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# DIPLOMARBEIT

Titel der Diplomarbeit

„Stability of microencapsulated omega-3 fatty acids in the food matrix“

Verfasserin

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# ABBREVIATIONS

| | |
|---|---|
| AA | arachidonic acid, *cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid |
| AAPH | 2,2'-azobis-(2-amidinopropane)-dihydrochloride |
| ABTS | 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) |
| ALA | α-linolenic acid, *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid |
| ANOVA | analysis of variance |
| AOCS | American oil chemists' society |
| AV | acid value |
| CD | conjugated dienes |
| CHD | coronary heart disease |
| CLA | conjugated linoleic acid |
| CoA | coenzyme A |
| CT | conjugated trienes |
| CVD | cardiovascular disease |
| D-A-CH | Germany-Austria-Switzerland (Nutrition Society) |
| DHA | *cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid |
| DPA | *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosapentaenoic acid |
| EFSA | European Food Safety Authority |
| EPA | *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid |
| EPG | ethanolamine phosphoglycerides |
| FAO | Food and Agriculture Organisation |
| FAME | fatty acid methyl esters |
| FDA | U.S. Food and Drug Administration |
| FRAP | ferric reducing-antioxidative power |
| GC | gas chromatography |
| GSH | glutathione |
| HDL | high density lipoprotein |
| HPLC | high performance liquid chromatography |
| ISO | International Organisation for Standardisation |
| IUPAC | International Union of Pure and Applied Chemistry |

| | |
|---|---|
| IV | iodine value |
| LA | linoleic acid, *cis*-9,*cis*-12-octadecadienoic acid |
| LCPUFA | long-chain polyunsaturated fatty acid |
| LDL | low density lipoprotein |
| LUV | large unilamellar vesicles |
| MTKI | Magyar Tejgazdasági Kísérleti Intézet (Hungarian Dairy Research Institute) |
| MUFA | monounsaturated fatty acid |
| NP | normal phase (HPLC) |
| ORAC | oxygen radical absorbance capacity |
| PUFA | polyunsaturated fatty acid |
| PG | prostaglandin |
| PS | phosphatidylserine |
| PV | peroxide value |
| RP | reversed phase (HPLC) |
| SACN | Scientific Advisory Committee on Nutrition |
| SFA | saturated fatty acid |
| SV | saponification value |
| TAC | total antioxidative capacity |
| TE | trolox equivalents |
| TEAC | trolox equivalent antioxidative capacity |
| TFA | trans-configurated fatty acid |
| ToM | Theory of Mind |
| TRAP | total radical-trapping antioxidative parameter |
| UFA | unsaturated fatty acid |
| VLDL | very low density lipoprotein |
| WHO | World Health Organisation |

# 1. AIM OF THE STUDY

This study addresses the question whether the microencapsulation of oils enriched with omega-3 fatty acids influences their stability when incorporated into foods.

A search on the keyword “omega-3” in the web portal www.sciencedirect.com yields the astonishing number of 424,937 papers. There exists a huge amount of literature on analysis techniques on omega-3, the rigorous determination of all the chemical and/or enzymatic ways of their degradation, even the identification of precise decomposition products of omega-3 (and other unsaturated fatty acids present in oils enriched with omega-3).

However, the literature is scarce in assessing the chemical stability of omega-3 when incorporated into foods, and even more limited when the omega-3 are microencapsulated. What seems to be a straightforward evaluation, i.e. analysis of pure oils, turns to be extremely complicated due to the complexity of the food matrices and the non-validity of conventional analysis methods for omega-3 oils in foods as microcapsules. Further, the few accumulated experience remains secret to the public due to the industrial nature of such kind of research.

One of the major commercial problems of this kind of functional foods is the development of fishy taste and smell over time. Apparently, the molecules responsible for this effect have been extensively characterized in controlled experiments, mainly with pure oils. However, the reality is that such chemical characterization has not been proven to be effective when omega-3 oils are incorporated in foods at the concentrations usually used in the food industry.

Secondary oxidation products of omega-3 (i.e., peroxides’ decomposition products) provoke (at least partially) the undesirable fishy smell and taste. However, the well-established tests to determine the oil quality (this study being focused on fish oil) as peroxide value, acid value, p-anisidine value, 2-thiobarbituric acid value, levels of conjugated dienes are of limited value in the assessment of the quality of foods enriched with omega-3 from fish oil (notably, these methods fail most of the time as well as definitive method of evaluation of omega-3 of microbial or vegetable origin). For example, C. Jacobsen found no

correlation between the organoleptic properties of mayonnaise enriched with omega-3 and the peroxide values; the undesirable smell and taste was only possible to be determined with panel testing [JACOBSEN, 1999]. Even Macfarlane et al. goes beyond the findings of Jacobsen: they showed that none of all the parameters abovementioned correlated with a trained taste panel response [MACFARLANE et al, 2001]. The experience at industrial level at GAT Food Essentials GmbH is coincident with Jacobsen and Macfarlane et al.

It is therefore the aim of this study to try to fill this essential gap in the current knowledge on functional foods enriched with microencapsulated omega-3, namely, the correct evaluation of their chemical stability and their corresponding organoleptic characteristics in functional foods in comparison to the use of non-microencapsulated omega-3.

# 2. FATTY ACIDS

Chemically a fatty acid is a carboxylic acid with an aliphatic tail consisting of at least 4 in dairy fat to 30 carbon atoms in some marine lipids. The most important fatty acids in human nutrition are straight chained and contain an even number of carbon atoms (as explained later the preference of an even number is due to the predominant route of biosynthesis in plants and animals).

Dietary fat comprises of tocopherols, cholesterol, phytosterols, fatty acids and many other substances. The chemical compounds in which fatty acids are mainly present in the diet are the triglycerides, which consist of approximately 95% of fatty acids. This is due to the fact that in plants and animals fatty acids are present in the form of triglycerides in storage tissues because of their relative chemical inactivity. Monoglycerides and diglycerides, together with free fatty acids, contribute as well to the dietary fat.

Whether in the form of mono-, di- or triglycerides, the metabolic route they follow in the human body results in the saponification catalysed by lipases of such glycerides to free fatty acids. Conversely, the fatty acids in the human body may be present in its free acid form, in the form of glycerides, as phospholipids, glycolipids or sphingolipids. In the body fatty acids are present in biological membranes, in fats deposits and blood lipids.

Hereinafter, any reference to fatty acids shall be understood to include as well their other chemical forms in which they are present. Unless explicitly mentioned, data is interpreted towards biological systems.

## 2.1 Nomenclature and structure of fatty acids

### 2.1.1 Nomenclature

For the nomenclature of fatty acids different systems are used. The international standard is that of the IUPAC (International Union of Pure and Applied Chemistry). The basis for the systematic nomenclature of fatty acids is the number of carbon atoms. Also the number and position of double bonds relative to the carboxyl group is referred. The carbon atom of the carboxyl group is

considered as number 1. The double bonds are identified by the lower number of the joined carbon atoms. So the correct IUPAC name for i.e. α-linolenic acid (ALA) is *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid. [IUPAC – IUB Commission on Biochemical Nomenclature, 1978]

The IUPAC nomenclature system is sometimes replaced by a more straightforward nomenclature. Therefore for most naturally occurring fatty acids "trivial" or historical names, i.e. α-linolenic acid and shorthand notations are frequently in use. Shorthand notations adopt the form of carbon atom to double bond (C:D, i.e. ALA 18:3).

Nutritionists and biochemists also often use the "n minus" or the "omega" system for naturally occurring *cis*-unsaturated fatty acids. This system refers to the double bond closest to the methyl end of the carbon chain (i.e. ALA 18:3 n-3 or 18:3 ω-3). So this system starts counting on the non-carboxylic end of the molecule when compared with the IUPAC nomenclature. It is only the first double bond mentioned and also the configuration of the double bond is not mentioned. This system is not in use for *trans*-configurated fatty acids.

Another system is the delta (Δ) system which numbers the carbon atoms with the beginning at the carboxyl group and also shows the configuration of the double bond (i.e. ALA 18:3 Δ9c,12c,15c). [RATANYAKE, 2009]

## 2.1.2 Structure

### 2.1.2.1 Saturated fatty acids (SFAs)

Saturated fatty acids contain no carbon-carbon double bonds and have an unbranched structure. They mainly occur with chains from 4 – 24 carbon atoms and can be divided into subclasses.

- Short-chain fatty acids

  Fatty acids with 4 to 6 carbon atoms are called short-chain fatty acids. The most important are butyric acid (C4:0) and caproic acid (C6:0), which occur in milk fat. They are usually not found in vegetable oils.

- Medium-chain fatty acids

  These are the fatty acids with a chain length from 8 to 12 carbon atoms. They occur in milk fats and vegetable oils. For example lauric acid (C12:0) is present in coconut and palm kernel oil.

- Long-chain fatty acids

  Long-chain fatty acids cover the fatty acids with 14 – 20 carbon atoms. The most important are palmitic acid (C16:0) and stearic acid (C18:0). Palmitic acid occurs in more or less every fat. Stearic acid is present in most vegetable fats but is significant only in cocoa butter and shea butter. It also occurs in most animal fats and is a major component of tallow of ruminants.

- Very-long-chain fatty acids

  These are all fatty acids with 22 or more carbon atoms. Fatty acids with 22 and 24 carbon atoms occur in small amounts in most dietary fats.

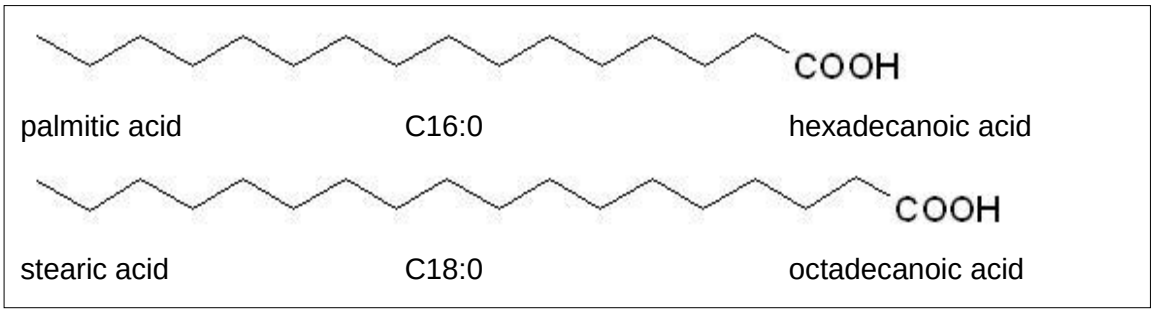



Figure 1: Examples of saturated fatty acids

SFAs are the least reactive and therefore the most stable fatty acids. The melting point increases with chain length.

2.1.2.2 Monounsaturated fatty acids (MUFAs)

In general they are even numbered and have 14 to 24 carbon atoms. The most common and most widely distributed MUFA is oleic acid (C18:1 9c; C18:1 ω9), wherein the double bond is at the Δ9 position. Another mentionable monounsaturated fatty acid is erucic acid (C22:1,13c; C22:1 ω9). It occurs in higher levels in plants in the family Brassicaceae like rape and mustard and therefore also in rapeseed oil and mustard seed oil. In newer varieties of rape it

is removed because it showed negative health effects in several animal models. MUFAs with more than 22 carbon atoms are rare in the human diet.

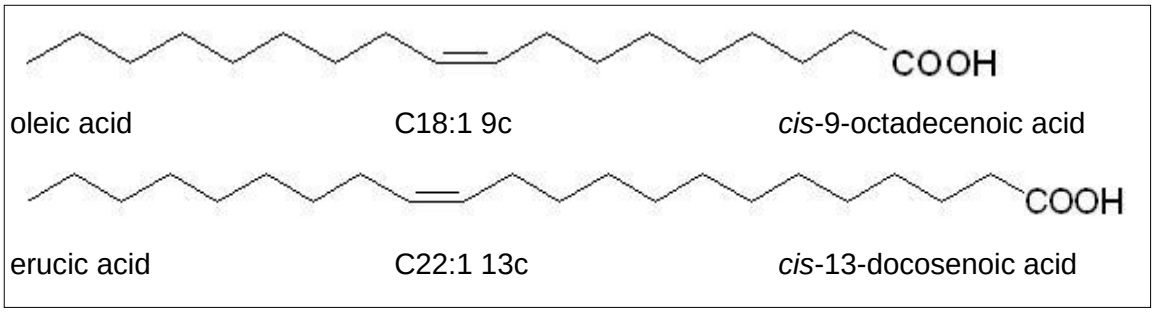


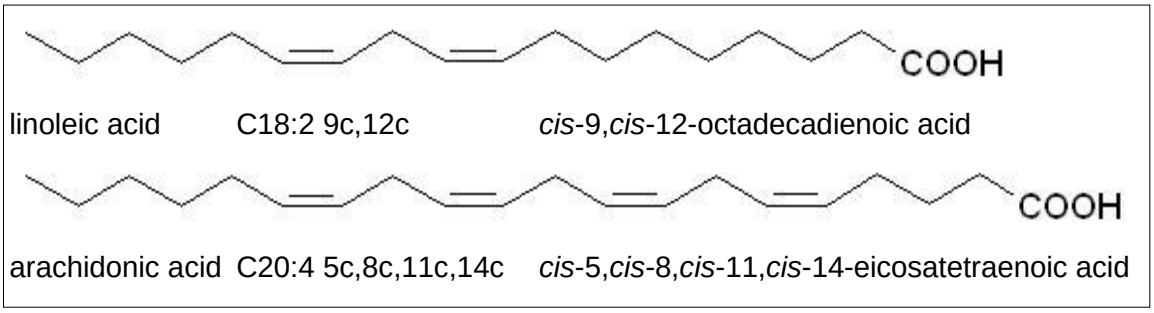

Figure 2: Examples of monounsaturated fatty acids

MUFAs are chemically more reactive than SFAs because of their carbon-carbon double bond. The melting point decreases with increasing of double bonds.

2.1.2.3 Omega-6 polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) have at least more than one carbon-carbon double bond. Omega-6 or n-6 PUFAs show the first double bond on the sixth carbon atom counted from the methyl end of the carbon chain. Linoleic acid (LA) is the parent of the omega-6 fatty acids. It is present in almost every dietary fat and is the major component of some vegetable fats like sunflower and safflower oil. Due to its wide distribution many people over consume LA at the expense of the intake of omega-3 fatty acids.

LA is essential for humans and can be desaturated and elongated to other omega-6 PUFAs (see Figure 3). The physiologically most important of these synthesised fatty acids are dihomo-gamma linolenic acid and arachidonic acid (AA). It is to mention that the conversion rate of LA to AA is only approximately 10%. Arachidonic acid is also present in meat and egg lipids.




linoleic acid C18:2 9c,12c *cis*-9,*cis*-12-octadecadienoic acid

arachidonic acid C20:4 5c,8c,11c,14c *cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid

Figure 3: Examples of omega-6 fatty acids

2.1.2.4 Omega-3 polyunsaturated fatty acids

Omega-3 PUFAs show their first double bond on the third carbon atom counted from the methyl end of the carbon chain. α-linolenic acid (ALA) is the parent of the omega-3 PUFAs. It is primary present in plants like in flaxseed oil in high amounts and also readily available in canola and soybean oil. From ALA the other omega-3 PUFAs like EPA, DPA and DHA can be formed through desaturation and elongation. Therefore the same enzyme systems as for the synthesis of the long chain omega-6 fatty acids are needed.

Other very important omega-3 PUFAs are EPA (all *cis*-5,8,11,14,17-eicosapentaenoic acid), DPA (all *cis*-4,7,10,13,16-docosapentaenoic acid) and DHA (all *cis*-4,7,10,13,16,19-docosahexaenoic acid). These are major components in marine lipids. Marine fish like mackerel, salmon, sardine, herring and smelt contain very high amounts of these long-chain PUFAs. Today also algal oils and single cell oils with considerable amounts of omega-3 PUFAs become available.

The high importance of EPA and DHA is founded in their functions in the human body. EPA is the precursor for prostaglandins and thromboxanes, DHA is the precursor for docosanoids. DHA is also highly present in retinal cells, brain cells and cardiomyocytes, and very important for the brain development.

ALA is, besides LA, the other essential FA for humans.

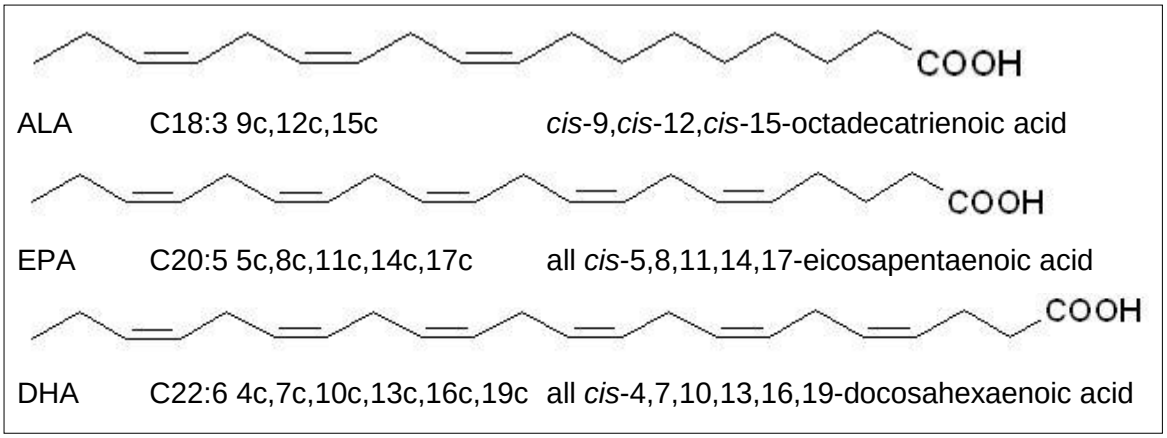



Figure 4: Examples of omega-3 fatty acids

2.1.2.5 *Trans*-fatty acids (TFAs)

Most of the naturally occurring unsaturated fatty acids are *cis*-configurated, because the enzyme systems in plant and animal tissue for the elongation and desaturation of fatty acids are only capable of building *cis*-configurated unsaturated fatty acids. However there exist also *trans*-fatty acids in nature. Small amounts are enclosed in ruminant deposits and milk fats because they are produced from hydrogenation of unsaturated fatty acids through bacterial fermentation in the ruminant's stomach. TFAs are also built through technical treatment as partial or hydrogenation of fats for blends for margarine, shortenings or deep fat frying. They can also be formed during the refining process of vegetable oils.

The most important TFA is the *trans*-isomer of oleic acid. The double bond occurs between the positions Δ4 and Δ16. The most natural *trans*-18:1-fatty acid is vaccenic acid (C18:1 11t).

Studies showed a positive correlation between the intake of *trans*-fatty acids and coronary heart disease. *Trans*-fatty acids also have a negative impact on the LDL/HDL ratio because they increase the levels of LDL and decrease the levels of HDL. [HUNTER, 2005; ELMADFA and LEITZMANN, 2004]

Based on the outcome of these studies the EFSA (European Food Safety Authority) recommends that the intake of *trans*-fatty acids should be as low as possible. The FDA (U.S. Food and Drug Administration) issued a final rule requiring the mandatory declaration of the amount of *trans*-fatty acids on the nutritional label of foods and dietary supplements. The D-A-CH nutrition society

recommends that the amount of *trans*-fatty acids in the diet should be lower than 1% of the total energy intake per day, whereas neither the EFSA nor the FDA set an upper limit or a daily recommended value. The EU is currently working on a new legislation for nutrition labeling for foodstuffs which may be include also a mandatory declaration of fatty acids on the nutritional label of foods and dietary supplements. In Austria legislation already limits the content of *trans*-fatty acids up to 2% of the total fat content of foodstuffs. [FDA, 2003; MOSS, 2006; DGE, 2008; REPUBLIK ÖSTERREICH, 2009; EFSA, 2010]

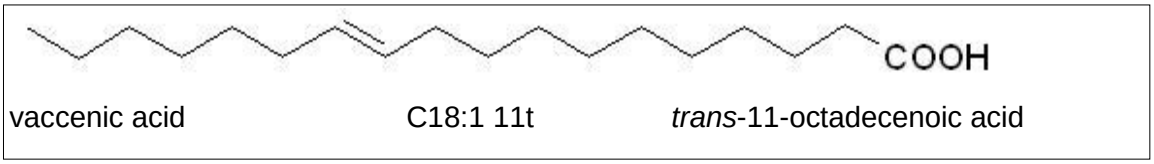
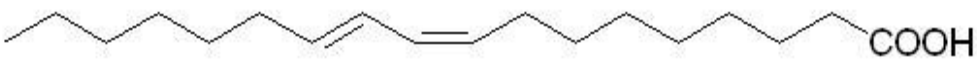



Figure 5: Example of *trans*-configurated unsaturated fatty acids

Exceptions are the isomers of conjugated linoleic acid (CLA). Discovered accidentally when searching for carcinogens from overcooked meat, the CLA isomers exhibited anti-carcinogenic instead of the prospected pro-carcinogenic properties. These properties could only be detected in cell lines. [PARIZA and HARGRAVE, 1985]

CLA refers to a group of stereo- and positional isomers of linoleic acid (C18:2 9c,12c). The predominantly positions for the double bonds are the carbon atoms 8 and 10, 9 and 11, 10 and 12, or 11 and 13. In the diet the most common is the *cis*-9,*trans*-12 isomer with more than 90%. Health benefits have been discovered through animal studies for mainly two of its isomers: *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecacienoic acid. [BHATTACHARYA et al, 2006]




conjugated linoleic acid C18:2 9c,11t *cis*-9,*trans*-11-octadecadienoic acid


conjugated linoleic acid C18:2 10t,12c *trans*-10,cis-12-octadecadienoic acid

Figure 6: Examples of CLAs

The discovery of further benefits as body fat reduction, positive impact on insulin resistance, bone health and cardiovascular health in cell line and animal studies still need to be confirmed in human intervention trials.

## 2.2 Biosynthesis of fatty acids

### 2.2.1 Biosynthesis of saturated fatty acids

The biosynthesis of fatty acids takes place in the cytosol of the cells of all organisms. In the beginning a molecule of acetyl-CoA is transformed to malonyl-CoA through carboxylation. Then fragments of 2 carbon atoms coming from the malonyl-CoA molecule are added to another molecule of acetyl-CoA. This reaction is catalysed through a multi enzyme complex, the fatty acid synthase. After the addition of acetyl-CoA and malonyl-CoA on the two SH-groups condensation, reduction and dehydrogenation lead to a chain of carbon atoms. This reaction is carried out until the chain reaches a length of 16 carbon atoms. Then the acyl-compound is hydrolysed to palmitic acid (C16:0) and added to coenzyme A. Elongation further than to a chain length of 16 carbon atoms is carried out in the endoplasmic reticulum. [ELMADFA and LEITZMANN, 2004]

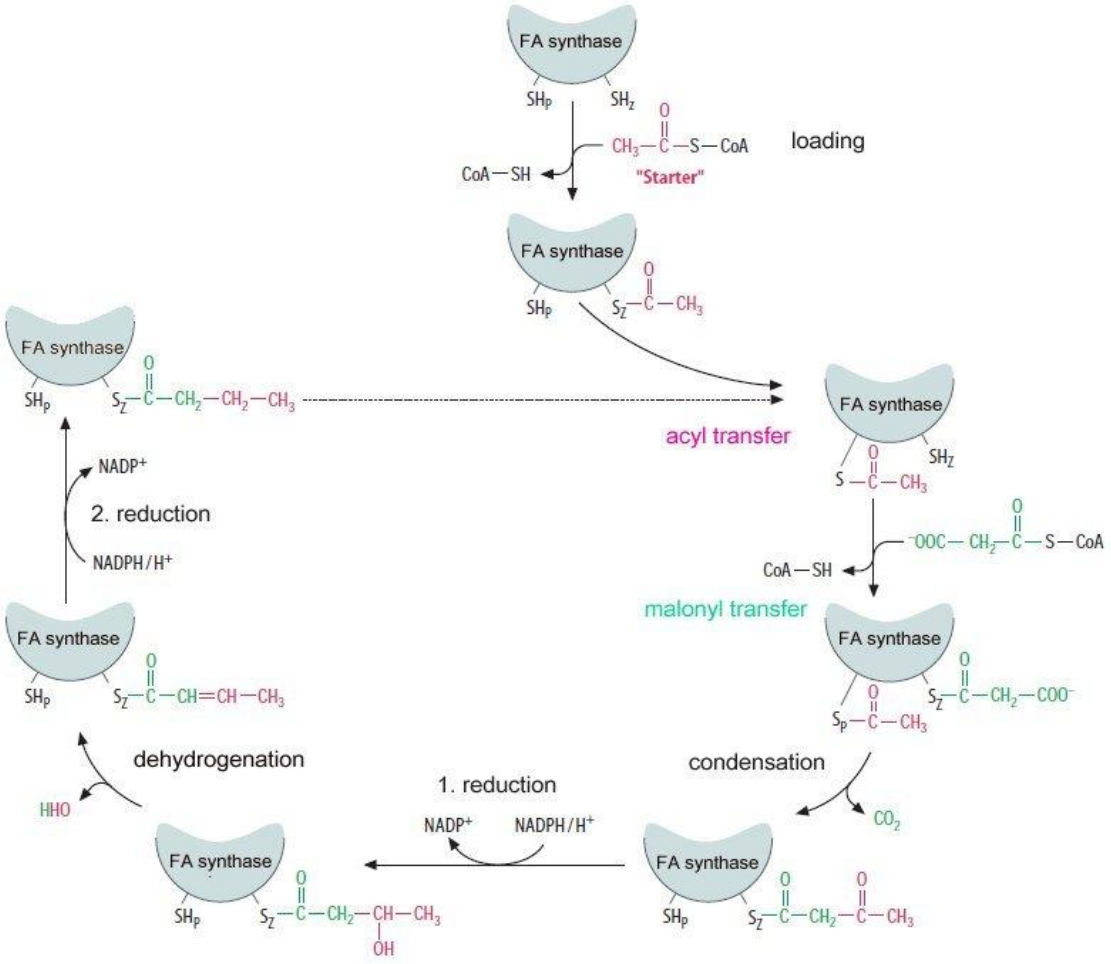



Figure 7: Biosynthesis of saturated fatty acids [modified from LÖFFLER, 2002]

### 2.2.2 Biosynthesis of unsaturated fatty acids

Unsaturated fatty acids can be built via two different paths. The first possibility is the dehydrogenation of saturated fatty acids with the same chain length. The second path is an elongation of the carbon chain of medium-chain fatty acids.

The dehydrogenation of stearic acid to oleic acid takes place in tissues of liver and muscle via the same enzyme complex which also converts palmitic acid to palmitoleic acid (C16:1 9c; C16:1 ω7) in the microsomes. Animal cells contain desaturases which only can add double bonds between the carboxyl group and the ninth carbon atom of the fatty acid. Therefore the fatty acids LA (omega-6) and ALA (omega-3) are essential.

### 2.2.3 Biosynthesis of polyunsaturated fatty acids

The biosynthesis of PUFAs is divided into two reactions: first an elongation of the carbon chain from 16 carbon atoms to 22 carbon atoms is carried out. Then cis-configurated double bonds are built in the alkane chains. The elongation of the chain is localised in the membranes of the endoplasmic reticulum. The desaturation and the elongation proceed alternately.

Linoleic acid (ω6) and α-linolenic acid (ω3) can't be synthesised by the human or animal body because these organisms lack of the enzymes that are necessary for the addition of doube bonds between the ninth carbon atom and the methyl end of the fatty acid. The desaturation of linoleic acid to α-linolenic acid just occurs in the chloroplasts of marine algae and green leaves.

Through elongation and desaturation of α-linolenic acid EPA and DHA can be built. For this animal organisms are also capable although this process is not efficient especially in infants. EPA and DHA accumulate in cold water fishes because they have a high intake through marine algae.

Another problem which occurs when EPA and DHA are converted from ALA is the use of the same enzyme system which is needed for the conversion of LA to arachidonic acid. Due to the modern western diet the intake of LA is much higher than the intake of ALA. Therefore the enzymes like Δ6-desaturase, Δ6-elongase and Δ5-desaturase are consumed for the conversion of omega-6 fatty acids instead of omega-3 fatty acids. The efficiency of the overall conversion of ALA is 0.2% to EPA and 0.05% to DHA (via EPA and DPA). One explanation may be the high amount of ALA which is β-oxidised to acetyl-CoA and used for the synthesis of cholesterol, SFA and MUFAs or metabolised to carbon dioxide ($CO_2$). Because of these facts it might be better to increase the intake of EPA and DHA through the daily diet. [RATNAYAKE and GALLI, 2009; ELMADFA and LEITZMANN, 2004]

For the detailed description of the synthesis of EPA and DHA view Figure 8.

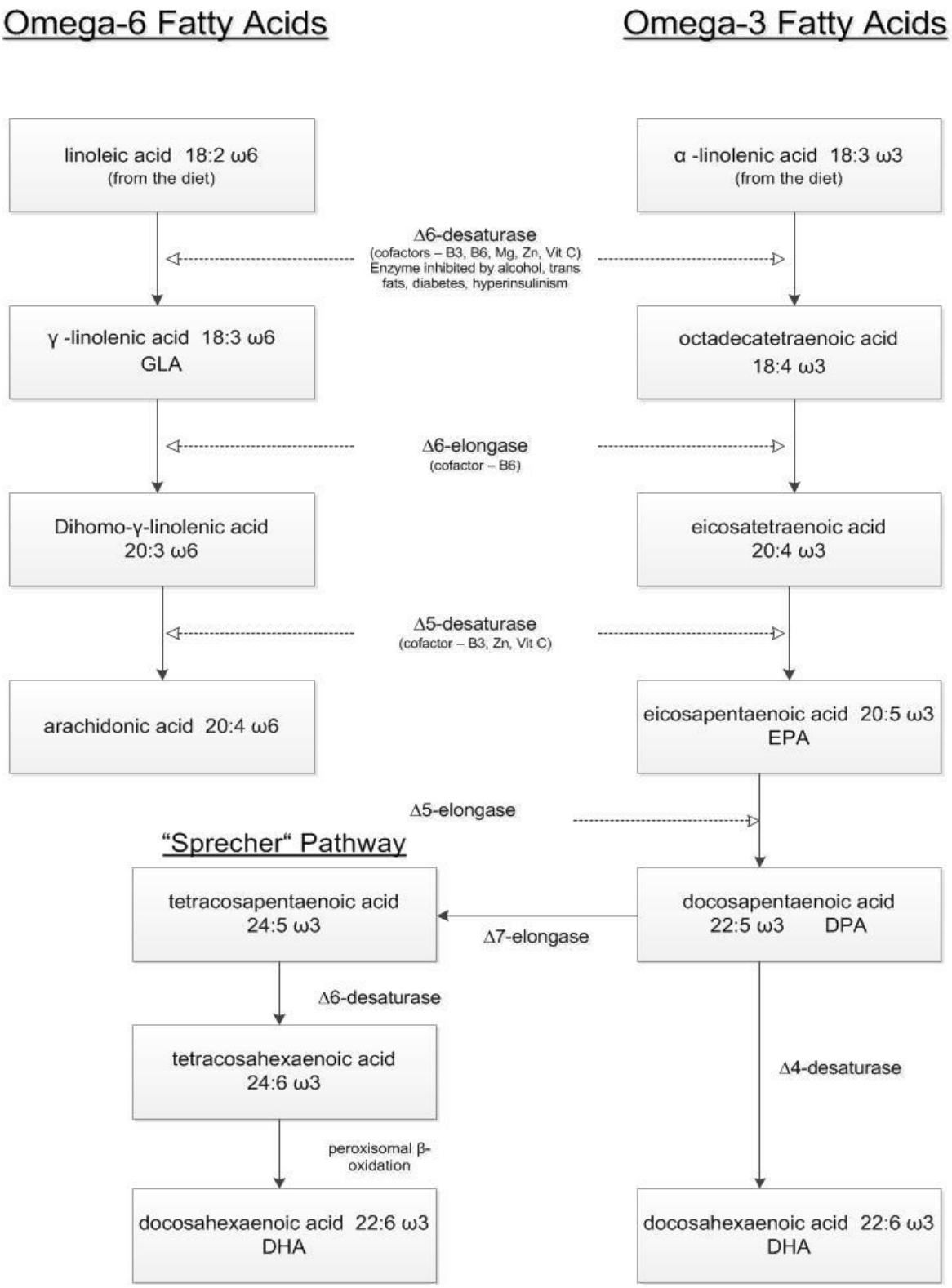



Figure 8: Essential fatty acid metabolism in the human body: desaturation and elongation of omega-3 and omega-6 fatty acids [according to THORNE RESEARCH, INC., 2009]

## 2.3 Omega-3 fatty acids

### 2.3.1 Physiological importance of omega-3 fatty acids

2.3.1.1 Distribution of omega-3 fatty acids in the human body

It is known that long-chain PUFAs are incorporated in cell membranes. They account for 21-36% of the fatty acids in these membranes. Especially to mention here is the distribution of fatty acids in the human brain. The human brain contains 50-60% of its dry weight as lipid and of these about 35% are PUFAs. But also the testis and the placenta are high in PUFAs. DHA is specific in its distribution in the different tissues. It is very abundant in the cell membranes of the brain, e.g. the gray matter, and of the retina especially in the rod photoreceptors. DHA is also high in phosphatidylserine and phosphatidylethanolamine of membranes in general and of course in synaptosomal membranes. [LAURITZEN et. al, 2001]

Table 1 gives an overview of the distribution of omega-3 fatty acids in various tissues.

Table 1: The fatty acids composition of various tissues for humans living in Western societies [according to LAURITZEN et.al, 2001]

| **wt% of tissues** | **fatty acids** | | | | | |
|---|---|---|---|---|---|---|
| | 18:3 ω-3 | 20:5 ω-3 | 22:6 ω-3 | total PUFAs | total LCPUFAs | total ω-3 |
| **adipose tissue** | 0.8 | traces | 0.3 | 13.1 | 1.1 | 1.9 |
| **red blood cells** | not detectable | 0.7 | 3.2 | 33.3 | 24.0 | 5.7 |
| **placenta** | no data | 0.1 | 4.8 | 44.4 | 34.2 | 6.3 |
| **liver** | 0.3 | 0.4 | 3.4 | 32.0 | 14.2 | 4.6 |
| **testis** | 0.7 | no data | 8.5 | 30.7 | 24.4 | 9.2 |
| **brain cerebrum** | traces | traces | 7.2 | 23.4 | 22.8 | 7.4 |
| **retina** | not detectable | 0.1 | 19.7 | 37.2 | 35.5 | 21.1 |

#### 2.3.1.2 Dietary Requirements

Absolute amounts of fat and individual fatty acids which are compulsory for human health and metabolic integrity cannot be determined because of insufficient data on this field of research.

For omega-3 fatty acids some points are clear. ALA is an essential fatty acid because of its role as precursor for EPA and DHA. The mean ALA intakes for adults in the European population are not associated with any signs of deficiency (0.7 – 2.3 g/day; 0.4 – 0.8 E%). For infant formula the same amounts as delivered normally by human milk are requested (0.5 – 1.0 % of fat as omega-3 fatty acids). [EFSA, 2010]

The conversion from ALA to EPA and DHA is very low. According Hussein et al. the estimated conversion from ALA to EPA and DHA combined is less than 0.4% and the conversion from ALA to DHA is less than 0.1%. [HUSSEIN et al., 2005] Other sources estimate the conversion to be about 0.2 % to EPA and 0.05 % to DHA. [PAWLOWSKY et al., 2001] It is also known that the conversion rate is increased by estrogens so that women convert twice as much ALA to DHA than men. [BURDGE and WOOTTON, 2002]

The requirements of pregnant women increase during the last trimester of pregnancy because of the accumulation to accommodate the needs of the infant for deposition of DHA in the brain and in the retina (about 10g) and also during six months of lactation (12 – 14g). Therefore they have an additional requirement of 90 – 100 mg/day over their basic DHA requirement. [SACN, 2004]

#### 2.3.1.3 Serum lipids and lipoproteins

EPA and DHA lower serum triacylglycerol concentrations compared to other fatty acids. This effect is also evident in patients with increased serum triacylglycerol levels. These fatty acids also slightly increase LDL and HDL cholesterol. The effects of fish oil fatty acids on the serum lipoprotein profile are small at daily intakes <1g/d. [BALK et al., 2006]

There is also the effect of decreasing VLDL concentrations [HARRIS, 1997].

Apparently the intake of omega-3 fatty acids from fish oil promotes a change in lipoprotein fatty acid composition. This may affect some physiological events associated with atherosclerosis. The benefits of EPA and DHA may be increased according to cellular events such as reductions in fibrinogen, platelet-derived growth factor, platelet activating factor and increases in tissue plasminogen activator and endothelial-derived relaxation factor. [LAYNE et al., 1996]

There is also evidence that the plasma level of EPA increases by provision of its precursor ALA in form of flaxseed oil capsules. Harper et al. showed in their study a significant increase in EPA and DPA but not in DHA plasma lipids with a relatively low dose of ALA (3g/d), which can also be obtained through a reasonable diet containing ALA-rich nuts, cereals and oils. [HARPER et al, 2006]

#### 2.3.1.4 Inflammation and immune function

Prostaglandins, prostacyclins, thromboxanes and leukotrienes and hydroxyfatty acids converted out of the omega-6 fatty acids like arachidonic acid act pro-inflammatory whereas prostaglandins, prostacyclins, thromboxanes and leukotrienes and hydroxyl fatty acids established out of omega-3 fatty acids like EPA and DHA act anti-inflammatory.

EPA and DHA inhibit the pro-inflammatory interleukins, tumour necrosis factor α and the 2 series of inflammatory PGs when they are released by an injured cell membrane. Improvements in joint pain and function when suffering on chronic inflammatory conditions are reported after application of ω-3 fatty acids. [MAROON and BOST, 2006]

As omega-3 fatty acids compete with omega-6 fatty acids for the same enzyme systems for conversion to eicosanoids like prostaglandins, thromboxanes and leukotrienes, a fish oil supplementation shows decreased production of omega-6 related prostaglandins and thromboxanes by inflammatory cells and increased production of the omega-3 related anti-inflammatory eicosanoids. Long chain omega-3 PUFAs also affect the cell surface expression of adhesion molecules which leads to a different reaction. [CALDER, 2006]

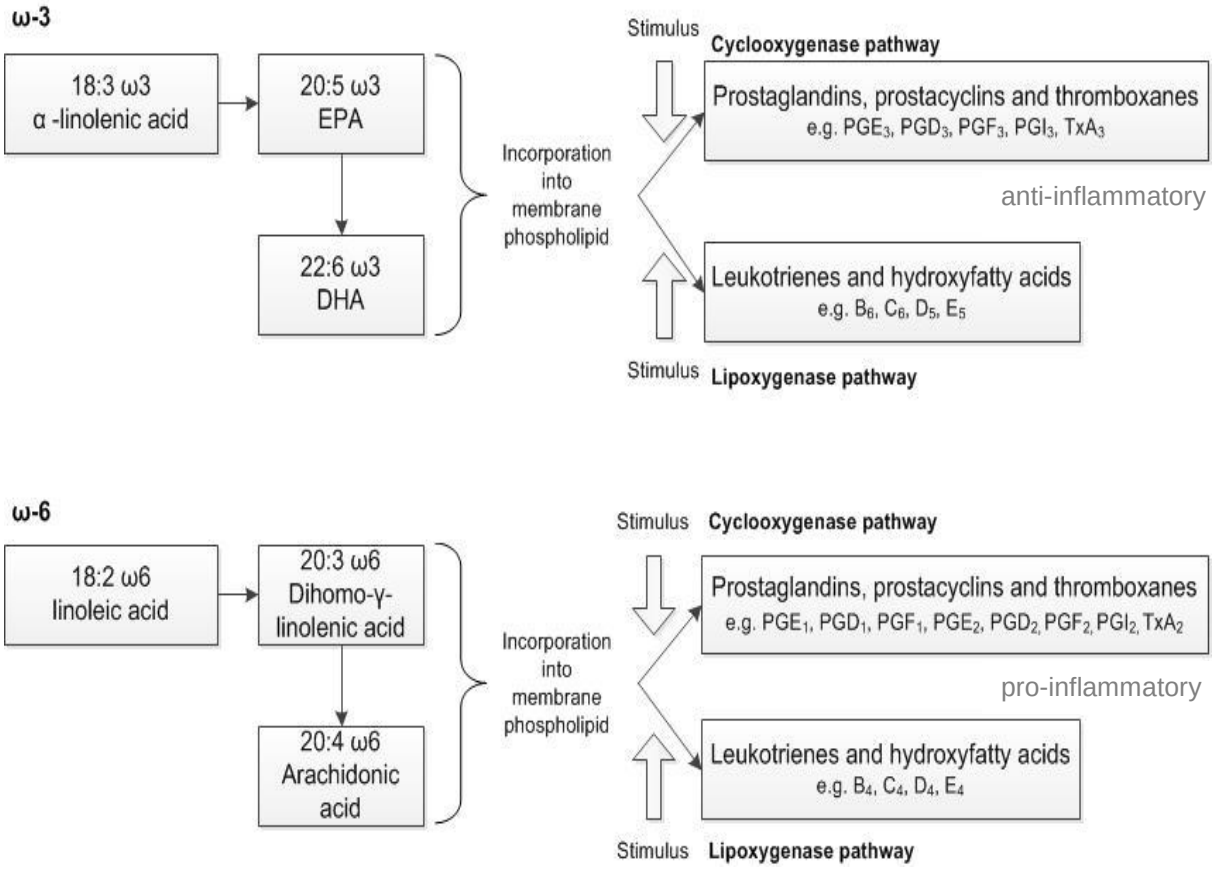



Figure 9: Conversion of eicosanoids [according to LUNN and THEOBALD, 2006]

### 2.3.1.5 Cardiovascular disease

CVD, including coronary heart disease (CHD), stroke and other diseases of the cardiovascular system, is one of the leading causes of mortality and morbidity worldwide. It is characterised by hardening and narrowing of the blood vessels, the atherosclerosis, as well as development of blood clots called thrombosis. There are various risk factors including the not modifiable like age, sex and ethnicity and the influenceable like smoking, physical inactivity, hypertension, abnormal blood lipids, obesity and diabetes.

Unsaturated fatty acids reduce the risk of CVD but the precise mechanisms are not clear yet.

According several studies including cohort studies [HU et al., 2002], and intervention trials with fish oil supplements [GISSI-Prevenzione Investigators, 1999] there is supportive evidence that the LCPUFAs EPA and DHA protect against fatal heart disease. They also affect the platelet aggregation due to the

different eicosanoids synthesised out of EPA and AA. Therefore population-wide recommendations have been established to support the intakes. It gets more and more obvious that ALA may play a role in preventing CVD. But it is not clear what association exists between CVD risk and ALA intake. [LUNN and THEOBALD, 2006]

In clinical trials there was no evidence that an increased intake of fish beyond one or two servings per week is likely to improve the primary prevention of CVD. There was also no association found between the incidence of myocardial infarction and fish oil intake. [STONE, 1996]

The GISSI-Prevenzione study showed that the supplementation of 1g omega-3 fatty acids per day (this provides 850-882mg of EPA and DHA ethyl esters) reduced the primary end point, which was set as death, nonfatal myocardial infarction, and stroke, by 15% and the risk of death by any cause by 20% significantly. This trial was carried out for 3.5 years. Although the mechanisms of action and the optimum dosage for prevention are not yet clear omega-3 fatty acids should be considerate for life-prolonging therapies for cardiovascular diseases. [GISSI-Prevenzione Investigators, 1999]

As the GISSI-Prevenzione study just showed a significant effect of omega-3 fatty acids in secondary prevention, the GISSI-HF study also investigated the effect of omega-3 fatty acids in primary prevention. However, the treatment with EPA didn’t show a significant reduction in major coronary events. [GISSI-HF Investigators, 2008]

Especially in primary prevention there is essential need for further research to clarify evidence for the preventive effect of omega-3 fatty acids.

#### 2.3.1.6 Cancer

A recent study showed the effect of fish consumption and omega-3 fatty acid intake on breast cancer in pre- and post menopausal women in South Korea. According to their results the consumption of fatty fish correlates positive with a reduced risk of breast cancer. Also the intake of omega-3 fatty acids showed a reduction of postmenopausal breast cancer. [KIM et al., 2009]

Thiébaut et.al didn't find a significant impact of a high intake of long-chain omega-3 PUFAs on the risk of breast cancer in their prospective cohort study. But there was evidence that an increased ratio omega-3 to omega-6 PUFAs can decrease the risk of breast cancer. This may be referred to the competition of omega-3 and omega-6 fatty acids in the formation of eicosanoids. [THIÉBAUT et al., 2009]

They have also a positive influence on the expression of colon carcinomas. But there are large-scale studies needed to detect the mechanisms on which their anti-carcinogenic effects are based on. [KAPOOR, 2009 and CHAPKIN et al., 2007]

#### 2.3.1.7 Retina development and function

During late fetal development and shortly after birth omega-3 fatty acids accumulate in the retina, especially in the membranes of the photoreceptor cells. More than 50% of the fatty acids in the phospholipids of outer limiting membrane of the retina are represented by DHA. [STONE et al., 1979]

A lack of DHA is related with impaired visual function, such as the acuity of vision, because DHA is known to be a fundamental component of the membrane-linked phosphoglycerides of the retina. [BIRCH et al., 1993 and BIRCH et al., 1998]

DHA may has an influence on the photochemical activity of rhodopsine. Therefore it is determining the threshold of the light perception and activates chemical reactions, which are responsible for activation of nerve impulses. [HAHN et al., 2002]

#### 2.3.1.8 Brain development and function

A characteristic feature of the mammalian brain to mention is the high concentrations of DHA in the ethanolamine phosphoglycerides (EPG) and phosphatidylserine (PS). This is even shown among herbivores although they have low concentrations of DHA in plasma and hepatic lipids. There is also

strong evidence that the brain has the ability to concentrate DHA, may localised in the capillary endothelium, which contains high amounts of DHA.

Functions of lipid-bound DHA in the membrane include properties of the hydrophobic membrane core, like a high flexibility and direct interaction with membrane proteins, which impact signal transduction, neurotransmission and formation of lipid rafts. Unesterified DHA can be metabolised to neuroprotective metabolites and have a role in regulating gene expression, like the inhibition of oxidative stress-induced induction of proinflammatory genes and apoptosis in the brain and the retina. [INNIS, 2007]

Birberg-Thornberg et al. showed a positive correlation between DHA in maternal milk and the development of the ability to represent mental states like beliefs and intentions referred to as the Theory of mind (ToM). The value of the study is limited due to the small number of participants but correlate with previous findings according to omega-3 LCPUFAs and the intelligence of children. [BIRBERG-THORNBERG, 2006]

Broadhurst et al. found evidence that the preparation of LCPUFAs for the early human and therefore could have supported a rapid cerebral cortex enlargement without an enormous increase in body mass. So it is postulated that a diet chronically-low in LCPUFAs will lead to suboptimal brain development. There is evidence today that a lack of DHA *in utero* and also in infant nutrition results in a lower intelligence and also in depression and attention-deficit hyperactivity disorder in later years. [BROADHURST, 1998]

However, the development of intelligence is a field which needs much more studies not only in association with omega-3 PUFAs.

#### 2.3.1.9 Mental Health

With omega-3 fatty acids a new family of therapeutic substances for the psychiatrics emerged. They have to be seen as alternative or addition to present applied possibilities like antidepressants. Many studies show a negative correlation between the intake of fish oil or omega-3 fatty acids and the depression score. [EDWARDS et al., 1998]

Also a low amount of omega-3 fatty acids in serum and in the erythrocytes of patients with major depressions was measured. [MAES et al., 1996 and EDWARDS et al., 1998]

Low fish and DHA consumption is fairly linked with the risk of Alzheimer's disease. Also lower blood levels of DHA are contributed to a higher risk of cognitive decline and Alzheimer's disease. But more research is definitely needed. The focus should be how DHA is biologically linked to Alzheimer's disease. This knowledge is necessary to find a useful role for DHA as nutritional supplements for protecting against some forms of dementia. It is also not clear if DHA has just a role in prevention of Alzheimer's disease or could be used to treat it. [CUNNANE et al., 2009]

Patients with the attention deficit disorder show lower plasma levels of EPA and DHA. To treat this disorder the patients usually have to take substances which increase the neurotransmitter dopamine in the brain. Omega-3 fatty acids may have the same effect. [ANTALIS et al., 2006]

According to a recent meta-analysis there is good evidence that DHA is neuroprotective via many different mechanisms. It reduces arachidonic acid and its metabolites like the prostaglandins. DHA also increases activity of antioxidative enzymes like catalase and GSH peroxidase and improves the synaptic membrane fluidity. Compared to many new medications DHA has outstanding benefits like long-term safety, minimal side effects and relative low costs. [COLE et al., 2009]

#### 2.3.1.10 Foetal and infant development

An adequate supply of long chain omega-3 PUFAs before and throughout pregnancy is very important to support neurological development and cognitive function of the growing fetus. [LUNN and THEOBALD, 2006]

Before birth placental transfer provides all the required omega-3 fatty acids. Therefore the plasma level of DHA, although higher in the foetus than in the mother, is influenced by the maternal diet. The placental fatty acid transfer is dependent on maternal fatty acids, but is not regulated to protect the foetus from low maternal DHA. [INNIS, 2008]

DHA and other omega-3 fatty acids vary widely in human milk according to differences in the maternal diet. Vegan and vegetarian diets lead to very low concentrations of DHA. Increasing the maternal DHA intake can increase the amount of DHA in the human milk. [INNIS, 2008]

#### 2.3.1.11 Body weight and energy balance

For the effect of omega-3 fatty acids on the body weight and energy balance the results of different studies vary widely. According to Couet et al. omega-3 fatty acids from fish oil have the ability to reduce the body fat mass of healthy adults significantly and also increase the fat-free calcium-free mass. The resting metabolic rate increased also in this study during fish oil supplementation. They recommend a daily intake of 6 g fish oil per day to gain these results. [COUET et al., 1997]

Kratz et al. showed in their study the contrary. In their study with overweight adults the increased dietary omega-3 content to 3.6% of total energy didn't have any effect on the body weight or fat mass. [KRATZ et al., 2009]

### 2.3.2 Sources of omega-3 fatty acids

#### 2.3.2.1 Natural Sources

ALA is present in vegetable oils like flaxseed oil and in some green vegetables like broccoli. For long chain omega-3 PUFAs fish and fish oils are the main source in the human diet. But in these the LCPUFA also come originally from plant sources, such as phytoplankton like *Schizochytrium sp.* Now also the fermentation of microalgae and algae-like microorganisms are considered as new sources of omega-3 PUFAs.

Detailed contents of the different PUFAs in foods are shown in Table 2.

Table 2: Omega-3 PUFA contents of different natural sources [according to LI et al, 2003]

| | **content** | | | | |
|---|---|---|---|---|---|
| **fish [mg/100mg edible flesh]** | **ALA** | **EPA** | **DPA** | **DHA** | **total** |
| golden bream | 3 | 43 | 34 | 168 | 247 |
| sea mullet | 120 | 1318 | 635 | 286 | 2359 |
| atlantic salmon | 80 | 472 | 439 | 1147 | 2138 |
| cultivated rainbow trout | 35 | 53 | 26 | 442 | 556 |
| southern bluefin tuna | 3 | 230 | 115 | 804 | 1152 |
| **lean meat [mg/100g product]** | **ALA** | **EPA** | **DPA** | **DHA** | **total** |
| beef | 16 ± 2 | 16 ± 2 | 23 ± 4 | 3 ± 1 | 58 ± 9 |
| pork | 8 ± 2 | 3 ± 1 | 7 ± 1 | 3 ± 1 | 21 ± 4 |
| chicken | 16 ± 8 | 4 ± 0 | 8 ± 2 | 6 ± 2 | 34 ± 11 |
| lamb | 65 ± 18 | 23 ± 4 | 22 ± 1 | 8 ± 2 | 118 ± 2 |
| **vegetables [mg/100g fresh product]** | **ALA** | **EPA** | **DPA** | **DHA** | **total** |
| broccoli | 110 ± 16 | - | - | - | 114 ± 17 |
| spinach | 129 ± 16 | - | - | - | 150 ± 19 |
| watercress | 180 ± 71 | - | - | - | 225 ± 83 |
| **vegetable oils [mg/100g oil]** | **ALA** | **EPA** | **DPA** | **DHA** | **total** |
| flaxseed oil | 60000 | - | - | - | - |
| olive oil | 600 | - | - | - | - |
| safflower oil | 400 | - | - | - | - |

Further it is to mention that the modern Western diets show an imbalance between omega-3 and omega-6 fatty acids. This is a result of the modern agribusiness, the modern agriculture with the high consumption of vegetable oils containing high amounts of omega-6 fatty acids, and the modern aquaculture which also leads to a decrease of omega-3 fatty acids in cultivated fish compared to wild fish. [GUNSTONE, 2007; SIMOPOULOS, 1999]

The balance of omega-6 and omega-3 fatty acids is very important for homeostasis and normal development. The ratio should at least be 5:1 to ensure an appropriate intake of both. [DGE, 2008]

#### 2.3.2.2 Fortification

Fortification of food products is based on the proposed effect of the biologically active ingredient. On the European market there are many products that are enriched with omega-3 fatty acids. The range reaches from margarine to milk and from juices and shots to cookies and bread. Mostly the products contain amounts that ensure that the adequate intake of omega-3 fatty acids could be reached with one serving of the product. Mostly the products are not only enriched with plain fish oil but with stabilised fish oil e.g. by microencapsulation.
In 2009 the sales of omega-3 products grew 42% despite of the recession [WILLIAM REED BUSINESS MEDIAS, 2010]. One reason for this is, amongst others, the consumer's increasing interest in healthy diet. The largest part of the market is accounted by fortificated dairy products.

#### 2.3.2.3 Supplements

Fish oil supplements rich in omega-3 polyunsaturated fatty acids are widely used because most people do not reach the adequate intake of 250mg EPA & DHA per day [EFSA, 2010]. They often deliver high dosages of omega-3 PUFAs, sometimes twice as high as the recommended intake values. Sometimes they also can cause negative side effects such as "fish burps" or gastro-intestinal problems like diarrhea. So although they can help you to achieve the adequate intake value, a natural source is always the better decision because also the bioavailability can be lower in supplements than in real food. [KRIS-ETHERTON and HILL, 2008)

### 2.3.3 Average fat intake in Austria

The average fat intake of adults in Austria is with 36-38% of the total energy intake too high. For children the fat intake is within in the range of the recommendations (30-35% of the total energy intake). For the polyunsaturated fatty acids the intake is for children a little less than recommended, for adults the intake is appropriate. [ELMADFA et al, 2009]

Table 3: Intake of fat and fatty acids among children and adults in Austria [according to ELMADFA et al, 2009]

| | Fat total, E% | PUFA, E% | ALA, g/day | ALA, E% | EPA, g/day | EPA, E% | DHA, g/day | DHA, E% |
|---|---|---|---|---|---|---|---|---|
| **Men 18-65years** | 37 | 8 | 1.50 | 0.50 | 0.08 | 0.03 | 0.20 | 0.07 |
| **Women 18-65 years** | 37 | 8 | 1.30 | 0.60 | 0.07 | 0.03 | 0.20 | 0.09 |
| **Boys 7-10 years** | 34 | 6 | 0.80 | 0.40 | 0.02 | NA | 0.07 | NA |
| **Girls 7-10 years** | 34 | 6 | 0.70 | 0.40 | 0.02 | NA | 0.07 | NA |
| **Boys 11-14 years** | 34 | 6 | 1.20 | 0.60 | 0.03 | NA | 0.10 | NA |
| **Girls 11-14 years** | 34 | 6 | 1.00 | 0.50 | 0.03 | NA | 0.09 | NA |

## 2.3.4 Recommendations for the fat intake

### 2.3.4.1 D-A-CH

The nutrition societies of Germany, Austria and Switzerland have created together reference values for the nutrient intake. For fat in general they published a guideline referring to a fat intake lower or equal to 30% of the daily total energy intake.

For PUFAs, especially for EPA and DHA, the nutrition societies set an estimated value of 250 mg EPA+DHA per day to achieve the promised prevention against coronary heart disease. For pregnant and breast-feeding women an advice of an intake of 200 mg DHA per day is given to ensure an optimal development of the nervous system of the foetus. [DGE, 2008]

### 2.3.4.2 WHO/FAO

The joint experts of FAO/WHO set an acceptable macronutrient distribution range for adults with 20-35% fat intake of the total energy intake. For infants an adequate intake of 35-60% fat intake of the total energy intake.

For omega-3 PUFAs a range is given with 250 mg – 2 g per day. The higher value refers to secondary prevention of CHD. For infants (0-24months) an adequate intake of 10-12 mg DHA per kg body weight is recommended. For

children from 2 years to 10 years the values for an adequate intake of EPA & DHA are bridged from the infant value to the adult value with 100-150 mg per day to 200-250 mg per day. There is no need of a special ratio of ω-6 to ω-3 fatty acids as long the intake is within the recommendations. [WHO/FAO, 2008]

#### 2.3.4.3 EFSA

The EFSA Panel set a recommended daily intake for adults of 20-35% fat of the total energy intake. For children up to 3 years they recommended 35-40% fat of the total energy intake.

For omega-3 PUFAs they don't set a daily reference value, but for EPA & DHA an adequate intake of 250 mg per day is important for adults. For children up to 1 year the intake should exceed 100 mg DHA per day. [EFSA, 2010]

In the recent months the EFSA panel published the first opinions on submitted health claims referring to general health functions of omega-3 fatty acids. So far the EFSA panel published positive opinions on claims referring to the influence of omega-3 fatty acids on blood pressure and blood lipids. The European Commission already published a health claim referring to children's health. [EFSA, 2009; EUROPEAN COMMISSION, 2009]

## 2.4 Stability of polyunsaturated fatty acids

The most important impact on the stability of polyunsaturated fish oil is lipid peroxidation. Due to their high amount of unsaturated carbon atoms the probability of oxidation is very high.

Figure 10 shows the schematic reaction of lipid peroxidation. In the beginning a hydrogen radical is separated from an unsaturated fatty acid. This leads to an unstable carbon radical. Molecular rearrangement occurs and this causes a conjugated diene. Then the carbon radical reacts with oxygen and a peroxyl radical is formed. Formerly built radicals react with the peroxyl radical and a lipid peroxide is formed. As there are always new radicals formed throughout this reaction a chain reaction is the consequence which affects more fatty acids in each reaction step. Therefore the number of peroxides increases exponentially.

Some of the end products as 4-hydroxynonenal are responsible for the bad taste and smell rancid oils develop.

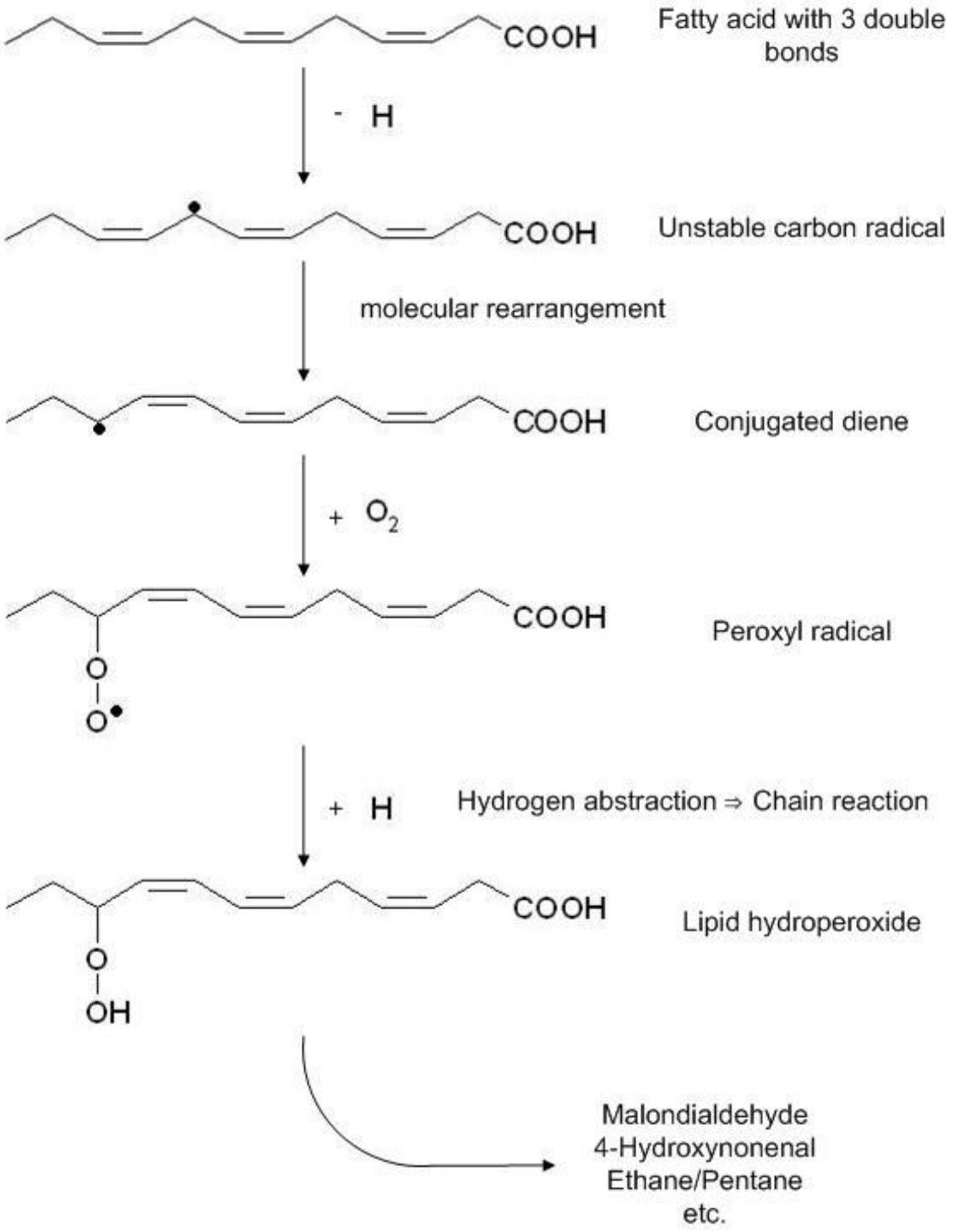



Figure 10: Schematic reaction lipid peroxidation [according to EBERMANN and ELMADFA, 2008]

Many studies have examined the stability and shelf life of plain fish oil. To show an example of such studies the study of Boran et al. is taken. In this study the oils of horse mackerel, shad, garfish and golden mullet were tested for their ester value (EV), acid value (AV), iodine value (IV), peroxide value (PV) and saponification value (SV) during 150 days storage at 4°C and -18°C. These fish are common for the Turkish market and contain all high amounts of polyunsaturated fatty acids. After the storage time garfish and shad show the greatest stability against oxidation and preserved their properties at -18°C for the whole storage period. Generally they showed said that the fish oils' shelf life was twice as long when they were stored at -18°C than at 4°C. [BORAN et al, 2006]

In the study of Let et al. the influence of fish oil quality on the oxidative stability of milk enriched with pure fish oil or a fish oil-rapeseed oil mixture was evaluated. This study shows that the storage temperature has important effects on the oxidation of the fish oil enriched milk emulsions. Best results were gained with the mixture of fish oil and rapeseed oil. [LET et al, 2005]

A study from Skall Nielsen et al. showed a very good stability of fish oil in drinking yoghurt. There was no oxidative instability observed in drinking yoghurt enriched with 2.0 % fish oil after more than 2 weeks storage at 2 °C. Also the sensory evaluation showed no fishy off-flavour during the storage time. These results may be attributed to the low pH of the yoghurt. [SKALL NIELSEN et al, 2007]

Serfert et al. showed that microencapsulation by spray drying can stabilise fish oil but it has to be considered that this multiphase system has very heterogeneous characteristics. The stability can also be influenced by oxidation that may occur already during the spray drying process. This can lead to high values of peroxides and hydroperoxides and therefore to a decrease of storage stability. [SERFERT et al, 2009]

## 2.5 Analysis of polyunsaturated fatty acids

### 2.5.1 Analysis by gas chromatography (GC)

Analysis of fatty acids by as chromatography is the most common way of qualification and quantification of the fatty acid pattern of oils or other foods. It enables a complete analysis in a relatively short time. For the analysis by gas chromatography the fatty acids have to be converted into fatty acid methyl esters (FAME). This is carried out with a surplus of methanol and is either catalysed by sodium methoxide or borontrifluoride. Early methods included also diazomethane as catalyst. As stationary phase polar polyester or fused silica capillary columns are in use. As carrier gas helium is the choice because it is much safer than hydrogen and retention times do not change when the speed of the gas flow changes.

Several detectors, like flame ionisation detector (FID), electron capture detector (ECD) or mass spectrometry (MS), can be used for the analysis of fatty acids, but in most studies the flame ionisation detector is used. GC/MS detection is often used for the analysis of trace amounts. [CHRISTIE, 2003; PETROVIĆ et al, 2010]

### 2.5.2 Analysis of fatty acids by high performance liquid chromatography (HPLC)

In recent years the advantages of HPLC in the analysis of fatty acids are recognised. It is widely used to analyze compounds which are highly volatile like decomposition products of polyunsaturated fatty acids. Separation modes can be divided into reversed phase (RP), which is commonly used for the separation of organic acids, hydroxyl-polyunsaturated fatty acids or other polar lipids, and normal phase (NP), which is used for the analysis of free fatty acids and other non-polar lipids. Both modes show no limitation for MS as detector. One advantage of HPLC compared with GC is the wide range of different derivates that can be used for analysis. The choice of derivatives depends on the detector that will be used., e.g. methylated derivatives for FID, UV-absorbent derivatives

for UV-VIS detector, fluorescent derivatives for fluorescence detector. Besides MS also UV-VIS detectors, fluorescence detectors or $^{1}H$ NMR (nuclear magnetic resonance spectroscopy) can be used.
Another form of HPLC which is used for separation and quantitation of cis/trans fatty acids or FAME positional isomers is silver ion chromatography ($Ag^{+}$-HPLC). [SAJIKI and YONEKUBO, 2002; RUIZ-RODRIGUES et al, 2010]

### 2.5.3 Chemical constants

Before modern analytical methods like gas chromatography or HPLC were developed many chemical constants were used for identification and determination of the composition and quality of fats and oils. Whereas most of these constants are not necessary anymore some of them are still used due to their information value and their simple use. [MATISSEK, STEINER, and FISCHER, 2010]

#### 2.5.3.1 Saponification value (SV)

The SV is a measure for the bound and free fatty acids in a fat or oil and is linked to the average molecular weight of the fatty acids. The lower the average molecular weight the higher is the SV. The SV can be used for the determination of purity of certain fats and oils.
Definition: The SV refers to the amount of KOH in mg, which is necessary for the saponification of 1 g fat.

#### 2.5.3.2 Iodine value (IV)

The iodine value is a measure for the amount of unsaturation contained in a fat or oil. The higher the IV is the higher is the amount of double bonds in a fat. Therefore the IV can be used for determination of purity and identity of fats and oils. Besides the unsaturated fatty acids the unsaturated fat-accompanying substances are also detected.
Definition: The IV refers to the amount of halogens in g, based on iodine, which is bound by 100 g of fat or fatty acids.

#### 2.5.3.3 Acid value (AV)

The acid value is a measure for the amount of free acids in fats, oils or fatty acids. Besides fatty acids also mineral acids can be detected. Acids linked to glycerol are not detected by this method. The AV is used as purity measurement and also refinement of oils can be detected by this method. Unrefined oils, such as virgin and cold pressed oils show an AV up to 10 whereas refined oils usually have an AV lower than 0.2.

Definition: The AV refers to the amount of KOH in mg, which is necessary for the neutralization of the free acids contained in 1 g fat oil or fatty acids.

#### 2.5.3.4 Peroxide value (PV)

Hydroperoxide (ROOH) formation is one of the first steps of lipid oxidation. Peroxides are intermediate products and could lead to the formation of volatile compounds like hexanal, heptanal acetaldehyde. The hydroperoxides react with triplet or singlet oxygen. Triplet oxygen is a di-radical molecule that reacts directly with molecules in singlet state and as well with molecules in doublet-state such as radicals. Singlet oxygen is a non-radical and electrophilic molecule and can react directly with electron-rich double bond containing compounds like oleic acid, LA or ALA.

The formation of hydroperoxides is an autocatalytic reaction, which means that at least one of the products is also a reactant. The hydroperoxides are decomposed to low molecular weight volatile compounds, e.g. aldehydes, ketones, alcohols, acids and hydrocarbons. The aldehydes are responsible for the rancid flavour of oxidised oils but there are approximately 150 volatile compounds built during oxidation of vegetable oils. [HAHM and MIN, 1995]

The peroxide value is a measure for the peroxidic bond oxygen in fats or oils.

Definition: The PV refers to the amount of active oxygen in 1 kg of sample.

## 2.5.4 UV-VIS Spectrophotometric analysis

### 2.5.4.1 Conjugated dienes

One of the first steps in the oxidation of linoleic acid or higher PUFAs is a shifting of double bonds. Pentadienyl radical is formed during oxidation. This is able to shift into two possible conjugated dienes (CD). These could react with molecular oxygen to hydroperoxides. The increase of double bond displacement correlates with the degree of peroxidation in an unsaturated oil.
As these conjugated structures absorb strongly at wavelengths of 232 – 234 nm they can be measured easily. The CD value represents the percentage of conjugated dienoic acid in an unsaturated oil and is an indication of primary oxidation products. The CD value does not increase further when a certain concentration is reached.
The amount of PUFAs in an oil and CD correlate positively. But the magnitude in the change of the CD cannot be related to degree of oxidation because this is highly influenced by the plant species and the fatty acid profile. [NOOR and AUGUSTIN, 1984]
The CD value can be used as a relative measurement of oxidation in a particular oil with known fatty acid composition. [WHITE, 1995]

### 2.5.4.2 Conjugated trienes

Conjugated trienes (CT), which can be measured with the same system and sample preparation as CD at a wavelength of 268 nm, ethylenic diketones, conjugated ketodienes and dienals are secondary oxidation products. The CT value represents the percentage of conjugated trienoic acid in an unsaturated oil and is an indication of secondary oxidation products. [WHITE, 1995]

## 2.5.5 Fluorometric analysis - ORAC assay

The ORAC (oxygen radical absorbance capacity) assay is used to measure the antioxidative capacity of various materials such as essential oils, dietary supplements or spices. The basic assay bases upon the reaction of a peroxyl radical with a fluorescent probe and the formation of a nonfluorescent product. The peroxide radicals are formed by AAPH. The reaction is quantified by the

measurement of the change in fluorescence. The results are expressed as Trolox equivalents. Trolox is a water soluble vitamin E analog.

The relevance of the antioxidative capacity gets widely accepted in the food industry. [BENTAYEB et al., 2009]

# 3. MICROENCAPSULATION TECHNOLOGY

Microencapsulation is defined as ‘the technology of packaging solid, liquid and gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time’. [CHAMPAGNE and FUSTIER, 2007] Originally this technology was used for the pharmaceutical industry to stabilise sensitive active ingredients. But in recent years it gets more and more important for the food industry especially for the sector of functional foods. The addition of active ingredients to functional foods is often challenging because of the stability of these ingredients during production and storage of the final product. Another point is the matter of taste in the final product. The active compounds should not affect the desired or expected taste of the food. Microencapsulation is also useful for the controlled release of the nutrients in the gastrointestinal system. [CHAMPAGNE and FUSTIER, 2007]

There are several techniques for microencapsulation of active ingredients especially of oils rich in omega-3 LCPUFAs. They are differentiated by the way of building up the carrier matrix surrounding the core material.

- Microencapsulation by spray drying

  Spray drying is in use for microencapsulation of flavours and lipids for decades. The process is divided in 4 stages: (1) preparation of the emulsion to be processed, (2) homogenisation, (3) atomisation of the emulsion and (4) dehydration of the atomised particles.

  The core material is dispersed into a solution of the wall material. This dispersion has to be heated and homogenised and depending on the emulsifying properties of the coating agent a emulsifier must be added to form a stable emulsion. Then the oil-in-water emulsion is atomised into a hot air stream to evaporate the solvent. Due to the short time exposure to the heat the core temperature could be kept below 40°C in spite of the high temperatures used in the process of spray drying.

  The chemical functionality is determined by the used wall materials. The criteria are compatibility with the food product, appropriate particle size,

appropriate release, mechanical strength etc. The coating materials can be divided into 3 major groups: carbohydrates, gums and proteins. Carbohydrates such as maltodextrins, starches or pectin produce stable emulsions and exhibit low viscosity, which is necessary for a low particle size.

Gums are used due to their emulsion stabilisation properties and their film forming around the core material. Most used among all gums is gum Arabic due to its outstanding emulsification properties.

Out of the huge amount of proteins milk or whey protein and gelatine is mostly used for microencapsulation by spray drying.

Spray drying is the most common and cheapest microencapsulation technology. [GHARSALLAOUI et al, 2007]

Spray drying is a widely used form to microencapsulate omega-3 rich oils like fish oil.

- Microencapsulation by fluidized bed technology

  The aim of this technology is to apply a uniform shell onto solid particles. The advantage of this technique is the possibility to use any kind of shell material, which enlarges the controlled released possibilities. As coating formulations aqueous solutions of hydrocolloids like gums and proteins, ethanolic solutions of synthetic polymers and melted fats have been used. It is also possible to use the fluidized bed technology as a second step of spray drying to enlarge the shelf life of the microcapsules. [GOUIN, 2004]

- Microencapsulation by spray cooling / chilling

  This technology is routinely used for enzymes, flavours and organic and inorganic salts to improve their heat stability, delay release in wet environments or to convert liquid hydrophilic ingredients to powders.

  It is more a ‘matrix’ encapsulation than a ‘true’ encapsulation. As a ‘matrix’ encapsulation an encapsulation is described where the particles of the active ingredient are incorporated in a fat matrix in contrast to a ‘true’ encapsulation which has a core/shell type of microcapsules.

  The release cannot be controlled in the way it is possible with other types of microencapsulation, because the encapsulated ingredient is released as

soon as the capsules come in contact with the food stuff. Further factors that affect the release are osmotic forces, slow diffusion of water through the shell imperfections, mechanical disruption of the particles and others. [GOUIN, 2004]

- Microencapsulation by extrusion

  Extrusion microencapsulation is used for volatile and unstable flavours which leads to a very long shelf life compared to unencapsulated flavour compounds like citrus oils. The used glassy carbohydrates build an almost impermeable barrier against atmosphere oxygen.

  A mass of modified starch, glycerol and water is mixed and heated up to 100°C. When it has cooled down the sensitive ingredient, usually a volatile flavour is added and then the exiting ropes are cut into pieces.

  This technique offers a high payload – about 40%, which is twice as the payload of spray dried flavours – and a long shelf life of about 5 years. The disadvantages are the high costs and relatively high particles, which limits their application in foods where mouthfeel is a crucial factor. [GOUIN, 2004]

- Liposome microencapsulation

  Liposome microencapsulation was mostly used for pharmaceutical products until recently also the advantages for the food industry became obvious. The most appropriate liposomes for food industry are the large unilamellar vesicles (LUV). They show high encapsulation efficiency, a good stability over time and are easy to produce. The advantage of the liposome microencapsulation technology is the targeted delivery of active ingredient in specific parts of the foodstuff. Vitamin C encapsulated with this technique shows a higher shelf life since it is protected against prooxidants like copper. [KIRBY et al., 1991]

- Microencapsulation by coacervation

  This technology offers the advantage of a payload up to 99% and a variety of application areas like the encapsulation of flavour oils, fish oils, enzymes and vitamins. The concept of simple coacervation is based on the phase separation of hydrocholloids from an initial solution and a deposition of the newly formed coacervate phase around the active ingredient. The shell can afterwards be crosslinked with chemicals or enzymes. Coacervation systems that exhibit good properties are gelatin/gum arabic system, carrageenan, gelatin/carboxymethylcellulose system or starch.

  Complex coacervation involves the use of two oppositely charged hydrophilic colloids. In the case of complex coacervation the phase separation occurs because of the repulsion of the oppositely charged molecules.

  An advanced form is the formation of multi-layered coacervated microcapsules which final layer shells can reach a thickness up to 100µm.

  A problem in getting this technique into the food industry is the high cost of the process. Some problems can also occur with the use of chemicals as crosslinking agents due to the food legislation in some countries which often limits the use of such chemicals. [GOUIN, 2004]

- Microencapsulation by multiple emulsion ( wowCAPS® technology)

  This is a continuous multi-microencapsulation process with interfacial and in situ polymerisation of active ingredients.

  The first step is the addition of a water phase containing a polymerisation initiator to an oil phase (both of the phases are capable of containing a biologically active ingredient) so that an emulsion is built. As a second step a solution or dispersion of at least one hydrocholloid in water, e.g. alginates, is added to the emulsion to produce a phase inversion. Now a wall of the hydrocolloid is built around the new formed drops consisting a water in oil emulsion. This wall building is induced by a reaction of alginates with calcium, which is enclosed in the water phase of the emulsion. As an alginate consists of D-mannuronic acid and L-guluronic acid, which build monomeric blocks, the calcium reactivity is a result of calcium-induced

dimeric association of the G-blocks. Low amounts of calcium lead to temporary associations of the G-blocks, whereas higher amounts obtain stronger linkage of the chains.

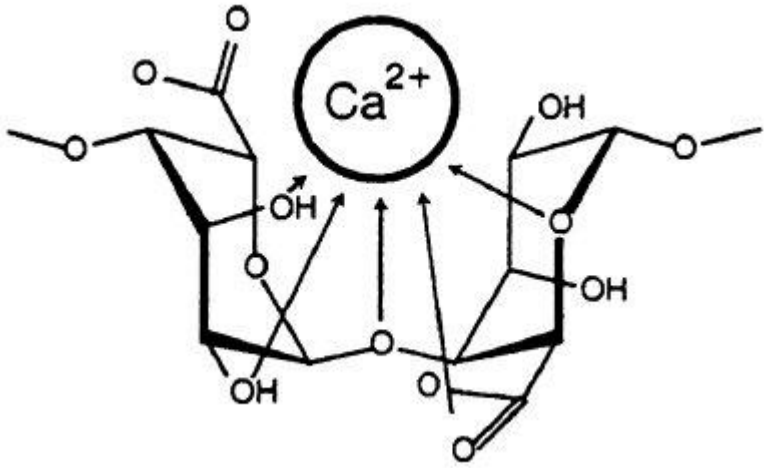



Figure 11: Calcium-induced association of L-guluronic acid [KHYMOS, 2006]

The third step is the addition of a suspension or solution of a protective colloid in water, which is deposited on the surface of the water in oil droplets to polymerise and cross-link with itself and the hydrocolloid. In a fourth step a primary surfactant is added, that allows a reduction of the size of the water in oil drops.

During a fifth step the partially formed microcapsules are deagglomerated and reagglomerated which can lead to enclosure of drops inside bigger drops. After the formation of the capsules the temperature is increased in order to strengthen the wall of the microcapsules. All steps are carried out under constant agitation.

In Figure 12 a built microcapsule is shown.

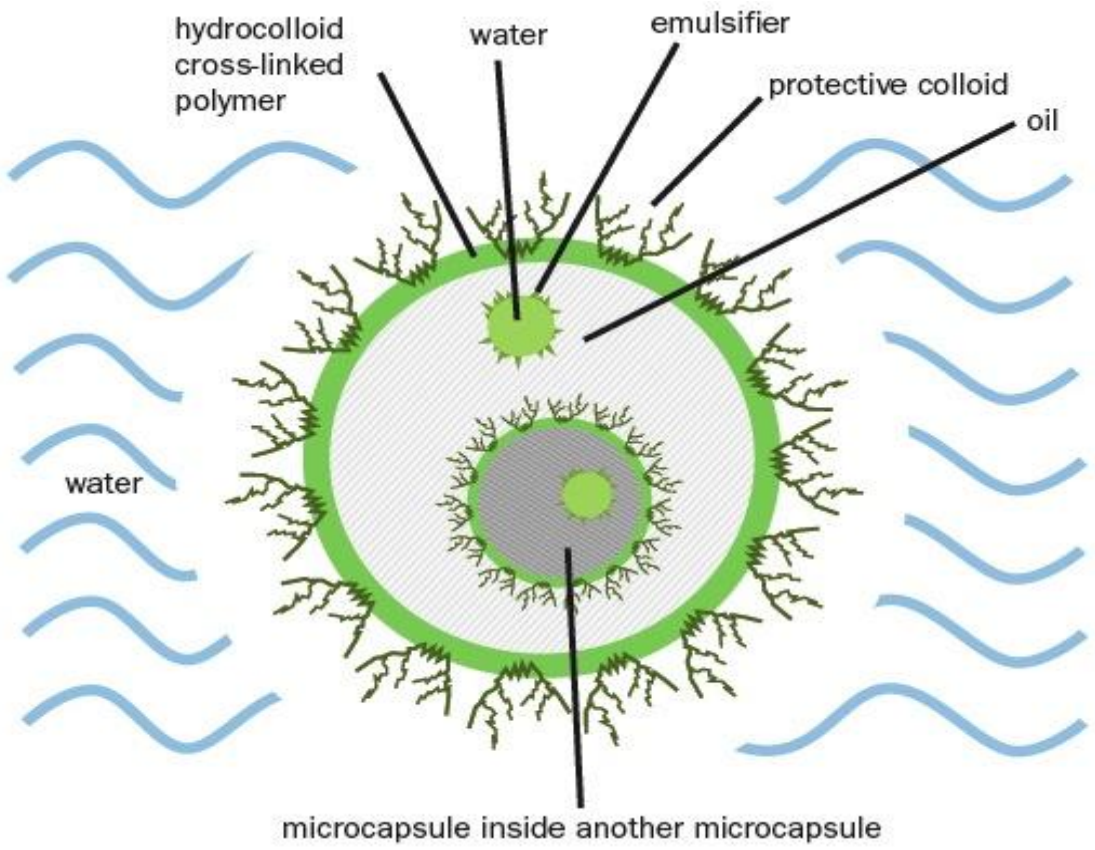



Figure 12: Schematic presentation of GAT wowCAPS® [according to CASAÑA et al, 2006]

The advantages of this technology are the possibility of a targeted release at a pH lower than 3 and the protection of the active ingredients against oxidation or degradation. [DRUSCH and MANNINO, 2009; CASAÑA et al, 2006]

In Table 4 compares the most established microencapsulation technologies for foods.

Table 4: Comparison of microencapsulation technologies [GAT Food Essentials, 2008]

| | **GATs Micro-encapsulation wowCAPS®** | **Matrix-encapsulation** | **Gelatine-encapsulation „coacervation“** |
|---|---|---|---|
| **Characteristics** | liquid, w/o/w system wowCAPS® | powder, (mostly) spray-dried | powder „powder loc“ |
| **Size of capsules** | 1 – 5 µm | 30 - 500 µm | > 60 µm |
| **Release of active ingredient** | targeted release with pH < 3.0 | gradual incomplete release at pH < 5.5 | gradual incomplete release in the small intestine by stomach acid and digestive enzymes |
| **Protection of active ingredient** | multiple micro-encapsulation system protected in shell of cross-linked biopolymers | sponge type matrix | double shell matrix, encapsulation of agglomeration of microcapsules |
| **Product stability** | very low free oil content (< 1%), high protection against oxidation | high surface oil levels (> 1%), low protection against oxidation | due to shell thickness high protection against oxidation |
| **Process stability** | resistant to common production requirements due to small particle size | unstable within common production requirements due to big particle size | suitable for dry mixing or addition to end product.; not suitable for high stress productions processes |
| **Technology** | natural, GMO-free ingredients no proteins added | carrier consisting of starch, proteins, pectins, sugars or other | fish or pork gelatin, formaldehyde as cross linker |

## 3.1 GAT Food Essentials GmbH

GAT Formulation was founded in 1999 as GAT Formulation GmbH. GAT started to provide R&D and analytical service to the agro industry. Since 2004 research in stabilisation of sensitive ingredients was performed. In 2006 a patent for the invented technology was published. The global sales and distributions part was expanded and offices in Latin America were founded. Since 2008 GAT Food Essentials GmbH is its own entity.

Various strategic partnerships with leading ingredients providers and long-term collaborations with food and beverage companies have enabled the company to become a recognised microencapsulation specialist. A growing number of food and beverage products containing ingredients using GAT's microencapsulation technology are available on the market all over the world.

The target market for GAT's wowCAPS® are long shelf life products with a high complexity of the food or beverage application such as low fat UHT milk or beverages with a low pH value such as orange juice.

In 2009 the company won the Frost & Sullivan 2009 Best Practices Award "European Functional Food & Beverage Microencapsulation Technology Innovation Award".

# 4. MATERIALS AND METHODS

## 4.1 Chemicals

Table 5 provides an overview of all chemicals used in this study.

Table 5: Chemicals used

| **Chemical** | **Supplier** | **Supplier's catalogue reference** | **used for:** |
|---|---|---|---|
| AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride) | Cayman, Ann Arbor | 82235 | ORAC assay |
| acetone CHROMASOLV for HPLC ≥ 99.9% | Sigma,Vienna | 270725 | analysis of the fatty acid pattern |
| acetonitrile | VWR, Vienna | 20060.320 | analysis of the fatty acid pattern |
| dichloromethane | Merck, Vienna | 8.22271 | analysis of the fatty acid pattern |
| dipotassium hydrogen phosphate | Sigma,Vienna | P 3786 | ORAC assay |
| *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester 98% | Sigma,Vienna | D 2659 | analysis of the fatty acid pattern |
| docosane, for synthesis, >98% | VWR, Vienna | 822046 | analysis of the fatty acid pattern |
| fluorescein (free acid) | Fluka, Vienna | 46955 | ORAC assay |
| n-hexane, HPLC grade | VWR, Vienna | 104391 | analysis of the fatty acid pattern |
| (+/-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid purum > 98.0% (HPLC) (Trolox) | Fluka, Vienna | 56510 | ORAC assay |
| methyl linolenate – methyl ester of linolenic acid, 99% | Sigma,Vienna | 23,526-1 | analysis of the fatty acid pattern |
| methyl all-*cis*-5,8,11,14,17-eicosapentaenoate | Sigma,Vienna | 17266 | analysis of the fatty acid pattern |
| O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBH) | Fluka, Vienna | 76735 | analysis of the fatty acid pattern |
| potassium dihydrogen phosphate | Merck, Vienna | 1.04873 | ORAC assay |
| 2-propanol | Merck, Vienna | 1.01040 | analysis of the fatty acid pattern |
| sodium methoxide 0.5M solution in methanol, A.C.S. reagent | Aldrich, Vienna | 40,306-7 | analysis of the fatty acid pattern |
| sodium sulphate | VWR, Vienna | 1.06649 | analysis of the fatty acid pattern |
| 2,2,4-trimethylpentane, ACS reagent ≥ 99.0% | Sigma,Vienna | 360597 | UV determination of conjugated dienes |
| 37 Components FAME Mix 10 mg/mL in $CH_2Cl_2$ | Supelco, Vienna | 47885-U | analysis of the fatty acid pattern |

## 4.2 Materials

Table 6 provides an overview of the materials used in this study.

Table 6: Materials used

| **Material** | **Supplier** | **Supplier's catalogue reference** | **used for:** |
|---|---|---|---|
| disposable syringe | VWR, Vienna | 6120109 | analysis of the fatty acid pattern |
| filter for syringe | VWR, Vienna | 117761Q | analysis of the fatty acid pattern |
| glass bottles, amber, 30 mL & 50 mL with screw cap | VWR, Vienna | 2151802 | analysis of the fatty acid pattern |
| 96-well assay plate, Optilux, non-sterile | VWR, Vienna | 353293 | ORAC assay |
| sample cups PP, sterile | VWR, Vienna | 2162690 | yogurt production |
| vials, amber, 32 x 11.5 mm | VWR, Vienna | 18022494 | analysis of the fatty acid pattern |

## 4.3 Apparatus

1. Agilent 6890N GC equipped with Flame Ionization Detector (FID) and PC system with GC ChemStation, Rev. A.09.03, Agilent Technologies Inc. (Inv. No. 03/09/34, GC-05)

2. Agilent 6890N GC/MSD equipped with MSD quadrupole 5973 inert mass selective detector and Flame Ionization Detector (FID), PC system with MSD ChemStation D.02.00.275, Agilent Technologies Inc. (Inv. No. 05/09/43, GC/MS-06)

3. Agilent HP 1100 HPLC equipped with a degasser (G1379A), quaternary pump (G1311A), autosampler (G1315B), PC system with ChemStation for LC 3D, Rev. A.10.02, Agilent Technologies Inc. (inv. No. 02/09/17, HPLC-04)

4. BMG Labtech FLUOstar OPTIMA and PC system with FLUOstar OPTIMA software 1.30-0, BMG Labtech GmbH (Inv. No. YY/09/94/02)

5. Eppendorf Research 500 – 5000 µL, Eppendorf (Inv. No. 09/05/68/01)

6. Eppendorf Varispenser plus 5 - 25 ml, Dispenser, Eppendorf (Inv. No. 07/05/57/01

7. Hach DR/4000U Spectrophotometer UV/VIS and HachLink 2000 software

8. Microscope Nikon Alphaphot 2 YS2 (Inv. No. 96/07/01/01) with camera Nikon Coolpix 950 (Inv. No. 00/07/03/01) and screen JVC TM 1700PN (Inv. No. 00/07/02/01)

9. Sartorius Competence electrical analytical balance CP225D (max. 40/80/220 g / d= 0.01/0,01/0.1 mg), Sartorius (Inv. No. 03/06/13/01)

10. Sartorius Gem plus electronical precision balance GP5202 (max. 5200g / d=0,01g), Sartorius (Inv. No. 04/06/15/01)

11. Ultrasonic bath Sonorex RK 510H / 9,0 L with thermostatic control, Bandelin (Inv. No. 04/08/56/01)

## 4.4 Samples

### 4.4.1 Formulation

The formulation wowCAPS® (in this study also referred to as “the formulation”) is a water- in oil- in water (w/o/w) microcapsule suspension containing min. 7w% EPA/DHA (calculated as free fatty acids from the actual presence in the form of glycerides). For this study 100 g samples from a 500 kg production lot are taken. They are used for analysis as well as for the application trials. The formulation was stored for the time of the experiment (up to 4 weeks) in a cool chamber at 3-6 °C or in an oven at 30°C or 70°C respectively. Immediately before each trial a fresh bag from the cool chamber is opened.

### 4.4.2 Oil

The fish oil rich in EPA/DHA (in this study also referred to as “the fish oil”) is derived from the species *Gadus morhua*. The content of EPA/DHA is min. 26w% (calculated as free fatty acids from the actual presence in the form of glycerides). It is provided by a supplier in 9 L bags and is refilled in sterile plastic cups for this study. It is used for analysis as well as for the preparation of the yoghurt samples. The oil is stored for the time of the experiment (up to 4 weeks) in the cool house at 3-6 °C or in an oven at 30°C and 70°C respectively. The nutritional information of the oil is derived from a standardised oil, but due to seasonal variation the content of EPA/DHA is likely to vary.

Table 7: Nutritional information of fish oil per 100 g

| | | |
|---|---|---|
| Energy | | 900 kcal |
| Fat | | |
| SFA | | 23 g |
| MUFA | | 35 g |
| PUFA | | 42 g |
| - of which: | ALA | 1 g |
| | EPA | 13 g |
| | DHA | 13 g |
| | Total ω-3 | 34 g |

### 4.4.3 Food applications

#### 4.4.3.1 Yoghurt

Yoghurt was chosen as food matrix for the thesis for its commercial interest as functional food enriched with omega-3.The yogurt is made in-house according the recipe in Table 8.

Table 8: Yoghurt recipe [in %]

| | **Yoghurt blank** | **Yoghurt containing wowCAPS®** | **Yoghurt containing fish oil** |
|---|---|---|---|
| Milk | 96.98 | 95.98 | 96.60 |
| Milk powder | 3.00 | 3.00 | 3.00 |
| Yoghurt cultures | 0.02 | 0.02 | 0.02 |
| Formulation wowCAPS® | - | 1.00 | - |
| Fish oil | - | - | 0.38 |

The yoghurt is freshly prepared in the laboratory. Milk is heated in a tempering beaker to 85 °C ± 2 °C. Skimmed milk powder is added to achieve a higher dry matter. When the milk reaches 85°C ± 2 °C the formulation (1%) and the oil (0.38%) respectively is added and the temperature is kept at 85 °C ± 2 °C for 5 minutes. Then the milk is cooled down to about 43 °C ± 2 °C and the yoghurt cultures (*Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus salivarius ssp. thermophilus*) are added. When they are distributed homogenously the yoghurt is filled in sterile cups and put into the oven for incubation at 41 °C ± 2 °C for 4 hours. Afterwards the cups are put in the cool chamber and stored before being analyzed. On each time point of analysis a fresh cup is taken out of the storage for evaluation.

#### 4.4.3.2 Cauliflower soup

Onions are roasted in olive oil and then 1 liter water is added. The cauliflowers are cut and cooked in the water. After adding of spices the cauliflowers are pureed. Then the formulation wowCAPS® (0.7%) is added. The cauliflower soup is analyzed initially and after 1 week storage in the cool house at 3-6 °C.

#### 4.4.3.3 Tomato sauce

Onions are roasted and one package of pure tomato sauce and one can of sliced tomatoes are added. Then herbs, salt and pepper are added and the sauce is cooked for 15 minutes. Then the formulation wowCAPS® (0.7%) is added and the sauce is stirred until the formulation is distributed homogenously. Then it is taken off the oven and filled in brown glass bottles for the one week storage. The tomato sauce is analyzed initially and after one week storage in the cool house at 3-6 °C.

#### 4.4.3.4 UHT milk

UHT milk with a fat content of 1.5% enriched with wowCAPS® from an industrial trial is delivered to GAT Food Essentials GmbH from a customer. According to GAT's recommendations the milk was enriched with 40 mg EPA/DHA per 250 mL. The shelf life study is conducted over 3 months at ambient temperature (~ 20 °C laboratory controlled).

## 4.5 Analysis of polyunsaturated fatty acids

### 4.5.1 Gas Chromatography (GC)

4.5.1.1 Gas Chromatography with Flame Ionization Detector (FID)

The GC-FID is used for the quantification of the omega-3 fatty acids in the application samples. The validated method for analysis of omega-3 fatty acids GAT-0604-FIO01-GC is used. With this method only the fatty acids bond in triglycerides are detected.

Chromatographic Parameters

| | |
|---|---|
| Column | SP2380 Fused Silica Column with stabilized cyanosilicone phase |
| Injector | 250 °C (split 1:100) |
| Column oven | Gradient programm:<br>start at 150°C hold for 20.0 minutes<br>at 10°C / minute to 240 °C hold for 6 minutes |
| Detector | FID (Flame Ionisation Detector) at 260 °C |
| Flow | 1.0 mL / minute |
| Carrier gas | Helium |
| Injection volume | 1.0 µL |
| Analysis time | 35.0 minutes |

Sample preparation and analysis

The sample preparation for this analysis is carried out 4 times for each sample.

- Fish oil

  In a bottle approximately 3.75 mg/mL fish oil in n-hexane are weighed accurately at 20 ºC. The sample is homogenized in an ultrasonic bath for 20 minutes. After homogenization the fatty acids are methylated using sodium methoxide and again homogenized in an ultrasonic bath for 30 minutes. The fatty acids methyl esters are analyzed by gas capillary chromatography (GC) using a fused silica capillary GC column with a

stabilized cyanosilicone phase. The fatty acid methyl esters are quantified using docosane as internal standard.

- Formulation wowCAPS®

  In a bottle approximately 5.0 mg/mL microencapsulated fish oil in n-hexane are weighed accurately at 20 ºC. The sample is extracted and homogenized in an ultrasonic bath for 20 minutes. After extraction from the sample the fatty acids are methylated using sodium methoxide and again homogenized in an ultrasonic bath for 30 minutes. The emerging fatty acid methyl esters are analyzed by gas capillary chromatography (GC) using a fused silica capillary GC column with a stabilized cyanosilicone phase. The fatty acid methyl esters are quantified using docosane as internal standard.

- Food samples containing wowCAPS®

  In a bottle approximately 12.5 mg/mL food sample containing microencapsulated fish oil is weighed at 20 ºC in n-hexane in the concentration, which is equal to the amount of formulation of microencapsulated fish oil. The sample is extracted in an ultrasonic bath for 20 minutes. After extraction from the sample the fatty acids are methylated using sodium methoxide and again homogenized in an ultrasonic bath for 30 minutes. The fatty acids methyl esters are analyzed by gas capillary chromatography (GC) using a fused silica capillary GC column with a stabilized cyanosilicone phase. The fatty acid methyl esters are quantified using docosane as internal standard.

Evaluation

Evaluation is carried out using GC ChemStation, Rev. A.09.03, Agilent Technologies Inc.

4.5.1.2 Gas Chromatography with Mass Sensitive detector (MSD)

The GC-MSD is used for the qualification of the omega-3 fatty acids in the fish oil, the wowCAPS® and the yoghurt samples.

Chromatographic Parameters

| | | |
|---|---|---|
| Column | SP2560 Fused Silica Column | |
| Injector | 250 °C (split 1:100) | |
| Column oven | Gradient program: | |
| | start at 140 °C hold for 5.0 minutes | |
| | at 2 °C / minute to 250 °C hold for 10 minutes | |
| Detector | MSD quadrupole (5973 inert mass selective detector) | |
| Mass parameters | EM voltage: | 1811 |
| | Acquisition mode: | Scan |
| | Scan Group 1 Low Mass: | 40.00 amu |
| | Scan Group 1 High Mass: | 650.00 amu |
| | Threshold: | 150 counts |
| | Sampling rate (2Ùn): | 2 |
| | Scans/sec: | 2.42 |
| | MS Timed Events Table | |
| | Event type: | Detector |
| | Detector on: | 9.00 min |
| | MS temperatures | |
| | MS source: | 230 °C |
| | MS quad: | 150 °C |
| Flow | 1.0 mL / minute | |
| Carrier gas | Helium | |
| Injection volume | 2.0 µL | |
| Analysis time | 60.0 minutes | |

Sample preparation and analysis

The sample preparation for this analysis is carried out 4 times for each sample.

- Standards of fatty acids

  Approximately 27 mg of the standards of methyl-linolenate, methyl-EPA and methyl-DHA are weighed accurately and dissolved and homogenized in 30 mL n-hexane in an ultrasonic bath. The 37

Components FAME Mix (10 mg/mL in $CH_2Cl_2$) is injected without further preparations.

- Fish oil

  Approximately 5 g of oil are weighed accurately in a brown glass bottle. Then 2 mL of dichloromethane and 1.5 mL of reagent solution (100 mg PFBH in 15 mL methanol) are added. The samples are mixed in an ultrasonic bath for 15 minutes at fixed temperature of 25 °C.

- Formulation wowCAPS®

  Approximately 13 g of formulation are weighed accurately in a brown glass bottle. Then 1 mL of 0.05 mol HCl is added and the sample is mixed in an ultrasonic bath. Then 10 mL of n-hexane are added and the sample is put in an ultrasonic bath for 15 minutes at fixed temperature of 25 °C. Then the sample is centrifugated and 10 mL of the n-hexane phase are taken and dried using a rotavapor. The remaining is dissolved in 2 mL dichloromethane and 1.5 mL reagent solution (100 mg PFBH in 15 mL methanol).

- Food samples containing wowCAPS®

  Approximately 1 g of yoghurt (blank, containing wowCAPS®, containing fish oil) is weighed and dissolved in 10 mL n-hexane in an ultrasonic bath. Then 3 mL of sodium methoxide is added and mixed in an ultrasonic bath for 15 minutes at fixed temperature of 25 °C.

For this sample preparation no internal standard was used since no quantification was done.

Evaluation

Evaluation is carried out using MSD ChemStation D.02.00.275, Agilent Technologies Inc.

### 4.5.2 High Performance Liquid Chromatography (HPLC)

4.5.1.2 Reverse Phase High Performance Liquid Chromatography with Diode Array Detector (DAD)

The HPLC analysis is carried out to detect degradation products of fatty acids. As most of the degradation products are volatile and unstable under high temperature they cannot be detected by gas chromatography. After literature review and a pre-test, in which the whole UV-VIS spectrum was measured, the wavelengths for detection were chosen accordingly.

Chromatographic Parameters

| | |
|---|---|
| Column | LiChroCART 250-4 LiChrospher $NH_2$ (5 µm) |
| Column oven | 20°C for 45 minutes |
| Detector | DAD (Diode Array Detector) at 216 nm, 234 nm, 275 nm, 288 nm, 342 nm |
| | Spectra 190-500 nm |
| Flow | 1.0 mL / minute |
| Mobile phase | n-hexane / acetonitrile (99.75 : 0.25) |
| Injection volume | 40.0 µL |
| Analysis time | 45.0 minutes |

Sample preparation and analysis

The sample preparation for this analysis is carried out 4 times for each sample. Approximately 1.14 g oil / 3 g formulation is weighed accurately in a brown glass bottle. 20 mL n-hexane is added and the sample is put in an ultrasonic bath for 20 minutes at fixed temperature of 25 °C. Then 2 mL sodium methoxide are added and the sample is put in an ultrasonic bath for another 30 minutes at fixed temperature of 25 °C. Then it is centrifugated and the upper phase is analyzed by HPLC.

Evaluation

Evaluation is carried out using ChemStation for LC 3D, Rev. A.10.02, Agilent Technologies Inc. The qualification is done according the 3-dimensional scale time/wavelength/absorbance.

### 4.5.3 Peroxide value (method according to Wheeler)

The peroxide value (PV) measures the amount of peroxides and hydroperoxides formed during early stages of lipid oxidation. In more advanced stages it is not reliable because hydroperoxides decompose further into other products.

The samples are sent to an extern laboratory and are analyzed as follows:
2.0 – 5.0 g of oil and 5.3 – 13.3 g of formulation respectively are dissolved in 50 mL isooctane/acetic acid solution (3:2, v/v) and 0.5 mL of saturated potassium iodide is added and the mixture is shaken for exactly one minute to form $I_2$:

$$ROOH + 2\ H^+ + 2\ I^- \rightarrow ROH + I_2 + H_2O$$

After the addition of 30 mL water the sample is titrated with 0.01 N sodium thiosulfate solution.

$$I_2 + 2\ S_2O_3^{2-} \rightarrow 2\ I^- + S_4O_6^{2-}$$

The PV is expressed in milliequivalents peroxide per kg of sample and is calculated as per EN ISO 3960:2007 [ISO, 2007]:

$$PV = c * v * 16 * 1000 / 2 * m / 8$$

c = Titrant concentration in mol/L
v = volume of tritrant in mL
m = weighed amount of sample in g

16 = molecular weight of oxygen

2 = as 2 moles of titrant correspond to 1 mol of sample

8 = for meq/kg

### 4.5.4 UV-VIS Spectrophotometric analysis

4.5.4.1 Conjugated dienes

Conjugated dienes (CD) are intermediate products in the early stages of oxidation. They react with oxygen to from conjugated hydroperoxides. The most representative absorption maximum is at 234 nm and can be measured spectrophotometrically.

56 mg of formulation with 2 mL of acidified water (pH 2) for breaking the capsules and 20 mg of oil respectively are weighed into a 20 mL volumetric flask which is filled with isooctane (2,2,4-trimethylpentane) up to the 20 mL mark. The flasks are put into an ultrasonic bath to obtain a homogenous distribution. The absorbance was measured using a Hach DR/4000U spectrophotometer at 234 nm against a blank of isooctane.
To ensure that there is no interference a test with oil and acidified water is carried out.

The CD values are calculated as described in the AOCS official method Ti 1a-64 [AOCS, 1998]:

$$CD, \% = 0.84\ [(A_s/b*c)-K_0]$$

$A_s$ = absorbency at 234 nm

b = cuvette length in cm

c = sample concentration in isooctane (g/L)

$K_0$ = absorptivity by acid or ester groups; 0.07 for esters, 0.03 for acids

4.5.4.2 Conjugated trienes

Conjugated trienes (CT) are intermediate products in the early stages of oxidation. They react with oxygen to from conjugated hydroperoxides. The most representative absorption maximum is at 268 nm and can be measured spectrophotometrically.

56 mg of formulation with 2mL of acidified water (pH 2) for breaking the capsules and 20 mg of oil respectively are weighed into a 20 mL volumetric flask which is filled with isooctane (2,2,4-trimethylpentane) up to the 20 mL mark. The flasks are put into an ultrasonic bath to obtain a homogenous distribution. The absorbance was measured using a Hach DR/4000U spectrophotometer at 268 nm against a blank of isooctane.
To ensure that there is no interference a test with oil and acidified water is carried out.

The CT values are calculated as described in the AOCS official method Ti 1a-64 [AOCS, 1998]:

$$CT, \% = 0.84\ [(A_s/b*c)-K_0]$$

$A_s$ = absorbency at 268 nm
b = cuvette length in cm
c = sample concentration in isooctane (g/L)
$K_0$ = absorptivity by acid or ester groups; 0.07 for esters, 0.03 for acids

## 4.5.5 Fluorometric analysis – ORAC assay

The ORAC (oxygen radical antioxidative capacity) assay is used to determine the antioxidative capacity of the tested samples. While this test is well developed for water soluble compounds, ORAC analysis for water-insoluble compounds is still lacking of adequate testing methodology. It measures the

decrease in the fluorescence of fluorescein as a result of its degradation when it is oxidatively damaged by a source of peroxyl radicals.

The automated ORAC assay is carried out on a FLUOstar OPTIMA with fluorescein as the “fluorescent source” and AAPH radical as the catalysator. The reaction is performed at 37 °C as the reaction is started by thermal decomposition of AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride). As fluorescein is sensitive to pH the solvent is a 75mMol phosphate buffer with pH 7.4. As standard trolox ((+/-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used in 5 different dilutions.
As the ORAC assay is very sensitive to oxidation the solvent and all reagents, standards and samples have to be made daily fresh before analysis.
The samples (75 mg of formulation, 28.5 mg of oil, 1800 mg of yoghurt, respectively) and standards (10 mg trolox) are diluted in 30 mL phosphate buffer solution and further dilutions are made. To enable the dilution of the oil an emulsion is prepared. The same emulsion without the oil is tested as blank. Then to each of the samples 150 µL of fluorescein is added and they are put in the FLUOstar OPTIMA to measure their antioxidative capacity. After 3 runs 25 µL of the reagent AAPH is added to catalyze the reaction of forming oxidative radicals. Then the decrease of the ability to fluoresce is measured.
The ORAC values are calculated by following formula and expressed as µMol trolox equivalents (TE).

$$\text{ORAC [µmol TE]} = \frac{(C_{Trolox} * (AUC_{Sample} - AUC_{Blank}) * k)}{(AUC_{Trolox} - AUC_{Blank})}$$

AUC = area under the curve
$C_{Trolox}$ = is the concentration of Trolox
k = sample dilution factor

Figure 13 shows an image of the evaluation of the ORAC essay as example.

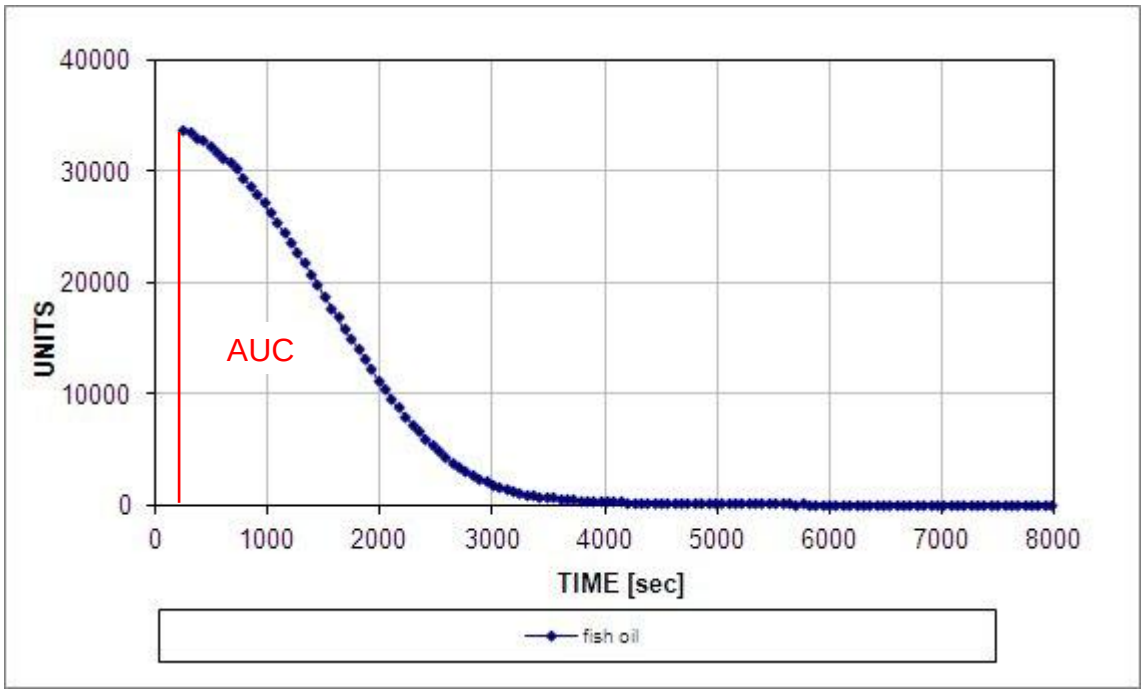



Figure 13: Image of ORAC essay evaluation

## 4.6 Sensory evaluation

For the sensory evaluation a panel of 8 persons of GAT was established according general guidelines for this purpose. The samples are indistinguishable to the panel and are taken from the cool house 3 hours before tasting to warm them up. The panel has to evaluate the samples on color, cloudiness, surface appearance, consistency, smell, taste, aftertaste and mouth feel. All these terms are used to determine the general impact, whereas smell, taste and aftertaste are used to determine off-flavor. The range of acceptability is chosen due to a long experience in this kind of tastings. The scale is set to 0 for least acceptability and 8 for highest acceptability. The scale of the overall acceptability is set to like, neutral and dislike.

As samples yoghurt, cauliflower soup, and tomato sauce are taken. One tasting per day is scheduled. The evaluation of the tastings is carried out with MS Excel.

## 4.7 Microscopic observation

Microscopic observation of the microencapsulated product and the food samples is carried out to show if the capsules undergo any changes throughout the production processes of the food samples and to assess microscopical changes in the food structure.

Sample preparation and analysis

- Formulation wowCAPS®

  20 g of deionized water are weighed in an amber glass bottle. 1 g of wowCAPS® is added and emulsified. A small drop of it is placed on the microscope slide and covered with a cover glass.
- Yoghurt containing wowCAPS®

  5 g of deionized water are weighed in an amber glass bottle. 5 g of yoghurt containing wowCAPS® is added and shaken by hand. A small drop of it is placed on the microscope slide and covered with a cover glass.
- Yoghurt containing fish oil

  5 g of deionized water are weighed in an amber glass bottle. 5 g of yoghurt containing fish oil is added and shaken by hand. A small drop of it is placed on the microscope slide and covered with a cover glass.
- Cauliflower soup, tomato sauce, UHT milk containing wowCAPS®

  5 g of deionized water are weighed in an amber glass bottle. 5 g of the sample containing wowCAPS® is added and homogenized by hand. A small drop of it is placed on the microscope slide and covered with a cover glass.

## 4.8 Statistical analysis

Statistical analysis has been performed with the software package OriginPro 8.0 SR0, v. 8.0724 (B724) running in a PC equipped with Windows 7 operative system.

Grubb's test for detecting outliers is used to find outliers in the data of the omega-3 analysis of the yoghurt containing wowCAPS®.

For the analysis of the omega-3 values in yoghurt One-Way ANOVA is used. To determine the significance of the values of the conjugated dienes and trienes One-Way ANOVA as well as the Fisher-Test is used.

For organoleptic evaluation One-Way ANOVA is used.

# 5. RESULTS & DISCUSSION

## 5.1 Analysis of polyunsaturated fatty acids in formulation, oil, and ω-3 fortified yogurt, cauliflower soup, tomato sauce and UHT milk

Evaluation of the analysis is limited to the evaluation of EPA and DHA as these are the primary components of marine fish oils. As the content of EPA and DHA is very high in these oils it is easier to analyze them in complex matrices. We assume that the organoleptic and chemical properties of the other ω-3 fatty acids follow the same pattern.

CHANGES OF ω-3 FATTY ACIDS OVER TIME

### 5.1.1 GC - FID

#### 5.1.1.1 Formulation wowCAPS®

Figure 14 shows the GC-FID chromatogram of the quantitative fatty acid analysis of the formulation wowCAPS®.

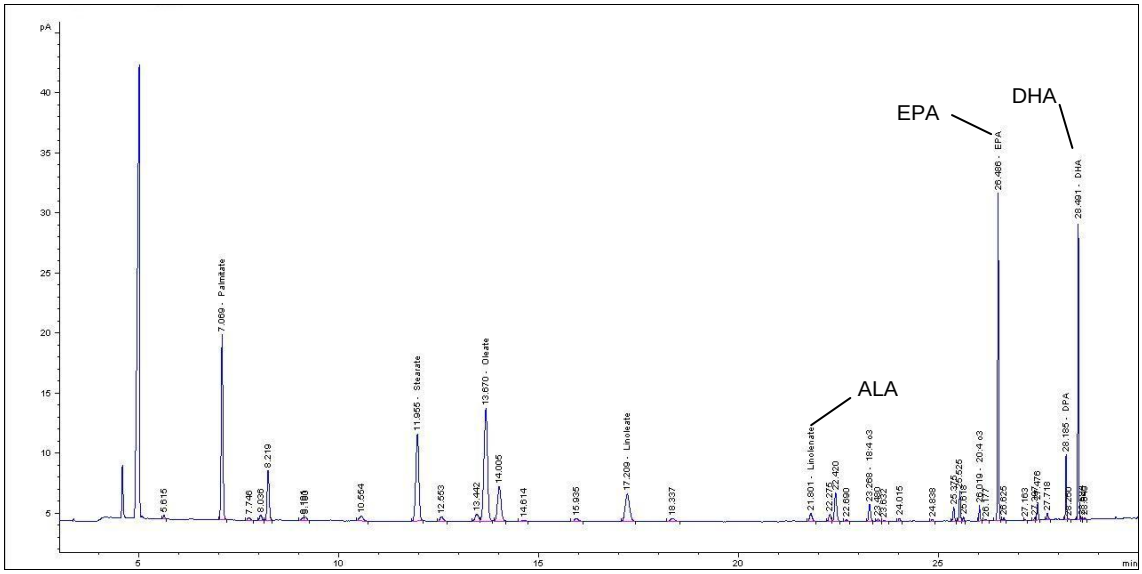



Figure 14: GC-FID chromatogram of formulation wowCAPS®

Figure 15 shows an outtake of the GC-FID chromatogram of the quantitative fatty acid analysis of the formulation wowCAPS®.

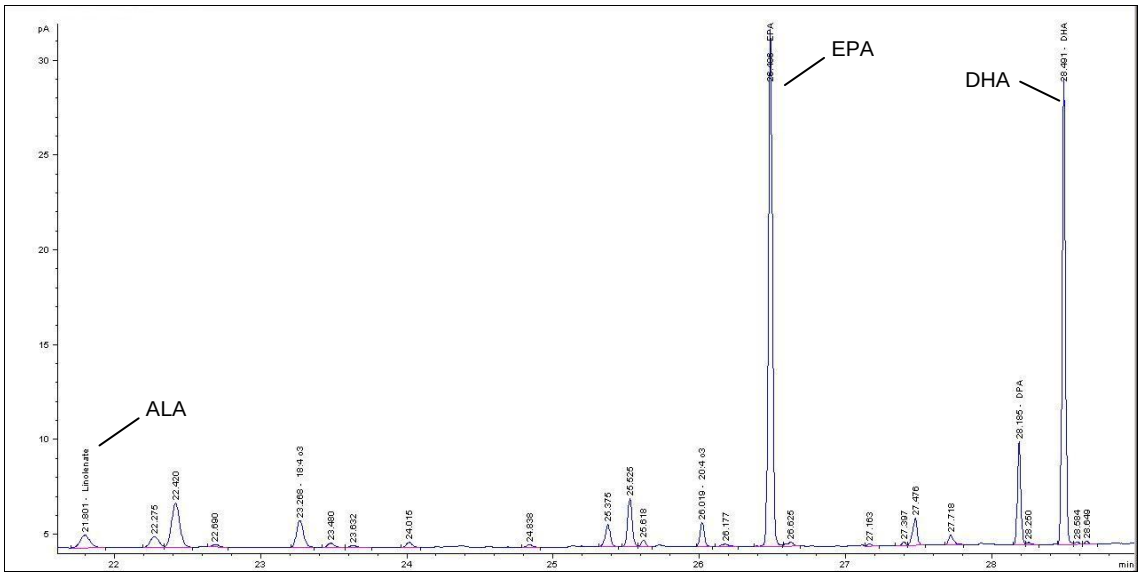



Figure 15: GC-FID chromatogram of formulation wowCAPS® (outtake)

Table 9 shows the results of the quantitative fatty acid analysis of wowCAPS® with GC-FID.

Table 9: Fatty acid analysis of formulation wowCAPS®

| **Omega-3 fatty acids** | **mg/100g** |
|---|---|
| all *cis*-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 290 |
| all *cis*-6,9,12,15-Octadecatetraenoic acid ω3 | 430 |
| all *cis*-8,11,14,17-Eicosatetraenoic acid ω3 | 230 |
| all *cis*-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 4630 |
| all *cis*-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 760 |
| all *cis*-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 3540 |
| TOTAL omega-3 | 9880 |
| EPA/DHA | 8170 |

5.1.1.2 Fish oil

Figure 16 shows the GC-FID chromatogram of the quantitative fatty acid analysis of the fish oil.

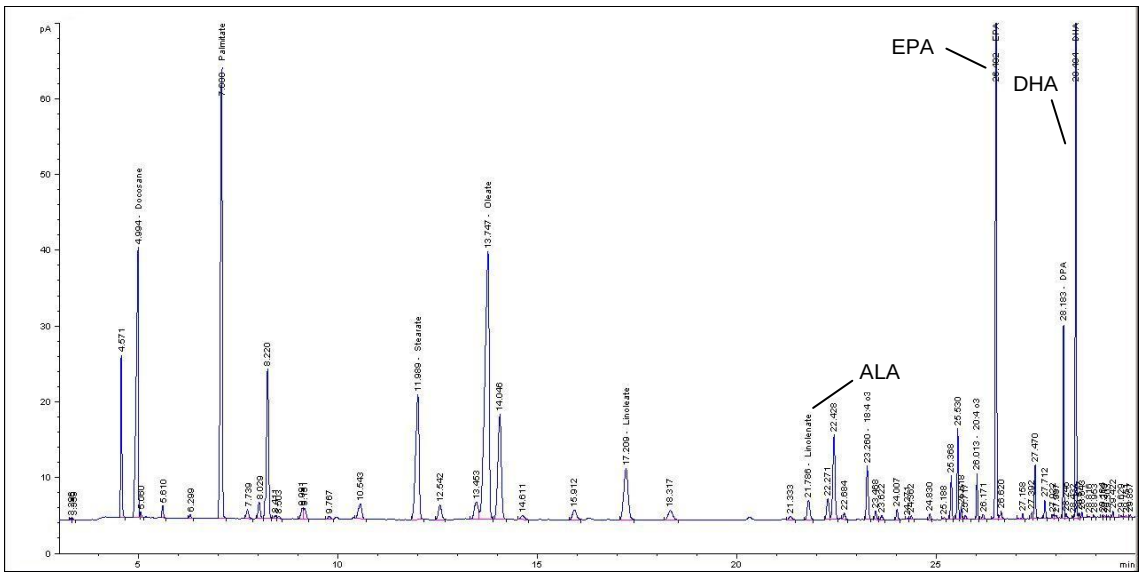



Figure 16: GC-FID chromatogram of fish oil

Figure 17 shows an outtake of the GC-FID chromatogram of the quantitative fatty acid analysis of the fish oil.

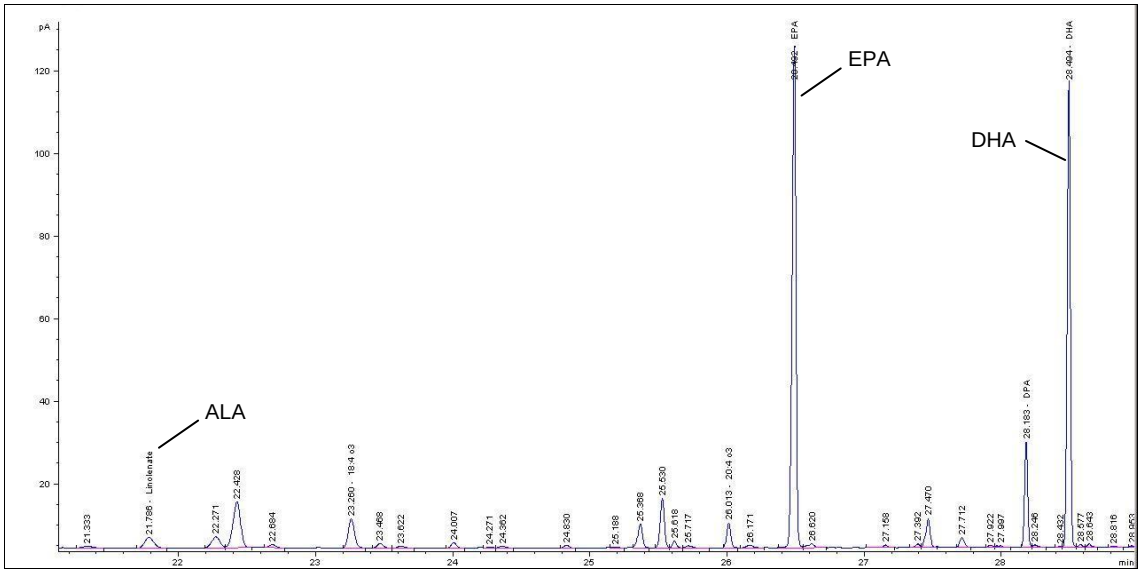



Figure 17: GC-FID chromatogram of fish oil (outtake)

Table 10 shows the results of the quantitative fatty acid analysis of fish oil with GC-FID.

Table 10: Fatty acid analysis of fish oil

| **Omega-3 fatty acids** | **mg/100g** |
|---|---|
| all *cis*-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 750 |
| all *cis*-6,9,12,15-Octadecatetraenoic acid ω3 | 1390 |
| all *cis*-8,11,14,17-Eicosatetraenoic acid ω3 | 750 |
| all *cis*-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 14230 |
| all *cis*-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 2400 |
| all *cis*-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 10970 |
| TOTAL omega-3 | 30490 |
| EPA/DHA | 25200 |

5.1.1.3 Yoghurt

Figure 18 shows the GC-FID chromatogram of the quantitative fatty acid analysis of the blank yoghurt at the time point of initial analysis.

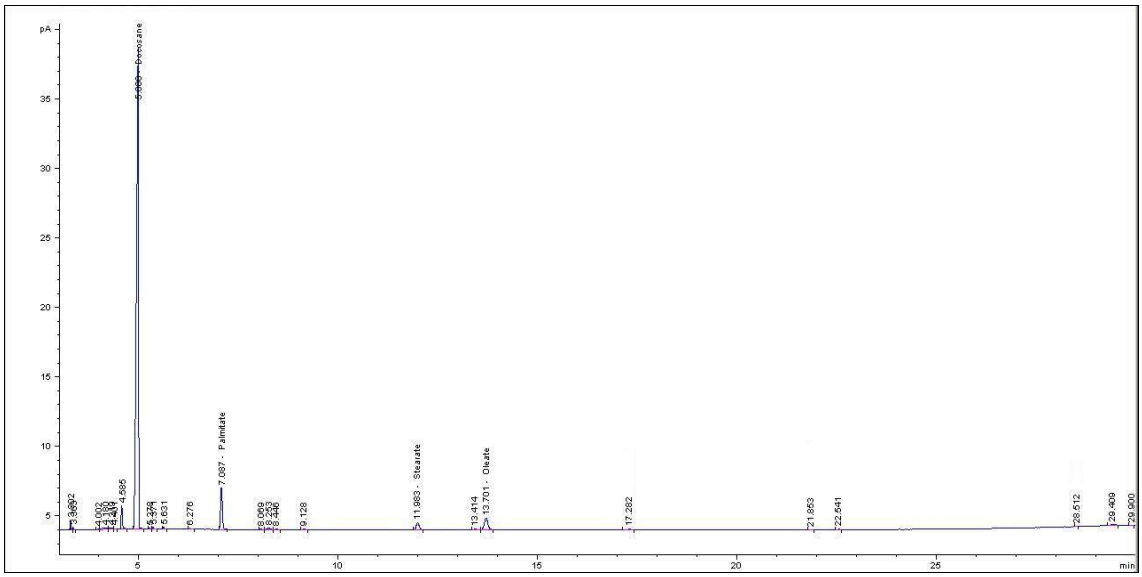



Figure 18: GC-FID chromatogram of yoghurt blank, initial

Figure 19 and Figure 20 show the GC-FID chromatograms of the quantitative fatty acid analysis of the yoghurt containing wowCAPS® at the time point of initial analysis (blue) and after 21 days storage at 3-6 °C (red).

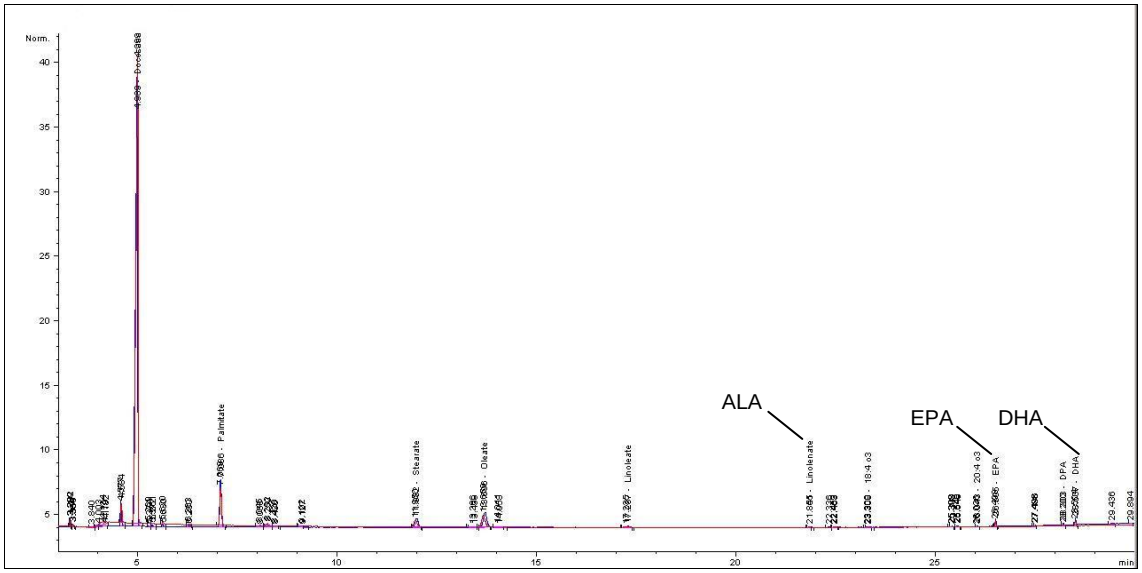



Figure 19: GC-FID chromatogram of yoghurt containing wowCAPS®

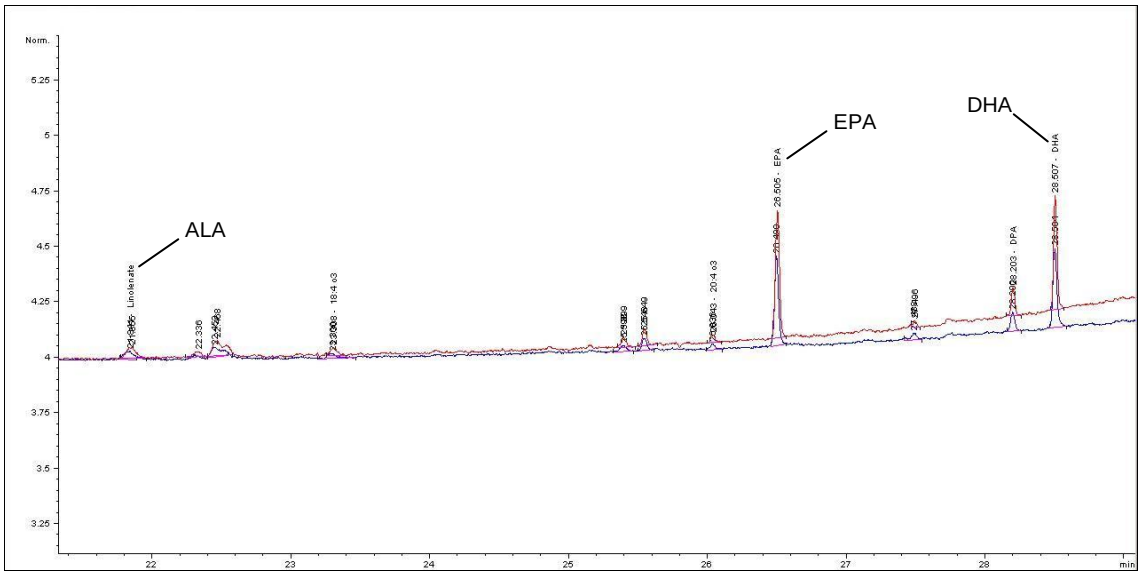



Figure 20: GC-FID chromatogram of yoghurt containing wowCAPS®

The changes in the base line of the different analyses are the results of unavoidable conversions of the apparatus.

In Table 11 the results of the quantitative fatty acid pattern analysis of the yoghurt enriched with wowCAPS® at each time point of the storage stability testing is shown. A deviation in both increase and decrease occurs throughout the storage time. This could be referred to an inhomogeneous distribution of the formulation in the milk prior to filling and fermentation. Further research on this topic would be necessary as the possibilities in the laboratory were limited with regard to the production process of yoghurt.

Table 11: Fatty acid analysis of homemade yoghurt enriched with wowCAPS® (n.d. – not detectable)

| Omega-3 fatty acids | Theory | initial / day 3 | day 4 | day 5 | day 6 | day 7 | day 10 | day 12 | day 13 | day 14 | day 17 | day 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g |
| all *cis*-9, 12, 15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 3.23 | 3.73 | 0.96 | 1.99 | 1.32 | 5.59 | 2.47 | 1.88 | 6.30 | 7.68 | 3.95 | 3.77 |
| all *cis*-6, 9, 12, 15-Octadecatetraenoic acid ω3 | 4.41 | 4.76 | 4.56 | 3.80 | 3.99 | 4.78 | 4.23 | 3.90 | 4.02 | n.d. | n.d. | n.d. |
| all *cis*-8, 11, 14, 17-Eicosatetraenoic acid ω3 | 2.30 | 3.16 | 2.84 | 3.41 | 2.99 | 3.20 | 3.18 | 3.03 | 2.68 | 2.41 | 2.44 | n.d. |
| all *cis*-5, 8, 11, 14, 17-Eicosapentaenoic acid ω3 (EPA) | 44.19 | 47.66 | 46.59 | 42.51 | 42.31 | 49.98 | 45.08 | 46.08 | 41.87 | 38.01 | 38.48 | 39.17 |
| all *cis*-7, 10, 13, 16, 19-Docosapentaenoic acid ω3 (DPA) | 7.13 | 7.61 | 7.47 | 7.10 | 7.81 | 8.39 | 8.65 | 8.02 | 7.89 | 7.82 | 7.47 | 8.07 |
| all *cis*-4, 7, 10, 13, 16, 19-Docosahexaenoic acid ω3 (DHA) | 34.84 | 31.63 | 30.94 | 27.73 | 28.65 | 34.94 | 32.88 | 34.27 | 32.74 | 25.85 | 29.16 | 30.18 |
| TOTAL omega-3 | 96.10 | 98.55 | 93.36 | 86.54 | 87.07 | 106.89 | 96.49 | 97.18 | 95.50 | 82.71 | 81.50 | 81.19 |
| Deviation to theory in % | - | 2.55 | - 2.85 | - 9.95 | - 9.40 | 11.29 | 0.41 | 1.13 | - 0.62 | - 13.93 | - 15.19 | - 14.92 |
| EPA/DHA | 79.03 | 79.29 | 77.53 | 70.24 | 70.96 | 84.92 | 77.96 | 80.35 | 74.61 | 63.86 | 67.64 | 69.35 |
| Deviation to theory in % | - | 0.33 | - 1.90 | - 11.12 | - 10.21 | 7.45 | - 1.35 | 1.67 | - 5.59 | - 19.20 | - 14.41 | - 12.25 |

Figure 21 and Figure 22 show the GC-FID chromatogram of the quantitative fatty acid analysis of the yoghurt containing fish oil at the time point of initial analysis (blue) and after 21 days storage at 3-6 °C (red).

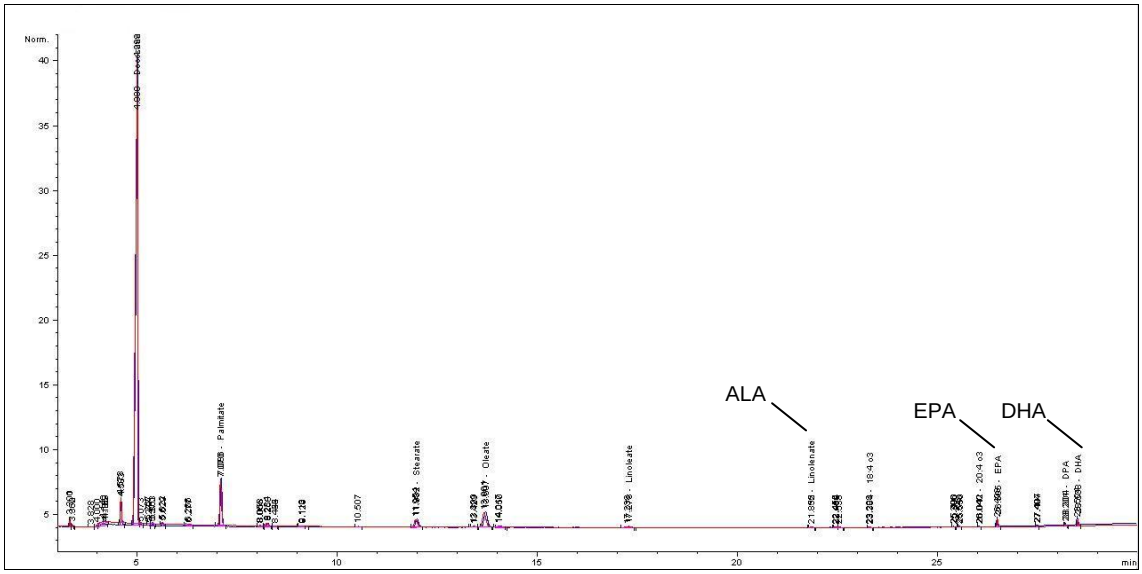



Figure 21: GC-FID chromatogram of yoghurt containing fish oil

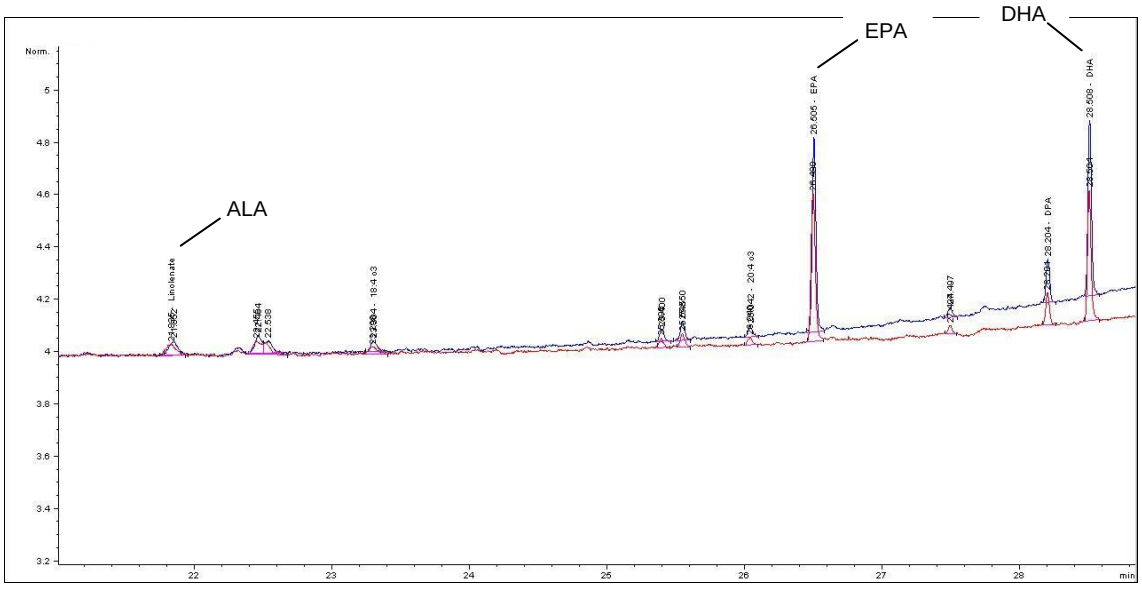



Figure 22: GC-FID chromatogram of yoghurt containing fish oil

In Table 12 the outcome of the quantitative fatty acid pattern analysis of the yoghurt enriched with fish oil at each time point of the storage stability testing is shown.

Table 12: Fatty acid analysis of homemade yoghurt enriched with fish oil (n.d. – not detectable)

| Omega-3 fatty acids | Theory | initial / day 3 | day 4 | day 5 | day 6 | day 7 | day 10 | day 12 | day 13 | day 14 | day 17 | day 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g | mg/100g |
| all *cis*-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 2.75 | 1.48 | 1.60 | 0.91 | 0.17 | 4.99 | 0.91 | 0.53 | 4.16 | 5.29 | 1.63 | 2.69 |
| all *cis*-6,9,12,15-Octadecatetraenoic acid ω3 | 5.20 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| all *cis*-8,11,14,17-Eicosatetraenoic acid ω3 | 2.78 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| all *cis*-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 53.98 | 30.21 | 17.06 | 21.60 | 35.57 | 33.06 | 21.70 | 13.79 | 12.35 | 19.97 | 19.50 | 11.42 |
| all *cis*-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 9.34 | 4.68 | 1.40 | 0.25 | 2.17 | 3.87 | 4.25 | 2.69 | 3.52 | 4.52 | 4.20 | 4.11 |
| all *cis*-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 41.79 | 17.45 | 6.14 | 11.81 | 10.31 | 17.15 | 10.30 | 8.43 | 7.67 | 11.34 | 12.68 | 8.62 |
| TOTAL omega-3 | 115.84 | 53.82 | 26.20 | 34.57 | 48.22 | 59.07 | 37.16 | 25.44 | 27.70 | 41.12 | 38.01 | 26.84 |
| Deviation to theory in % | - | - 53.54 | - 77.38 | - 70.16 | - 58.37 | - 49.01 | - 67.92 | - 78.04 | - 76.09 | - 64.50 | - 67.19 | - 76.83 |
| EPA/DHA | 95.77 | 47.66 | 23.20 | 33.41 | 45.88 | 50.21 | 32.00 | 22.22 | 20.02 | 31.31 | 32.18 | 20.04 |
| Deviation to theory in % | - | - 50.23 | - 75.76 | - 65.11 | - 52.09 | - 47.57 | - 66.59 | - 76.80 | - 79.10 | - 67.31 | - 66.40 | - 79.07 |

Statistical analysis of the stability of the omega-3 fatty acids incorporated into yogurt samples

The results shown in Table 11 and Table 12 have been subjected to a series of One-Way ANOVA statistical analysis.

Some of the omega-3 fatty acids, namely all *cis*-6,9,12,15-Octadecatetraenoic acid and all *cis*-8,11,14,17-Eicosatetraenoic acid have been excluded from the calculations, as they show interferences due to the presence of a high baseline caused by the food matrix (see Figure 20 and Figure 22). Similarly any outlier has been deleted from the analysis (day 4 and 5 for DPA; for the sake of having a balanced design such values have been disregarded in both set of data, the one with pure fish oil and the one with wowCAPS®). The verification of the condition of outlier has been performed with the online test http://www.graphpad.com/quickcalcs/Grubbs1.cfm at alpha = 0,01.

For the purpose of this work the analytical method GAT-0604-FIO01-GC used in this experimental part has shown to be precise and specific enough for a correct determination of the most important omega-3 from an industrial point of view, namely EPA and DHA [GAT FORMULATION, 2004]. The content in EPA and DHA in the final products is normally what is reflected in the label of the functional food as the claimed content of omega-3.

The One-Way ANOVA shows that there are significant differences ($p < 0.05$) in the EPA, DPA and DHA content depending on the form of application: in the yoghurt, the wowCAPS® formulation provides more stability over time for EPA, DPA and DHA than the simple addition of fish oil.

Additionally it has been observed (in the most aged yoghurt samples) that the fish oil, when added directly and not via wowCAPS®, separates in certain regions of the yoghurt. Although the samples of yoghurt were homogenized carefully before taking the aliquots for the analyses the wowCAPS® formulation provides more homogeneity in the distribution of the omega-3 in yoghurt samples than the simple addition of fish oil.

It is noted, that a relatively high variability of the results within the same type of dosage form has been observed, although this fact does not prevent to find statistically significant differences.

As the possibilities in the laboratory at GAT Food Essentials GmbH are limited when it comes to the production of food samples containing either wowCAPS® or fish oil further trials to confirm the findings of this study are necessary.

### 5.1.1.4 Cauliflower soup

Figure 23 and Figure 24 show the GC-FID chromatogram of the quantitative fatty acid analysis of cauliflower soup enriched with wowCAPS® at the time point of initial analysis (blue) and after 1 week storage at 3-6 °C (red).

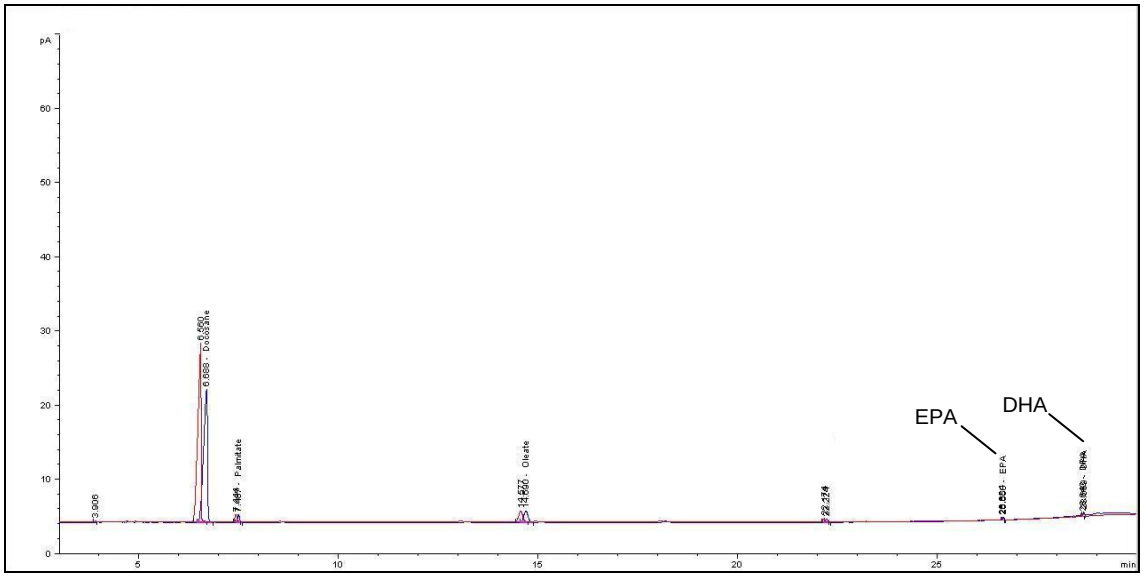



Figure 23: GC-FID chromatogram of cauliflower soup containing wowCAPS®

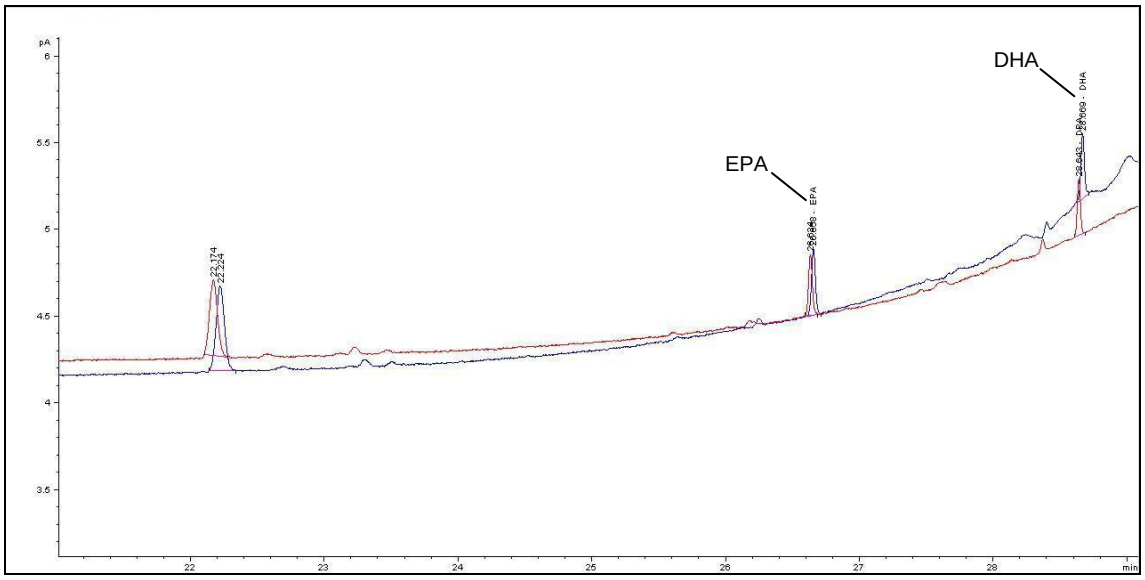



Figure 24: GC-FID chromatogram of cauliflower soup containing wowCAPS® after 1 week of storage

The olive oil used for the preparation of the cauliflower soup does not interfere with the analysis of the ω-3 fatty acids.

Table 13 shows the results of the fatty acid analysis of cauliflower soup enriched with wowCAPS® initially and after one week of storage at 3-6 °C.

Table 13: Fatty acid analysis of homemade cauliflower soup enriched with wowCAPS®

| **Omega-3 fatty acids** | **theory** | **initial** | **1 week** |
|---|---|---|---|
| | **mg/100 g** | **mg/100 g** | **mg/100 g** |
| all *cis*-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 2.03 | interferences | interferences |
| all *cis*-6,9,12,15-Octadecatetraenoic acid ω3 | 3.01 | not detectable | not detectable |
| all *cis*-8,11,14,17-Eicosatetraenoic acid ω3 | 1.61 | not detectable | not detectable |
| all *cis*-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 32.41 | 33.42 ± 0.92 | 32.61 ± 0.86 |
| all *cis*-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 5.32 | not detectable | not detectable |
| all *cis*-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 24.78 | 30.55 ± 2.05 | 24.46 ± 1.25 |
| TOTAL omega-3 | 69.16 | not calculable | not calculable |
| Deviation to the initial in % | - | - | not calculable |
| EPA/DHA | 57.19 | 63.97 | 57.07 |
| Deviation to the initial in % | - | - | -10.79 |

As some compounds interfered with the peak of ALA these values cannot be included.
No appraisable difference is detected between the initial values and the values after 1 week storage.

5.1.1.5 Tomato sauce

Figure 25 and Figure 26 show the GC-FID chromatogram of the quantitative fatty acid analysis of tomato sauce enriched with wowCAPS® at the time point of initial analysis (blue) and after 1 week storage at 3-6 °C (red).

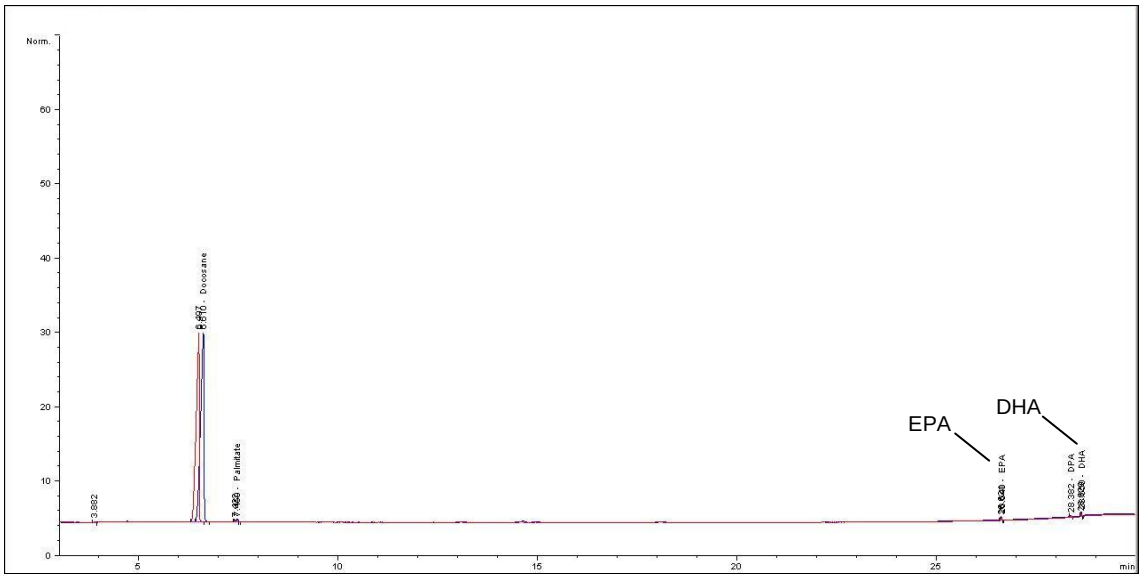



Figure 25: GC-FID chromatogram of tomato sauce containing wowCAPS®

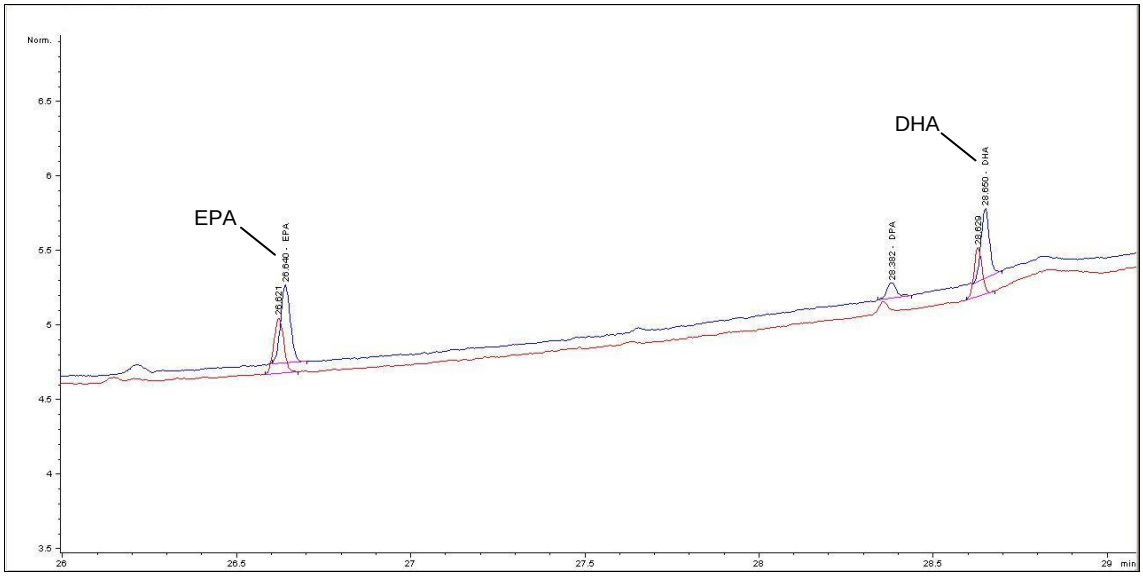



Figure 26: GC-FID chromatogram of tomato sauce containing wowCAPS® after 1 week of storage

Table 14 shows the results of the fatty acid analysis of tomato sauce enriched with wowCAPS® initially and after one week of storage at 3-6 °C.

Table 14: Fatty acid analysis of homemade tomato sauce enriched with wowCAPS®

| **Omega-3 fatty acids** | **theory** | **initial** | **1 week** |
|---|---|---|---|
| | **mg/100 g** | **mg/100 g** | **mg/100 g** |
| all *cis*-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 2.03 | not detectable | not detectable |
| all *cis*-6,9,12,15-Octadecatetraenoic acid ω3 | 3.01 | not detectable | not detectable |
| all *cis*-8,11,14,17-Eicosatetraenoic acid ω3 | 1.61 | not detectable | not detectable |
| all *cis*-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 32.41 | 31.31 ± 3.62 | 33.04 ± 1.09 |
| all *cis*-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 5.32 | 0.89 ± 2.53 | not detectable |
| all *cis*-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 24.78 | 23.63 ± 1.79 | 24.49 ± 1.79 |
| TOTAL omega-3 | 69.16 | 55.84 | 57.53 |
| Deviation to the initial in % | - | - | 3.04 |
| EPA/DHA | 57.19 | 54.94 | 57.53 |
| Deviation to the initial in % | - | - | 4.72 |

No significant difference is detected between the initial values and the values after 1 week storage.

5.1.1.7 UHT milk

Figure 27 and Figure 28 show the GC-FID chromatogram of the quantitative fatty acid analysis of UHT milk enriched with wowCAPS® at the time point of initial analysis (blue) and after 3 months storage at room temperature (red).

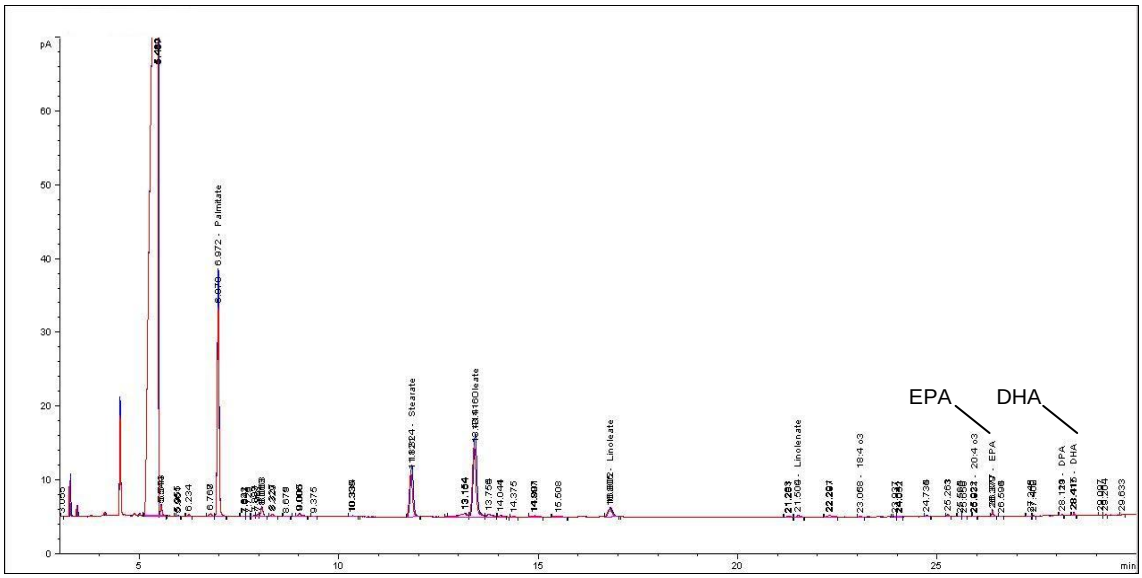



Figure 27: GC-FID chromatogram of UHT milk containing wowCAPS®

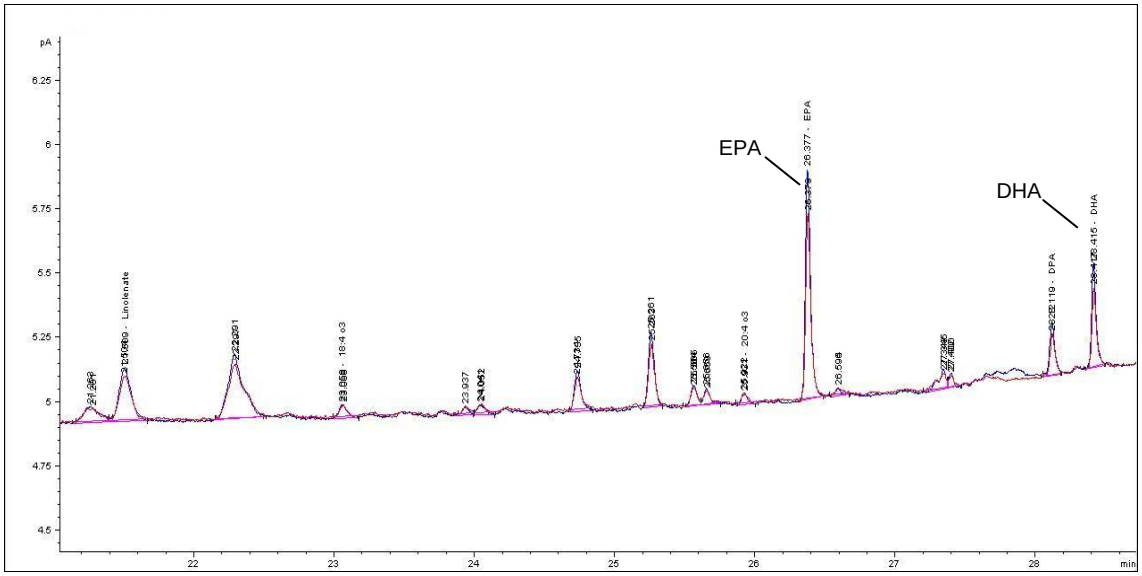



Figure 28: GC-FID chromatogram of UHT milk containing wowCAPS® after 3 months of storage

In Table 15 the results of the analysis of UHT milk enriched with wowCAPS® from an industrial trial are shown. Analysis was done initially, after 1 month and after 3 months of storage. The theoretical values are based on the Certificate of Analysis of the used formulation and the actual addition of this formulation. The high values of ALA might be influenced by the milk or other ingredients used.

Table 15: Fatty acid analysis of UHT milk enriched with wowCAPS® produced by customer

| **Omega-3 fatty acids** | **theory** | **initial** | **1 month** | **3 months** |
|---|---|---|---|---|
| | **mg/250 mL** | **mg/250 mL** | **mg/250 mL** | **mg/250 mL** |
| all cis-9,12,15-Octadecatrienoic acid ω3 (α-Linolenic acid) | 1.42 | 13.52 ±0.25 | 14.73 ±0.81 | 13.98 ±0.75 |
| all cis-6,9,12,15-Octadecatetraenoic acid ω3 | 2.11 | not detectable | not detectable | not detectable |
| all cis-8,11,14,17-Eicosatetraenoic acid ω3 | 1.12 | not detectable | not detectable | not detectable |
| all cis-5,8,11,14,17-Eicosapentaenoic acid ω3 (EPA) | 22.67 | 29.16 ±0.96 | 28.41 ±0.48 | 25.62 ±0.66 |
| all cis-7,10,13,16,19-Docosapentaenoic acid ω3 (DPA) | 3.72 | 5.48 ±0.14 | 6.56 ±0.40 | 5.68 ±0.36 |
| all cis-4,7,10,13,16,19-Docosahexaenoic acid ω3 (DHA) | 17.33 | 11.26 ±0.38 | 11.19 ±0.40 | 9.98 ±0.19 |
| TOTAL omega-3 | 48.37 | 59.42 | 60.89 | 55.26 |
| Deviation to the initial in % | - | - | -4.16 | -7.00 |
| EPA/DHA | 40.00 | 40.42 | 39.60 | 35.60 |
| Deviation to the initial in % | | - | -2.03 | -11.92 |

No significant difference is detected between the initial values and the values after 3 months storage.

CHANGES OF ω-3 FATTY ACIDS WITH TEMPERATURE

## 5.1.2 GC – MS

These analyses have been performed in order to confirm the molecular structure of the PUFAs and to assess if storage at elevated temperatures shows degradation products.

### 5.1.2.1 Standards of fatty acids

Figure 29 shows the GC-MS chromatogram of the analytical standard of methyl linolenate.

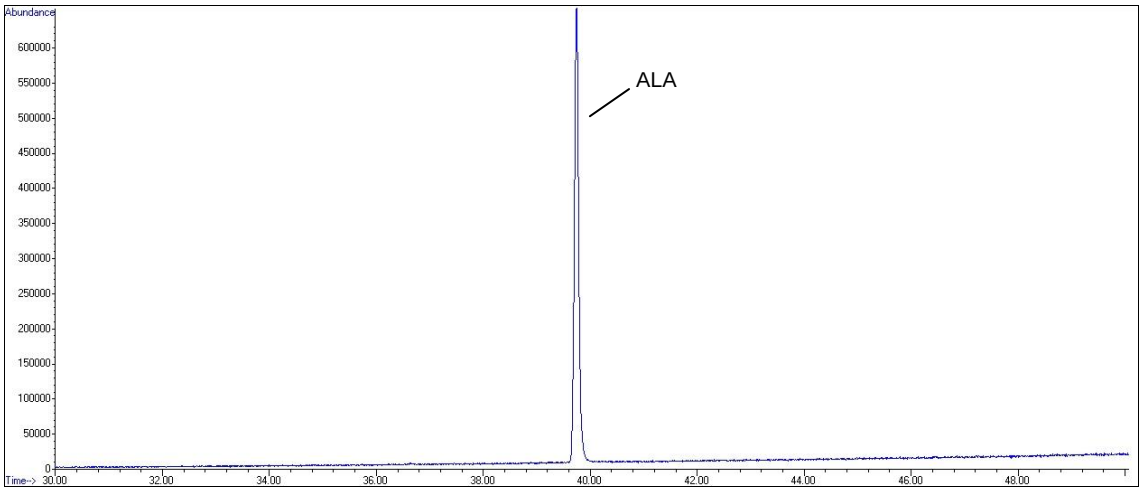



Figure 29: GC-MS chromatogram: methyl linolenate – methyl ester of linolenic acid, 99%

In Figure 30 the mass spectrum of methyl linolenate of the analytical standard and the database reference is shown.

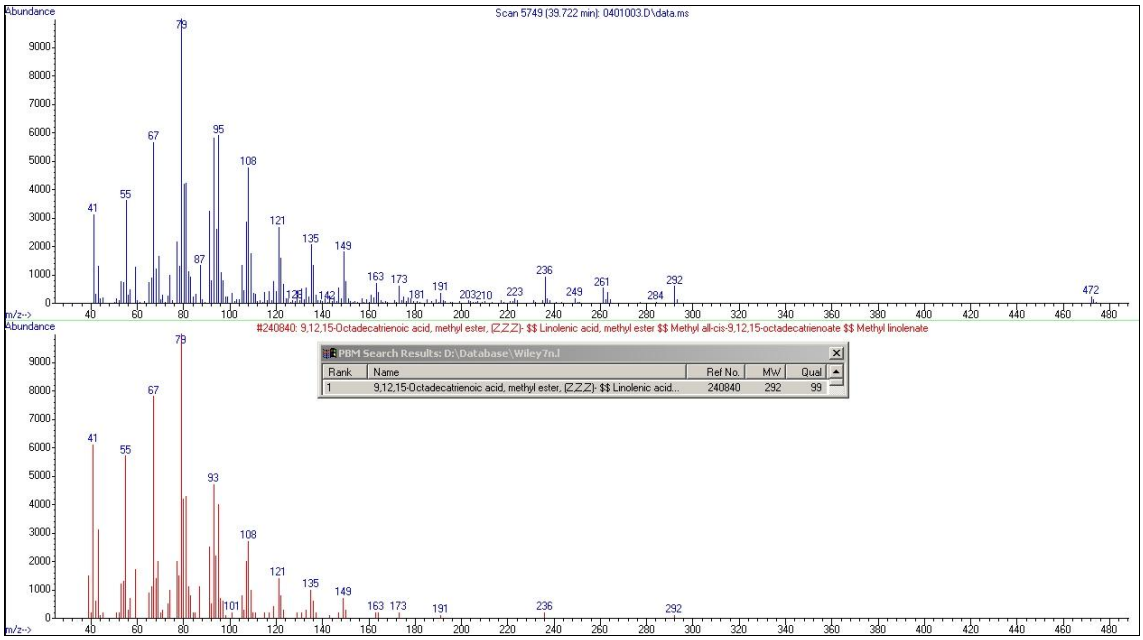



Figure 30: GC-MS spectrum: methyl linolenate – methyl ester of linolenic acid, 99%

Figure 31 shows the GC-MS chromatogram of methyl all-cis-5,8,11,14,17-Eicosapentaenoate.

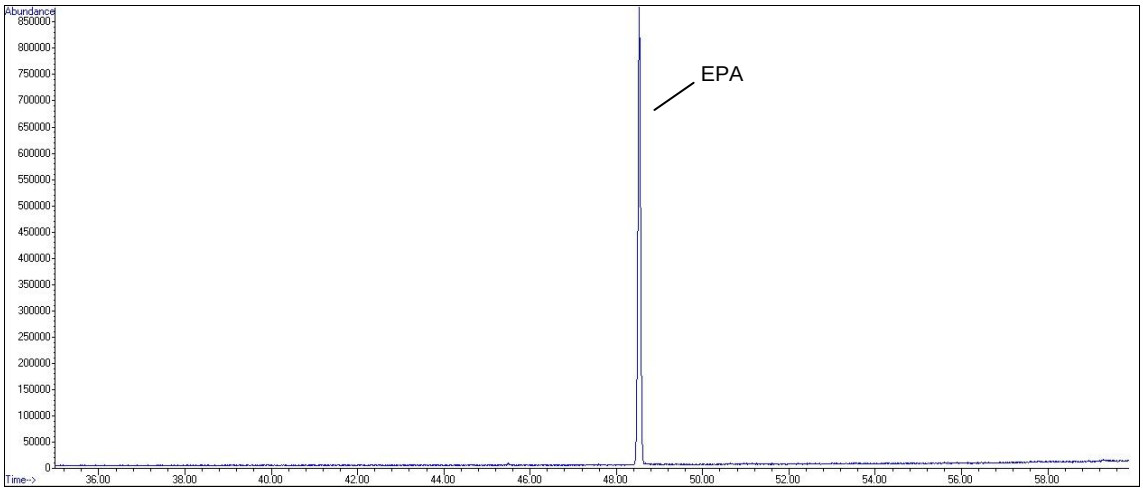



Figure 31: GC-MS chromatogram: methyl all-cis-5,8,11,14,17-Eicosapentaenoate

Figure 32 shows the mass spectrum of the injected analytical standard methyl all-*cis*-5,8,11,14,17-Eicosapentaenoate and the database reference.

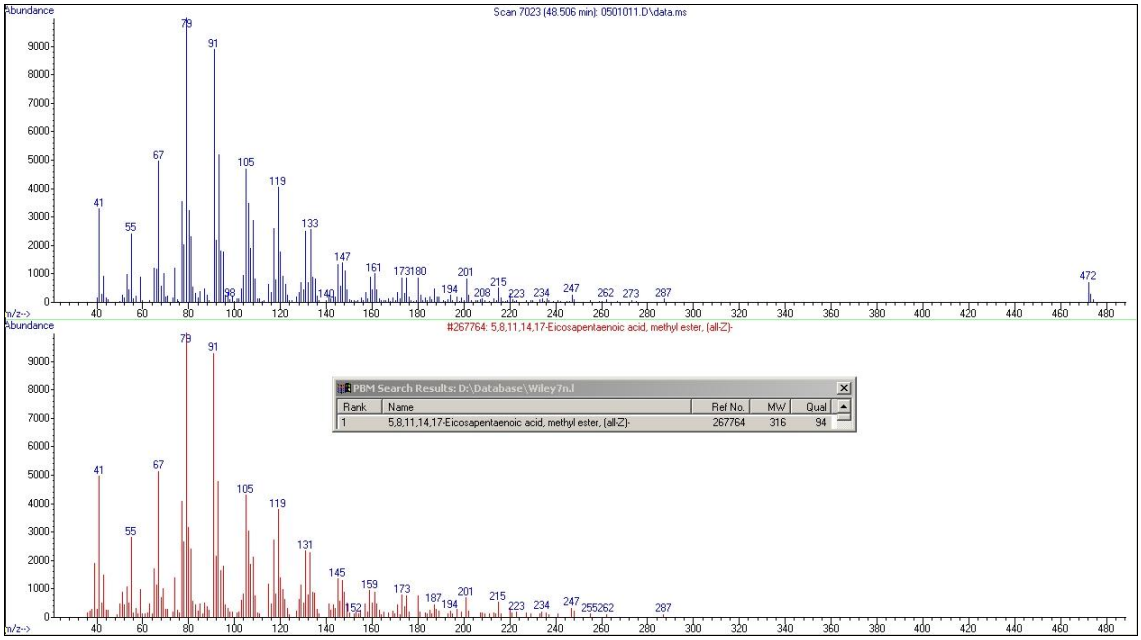



Figure 32: GC-MS spectrum: methyl all-cis-5,8,11,14,17-Eicosapentaenoate

Figure 33 shows the GC-MS chromatogram of *cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester.

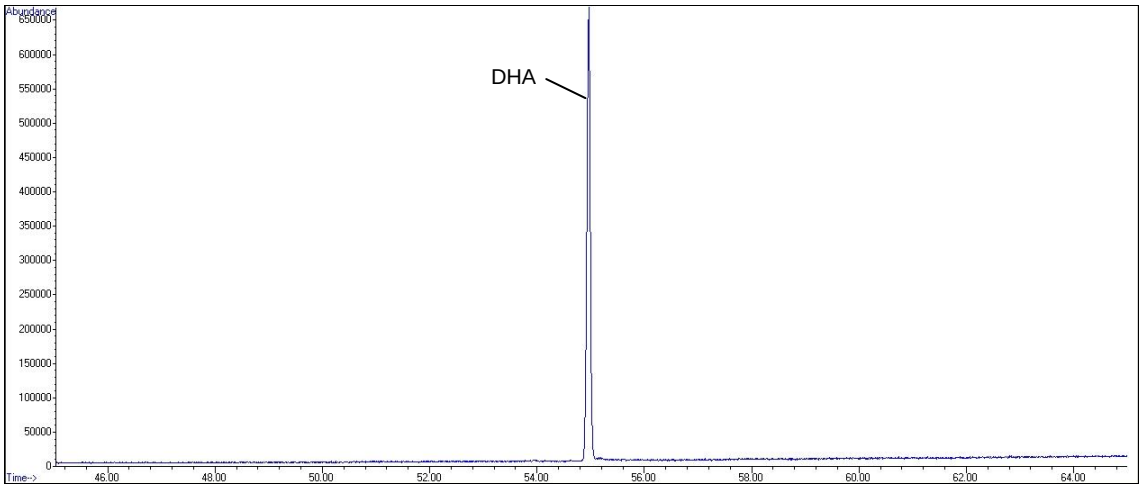



Figure 33: GC-MS chromatogram: cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester 98%

Figure 34 shows the mass spectrum of the injected analytical standard *cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester and the database reference.

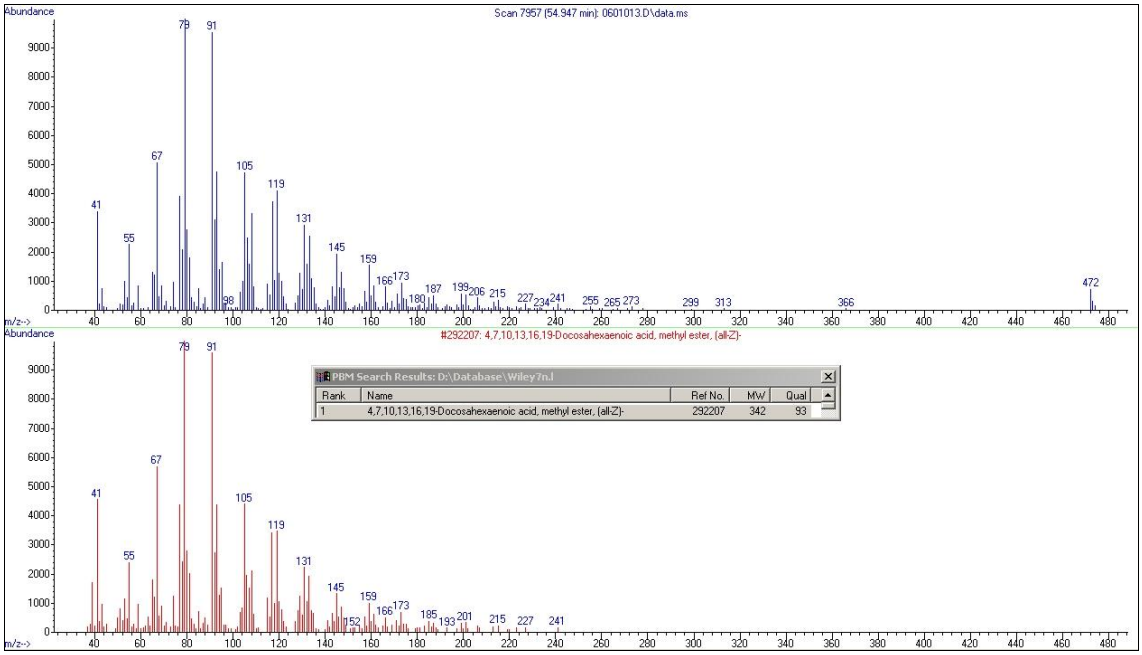



Figure 34: GC-MS spectrum: cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester 98%

Figure 35 shows the GC-MS chromatogram of the 37 components FAME Mix standard.

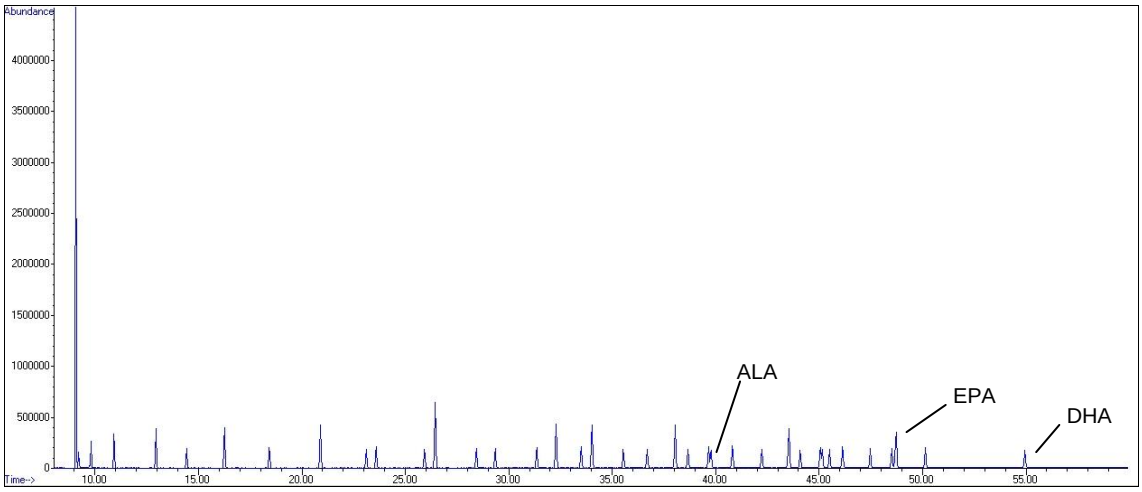



Figure 35: GC-MS chromatogram: 37 components FAME Mix 10 mg/mL in $CH_2Cl_2$

Figure 36 shows parts of the GC-MS chromatogram of the 37 components FAME Mix standard.

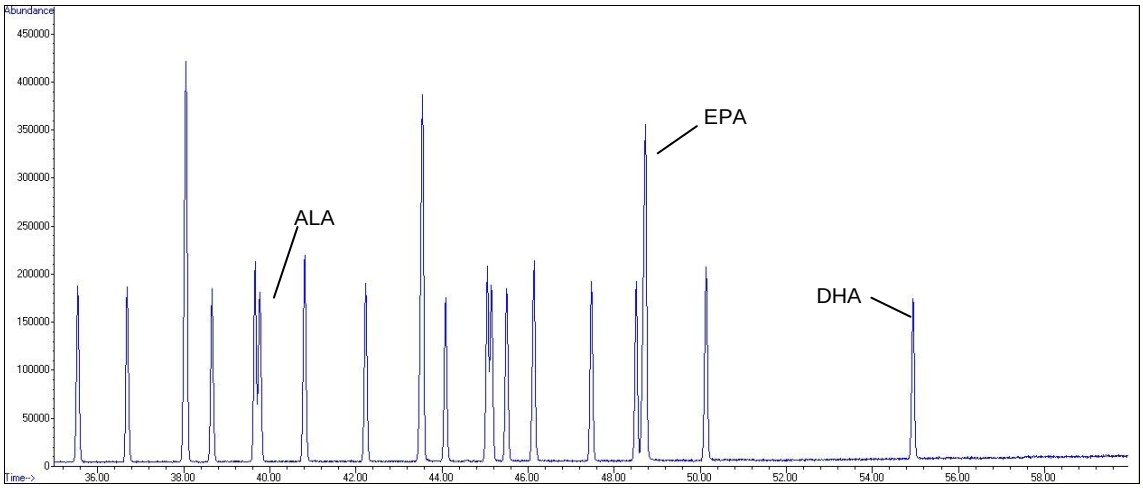



Figure 36: GC-MS chromatogram: 37 components FAME Mix 10 mg/mL in $CH_2Cl_2$ (outtake)

The comparison of the previously shown FA standards confirm the correct identification in the formulation wowCAPS® based on retention time and mass spectrum.

5.1.2.2 Formulation wowCAPS®

In Figure 37 the GC-MS chromatogram of the formulation wowCAPS® stored at 3-6 °C for 4 weeks is shown.

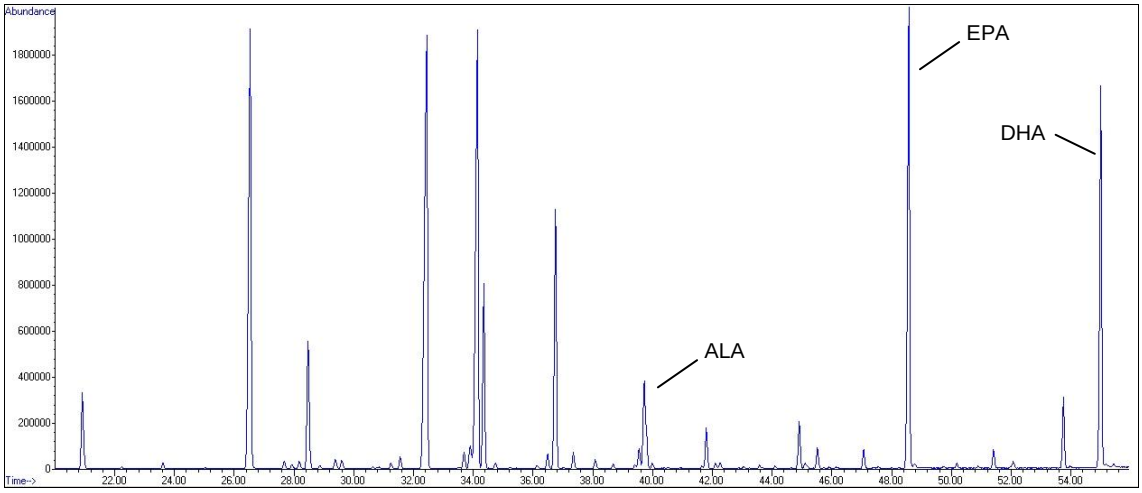



Figure 37: GC-MS chromatogram: wowCAPS® stored at 3-6 °C for 4 weeks

Figure 38 shows the GC-MS chromatogram of the formulation wowCAPS® stored at 30 °C for 4 weeks.

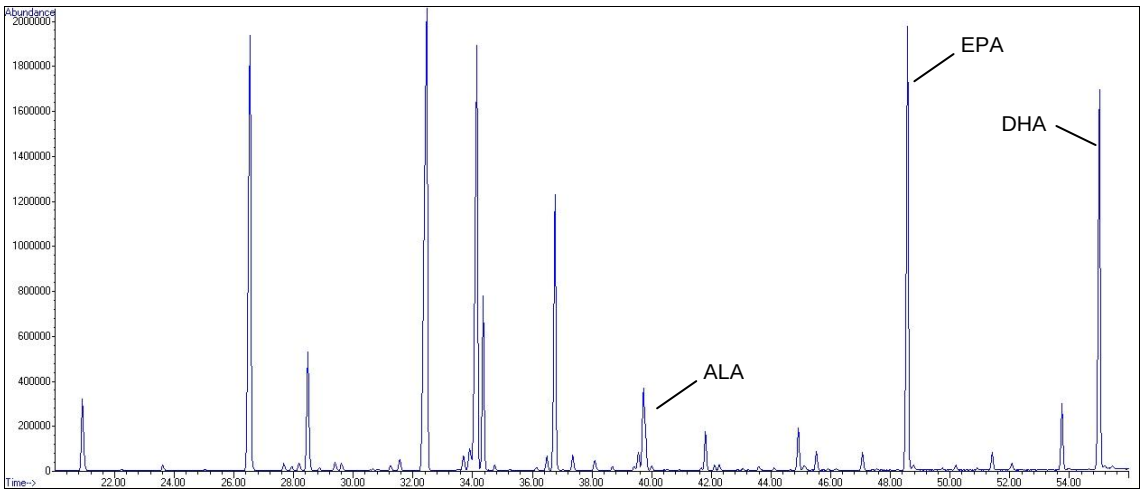



Figure 38: GC-MS chromatogram: wowCAPS® stored at 30 °C for 4 weeks

Figure 39 shows the GC-MS chromatogram of the formulation wowCAPS® stored at 70 °C for 4 weeks.

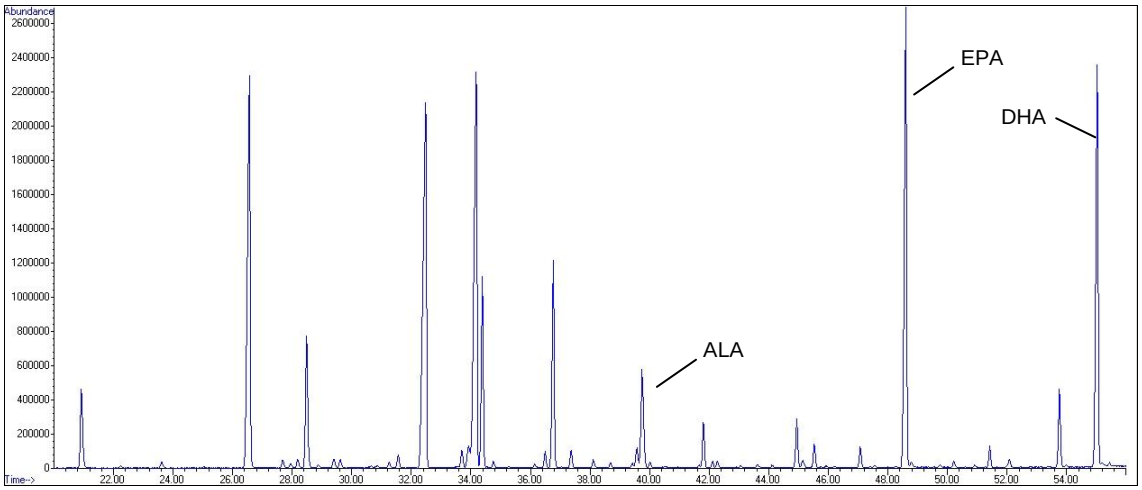



Figure 39: GC-MS chromatogram: wowCAPS® stored at 70 °C for 4 weeks

Figure 40 shows an overlay of the GC-MS chromatograms of the wowCAPS® stored at 3-6 °C, 30 °C and 70 °C for 4 weeks.

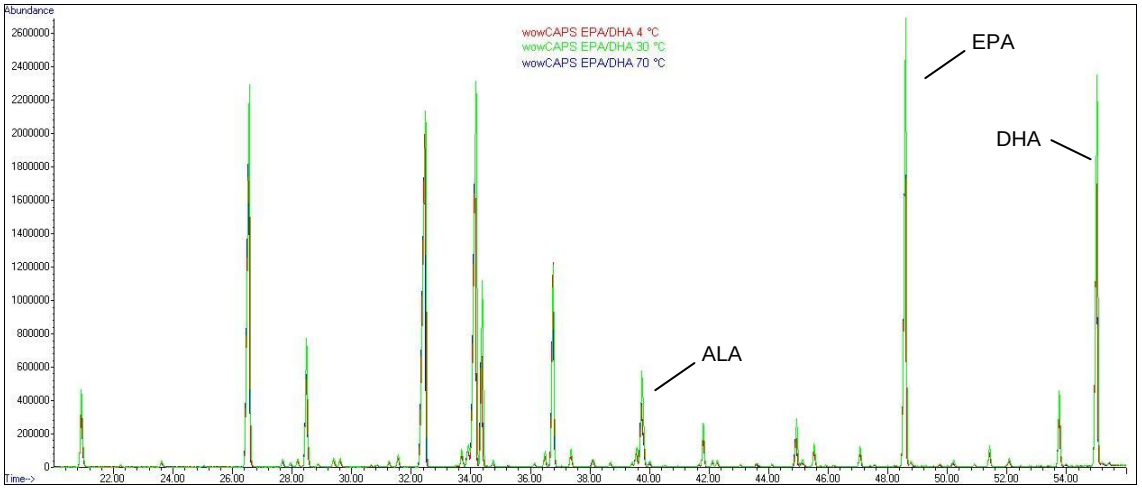



Figure 40: GC-MS chromatogram: comparison of wowCAPS® stored at 3-6 °C (red), 30 °C (green) and 70 °C (blue) for 4 weeks

The used method is valid for all samples because independently of the storage temperature the same peaks are identified.

It was decided to show the GC-MS spectra of the different fatty acids just for the standards and the fish oil as it is more clearly represented there.

5.1.2.3 Fish oil

Figure 41 shows the GC-MS chromatogram of the fish oil stored at 3-6 °C for 4 weeks.

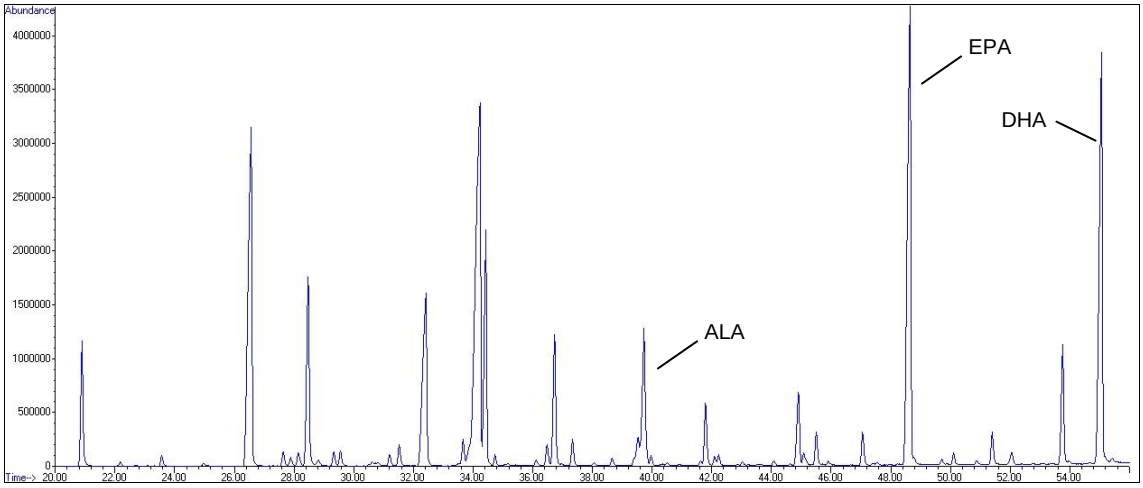



Figure 41: GC-MS chromatogram: fish oil stored at 3-6 °C for 4 weeks

Figure 42 shows the mass spectrum of EPA in the fish oil stored at 3-6 °C for 4 weeks.

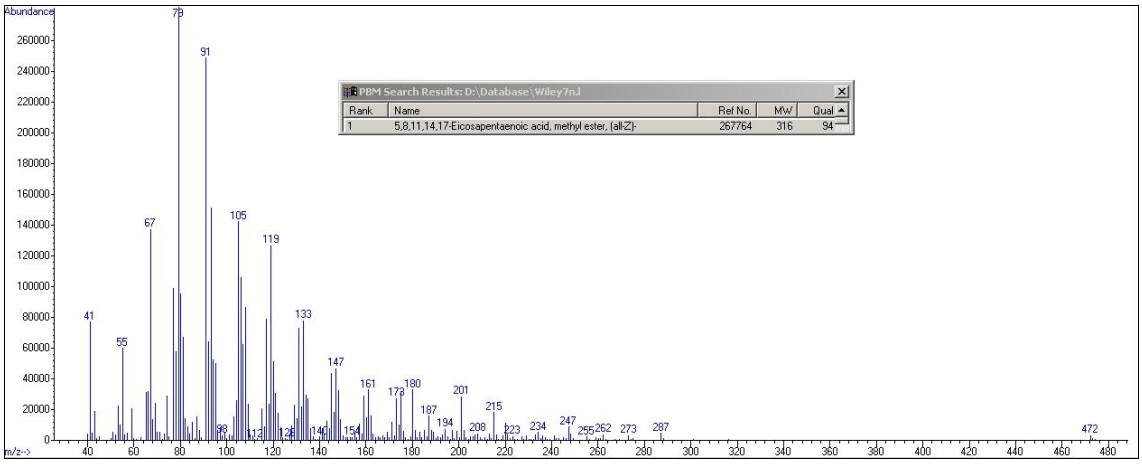



Figure 42: GC-MS spectrum EPA: fish oil stored at 3-6 °C for 4 weeks

Figure 43 shows the mass spectrum of DHA in the fish oil stored at 3-6 °C for 4 weeks.

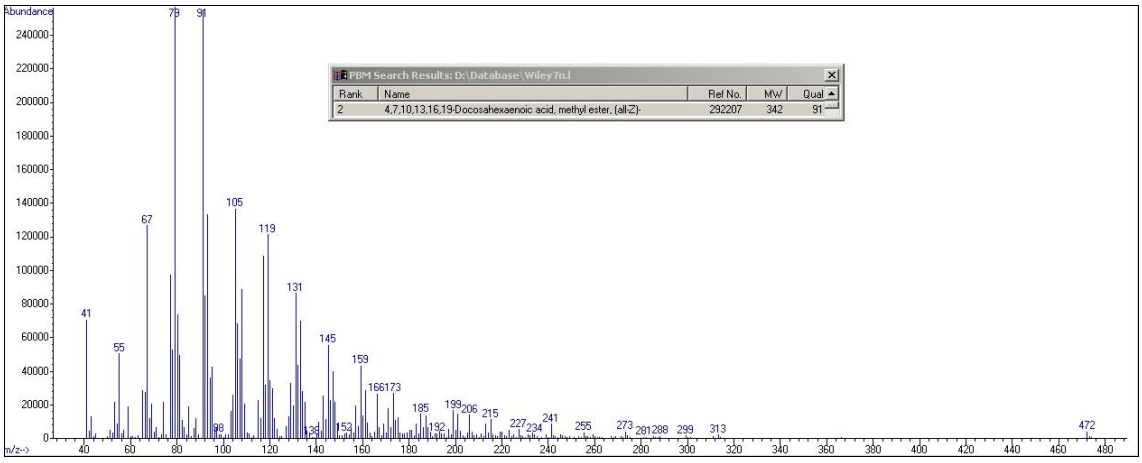



Figure 43: GC-MS spectrum DHA: fish oil stored at 3-6 °C for 4 weeks

Figure 44 shows the GC-MS chromatogram of the fish oil stored at 30 °C for 4 weeks.

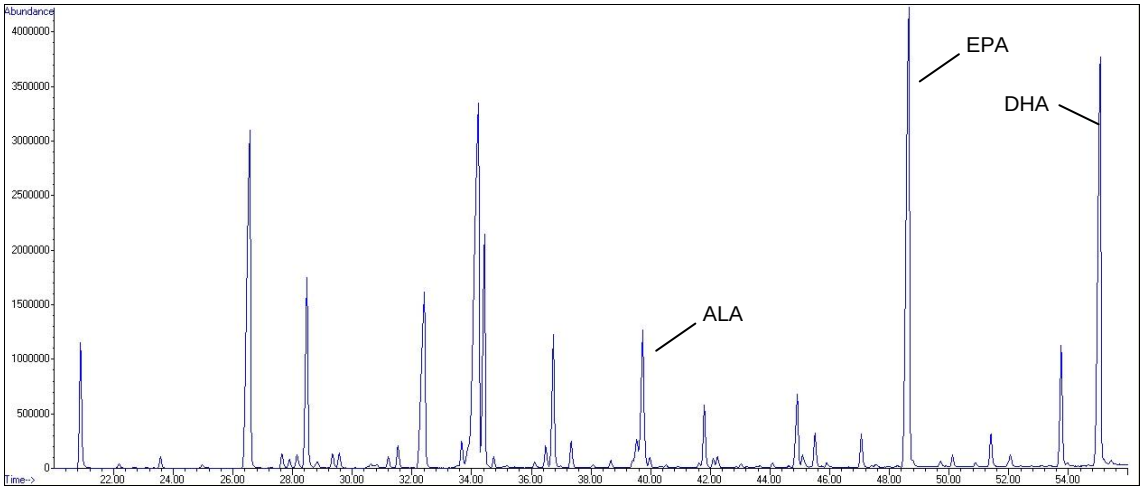



Figure 44: GC-MS chromatogram: fish oil stored at 30 °C for 4 weeks

Figure 45 shows the GC-MS chromatogram of the fish oil stored at 70 °C for 4 weeks.

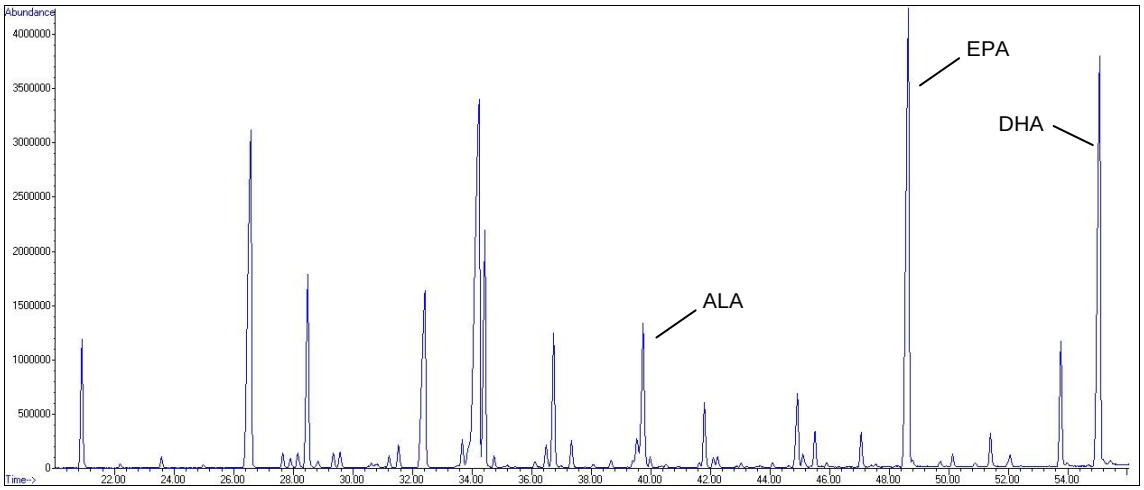



Figure 45: GC-MS chromatogram: fish oil stored at 70 °C for 4 weeks

Figure 46 shows the mass spectrum of EPA in the fish oil stored at 70 °C for 4 weeks.

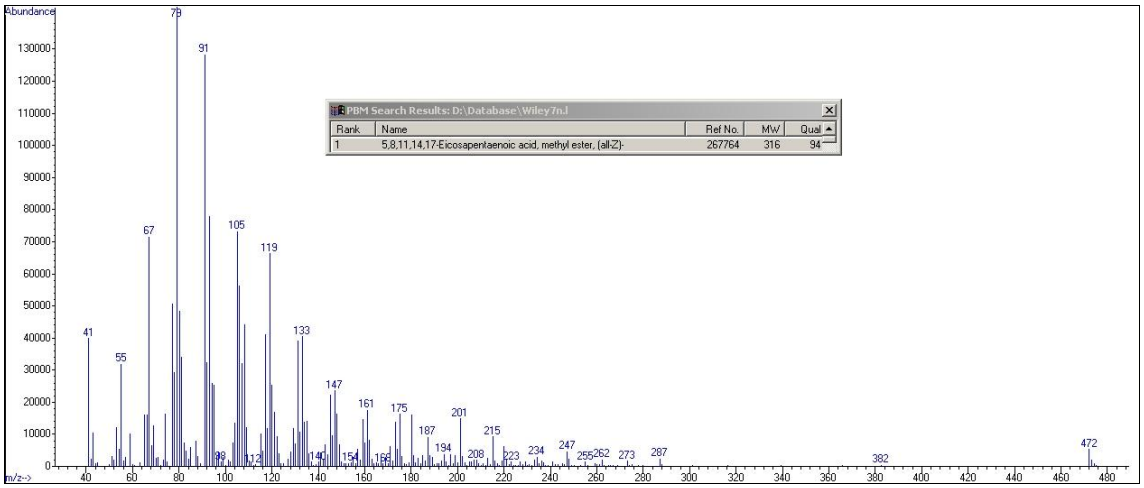



Figure 46: GC-MS spectrum EPA: fish oil stored at 70 °C

Figure 47 shows the mass spectrum of DHA in the fish oil stored at 70 °C for 4 weeks.

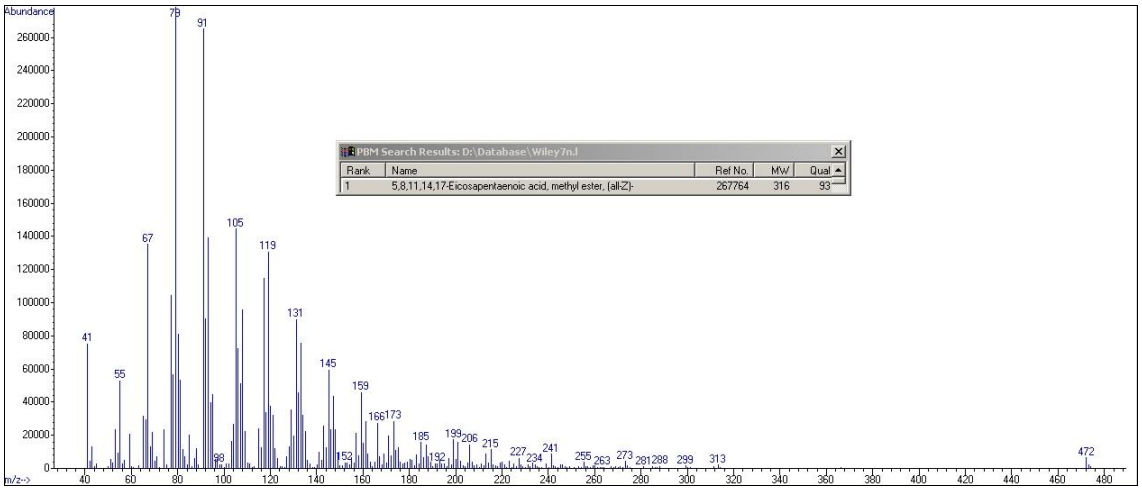



Figure 47: GC-MS spectrum DHA: fish oil stored at 70 °C

Figure 48 shows an overlay of the GC-MS chromatograms of the fish oil stored at 3-6 °C, 30 °C and 70 °C for 4 weeks.

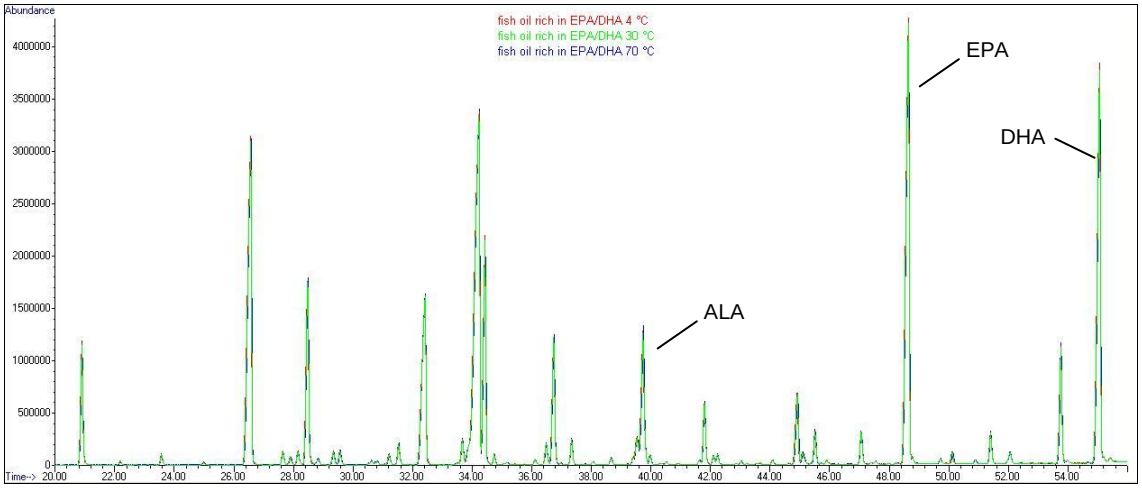



Figure 48: GC-MS chromatogram: comparison of fish oil stored at 3-6 °C (red), 30 °C (green) and 70 °C (blue) for 4 weeks

The used method is valid for all samples because independently of the storage temperature the same peaks are identified.

### 5.1.2.4 Yoghurt

Figure 49 shows the GC-MS chromatogram of a blank yoghurt sample.

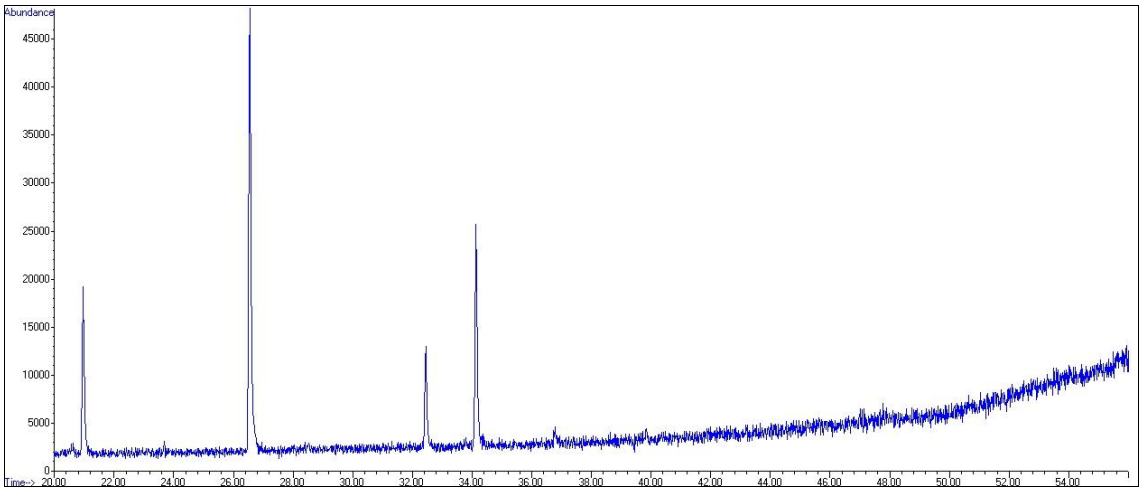



Figure 49: GC-MS chromatogram: yoghurt blank 3-6 °C

Figure 50 shows the GC-MS chromatogram of a yoghurt sample containing wowCAPS®. Only the peaks of ALA and EPA are visible since the random noise is too high to detect the peak of DHA.

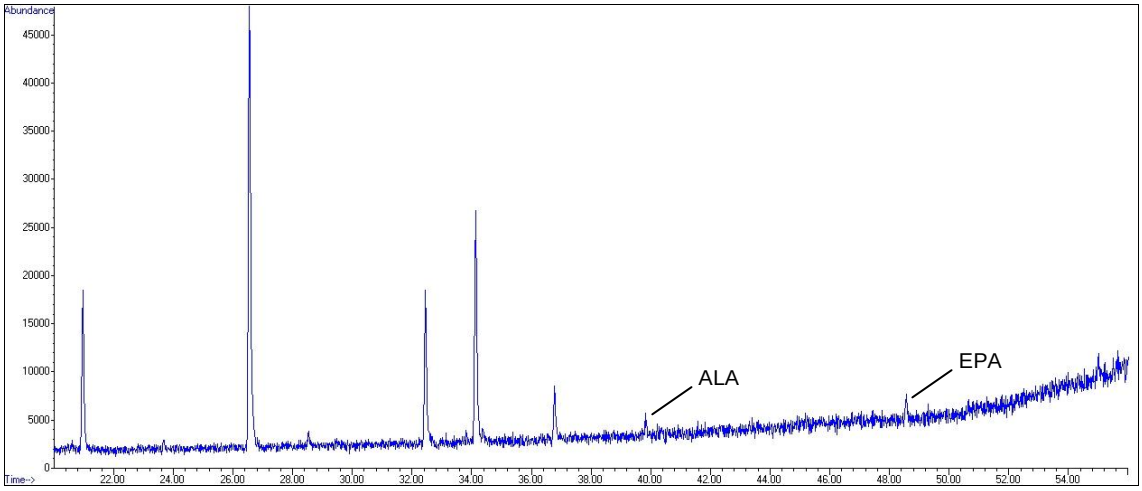



Figure 50: GC-MS chromatogram: yoghurt containing wowCAPS® 3-6 °C

Figure 51 shows the GC-MS chromatogram of a yoghurt sample fish oil. Only the peak of ALA is visible because since the random noise is too high to detect the peaks of EPA and DHA.

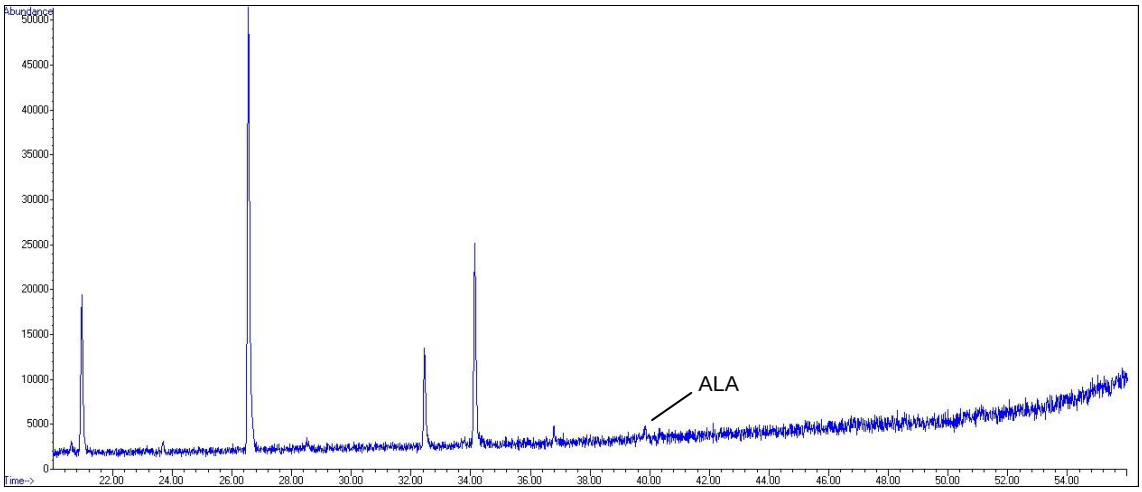



Figure 51: GC-MS chromatogram: yoghurt containing fish oil 3-6 °C

None of the used GC-MS analyses are able to reveal a significant decrease of any of the omega-3 FA due to increased storage temperature stress. Furthermore no decomposition products (e.g. aldehydes, ketones, etc.) could be detected in any sample.

### 5.1.3 HPLC – DAD

With a single wavelength display, or even with overlaid wavelengths display, it is very difficult to see differences among the tested samples. Therefore, various display options have been evaluated. The isoplot function shown below is not routinely used in the presentation of results from HPLC-DAD analysis, However, we have noted that is the best option to make differences visible. The reason is that the variations in absorption are difficult to be shown with individually selected wavelengths, since the baseline(s) are relatively high.

The parameters of the isoplot function are:
Fit: auto (higer intensity color: red; lower intensity color: blue)
Scale: log
Zero: 0.0 mAU

Note: The amount of chromatograms shown in this chapter has been limited for the sake of clarity. The tests have been performed at time zero, and for each sample (except UV irradiated) after 4 weeks at either 30 °C or 70 °C.

### 5.1.3.1 Formulation wowCAPS® at 30 °C and 70 °C

Figure 52 shows the chromatogram of the analysis of the formulation wowCAPS® stored at 30 °C for 4 weeks.

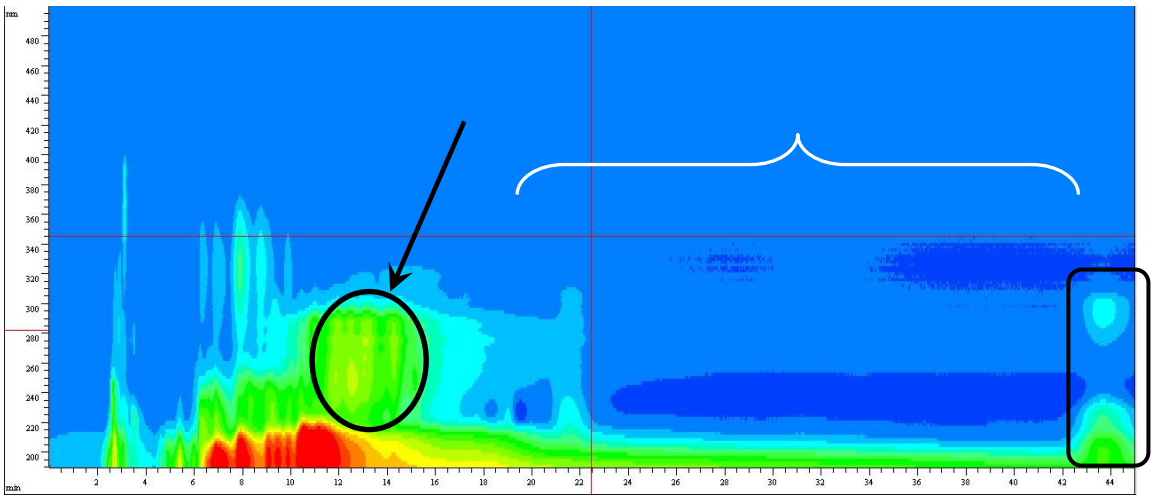


Figure 52: HPLC-DAD chromatogram (log): wowCAPS®, 4 weeks at 30 °C

Figure 53 shows the chromatogram of the analysis of the formulation wowCAPS® stored at 70 °C for 4 weeks.

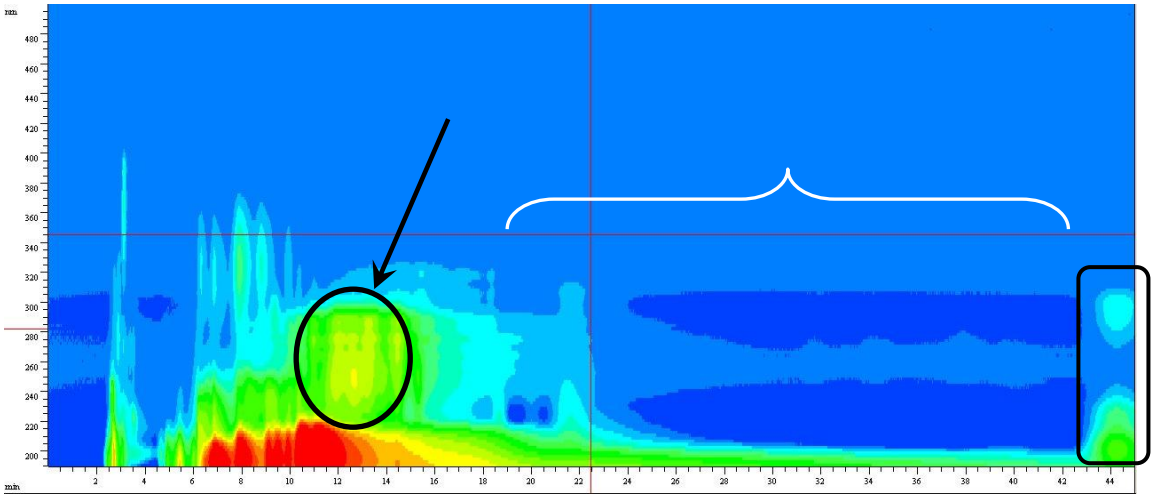


Figure 53: HPLC-DAD chromatogram (log): wowCAPS®, 4 weeks at 70 °C

The comparison of the plots shows a decrease in intensity of fatty acid signals from ca. min 24 to min 43 (under the white curly brace). This effect is

comparable in the experiments below with the fish oil whether treated with heat or UV irradiation to the extent that the higher the oxidation, the darker and broader is the blue area.

We can observe a slight increase in the area under the opened circle (pointed with the arrows). In the case of the wowCAPS, this increase between the 30 ºC and the 70 °C storage is mild but still appreciable.

The signals inside the black rectangle are specific for coformulants present in the wowCAPS® formulation and are to be disregarded.

5.1.3.2 Fish oil at different temperatures at 3-6 °C, 30 °C and 70 °C

Figure 54 show the chromatogram of the analysis of the fish oil stored at 3-6 °C for 4 weeks.

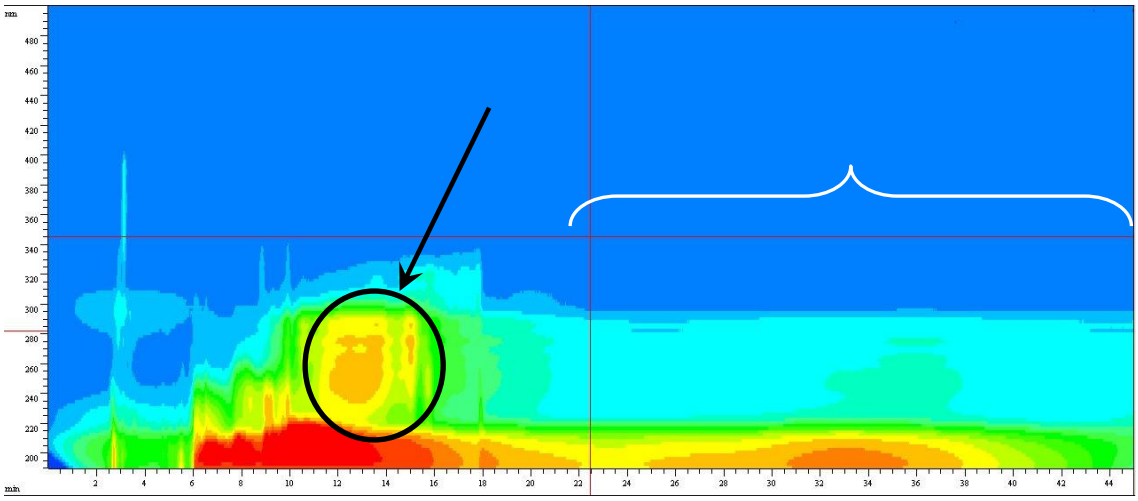


Figure 54: HPLC-DAD chromatogram (log): fish oil, 4 weeks at 3-6 °C

Figure 55 shows the chromatogram of the analysis of the fish oil stored at 30 °C for 4 weeks.

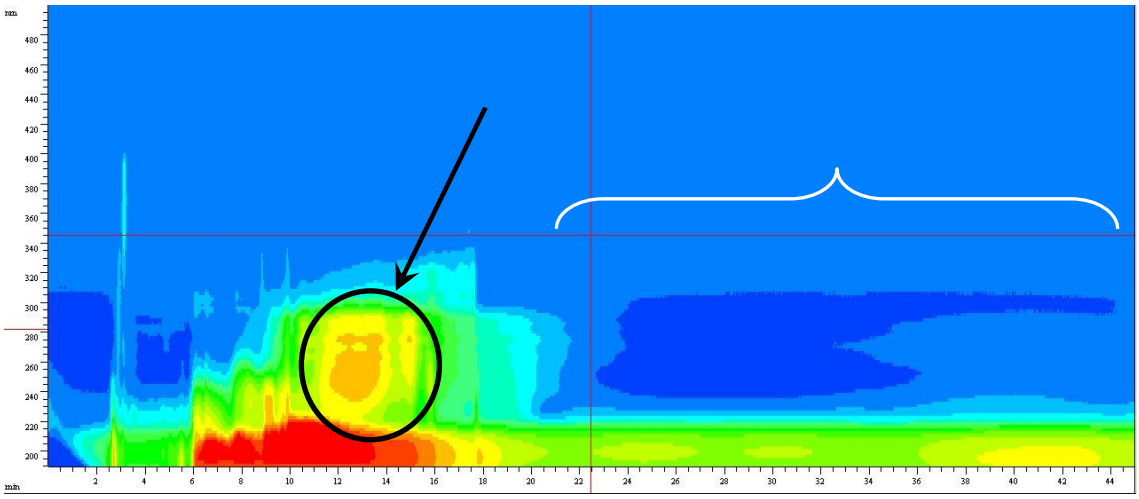


Figure 55: HPLC-DAD chromatogram (log): fish oil, 4 weeks at 30 °C

Figure 56 shows the chromatogram of the analysis of the fish oil stored at 70 °C for 4 weeks.

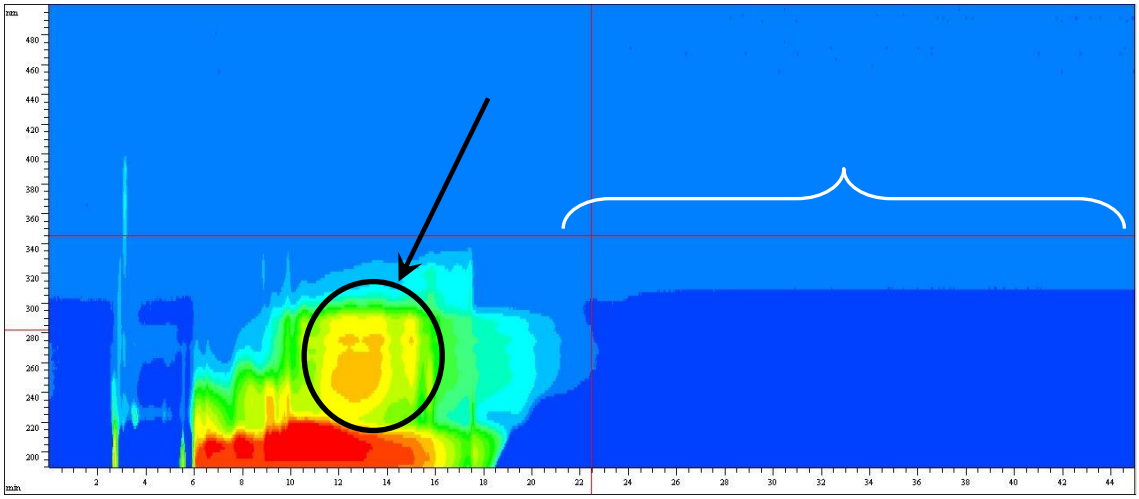


Figure 56: HPLC-DAD chromatogram (log): fish oil, 4 weeks at 70 °C

### 5.1.3.3 Fish oil irradiated with UV at 254 nm

Figure 57 shows the chromatogram of the analysis of the fish oil treated with UV at 254 nm for 5 hours.

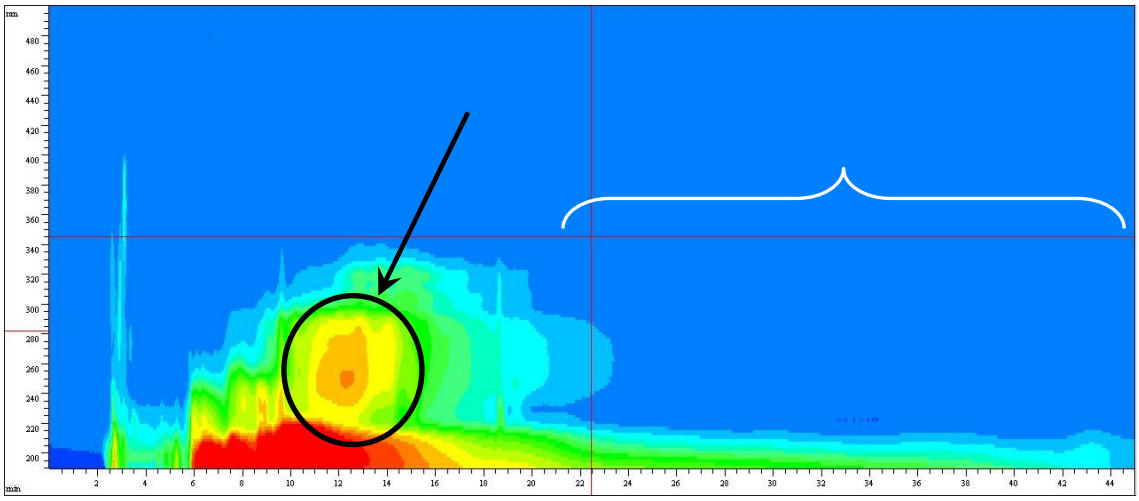


Figure 57: HPLC-DAD chromatogram (log): fish oil, UV 254 nm

Remarkably, the spot marked with a circle shows a deeper (towards the red end) color in the area that for the 3 experiment sets seems to indicate a marker for oxidation.

It seems that instead of showing a higher absorption at 234 nm (conjugated dienes) or at 268 nm (conjugated trienes), in these experiments, the increase of oxidation is better observed at the range 240 – 250 nm (with a maximum at 245 nm).

The reason behind this preferential wavelength when evaluating oxidized products remains unknown in this study. It is hypothesized that it must correspond to a (some) fatty acid(s) or fatty acid derivative(s) whose formation is independent from a plain free radical induced oxidation (UV irradiation) or a temperature induced oxidation. But it is to say that this hypothesis is very unlikely and needs to be proved.

### 5.1.4 Peroxide value

The determination of the peroxide value was carried out by the The Hungarian Dairy Research Institute (MTKI), Mosonmagyaróvár (Hungary) according the method ISO 3960:2007. The testing of the peroxide value was carried out in duplicate.

The self-made yoghurts were stored for 9 weeks at 3-6 °C and 30 °C and then sent for analysis to the external MTKI laboratory. The results are shown in Table 16.

Table 16: Peroxide values of self-made yoghurts after 1 month storage

| **Sample** | **storage** | **peroxide value [meq/kg] (average of 2 measurements)** |
|---|---|---|
| Yoghurt Blank | 3-6 °C | not detectable |
| Yoghurt Blank | 30 °C | not detectable |
| Yoghurt containing wowCAPS® | 3-6 °C | not detectable |
| Yoghurt containing wowCAPS® | 30 °C | not detectable |
| Yoghurt containing fish oil | 3-6 °C | not detectable |
| Yoghurt containing fish oil | 30 °C | 1.08 |

The results in Table 16 show that only in the sample with the plain oil, stored at 30 °C to simulate an accelerated storage, a peroxide value of 1.08 meq/kg could be detected.

The fish oil and the wowCAPS® were stored at 3-6 °C, 30 °C and 70 °C for one week to simulate an accelerated storage and then sent for analysis to the external MTKI laboratory. The results are shown in 17.

Table 17: Peroxide values of formulations and oils

| **Sample** | **storage** | **peroxide value [meq/kg] (average of 2 measurements)** |
|---|---|---|
| wowCAPS® | 3-6 °C | not detectable |
| wowCAPS® | 30 °C | not detectable |
| wowCAPS® | 70 °C | 0.65 |
| Fish oil | 3-6 °C | not detectable |
| Fish oil | 30 °C | not detectable |
| Fish oil | 70 °C | 3.99 |

The results in Table 17 show that in the fish oil sample stored at 70 °C a higher peroxide value is detected than in the sample of wowCAPS® stored at 70 °C. From this can be said that the formulation wowCAPS® is a protection for the fish oil against oxidation. The values were expected to be higher.

### 5.1.5 UV-VIS Spectrophotometric analysis

#### 5.1.5.1 Conjugated dienes

Storage of the yoghurt at 30 °C caused an increase of conjugated dienes (CD) compared with the yoghurt stored at 3-6 °C. The yoghurts were stored at these temperatures for 9 weeks.

This occurred for the yoghurt enriched with the formulation wowCAPS® as well as for the yoghurt enriched with fish oil. The conjugated dienes values are higher in both samples of the yoghurt enriched with oil than in the yoghurt containing formulation.

Table 18: Conjugated dienes [%] of yoghurts containing formulation wowCAPS® and fish oil

| **Sample** | **Average (n=4) [%]** | **Standard Deviation (n=4)** |
|---|---|---|
| Yogurt containing wowCAPS® 3-6°C | 0.202 | 0.140 |
| Yogurt containing wowCAPS® 30°C | 0.890 | 0.132 |
| Yogurt containing fish oil 3-6°C | 0.674 | 0.176 |
| Yogurt containing fish oil 30°C | 1.617 | 0.135 |

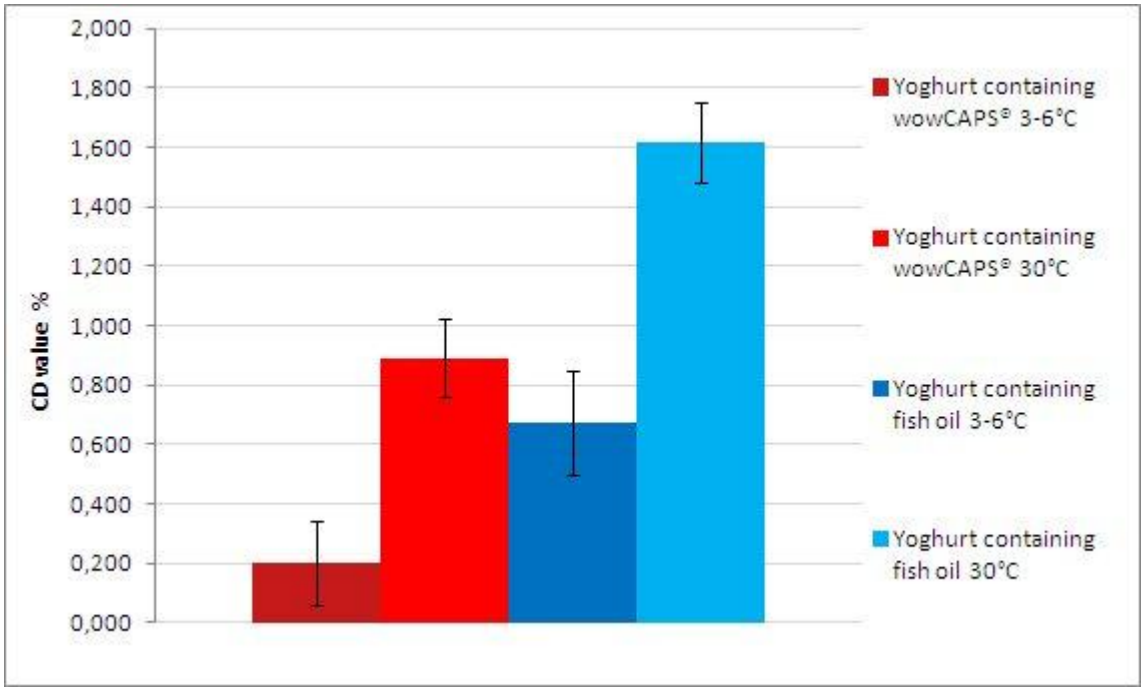



Figure 58: Conjugated diene values of yoghurts containing formulation wowCAPS® and fish oil

As seen in Figure 59 below the application of the oil into the yoghurt showed an high impact on conjugated dienes. Conjugated dienes in the yoghurt containing formulation wowCAPS® are significantly lower that in the yoghurt containing fish oil ($p < 0.05$).

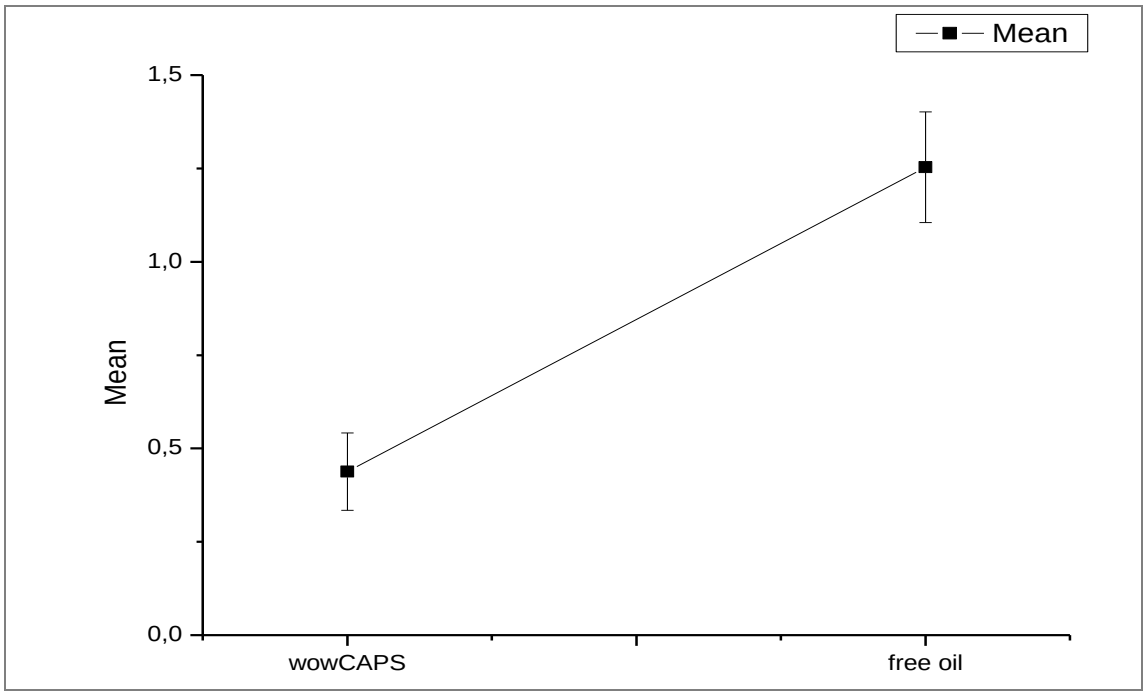



Figure 59: Means and standard errors of the conjugated dienes of the yogurt containing formulation wowCAPS® and the fish oil according the application form

In addition to the application form CD values increased with temperature ($p < 0.05$; Figure 60).

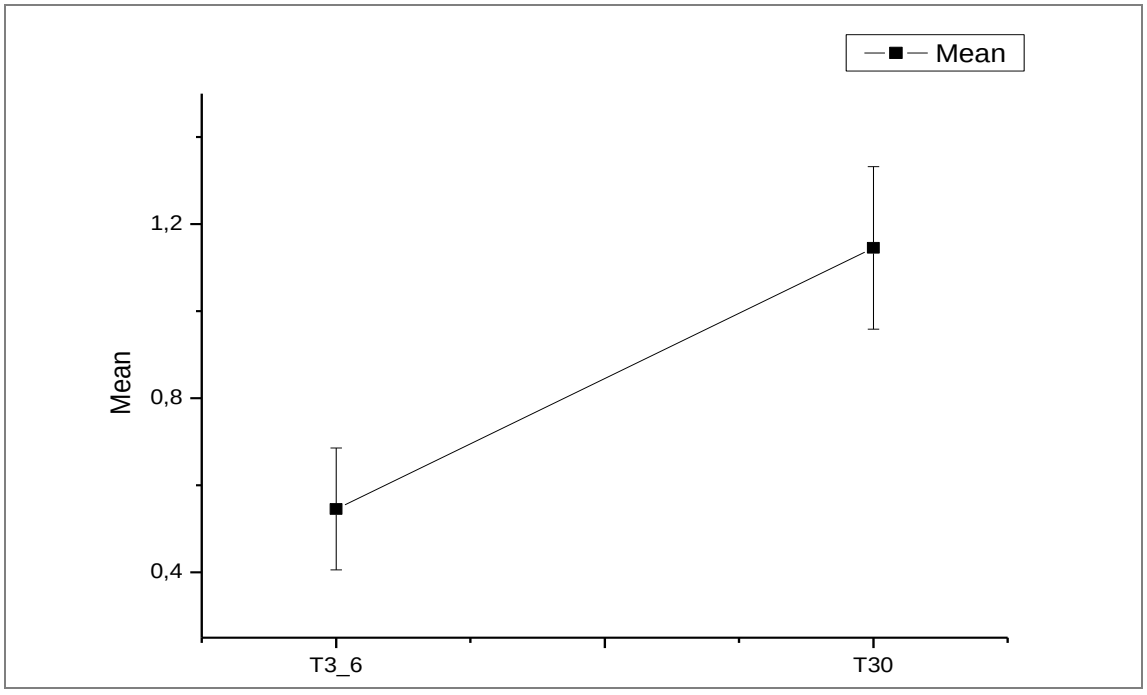



Figure 60: Means and standard errors of the conjugated dienes of the yoghurt containing formulation wowCAPS® and the fish oil analyzed according the treatment

The summary of CDs according to temperatureand UV treatment are shown in Table 19.

Table 19: Conjugated dienes [%] of the formulation wowCAPS® and the fish oil stored at different conditions

| Sample | Average (n=4) [%] | Standard Deviation (n=4) |
|---|---|---|
| wowCAPS® 3-6°C | 0.271 | 0.136 |
| wowCAPS® 30°C | 0.576 | 0.119 |
| wowCAPS® 70°C | 0.700 | 0.114 |
| wowCAPS® UV 254 nm | 0.531 | 0.092 |
| Fish oil 3-6°C | 0.499 | 0.086 |
| Fish oil 30° | 0.618 | 0.080 |
| Fish oil 70°C | 1.781 | 0.064 |
| Fish oil UV 254 nm | 0.634 | 0.082 |

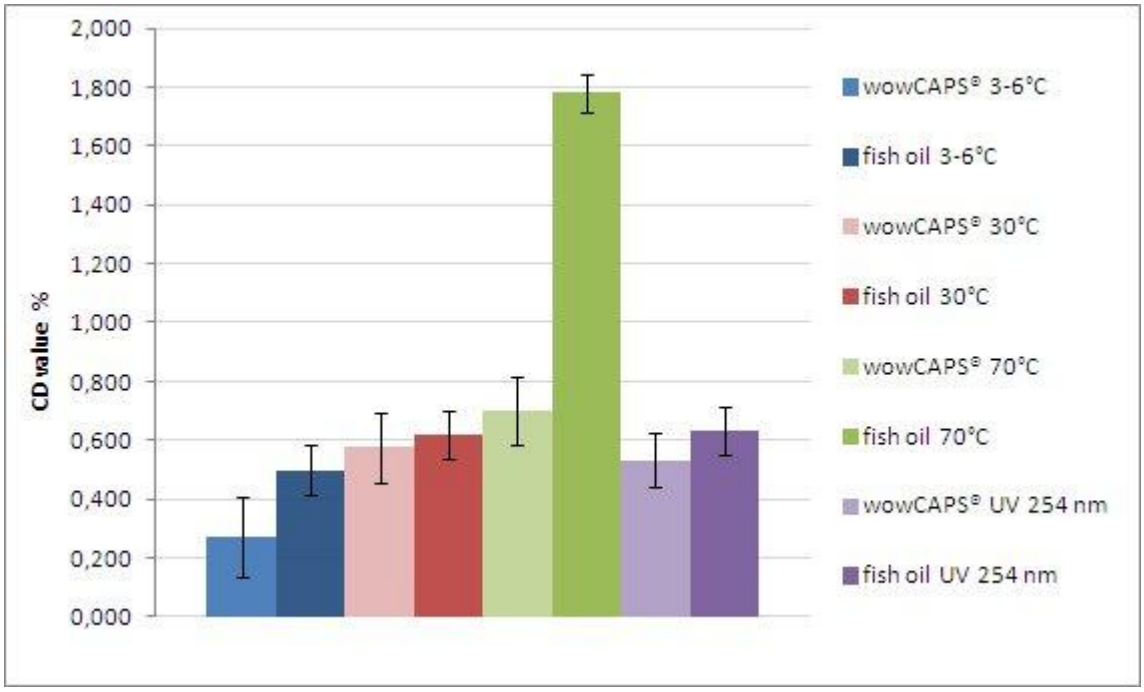



Figure 61: Conjugated dienes of the formulation wowCAPS® and the fish oil stored at different conditions

These trials showed an increase of CD in the oils and formulations stored at 30 °C and 70 °C and the oil and formulation treated with ultraviolett light at 254 nm wavelenght compared to the samples stored at 3-6 °C.

As seen in Figure 62 CD values in fish oil are higher than in the wowCAPS® ($p < 0.05$).

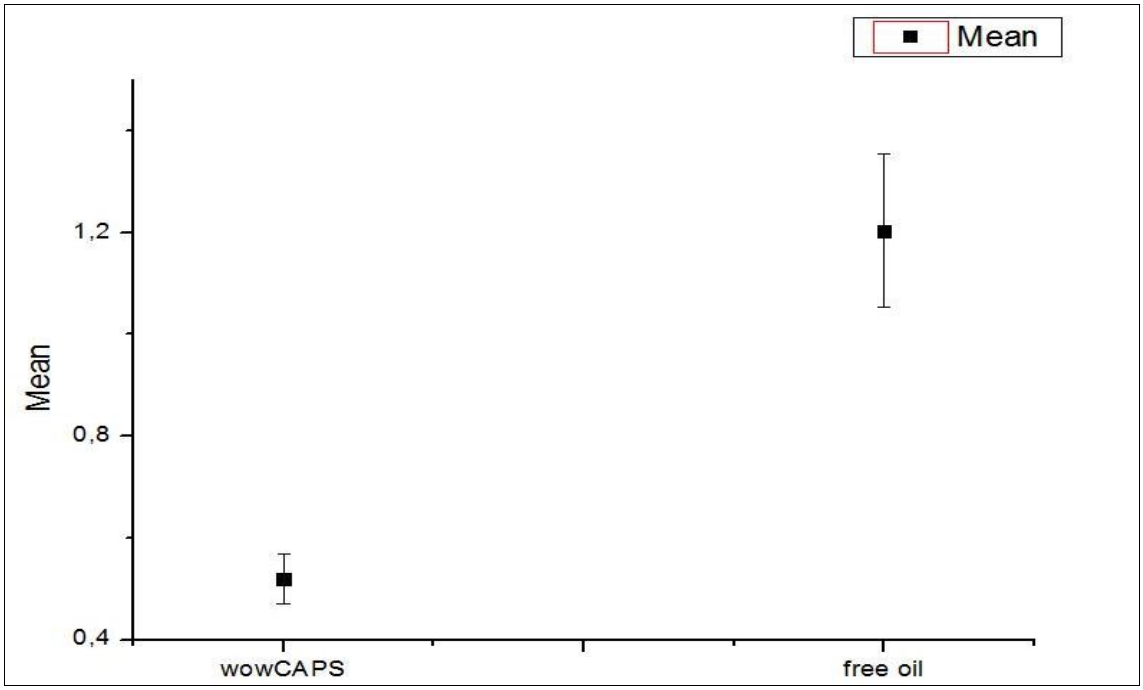



Figure 62: Means and standard errors of the conjugated dienes of the formulation wowCAPS® and the fish oil

As seen in Figure 63, CD values increased with temperature and are higher in the fish oil than in the wowCAPS® ($p < 0.05$). No significant difference was observed between the wowCAPS® and the fish oil after UV treatment at 254 nm (Figure 64).

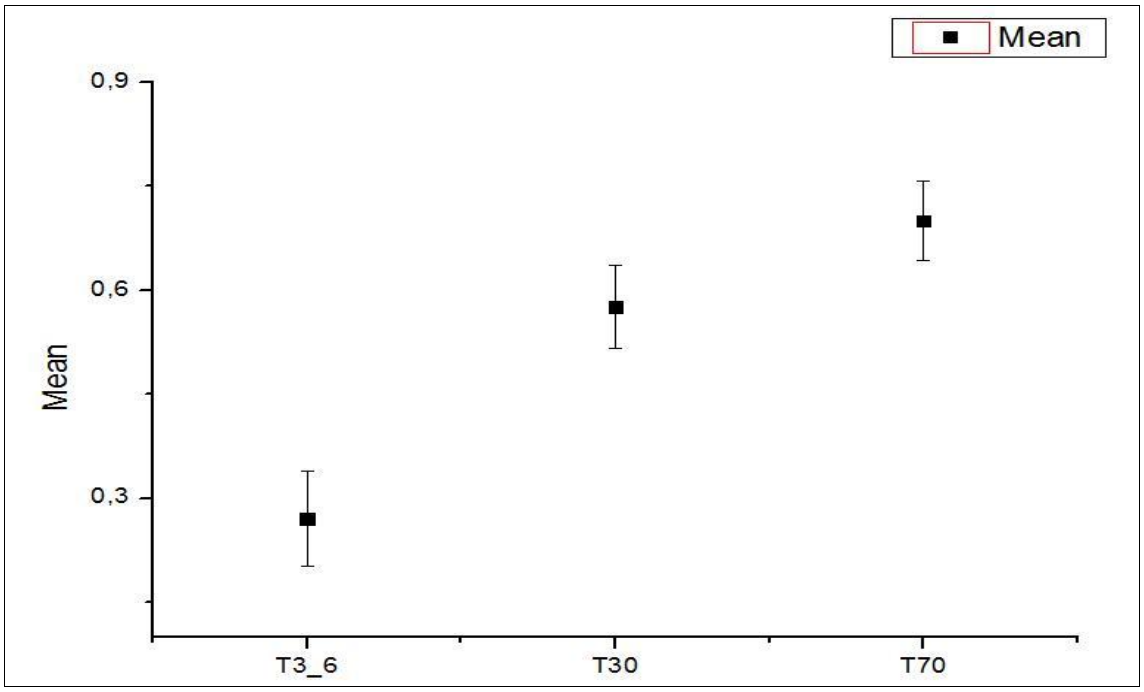



Figure 63: Means and standard errors of the conjugated dienes of the formulation wowCAPS® and the fish oil analyzed according to the storage temperature (T)

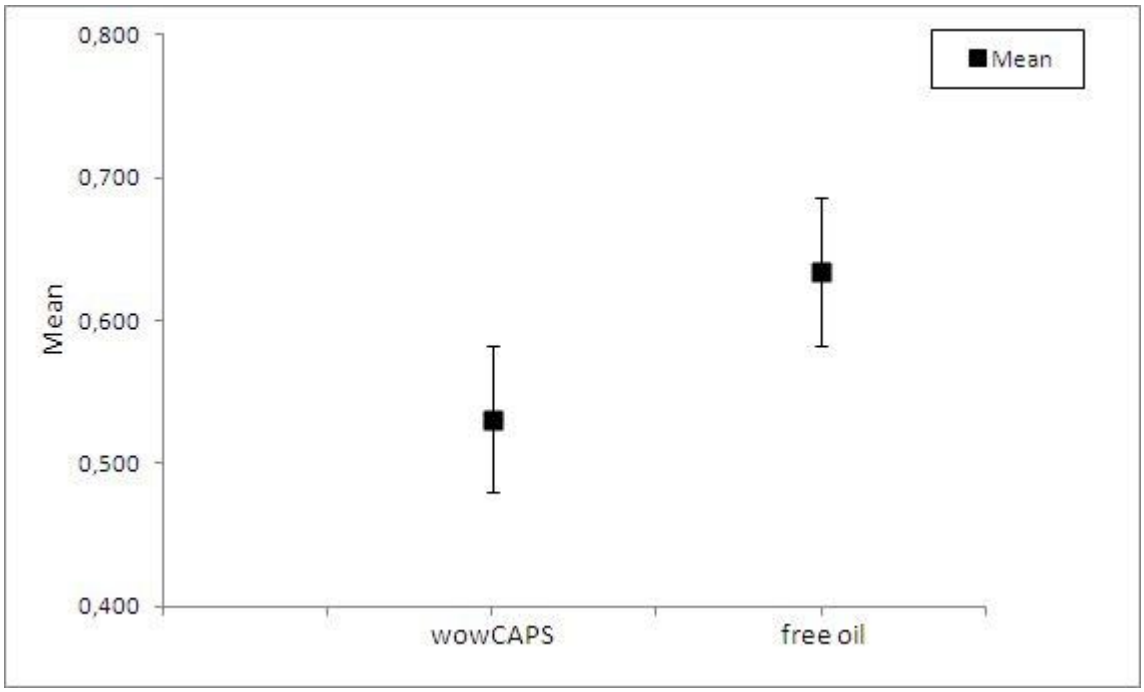



Figure 64: Means and standard errors of the conjugated dienes of the formulation wowCAPS® and the fish oil analyzed according UV irradiation

The formulation has a protective effect against oxidation for the fish oil.

### 5.1.5.2 Conjugated trienes

Storage of the yoghurt at 30 °C caused an increase in conjugated trienes (CT) compared with the yoghurt stored at 3-6 °C. The yoghurts were stored at these temperatures for 9 weeks.

This was detected in the yoghurt enriched with the formulation wowCAPS® as well as in the yoghurt enriched with the fish oil. The CTs are higher in both samples of the yoghurt enriched with oil than in the yoghurt containing the formulation.

Table 20: Conjugated trienes [%] of yoghurts containing formulation wowCAPS® and fish oil

| **Sample** | **Average (n=4) [%]** | **Standard Deviation (n=4)** |
|---|---|---|
| Yogurt containing wowCAPS® 3-6°C | 0.001 | 0.001 |
| Yogurt containing wowCAPS® 30°C | 0.122 | 0.100 |
| Yogurt containing fish oil 3-6°C | 0.163 | 0.092 |
| Yogurt containing fish oil 30°C | 0.329 | 0.108 |

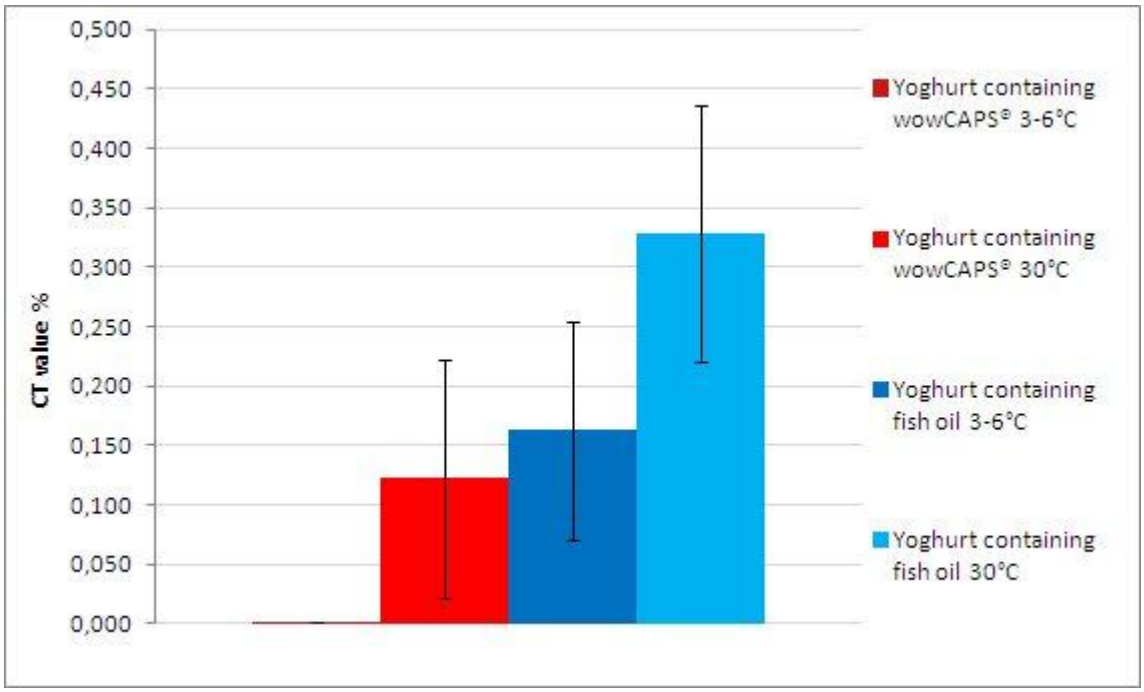



Figure 65: Conjugated triene values of yoghurts containing formulation wowCAPS® and fish oil

As seen in Figure 66 below the application of the oil into the yoghurt showed an high impact on conjugated trienes. Conjugated trienes in the yoghurt containing formulation wowCAPS® are significantly lower that in the yoghurt containing fish oil ($p < 0.05$).

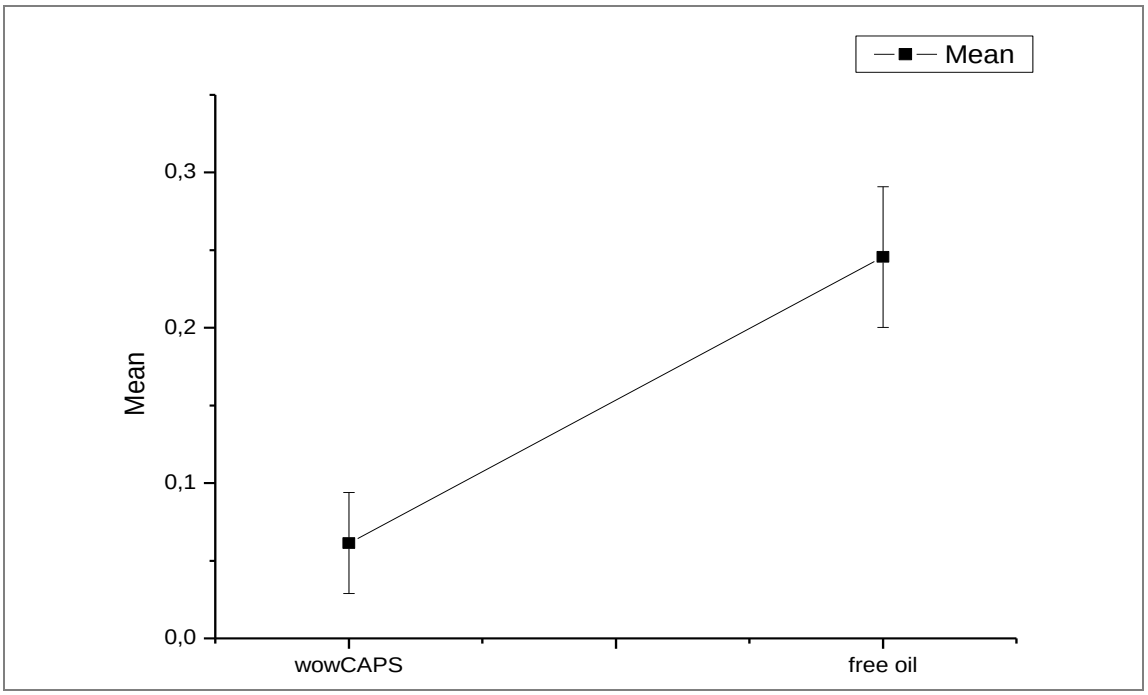



Figure 66: Means and standard errors of the conjugated trienes of the yogurt containing formulation wowCAPS® and the fish oil

In addition to the application form CT values increased with temperature ($p < 0.05$; Figure 67).

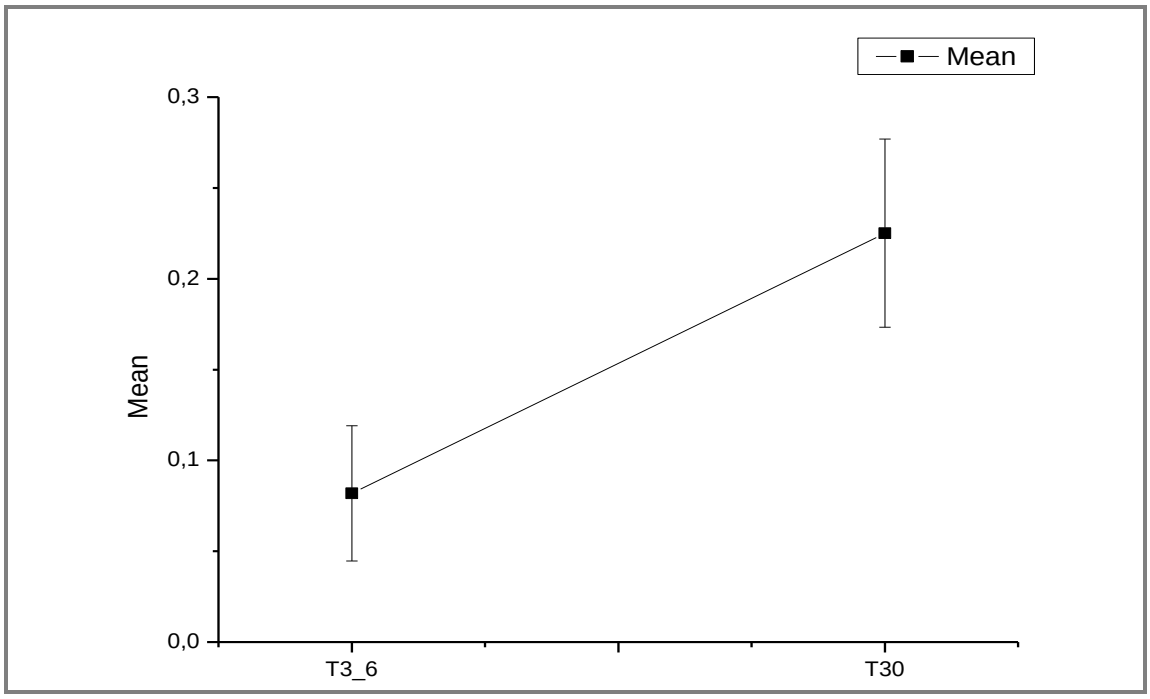



Figure 67: Means and standard errors of the conjugated trienes of the yoghurt containing formulation wowCAPS® and the fish oil analyzed according to the storage temperature

The summary of CTs according to temperature and UV treatment are shown in Table 21.

Table 21: Conjugated trienes [%] of the formulation wowCAPS® and the fish oil stored at different conditions

| **Sample** | **Average (n=4) [%]** | **Standard Deviation (n=4)** |
|---|---|---|
| wowCAPS® 3-6°C | 0.050 | 0.077 |
| wowCAPS® 30°C | 0.176 | 0.108 |
| wowCAPS® 70°C | 0.292 | 0.095 |
| wowCAPS® UV 254 nm | 0.190 | 0.102 |
| Fish oil 3-6°C | 0.349 | 0.094 |
| Fish oil 30° | 0.813 | 0.082 |
| Fish oil 70°C | 1.240 | 0.071 |
| Fish oil UV 254 nm | 0.003 | 0.001 |

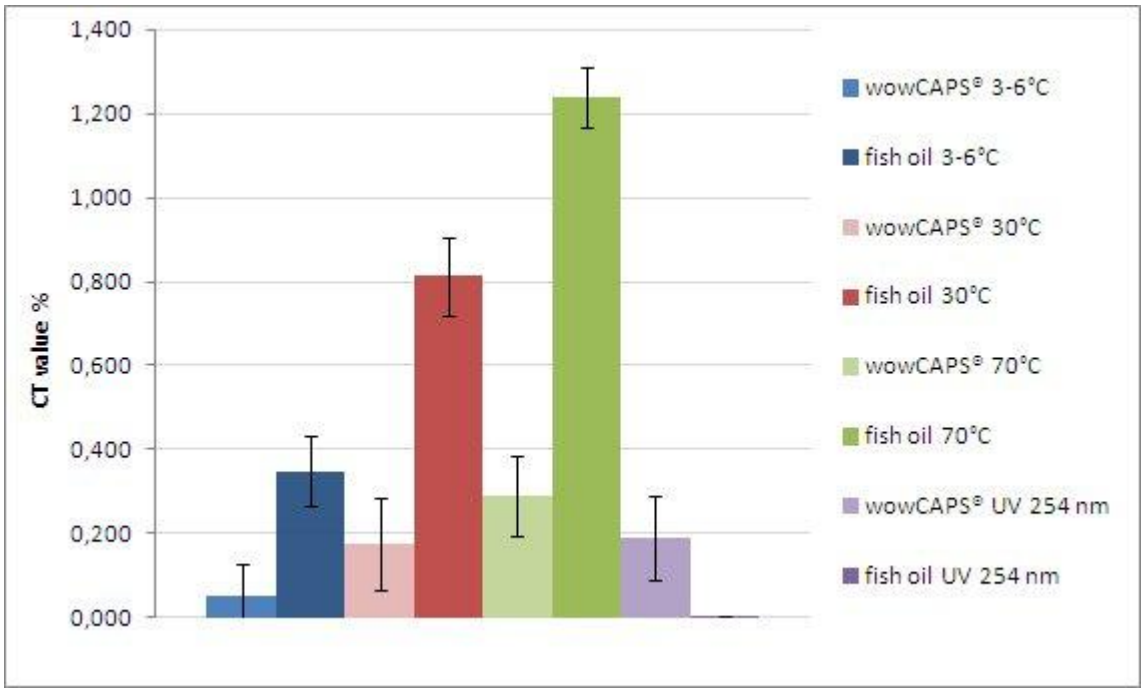



Figure 68: Conjugated trienes of the formulation wowCAPS® and the fish oil stored at different conditions

These trials showed an increase in CTs in the oils and formulations stored at 30 °C but particularly at 70 °C and the oil and formulation treated with ultraviolett light at 254 nm wavelenght compared with the samples stored at 3-6 °C.

As seen in Figure 69 below the results considering the dosage form showed an high impact of the dosage form on the degree of CTs. One-Way ANOVA analysis showed a significant difference ($p < 0.05$) between conjugated trienes of wowCAPS® and fish oil.

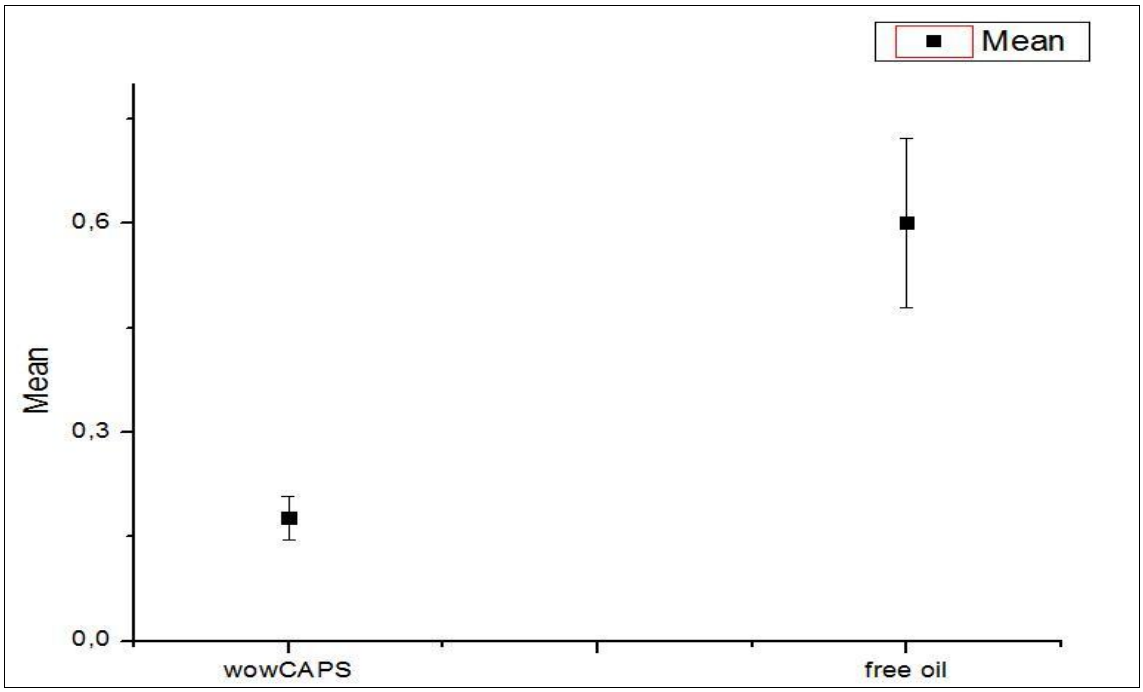



Figure 69: Means and standard errors of the conjugated trienes of the formulation wowCAPS® and the fish oil according the dosage form

As seen in Figure 70, CD values increased with temperature and are higher in the fish oil than in the wowCAPS® ($p < 0.05$).

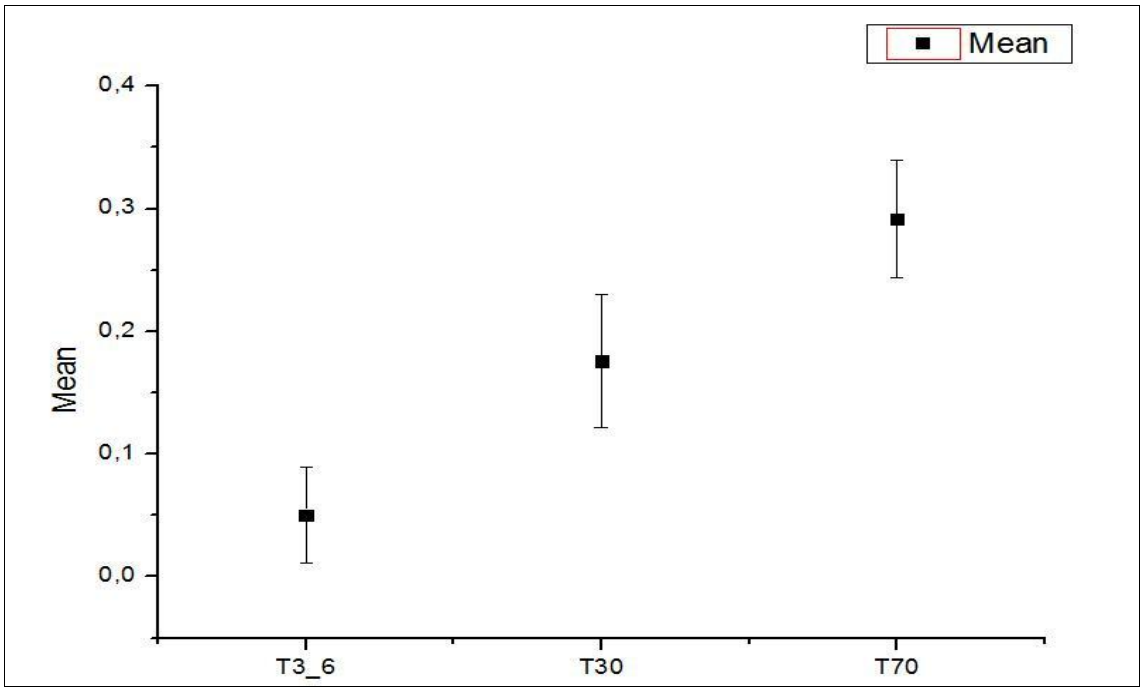



Figure 70: Means and standard errors of the conjugated trienes of the formulation wowCAPS® and the fish oil according to the storage temperature (T)

No significant difference was observed between the wowCAPS® and the fish oil after UV treatment at 254 nm (Figure 71).

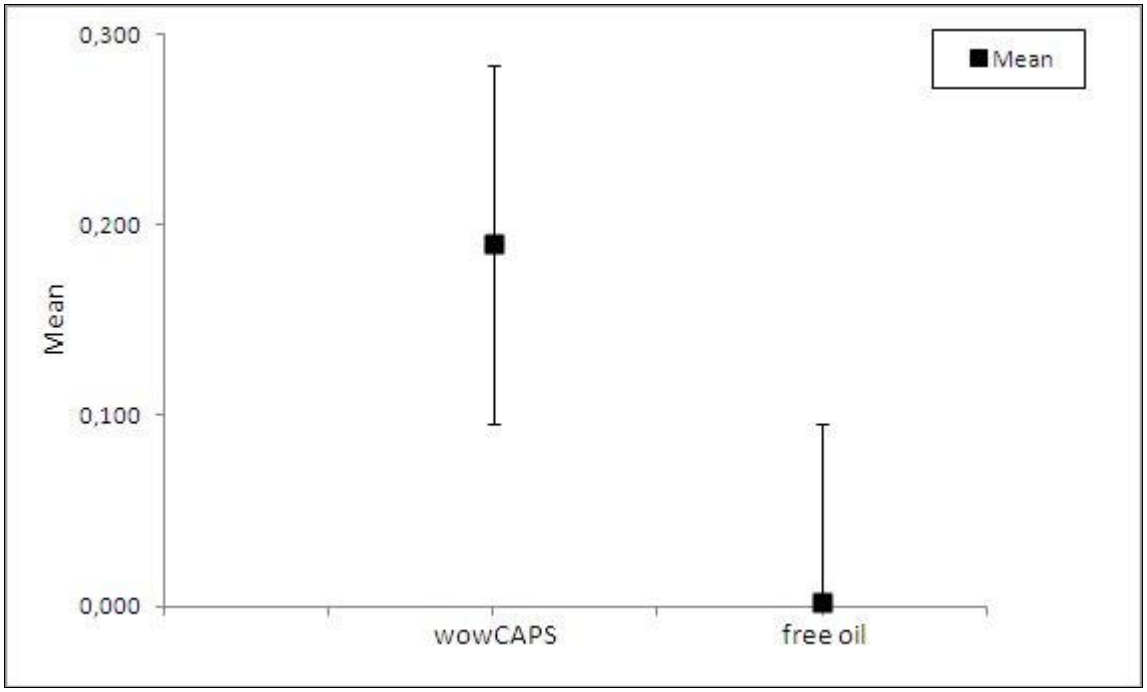



Figure 71: Means and standard errors of the conjugated trienes of the formulation wowCAPS® and the fish oil according UV irradiation

### 5.1.6 Fluorometric analysis – ORAC assay

In Table 22 the trolox equivalent measured with the ORAC assay is shown for the yogurt containing wowCAPS® and fish oil.

While the real quantity of antioxidants in the fish oil is not disclosed herein it affects both the test with free oil and with wowCAPS®. It is then reasonably not to consider the “baseline” antioxidant level of the pure fish oil

The ORAC assay is traditionally conceived for measuring the oxidation properties of water soluble substances; however, it can be applied as well to hydrophobic substances when properly dispersed in the hydrophilic media in which the samples are being tested.

The results are shown in Table 22.

Table 22: Trolox equivalent [µmol] of the yoghurt containing wowCAPS® and fish oil

| day | Trolox equivalent of yoghurt containing wowCAPS® (average of 2 measurements) [µmol] | Trolox equivalent of yoghurt containing fish oil (average of 2 measurements) [µmol] |
|---|---|---|
| 4 | 0.00165 | 0.00159 |
| 6 | 0.00209 | 0.00196 |
| 10 | 0.00239 | 0.00228 |
| 13 | 0.00214 | 0.00210 |
| 17 | 0.00228 | 0.00239 |
| 20 | 0.00259 | 0.00256 |

There is not a true independence of the values to apply a standard paired Student's t-test (since the physical effect happening in one value still is present in the next one). Therefore the analysis has been done according the non-parametric Paired-Sample Wilcoxon Signed Rank Test. At the level 0.05 the two distributions are not significantly different.

Therefore, it is concluded that the ORAC test is not capable to detect the influence (in the yogurt food matrix) of the microencapsulation of the omega-3 in the product wowCAPS®.

This does not necessarily mean that the microencapsulation has no effect on the oxidation / reduction properties of the yogurt, rather that if existent, they cannot be detected with the ORAC test. This result can be easily explained by the fact that the total influence of the omega-3 (and wowCAPS®) in the highly complex yogurt matrix, at the concentrations employed, is negligible.

In Table 23 and Figure 72 the Trolox equivalent measured with the ORAC assay is shown for the formulation wowCAPS® and the fish oil.

Table 23: Trolox equivalent [µmol] of the formulation wowCAPS® and the fish oil

| | Trolox equivalent of formulation wowCAPS® (average of 2 measurements) [µmol] | Trolox equivalent of the fish oil (average of 2 measurements) [µmol] |
|---|---|---|
| 3-6 °C | 0.00972 | 0.09137 |
| 70 °C | 0.00747 | 0.09647 |

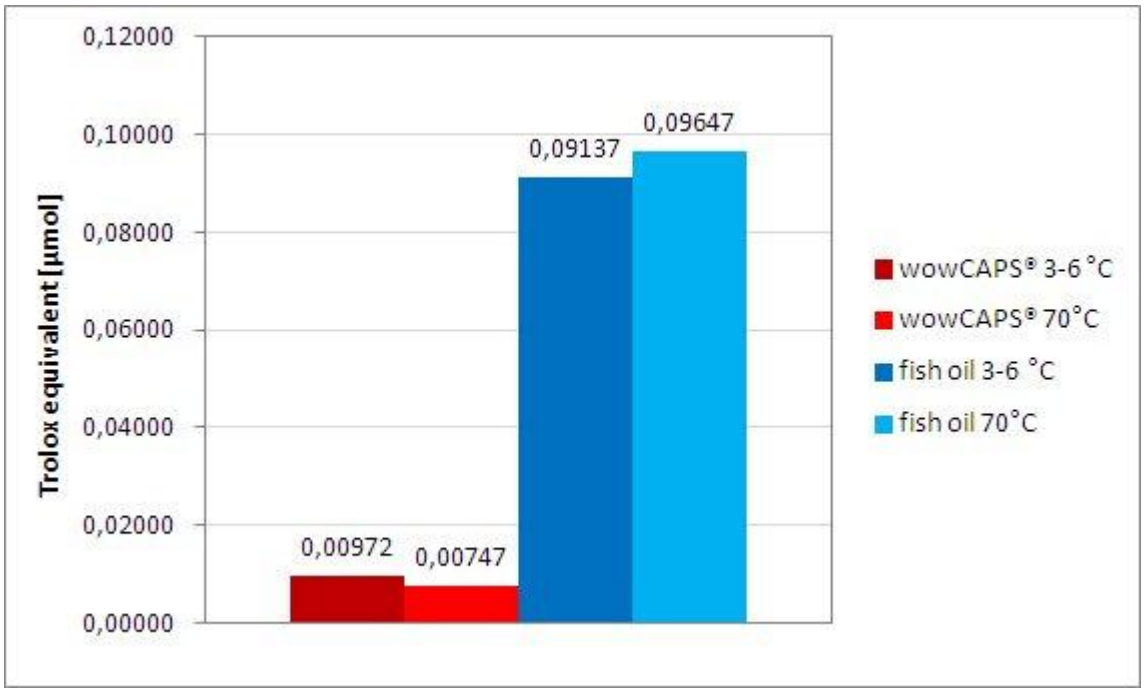



Figure 72: Trolox equivalent [µmol] of wowCAPS® and fish oil

The result shown in the Table 23 and in Figure 72 reflects a behaviour previously observed at GAT Food Essentials GmbH. The free fish oil appears to provide an antioxidant effect in much higher degree than the wowCAPS®.
However, this effect is believed to happen due to the faster oxidation of the PUFAs contained in the pure fish oil than that of fluorescein.
We believe that what we observe is due to a temporary protective effect of the omega-3 over fluorescein. Taking this into account the wowCAPS® formulation effects a remarkable isolation against oxidation.

## 5.2 Sensory evaluation

### 5.2.1 Yoghurt

The sensory evaluation was carried out once a week for 6 weeks with 8 participants. The values for taste and smell were counted together as off-flavor against the general impact of each sample. Then the averages of the 8 panelists of each tasting were compared.
There were no significant differences in between the panelists.

In Table 24 and Figure 73 (yogurt enriched with wowCAPS®) as well as in Table 25 and Figure 74 (yogurt enriched with fish oil) the outcomes of the tastings over the 6 weeks storage are shown.

Table 24: Outcome tasting of yoghurt enriched with wowCAPS®

| | **off-flavor [points]** | **general impact [points]** |
|---|---|---|
| initial | 7.8 ± 0.4 | 7.8 ± 0.4 |
| week 1 | 7.7 ± 0.4 | 7.3 ± 0.7 |
| week 2 | 7.3 ± 0.6 | 7.5 ± 0.4 |
| week 3 | 7.5 ± 0.4 | 7.7 ± 0.3 |
| week 4 | 7.3 ± 0.6 | 7.6 ± 0.4 |
| week 5 | 7.3 ± 0.6 | 7.8 ± 0.3 |

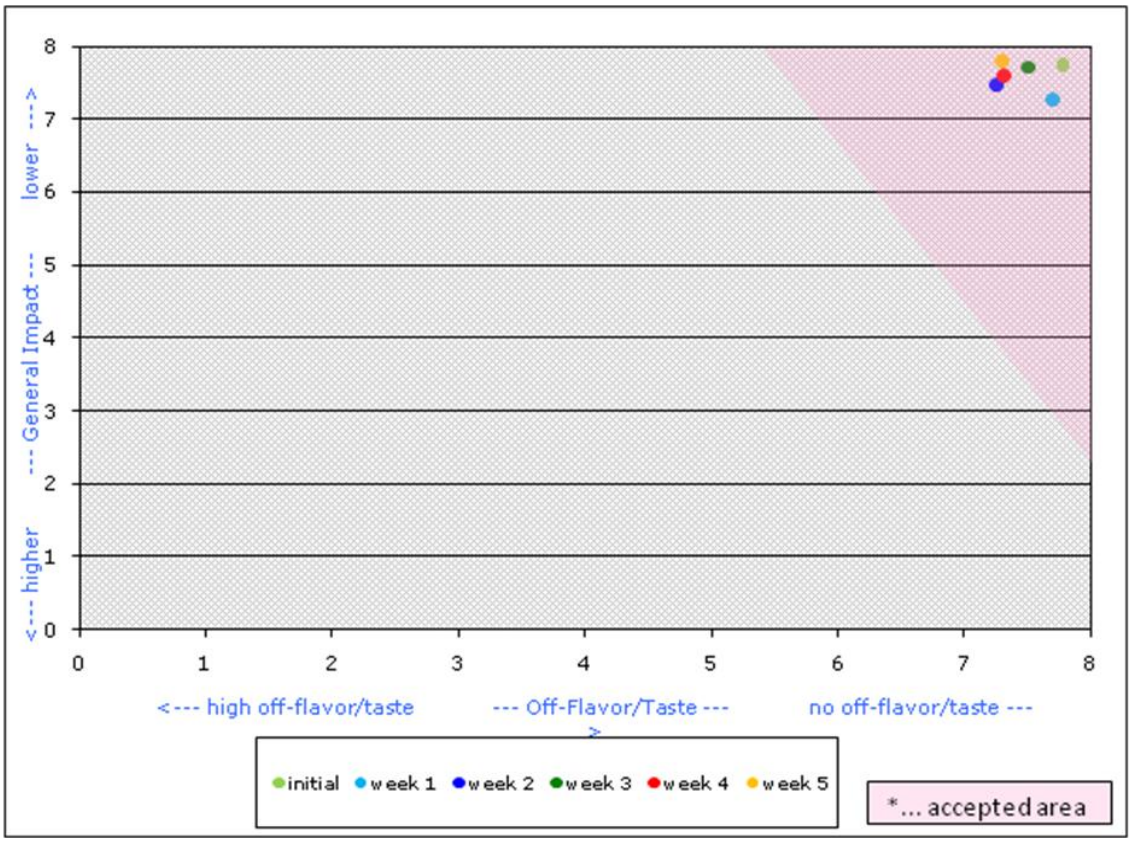



Figure 73: Outcome tasting of yoghurt enriched with wowCAPS®

One-Way ANOVA analysis on the off-flavor shows that there are no significant differences in between the off-flavor tested at any time when the oil is incorporated in the yogurt in the form of wowCAPS®.

Table 25: Outcome tasting of yoghurt enriched with fish oil

| | **off-flavor [points]** | **general impact [points]** |
|---|---|---|
| initial | 7.0 ± 0.6 | 7.0 ± 0.4 |
| week 1 | 6.8 ± 0.4 | 6.7 ± 0.6 |
| week 2 | 7.1 ± 0.3 | 6.6 ± 0.3 |
| week 3 | 6.9 ± 0.5 | 7.0 ± 0.4 |
| week 4 | 6.6 ± 0.4 | 6.5 ± 0.4 |
| week 5 | 6.3 ± 0.4 | 6.2 ± 0.3 |

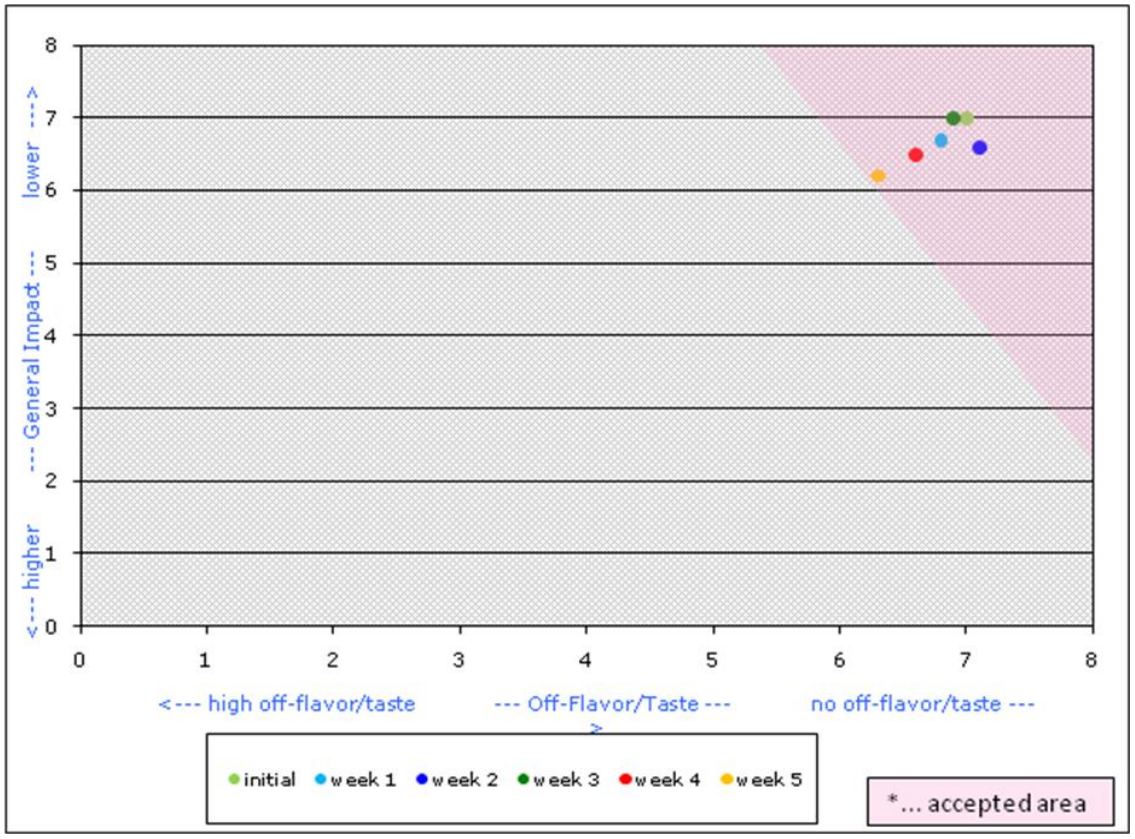



Figure 74: Outcome tasting of yoghurt enriched with fish oil

One-Way ANOVA analysis on the off-flavor shows that there are significant differences in between the initial value or the week 2 value and the week 5 ($p < 0.05$) when the oil is incorporated in pure form.

There was no significant difference in the off flavor of the yoghurt containing wowCAPS®. Over the storage time it gained better results than the yoghurt containing fish oil, which showed a significant difference in the terms of off-flavor over time. Nevertheless it is to mention that also the yoghurt containing fish oil remained in the accepted area throughout the evaluation time. Although the statistical results are definitive due to the limited resources for extended tests, it can be made the reasonable assumption that the enrichment with wowCAPS® is more suitable for yogurt than the enrichment with pure fish oil.

### 5.2.2 Cauliflower soup

The cauliflower soup was cooked and tasted immediately afterwards. For the tasting after 7 days it was heated in the microwave. No fishy or off-taste or smell could be detected in the cauliflower soup enriched with wowCAPS®. Each tasting was performed with 6 panelists. As these tastings were performed to test the acceptance of the cauliflower soup enriched with wowCAPS® there were no control samples for comparison. The values for taste and smell were counted together as off-flavor against the general impact of each sample. Then the mean values of the 6 panelists of the two tastings were compared.
The outcome of the tastings is shown in Table 26 and Figure 75.

Table 26: Outcome tasting of cauliflower soup enriched with wowCAPS®

| | **off-flavor [points]** | **general impact [points]** |
|---|---|---|
| initial | 7.8 ± 0.3 | 7.8 ± 0.3 |
| week 1 | 7.6 ± 0.3 | 7.5 ± 0.3 |

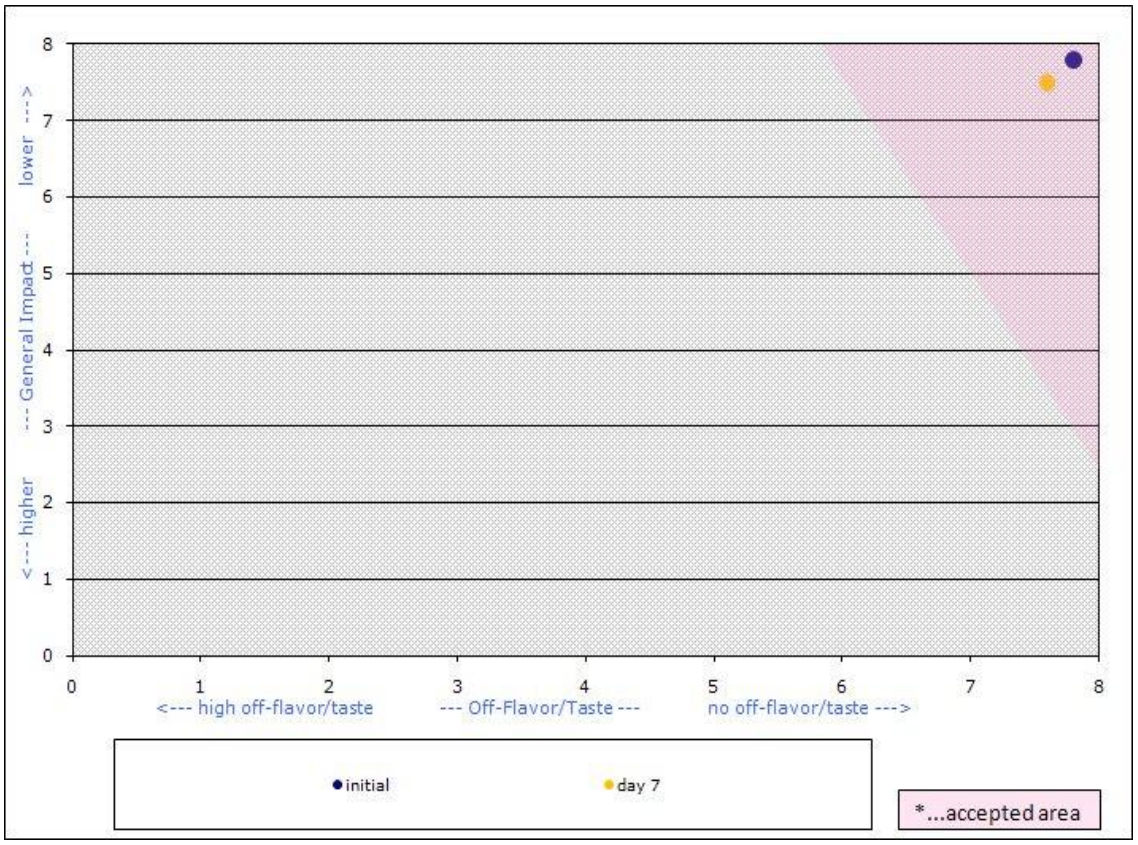



Figure 75: Outcome tasting of cauliflower soup enriched with wowCAPS®

There were no significant differences between initial levels and levela after 7 days. From this can be concluded that the food matrix cauliflower soup could be suitable for the enrichment with wowCAPS®.

### 5.2.3 Tomato sauce

The tomato sauce was tasted plain. For the tasting after 7 days it was heated in the microwave. No fishy or off-taste or smell could be detected in the tomato sauce enriched with wowCAPS®. Each tasting was performed with 8 panelists. As these tastings were performed to test the acceptance of the tomato sauce enriched with wowCAPS® there were no control samples for comparison. The values for taste and smell were counted together as off-flavor against the general impact of each sample. Then the mean values of the 8 panelists of the two tastings were compared.

The outcome of the tastings is shown in Table 27and Figure 76.

Table 27: Outcome tasting of tomato sauce enriched with wowCAPS®

| | off-flavor [points] | general impact [points] |
|---|---|---|
| initial | 7.6 ± 0.3 | 7.5 ± 0.4 |
| week 1 | 7.5 ± 0.4 | 7.1 ± 0.3 |

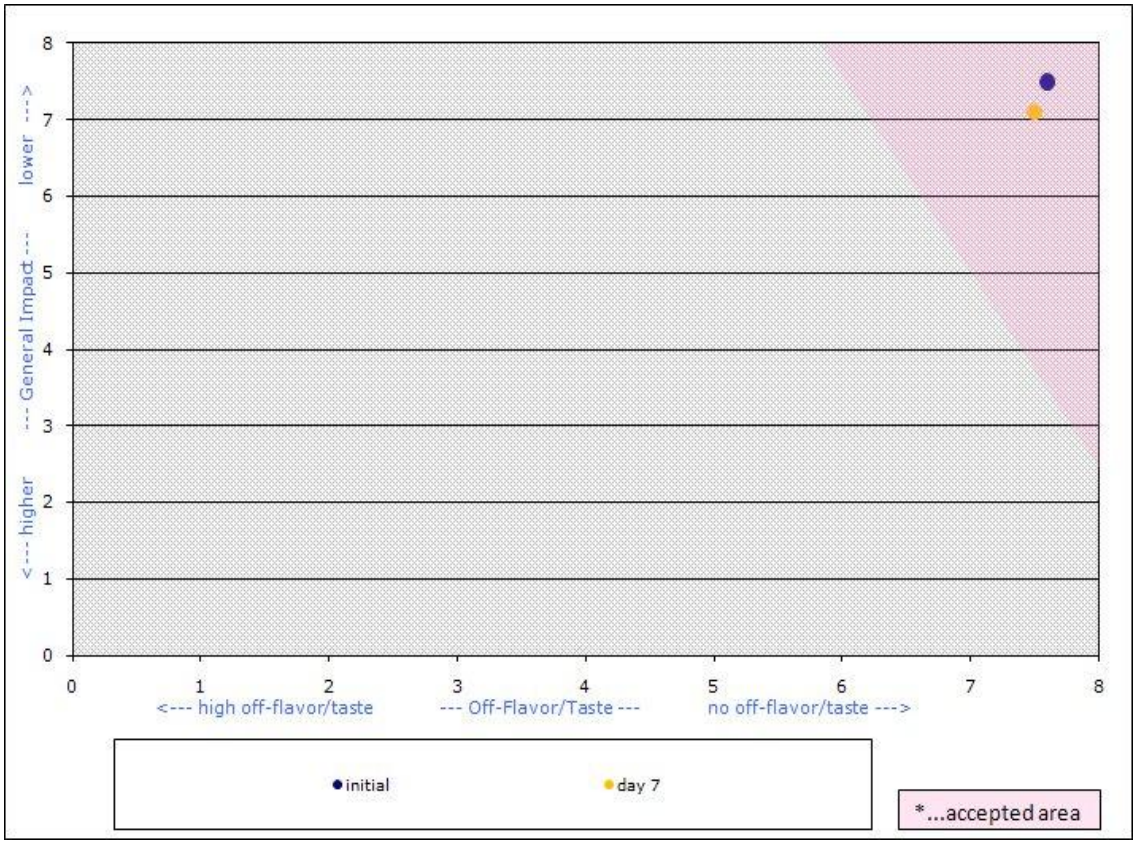



Figure 76: Outcome tasting of tomato sauce enriched with wowCAPS®

There were no significant differences between initial levels and levels after 7 days. From this it can be concluded that the food matrix tomato sauce could be suitable for the enrichment with wowCAPS®.

### 5.2.4 UHT milk

No fishy flavor could be detected in the UHT milk enriched with wowCAPS® in a descriptive tasting with 8 panelists.

As these tastings were performed to test the acceptance of the UHT milk enriched with wowCAPS® the panelists didn't get any control samples for

comparison. The values for taste and smell were counted together as off-flavor against the general impact of each sample. Then the mean values of the 8 panelists of each tasting were compared.

The outcome of the 3 tastings throughout the storage time is shown in Table 28 and Figure 77.

Table 28: Outcome tasting of UHT milk enriched with wowCAPS®

| | **off-flavor [points]** | **general impact [points]** |
|---|---|---|
| initial | 7.8 ± 0.2 | 6.9 ± 0.4 |
| 1 month | 7.7 ± 0.3 | 6.8 ± 0.2 |
| 3 months | 7.9 ± 0.2 | 6.7 ± 0.4 |

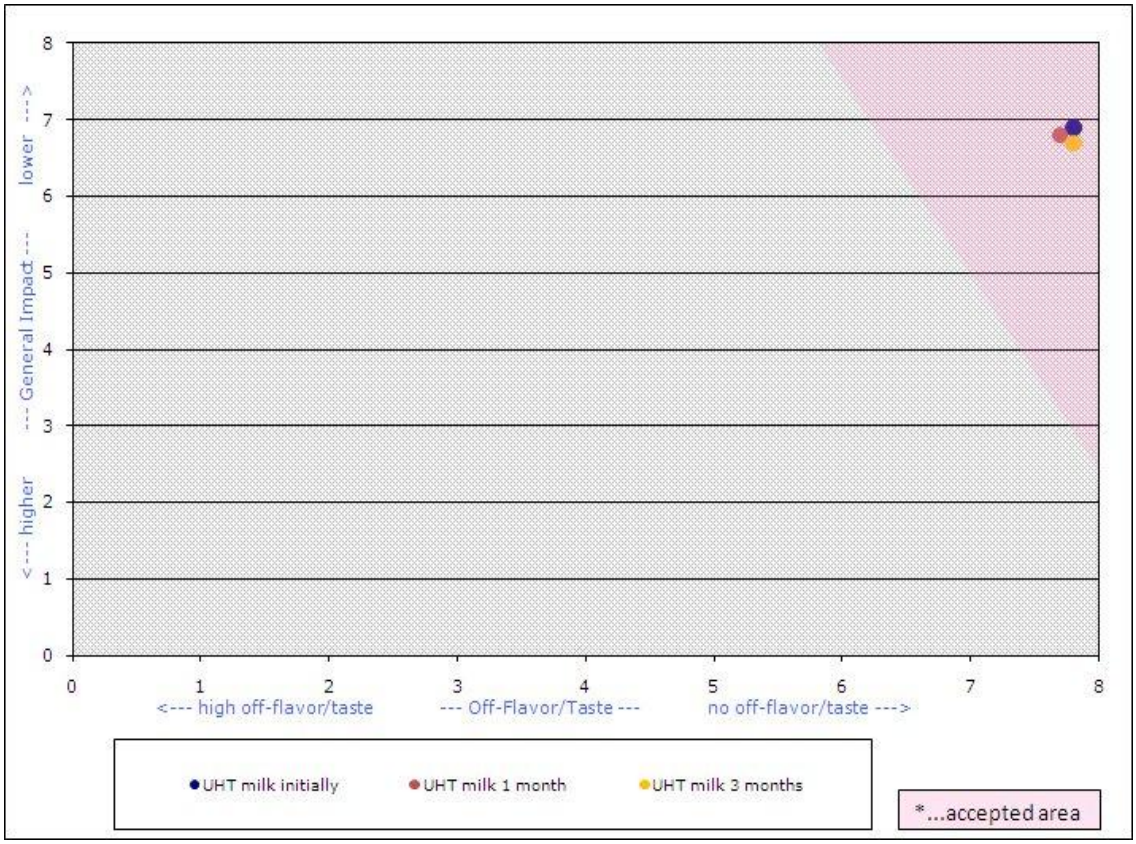



Figure 77: Outcome tasting of UHT milk enriched with wowCAPS®

There were no significant differences between initial levels and levels after 3 months. From this it can be concluded that the food matrix UHT milk could be suitable for the enrichment with wowCAPS®.

## 5.3 Microscopic observation

### 5.3.1 Formulation wowCAPS®

Figure 78 shows a picture of the microscopic observation of the formulation wowCAPS® in four hundredfold magnification. The picture shows intact capsules in different sizes

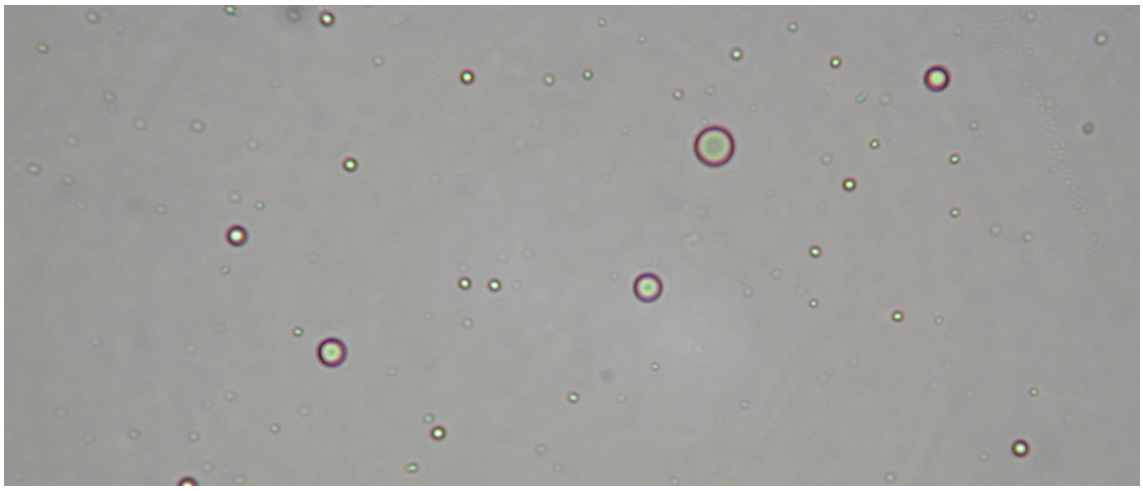


Figure 78: Microscopic observation of the formulation wowCAPS®, 1:400 magnification

Figure 79 shows a picture of the microscopic observation of the formulation wowCAPS® in thousand fold magnification. The picture shows intact capsules in different sizes.

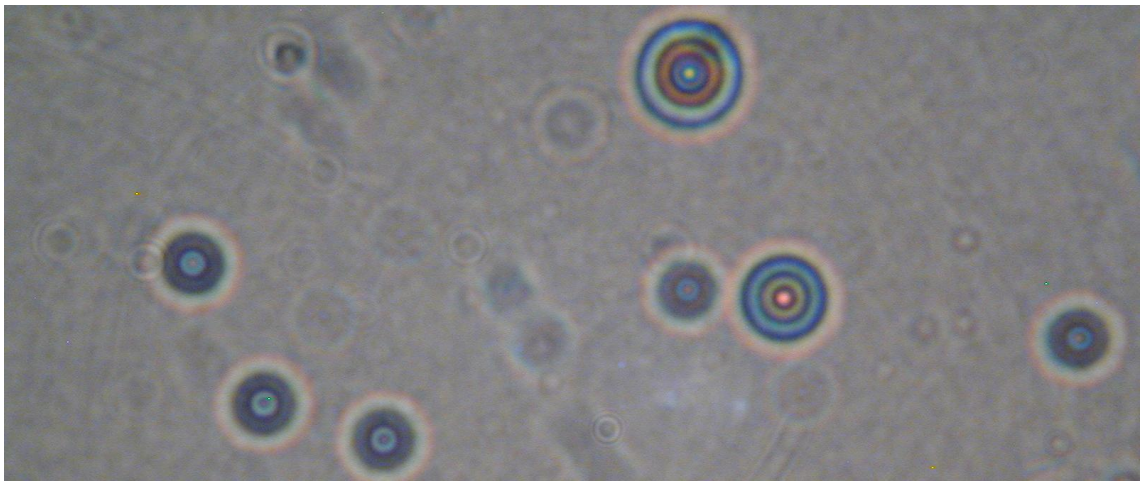


Figure 79: Microscopic observation of the formulation wowCAPS®, 1:1000 magnification

### 5.3.2 Yoghurt

#### 5.3.2.1 Yoghurt containing wowCAPS®

Figure 80 shows a picture of the microscopic observation of the yoghurt containing wowCAPS® on the day of the initial analysis in hundredfold magnification. Capsules are small and well distributed and marked with a red ring.

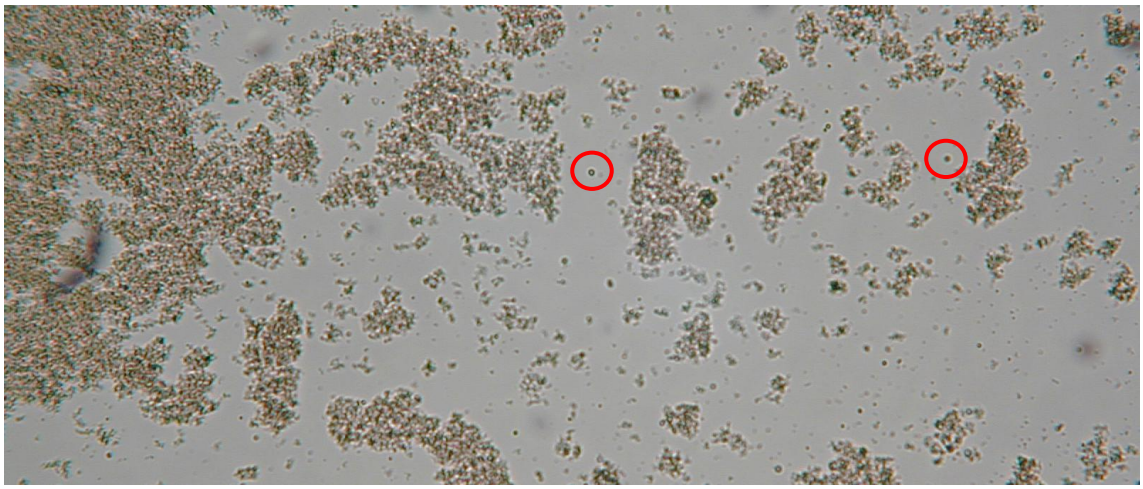


Figure 80: Microscopic observation of the yoghurt containing wowCAPS®, 1:100 magnification

Figure 81 shows a picture of the microscopic observation of the yoghurt containing wowCAPS® on the day of the initial analysis in four hundredfold magnification. Capsules are small and well distributed and marked with a red ring.

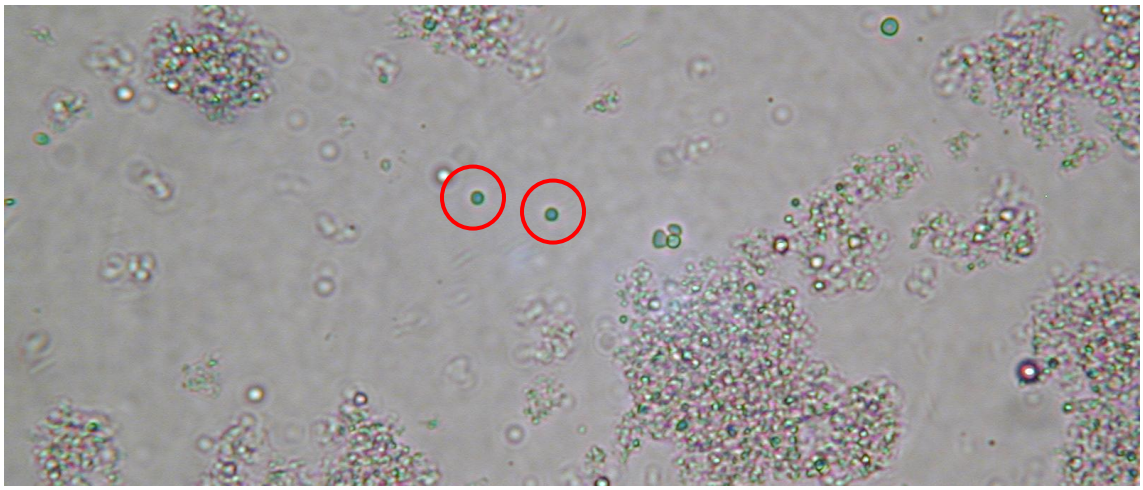


Figure 81: Microscopic observation of the yoghurt containing wowCAPS®, 1:400 magnification

Figure 82 shows a picture of the microscopic observation of the yoghurt containing wowCAPS® on the day of the initial analysis in thousand fold magnification. Capsules are small and well distributed and marked with a red ring.

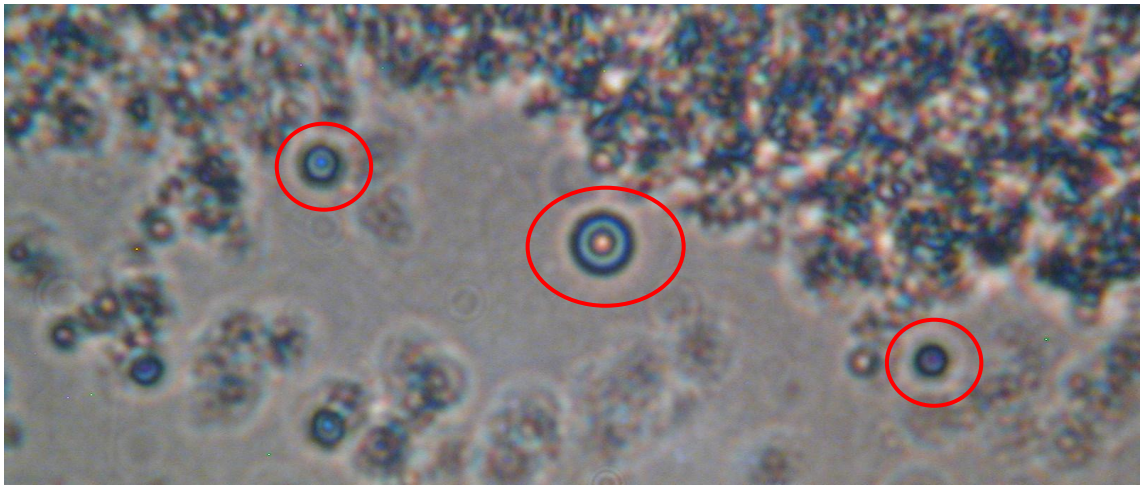


Figure 82: Microscopic observation of the yoghurt containing wowCAPS®, initially, 1:1000 magnification

Figure 83 shows a picture of the microscopic observation of the yoghurt containing wowCAPS® after 21 days of storage at 3-6 °C in thousand fold magnification. Capsules are small and well distributed and marked with a red ring.

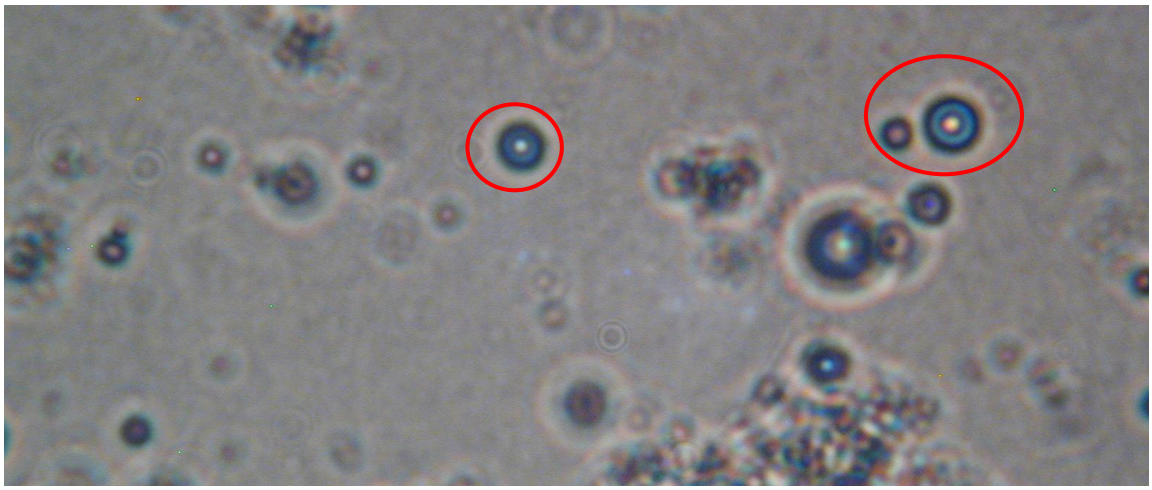


Figure 83: Microscopic observation of the yoghurt containing wowCAPS®, 21 days at 3-6 °C, 1:1000 magnification

From these pictures can be concluded that the microcapsules remain stable during the production process of yogurt and are not affected by the yogurt starter cultures.

### 5.3.2.2 Yoghurt containing fish oil

Figure 84 shows a picture of the microscopic observation of the yoghurt containing fish oil on the day of the initial analysis in hundredfold magnification. Oil droplets are clearly visible and marked with a blue ring.

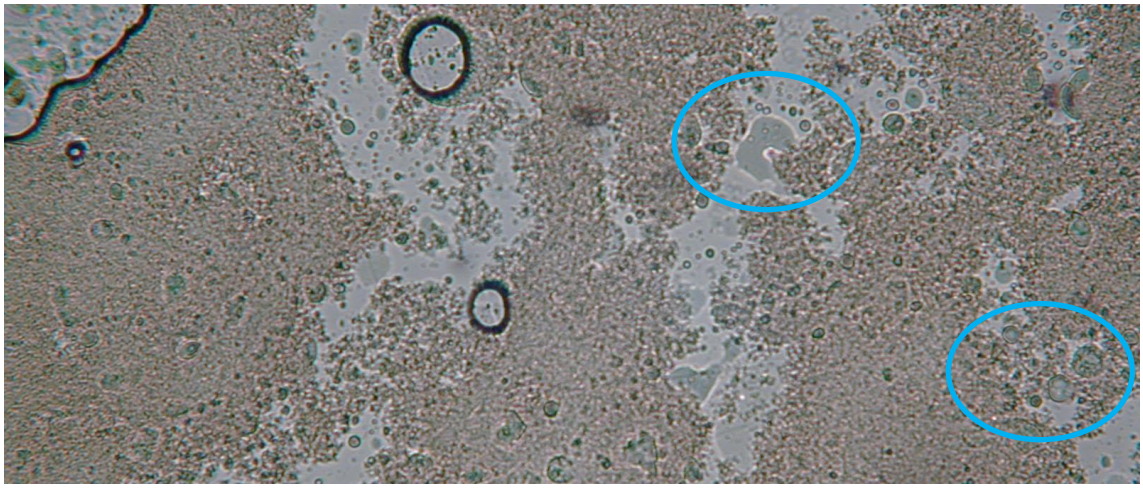


Figure 84: Microscopic observation of the yoghurt containing fish oil, 1:100 magnification

Figure 85 shows a picture of the microscopic observation of the yoghurt containing fish oil on the day of the initial analysis in four hundredfold magnification. Oil droplets are clearly visible and marked with a blue ring.

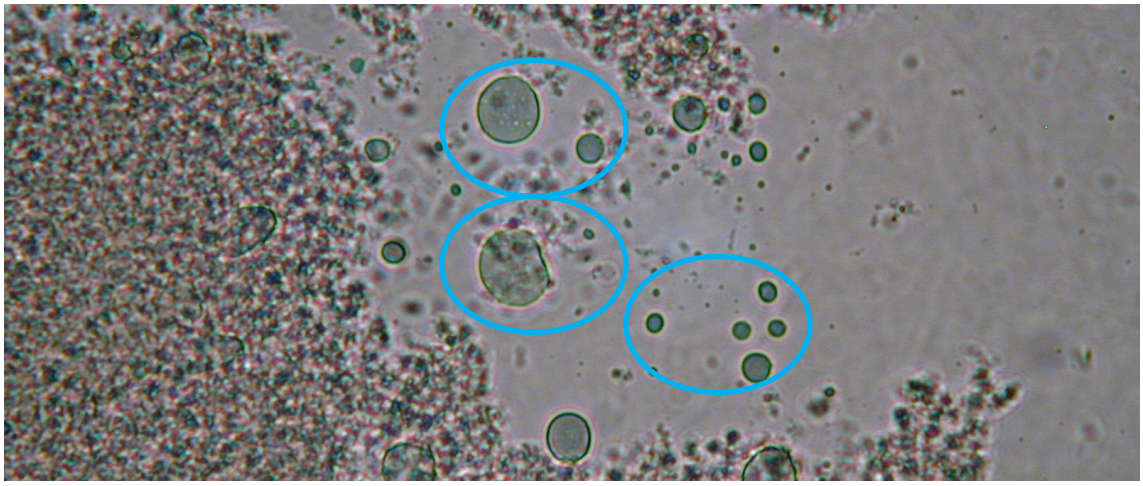


Figure 85: Microscopic observation of the yoghurt containing fish oil, 1:400 magnification

Figure 86 shows a picture of the microscopic observation of the yoghurt containing fish oil on the day of the initial analysis in thousand fold magnification. Oil droplets are clearly visible and marked with a blue ring.

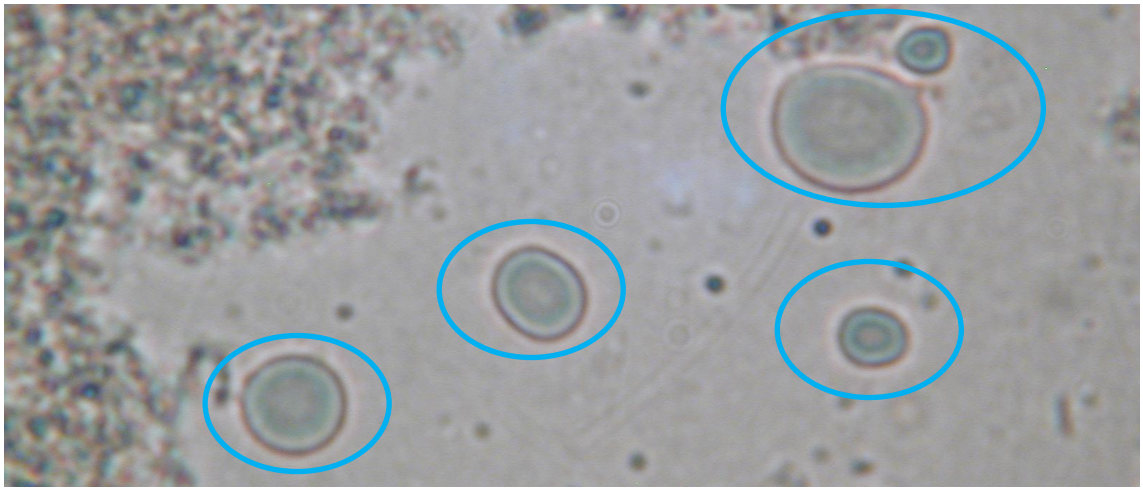


Figure 86: Microscopic observation of the yoghurt containing fish oil, initially, 1:1000 magnification

Figure 87 shows a picture of the microscopic observation of the yoghurt containing fish oil after 21 days of storage at 3-6 °C in thousand fold magnification. Oil droplets are clearly visible and marked with a blue ring.

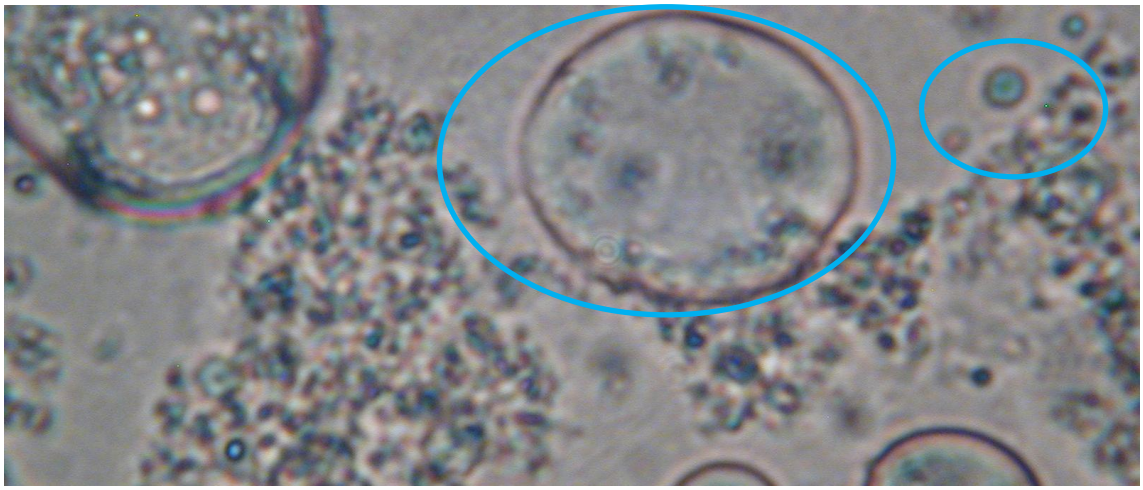


Figure 87: Microscopic observation of the yoghurt containing fish oil, 21 days at 3-6 °C, 1:1000 magnification

### 5.3.3 Cauliflower soup

Figure 88 shows a picture of the microscopic observation of cauliflower soup containing wowCAPS® in four hundredfold magnification. The picture shows intact capsules in different sizes. Some capsules are marked with a red ring for better detection.

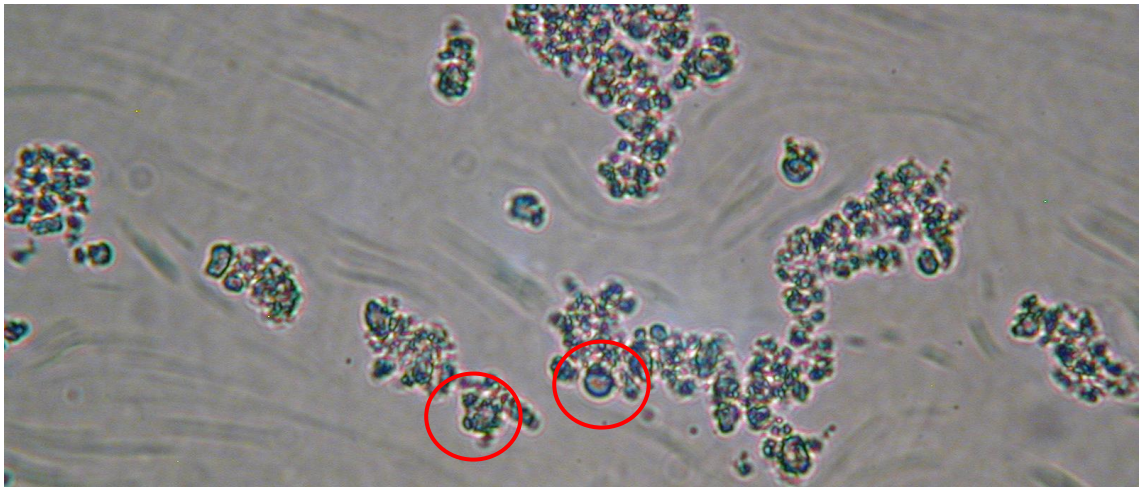


Figure 88: Microscopic observation of the cauliflower soup containing wowCAPS®, 1:400 magnification

Figure 89 shows a picture of the microscopic observation of cauliflower soup containing wowCAPS® in thousand fold magnification. The picture shows intact

capsules in different sizes. Some capsules are marked with a red ring for better detection.

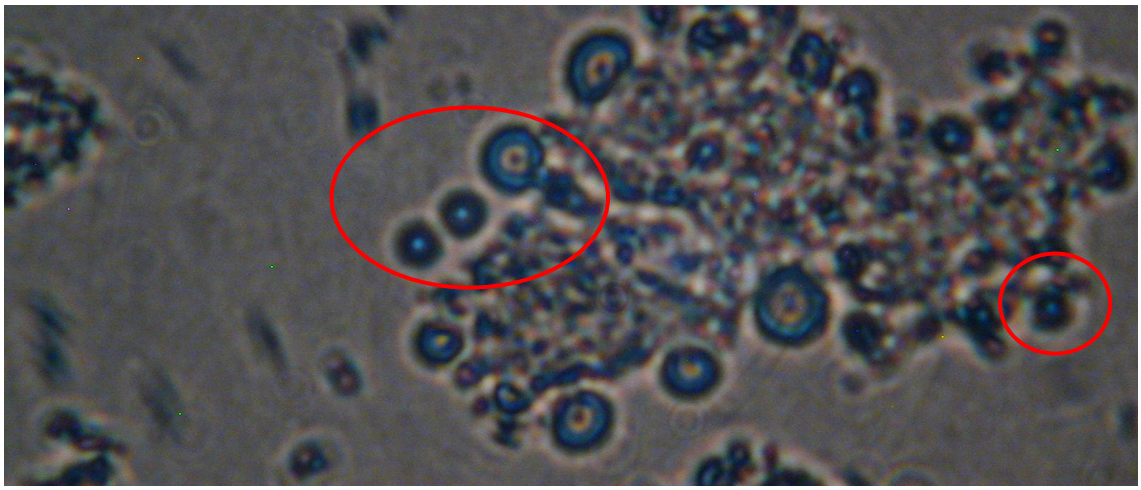


Figure 89: Microscopic observation of the cauliflower soup containing wowCAPS®, 1:1000 magnification

From these pictures can be concluded that the microcapsules remain stable during the production process of cauliflower soup.

### 5.3.4 Tomato sauce

Figure 90 shows a picture of the microscopic observation of tomato sauce containing wowCAPS® in four hundredfold magnification. The picture shows intact and well distributed capsules in different sizes. Some capsules are marked with a red ring for better detection

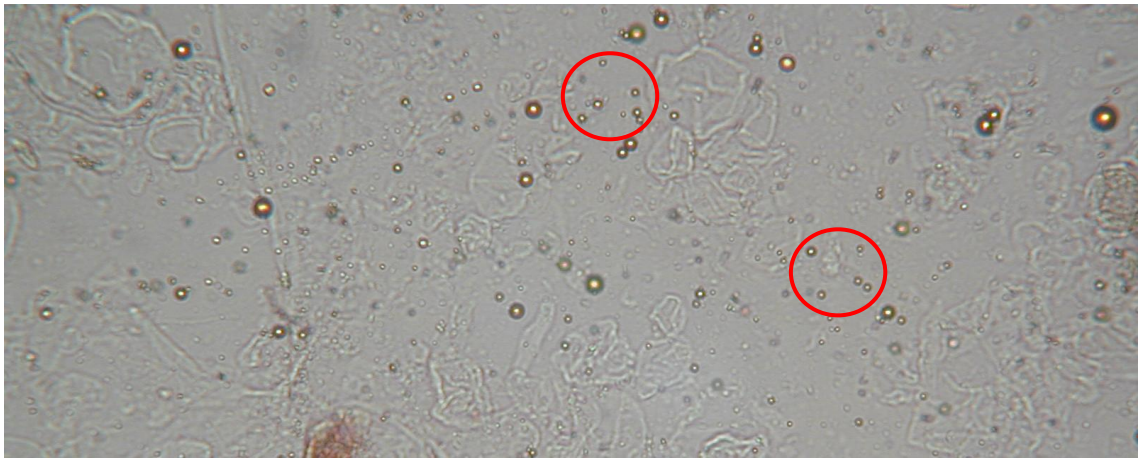


Figure 90: Microscopic observation of the tomato sauce containing wowCAPS®, 1:400 magnification

Figure 91 shows a picture of the microscopic observation of tomato sauce containing wowCAPS® in thousand fold magnification. The picture shows intact and well distributed capsules in different sizes. Some capsules are marked with a red ring for better detection.

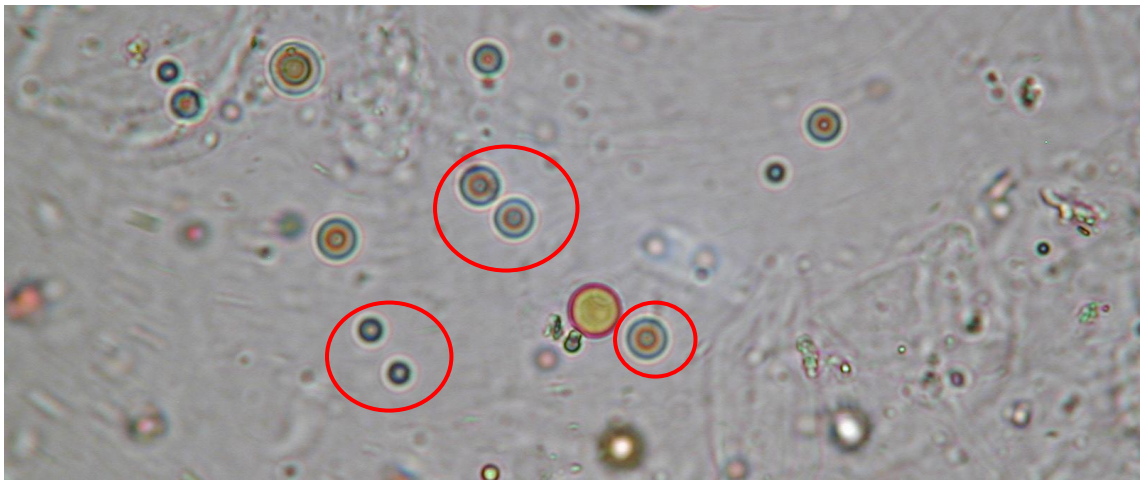


Figure 91: Microscopic observation of the tomato sauce containing wowCAPS®, 1:1000 magnification

From these pictures can be concluded that the microcapsules remain stable during the production process of tomato sauce.

### 5.3.5 UHT milk

Figure 92 shows a picture of the microscopic observation of the UHT milk enriched with wowCAPS® at the time point of the initial analysis in four hundredfold magnification. The picture shows intact and well distributed capsules in different sizes. In the background the milk micelles are visible.

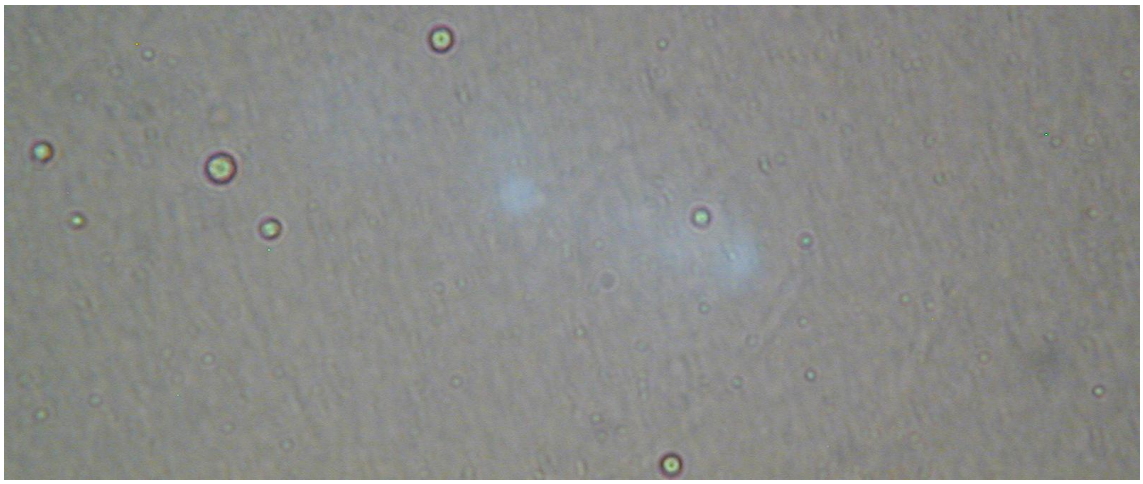


Figure 92: Microscopic observation of the UHT milk enriched with wowCAPS®, initially, RT, 1:400 magnification

Figure 93 shows a picture of the microscopic observation of the UHT milk enriched with wowCAPS® at the time point of initial analysis in thousand fold magnifictation. The picture shows intact and well distributed capsules in different sizes. In the background the milk micelles are visible.

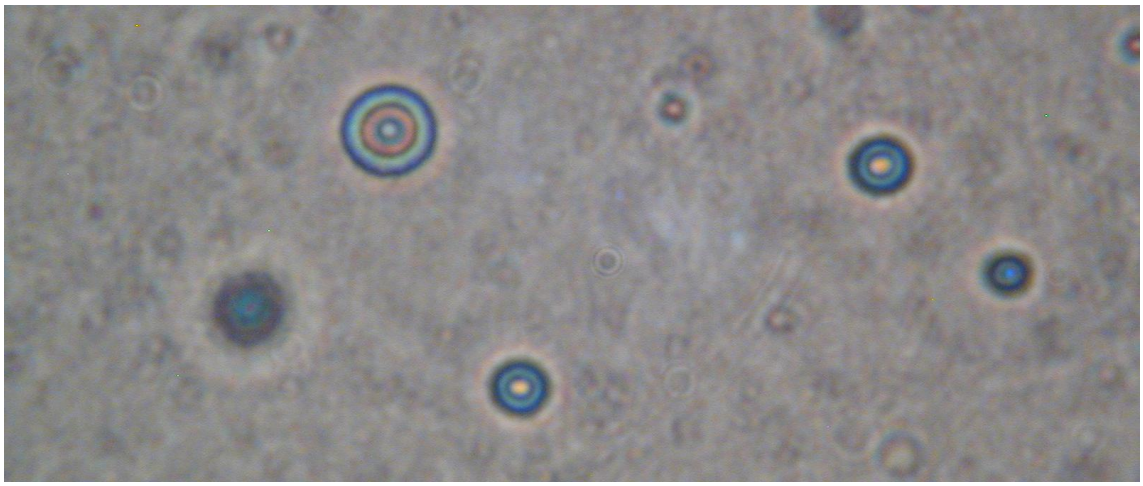


Figure 93: Microscopic observation of the UHT milk enriched with wowCAPS®, initially, RT, 1:1000 magnification

Figure 94 shows a picture of the microscopic observation of the UHT milk enriched with wowCAPS® after 3 months storage at room temperature in four hundredfold magnification. The picture shows intact and well distributed capsules in different sizes. In the background the milk micelles are visible.

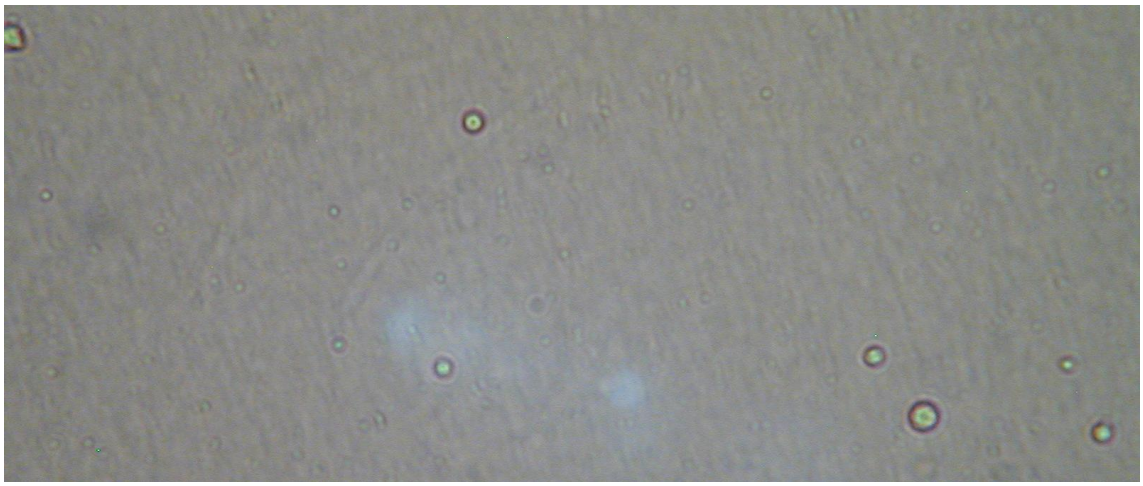


Figure 94: Microscopic observation of the UHT milk enriched with wowCAPS®, 3 months, RT, 1:400 magnification

Figure 95 shows a picture of the microscopic observation of the UHT milk enriched with wowCAPS® after 3 months storage at room temperature in thousand fold magnifictation. The picture shows intact and well distributed capsules in different sizes. In the background the milk micelles are visible.

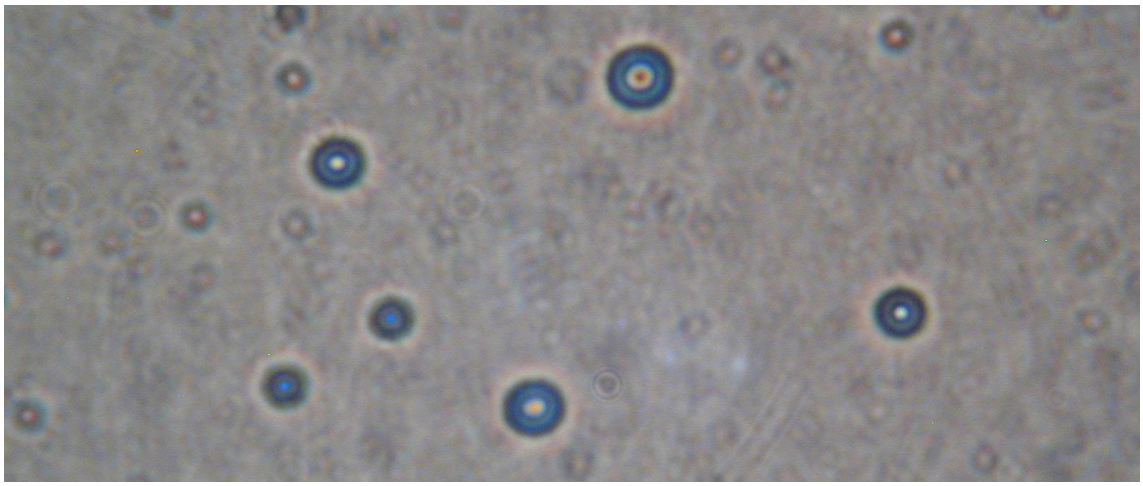


Figure 95: Microscopic observation of the UHT milk enriched with wowCAPS®, 3 months, RT, 1:1000 magnification

From these pictures can be concluded that the microcapsules remain stable during the production process of UHT milk.

# 6. SUMMARY

This study addresses how the microencapsulation of oils enriched with omega-3 fatty acids helps to improve their stability when incorporated into foods.

The current available literature is limited regarding a precise methodology for the analysis and interpretation of data when analyzing functional foods with microencapsulated omega-3 fatty acids.

In order to assess the stability of microencapsulated omega-3 in foods, detection methods for the omega-3's enriched oils, for the concentrated formulation containing them (the formulation to be mixed with the food) and for the final food containing such microcapsules have been developed in this study. The oil used for this purpose was from the fish species *Gadus morhua* origin, with a content of EPA and DHA of approx. 26w%. The formulation object of this study has been the proprietary product wowCAPS® (with a content in EPA and DHA of ca. 7w%) owned by GAT Food Essentials GmbH. The foods selected for this work was yogurt, cauliflower soup, tomato sauce and UHT milk, enriched at different levels with either the fish oil mentioned above or with the wowCAPS® product.

The well-established tests in use to determine the oil quality such as peroxide value, acid value, p-anisidine value, 2-thiobarbituric acid value, levels of conjugated dienes have their limitations in the assessment of the quality of foods enriched with omega-3 from fish oil. Notably, these methods fail as well as the method to evaluation whether omega-3 are of microbial or vegetable origin. Therefore additional analysis have been employed to come closer to a less subjective evaluation of the quality of omega-3 fatty acids in the context of functional foods.

Contrary to what would have been expected, plain analysis of total amount of omega-3 fatty acids by means of HPLC or GC is unable to detect the small degree of decomposition of omega-3 (that is in turn enough for providing unacceptable organoleptic characteristics) when they are incorporated in complex food matrices.

Notably, the analytical method to assess the omega-3 fatty acids used in the experimental part has shown to be precise and specific enough for a correct determination of the most important omega-3 from an industrial point of view, namely EPA and DHA. The content of EPA and DHA in the final products is usually what is reflected in the functional label as the claimed content of omega-3. Therefore, although the method shows not to be valid for all fatty acids analyzed, it is still of full applicability for industrial purposes, and for the purpose of the present work.

When also considering the other results (e.g., peroxide values, ORAC tests and most importantly, the organoleptic panel tasting), it could be considered that there is an effective protection of the wowCAPS® formulation.

# 7. ZUSAMMENFASSUNG

Ziel dieser Diplomarbeit war festzustellen wie die Mikroverkapselung die Stabilität von omega-3 Fettsäuren in angereicherten Lebensmitteln verbessert.

In der vorhandenen Literatur finden sich wenig präzise Methoden für die Analyse und Auswertung von Functional Foods, die mit mikroverkapselten omega-3 Fettsäuren angereichert wurden.

Um die Stabilität von mikroverkapselten omega-3 Fettsäuren in Lebensmitteln beurteilen zu können wurden in dieser Arbeit Analysemethoden entwickelt, die sowohl für das omega-3 reiche Öl, die konzentrierte Formulierung als auch für die angereicherten Lebensmittel anwendbar sind. Das verwendete Öl wurde ausschließlich aus der Fischspezies Gadus morhua gewonnen und hat einen Gehalt an EPA/DHA von 26%w. Die in dieser Arbeit verwendete Mikroverkapselung wowCAPS®, mit einem Gehalt an EPA und DHA von ca. 7%w, ist Eigentum der Firma GAT Food Essentials GmbH. Die verwendeten Lebensmittel Jogurt, Karfiolsuppe, Tomatensauce und UHT-Milch wurden mit unterschiedlichen Konzentrationen an Fischöl bzw. dem Produkt wowCAPS® angereichert.

Die gängigen Tests zur Evaluierung der Ölqualität, wie zum Beispiel Peroxidzahl, Säurezahl, p-Anisidinzahl, Thiobarbitursäuretest, Menge an konjugierten Dienen, sind für die Analyse mit omega-3 angereicherter Lebensmittel nur bedingt anwendbar. Auch für die Evaluierung von omega-3 Fettsäuren tierischer oder mikrobieller Herkunft sind diese Methoden nicht unbedingt geeignet. Aus diesem Grund wurden weitere Analysen durchgeführt um eine weniger subjektive Evaluierung der Qualität von omega-3 Fettsäuren in Functional Foods zu erreichen.

Im Gegensatz zu den Erwartungen war es weder mittels HPLC- noch mittels GC-Analyse möglich den geringen Grad des Abbaus der omega-3 Fettsäuren im Lebensmittel zu detektieren obwohl dieser bereits in geringem Maße die organolpetischen Eigenschaften beeinträchtigt.

Die Methode zur Analyse der omega-3 Fettsäuren, die im experimentellen Teil verwendet wurde, hat gezeigt, dass sie präzise und spezifisch genug ist um die

omega-3 Fettsäuren zu detektieren, die in der Industrie am wichtigsten sind, EPA und DHA. Der Gehalt an EPA und DHA ist in den meisten Fällen auf den Etiketten der angereicherten Lebensmittel ausgewiesen. Daher ist die Methode, obwohl sie nicht für alle analysierten Fettsäuren gültig zu sein scheint, sehr wohl geeignet für industrielle Zwecke und die Zwecke dieser Arbeit.

Im Hinblick auf die Resultate der weiteren Analysen (z.B. Peroxidezahl, ORAC-Tests und sensorische Tests), kann auf einen effektiven Schutz durch die wowCAPS®-Formulierung geschlossen werden.

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# 9. APPENDIX

## 9.1 Evaluation sheet for sensory evaluation

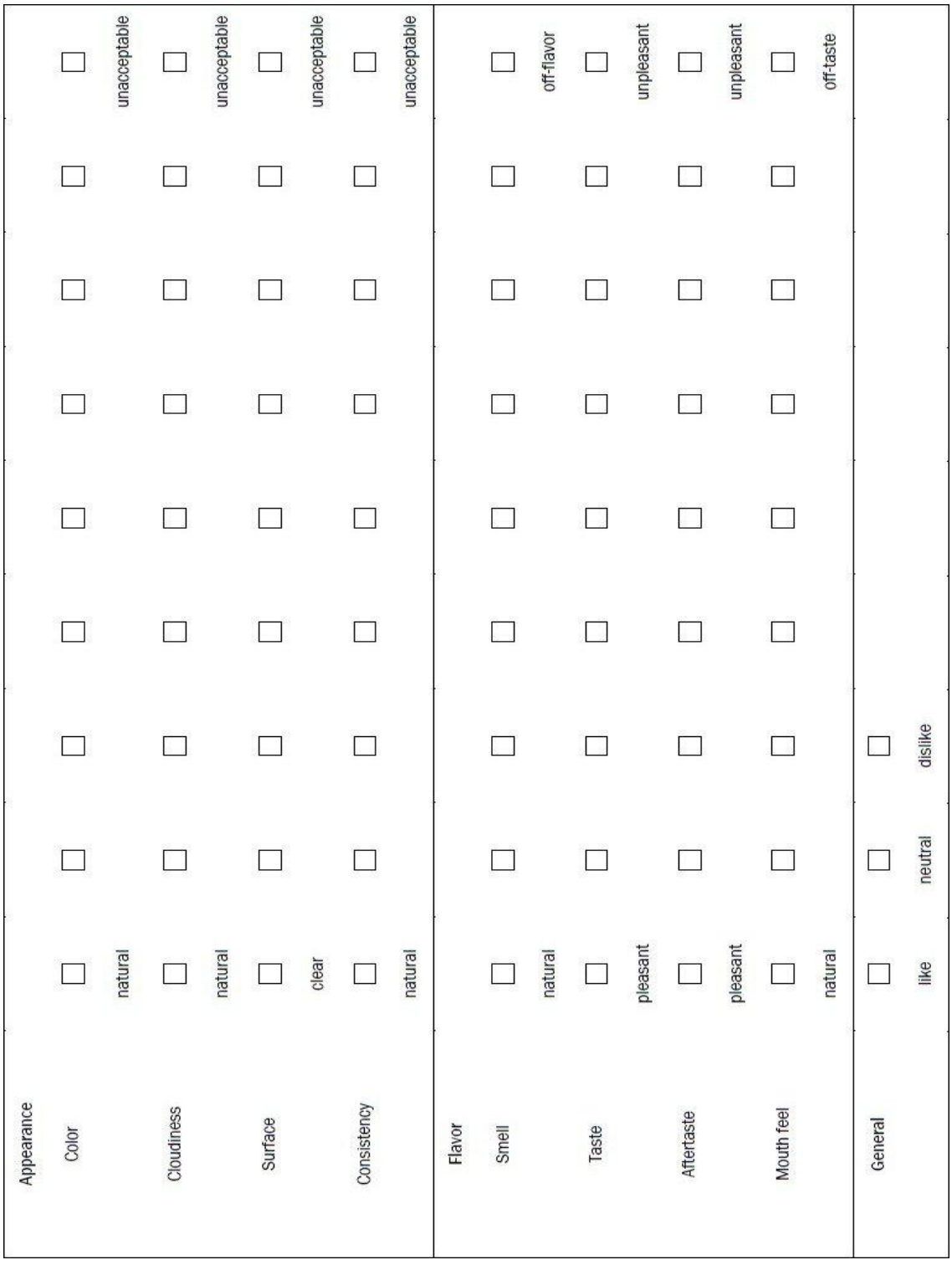


Figure 96: Evaluation sheet for sensory evaluation

# LEBENSLAUF

1. Persönliche Daten

   Rebecca Albrecht
   geboren am 07. Mai 1985 in Wiener Neustadt
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2. Ausbildung

| | |
|---|---|
| 09/1995 – 06/2003 | BRG Wiener Neustadt, BRG mit naturwissenschaftlichem Schwerpunkt Matura mit ausgezeichnetem Erfolg |
| 10/2003 – 02/2009 | Studium Übersetzen und Dolmetschen, Universität Wien |
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3. Berufliche Erfahrung und Praktika

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| 04/2004 – 09/2007 | geringfügige Beschäftigung in der Buchhaltung, Porsche Wien Liesing, Wien |
| 03/2008 | Praktikum, IMSB Austria, Wien |
| 05/2008 – 06/2008 | geringfügige Beschäftigung im Service, Hohe Wand Wiese, Wien |
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