possible inclusion of other interesting oils in tablets. An example can be algal oil instead of fish oil, for a full vegetarian alternative for the relatively large population of vegetarians (like most of India and surrounding countries).

A new stability study on TG 30% (w/w) products is initiated, and will be extended when possible. The study will include powder samples both stored in room temperature and below 8^{o}C, all in original container (aluminium bag in cardboard box, 20 kg). The intention behind the study is to establish shelf-life of the powder as raw material under different storage conditions. When this powder was included in the stability studies presented in this work, the main intention was to compare the stability of powder, tablet cores and coated tablets, or to study effect of antioxidants or process conditions.

A new long-term stability study of the coated tablets is also planned, as observations suggest that powder produced on a larger scale has better oxidative stability and it is therefore expected that the resulting tablets will be more stable than shown in this work. The increased stability is mainly attributed to the lower moisture content of powders produced on a larger scale. It is possible to achieve down to 7-8% RH in up-scaled spray granulators.

A supplementary bioavailability study with an optimized study design should be performed in order to maximize the output of the research done. A study confirming whether or not the omega-3 tablet could be labelled reflux-free would imply a quite large data set (50+ participants) and a carefully chosen product to compare with. However, the effort necessary in such a study would naturally defend itself if it should prove attainable to advertise the

product as reflux-free. Then advertising directed at pregnant women could significantly contribute to ensure the omega-3 supplement in such an important target group.

References

1. Rizos, E.C., et al., *Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: A systematic review and meta-analysis.* JAMA, 2012. **308**(10): p. 1024-1033.
2. Riediger, N.D., et al., *A Systemic Review of the Roles of n-3 Fatty Acids in Health and Disease.* Journal of the American Dietetic Association, 2009. **109**(4): p. 668-679.
3. Simopoulos, A.P., *Omega-3 fatty acids in health and disease and in growth and development.* The American journal of clinical nutrition, 1991. **54**(3): p. 438-463.
4. Karr, J.E., J.E. Alexander, and R.G. Winningham, *Omega-3 polyunsaturated fatty acids and cognition throughout the lifespan: a review.* Nutritional neuroscience, 2011. **14**(5): p. 216-225.
5. Whelan, J. and C. Rust, *Innovative Dietary Sources of N-3 Fatty Acids.* Annual review of nutrition, 2006. **26**(1): p. 75-103.
6. European Food Safety Authority. *Scientific Opinion on the substantiation of health claims related to eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and maintenance of normal cardiac function (ID 504, 506, 516, 527, 538, 703, 1128, 1317, 1324, 1325), maintenance of normal blood glucose concentrations (ID 566), maintenance of normal blood pressure (ID 506, 516, 703, 1317, 1324), maintenance of normal blood HDL-cholesterol concentrations (ID 506), maintenance of normal (fasting) blood concentrations of triglycerides (ID 506, 527, 538, 1317, 1324, 1325), maintenance of normal blood LDL-cholesterol concentrations (ID 527, 538, 1317, 1325, 4689), protection of the skin from photo-oxidative (UV-induced) damage (ID 530), improved absorption of EPA and DHA (ID 522, 523), contribution to the normal function of the immune system by decreasing the levels of eicosanoids, arachidonic acid-derived mediators and pro-inflammatory cytokines (ID 520, 2914), and "immunomodulating agent" (4690) pursuant to Article 13(1) of Regulation (EC) No 1924/20061*. 2010 12.2013]; Available from: http://www.efsa.europa.eu/en/efsajournal/doc/1796.pdf.
7. Frankel, E.N., *Control of oxidation*, in *Lipid oxidation*. 2005, The Oily Press- Woodhead Publishing. p. 187-206.
8. Hovorka, S.W. and C. Schoneich, *Oxidative degradation of pharmaceuticals: Theory, mechanisms and inhibition.* Journal of pharmaceutical sciences, 2001. **90**(3): p. 253-269.
9. GOED. *Global Organization of EPA and DHA; voluntary monograph for EPA and DHA, version 4*. 2015 05.2015 05.2015]; Available from: http://www.goedomega3.com/images/stories/files/goedmonograph.pdf.
10. *Monograph 1353; Omega-3-acid triglycerides* in *European Pharmacopoeia 5.0*. 2005.
11. Fassihi, A.R. and I. Kanfer, *Effect of Compressibility and Powder Flow Properties on Tablet Weight Variation.* Drug development and industrial pharmacy, 1986. **12**(11-13): p. 1947-1966.
12. Wells, J., *Pharmaceutical preformulation*, in *The science of dosage form design*, M.E. Aulton, Editor. 2002.
13. Leuenberger, H. and B.D. Rohera, *Fundamentals of powder compression. I. The compactibility and compressibility of pharmaceutical powders.* Pharmaceutical research, 1986. **3**(1): p. 12-22.
14. Jivraj, M., L.G. Martini, and C.M. Thomson, *An overview of the different excipients useful for the direct compression of tablets.* Pharmaceutical Science & Technology Today, 2000. **3**(2): p. 58-63.
15. Davis, M.E. and M.E. Brewster, *Cyclodextrin-based pharmaceutics: past, present and future.* Nature reviews. Drug discovery, 2004. **3**(12): p. 1023-1035.
16. Del Valle, E.M.M., *Cyclodextrins and their uses: a review.* Process biochemistry, 2004. **39**(9): p. 1033-1046.

17. Szente and J. Szejtli, *Cyclodextrins as food ingredients.* Trends in food science & technology, 2004. **15**(3): p. 137-142.
18. Brewster, M. and T. Loftsson, *Cyclodextrins as pharmaceutical solubilizers.* Advanced drug delivery reviews, 2007. **59**(7): p. 645-666.
19. Loftsson, T., et al., *Cyclodextrins in drug delivery.* Expert opinion on drug delivery, 2005. **2**(2): p. 335-351.
20. Ghorab, M., et al., *Tablet formulation containing meloxicam and β-cyclodextrin: Mechanical characterization and bioavailability evaluation.* AAPS PharmSciTech, 2004. **5**(4): p. 63-68.
21. ElShaboury, M.H., *Physical properties and dissolution profiles of tablets directly compressed with β-cyclodextrin.* International Journal of Pharmaceutics, 1990. **63**(2): p. 95-100.
22. Saleh, S.I., *Beta-cyclodextrin as a direct compression excipient compared to conventional ones.* Journal de pharmacie de Belgique, 1993. **48**(5): p. 371-377.
23. Szejtli, J. and L. Szente, *Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins.* European journal of pharmaceutics and biopharmaceutics, 2005. **61**(3): p. 115-125.
24. Shangraw, R., G.S. Pande, and P. Gala, *Characterization of the tableting properties of β-cyclodextrin and the effects of processing variables on inclusion complex formation, compactibility and dissolution.* Drug development and industrial pharmacy, 1992. **18**(17): p. 1831-1851.
25. de Castro, A., et al., *A new approach to the granulation of β-cyclodextrin inclusion complexes.* Chemical engineering journal, 2010. **164**(2): p. 316-321.
26. Summers, M. and M.E. Aulton, *Granulation*, in *Pharmaceutics- The science of dosage form design*, M.E. Aulton, Editor. 2002, Churchill Livingstone, Elsevier Limited. p. 372-374.
27. Aulton, M.E., *Drying*, in *Pharmaceutics- The science of dosage form design*, M.E. Aulton, Editor. 2002, Churchill Livingstone- Elsevier Limited. p. 389-390.
28. Wang, J., et al., *Lubrication in tablet formulations.* European journal of pharmaceutics and biopharmaceutics, 2010. **75**(1): p. 1-15.
29. Leuenberger, H., *The compressibility and compactibility of powder systems.* International Journal of Pharmaceutics, 1982. **12**(1): p. 41-55.
30. Perrault, M., F. Bertrand, and J. Chaouki, *An experimental investigation of the effect of the amount of lubricant on tablet properties.* Drug development and industrial pharmacy, 2011. **37**(2): p. 234-242.
31. Alderborn, G., *Tablets and compaction*, in *Pharmaceutics- The science of dosage form design*, M.E. Aulton, Editor. 2002, Churchill Livingstone- Elsevier Limited. p. 422-433.
32. Ohrem, L.H., et al., *Why is mannitol becoming more and more popular as a pharmaceutical excipient in solid dosage forms?* Pharmaceutical development and technology, 2014. **19**(3): p. 257-262.
33. Anwar, S. and B. Kunz, *The influence of drying methods on the stabilization of fish oil microcapsules: Comparison of spray granulation, spray drying, and freeze drying.* Journal of food engineering, 2011. **105**(2): p. 367-378.
34. Lopez-Nicolas, J.M., et al., *Use of'soluble lipids' for biochemical processes: linoleic acid-cyclodextrin inclusion complexes in aqueous solutions.* Biochem. J, 1995. **308**: p. 151-154.
35. Bru, R., J.M. López-Nicolás, and F. García-Carmona, *Aggregation of polyunsaturated fatty acids in the presence of cyclodextrins.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1995. **97**(3): p. 263-269.
36. Park, C.W., et al., *Inclusion complex of conjugated linoleic acid (CLA) with cyclodextrins.* Journal of agricultural and food chemistry, 2002. **50**(10): p. 2977-2983.
37. Furuta, T., *Microencapsulation of flavours and oil by Cyclodextrin.* Diss. Tottori University, nd Web.
38. Jyothirmayi, N., C.S. Ramadoss, and S. Divakar, *Nuclear magnetic resonance studies of cyclodextrin complexes of linoleic acid and arachidonic acid.* Journal of Agricultural and Food Chemistry, 1991. **39**(12): p. 2123-2127.

39. Parker, K.M. and A.M. Stalcup, *Affinity capillary electrophoresis and isothermal titration calorimetry for the determination of fatty acid binding with beta-cyclodextrin.* Journal of Chromatography A, 2008. **1204**(2): p. 171-182.
40. Szente, L., et al., ***Fatty acid-cyclodextrin complexes: Properties and applications.*** Journal of inclusion phenomena and molecular recognition in chemistry, 1993. **16**(4): p. 339-354.
41. Kolanowski, W., et al., *Microencapsulation of fish oil by spray drying-impact on oxidative stability. Part 1.* European food research and technology, 2006. **222**(3-4): p. 336-342.
42. Serfert, Y., S. Drusch, and K. Schwarz, *Chemical stabilisation of oils rich in long-chain polyunsaturated fatty acids during homogenisation, microencapsulation and storage.* Food chemistry, 2009. **113**(4): p. 1106-1112.
43. Kaushik, P., et al., *Microencapsulation of omega-3 fatty acids: A review of microencapsulation and characterization methods.* Journal of Functional Foods, 2015. **19**: p. 868-881.
44. Gibbs, B.F., et al., *Encapsulation in the food industry: a review.* International journal of food sciences and nutrition, 1999. **50**(3): p. 213-224.
45. Choi, M.-J., et al., *Morphological characterization of encapsulated fish oil with β-cyclodextrin and polycaprolactone.* Food research international, 2009. **42**(8): p. 989-997.
46. Choi, M.-J., et al., *Physical characteristics of fish oil encapsulated by β-cyclodextrin using an aggregation method or polycaprolactone using an emulsion–diffusion method.* Food chemistry, 2010. **119**(4): p. 1694-1703.
47. Rubio-Rodríguez, N., et al., *Production of omega-3 polyunsaturated fatty acid concentrates: A review.* Innovative Food Science & Emerging Technologies, 2010. **11**(1): p. 1-12.
48. KamalEldin, A. and N.V. Yanishlieva, *N-3 fatty acids for human nutrition: stability considerations.* European journal of lipid science and technology, 2002. **104**(12): p. 825-836.
49. Ke, P.J. and R.G. Ackman, *Bunsen coefficient for oxygen in marine oils at various temperatures determined by an exponential dilution method with a polarographic oxygen electrode.* Journal of the American Oil Chemists Society, 1973. **50**(11): p. 429-435.
50. Battino, R., T.R. Rettich, and T. Tominaga, *The Solubility of Oxygen and Ozone in Liquids.* Journal of Physical and Chemical Reference Data, 1983. **12**(2): p. 163-178.
51. Anwar, S., J. Weissbrodt, and B. Kunz, *Microencapsulation of Fish Oil by Spray Granulation and Fluid Bed Film Coating.* Journal of food science, 2010. **75**(6): p. E359-E371.
52. Pavlath, A.E. and W. Orts, *Edible Films and Coatings: Why, What, and How?*, in *Edible Films and Coatings for Food Applications*, M.E. Embuscado and K.C. Huber, Editors. 2009, Springer Science: USA. p. 1-23.
53. ICH, *Stability testing of new drug substances and products Q1A(R2)*. 2003, International Conference of Harmonization.
54. Kristott, J., *Fats and oils*, in *The stability and shelf-life of food*, D. Kilcast and P. Subramaniam, Editors. 2000, Taylor & Francis.
55. Regiert, M., T. Wimmer, and J.-P. Moldenhauer, *Application of gamma-cyclodextrin for the stabilization and/or dispersion of vegetable oils containing triglycerides of polyunsaturated acids.* Journal of inclusion phenomena and molecular recognition in chemistry, 1996(25): p. 213-216.
56. Yoshii, H., et al., *Oxidation Stability of Eicosapentaenoic and Docosahexaenoic Acid Included in Cyclodextrins*, in *Proceedings of the Eighth International Symposium on Cyclodextrins*, J. Szejtli and L. Szente, Editors. 1996, Springer Netherlands. p. 579-582.
57. Ghasemifard, S., G.M. Turchini, and A.J. Sinclair, *Omega-3 long chain fatty acid "bioavailability": a review of evidence and methodological considerations.* Progress in lipid research, 2014. **56**: p. 92-108.
58. Schuchardt, J.P. and A. Hahn, *Bioavailability of long-chain omega-3 fatty acids.* Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 2013. **89**(1): p. 1-8.
59. Kurowska, E.M., et al., *Bioavailability of omega-3 essential fatty acids from perilla seed oil.* Prostaglandins, leukotrienes and essential fatty acids, 2003. **68**(3): p. 207-212.

60. Lawson, L.D. and B.G. Hughes, *Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters.* Biochemical and biophysical research communications, 1988. **152**(1): p. 328-335.
61. Davidson, M.H., et al., *A novel omega-3 free fatty acid formulation has dramatically improved bioavailability during a low-fat diet compared with omega-3-acid ethyl esters: The ECLIPSE (Epanova® compared to Lovaza® in a pharmacokinetic single-dose evaluation) study.* Journal of clinical lipidology, 2012. **6**(6): p. 573-584.
62. Isolauri, J. and P. Laippala, *Prevalence of Symptoms Suggestive of Gastroesophageal Reflux Disease in an Adult Population.* Annals of medicine (Helsinki), 1995. **27**(1): p. 67-70.
63. Ronkainen, J., et al., *High prevalence of gastroesophageal reflux symptoms and esophagitis with or without symptoms in the general adult Swedish population: A Kalixanda study report.* Scandinavian journal of gastroenterology, 2005. **40**(3): p. 275-285.
64. Irie, T. and K. Uekama, *Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation.* Journal of pharmaceutical sciences, 1997. **86**(2): p. 147-162.
65. Stella, V.J. and Q. He, *Cyclodextrins.* Toxicologic pathology, 2008. **36**(1): p. 30-42.
66. Magnusdottir, A., M. Masson, and T. Loftsson, *Cyclodextrins.* J. Incl. Phenom. Macroc. Chem., 2002(44): p. 213-218.
67. Kubota, Y., et al., *Absorption, Distribution and Excretion of. BETA.-Cyclodextrin and Glucosyl-. BETA.-cyclodextrin in Rats.* Biological and Pharmaceutical Bulletin, 1996. **19**(8): p. 1068-1072.
68. Bellringer, M.E., et al., *β-Cyclodextrin: 52-week toxicity studies in the rat and dog.* Food and chemical toxicology, 1995. **33**(5): p. 367-376.