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Development of Nanoemulsion-based Delivery Systems for Evaluation of Triglycerides Bioactivity in *Caenorhabditis Elegans*

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**DEVELOPMENT OF NANOEMULSION-BASED DELIVERY SYSTEMS FOR
EVALUATION OF TRIGLYCERIDES BIOACTIVITY IN CAERNOHABDITIS
ELEGANS**

A Thesis Presented

by

JOSE DANIEL COLMENARES ARAQUE

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTERS OF SCIENCE

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Food Science

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ABSTRACT

DEVELOPMENT OF NANOEMULSION-BASED DELIVERY SYSTEMS FOR EVALUATION OF TRIGLYCERIDES BIOACTIVITY IN CAERNOHABDITIS ELEGANS

SEPTEMBER 2015

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To a large extent, free fatty acids have been studied using the nematode *C. elegans*. However, fatty acids mostly occur in triglyceride form, which may affect their digestibility and bioactive effect. A method to evaluate bioactive fatty acids in triglyceride form using *C. elegans* represents a more realistic approach and offers several advantages; yet, the use of *C. elegans* for the study of bioactive triglycerides is currently limited because of their feeding behavior (selectivity for *Escherichia coli* OP50) and their hydrophilic growth media. The purpose of this research is to develop a method to deliver hydrophobic bioactives directly into the digestive system of *C. elegans*. Nanoemulsions prepared by high energy methods with a wide range of particle size were ingested by *C. elegans*. The ingestion level was dependent on particle size and the oil concentration on the growth media. Dietary triglycerides did not influence significantly the fatty acid composition or life span in *C. elegans*. In summary, this research evidences that *C. elegans* can ingest triglyceride containing nanoemulsions with different particle size, an alternative method that enables *C. elegans* for evaluation of hydrophobic bioactives.

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CHAPTER 1

INTRODUCTION

Obesity is a metabolic disorder related to energy homeostasis; and develops when energy intake is higher than energy expenditure. The prevalence of overweight and obesity is increasing especially between young children and teenager. In fact, by 2013, 23.8% of boys and 22.6% of girls in developed countries were overweight (Gakidou 2014).

Feeding behavior has a significant impact on obesity. Overconsumption of carbohydrates and triglycerides, in combination with sedentarily, are promoting factors for body weight gain. In a large extend, much research on novel components has been done to find alternatives for prevention or treatment obesity. Particularly, bioactive free fatty acids have been evaluated at *in vitro* and *in vivo* levels with promising results; however, fatty acids mostly occur in triglyceride form in food. The digestion of a fatty acid in triglyceride form greatly depends on geometrical and physical-chemical properties of the fatty acids, their location within the glycerol backbone and the enzymatic environment for digestion. Consequently, the bioactive effect of a fatty acid in triglyceride form may differ from the effect when it is present in free form. Therefore, this research is intended to develop a method to evaluate bioactive fatty acids in triglyceride form, using the model *Caernohabditis elegans*.

C. elegans has been widely used as *in vivo* model for evaluation of bioactive fatty acids. This model offers several advantages (Pellettieri 2014); *C. elegans* are simple hermaphrodites with a short life cycle (3 days for adulthood) and a large brood size

(approximately 300 progeny). Their transparent body allows see their internal biology and they are easy and inexpensive to maintain in a lab. However, the use of *C. elegans* for the study of digestion and absorption of triglycerides is currently limited because of their feeding behavior (selectivity for *Escherichia coli* OP50) and their hydrophilic growth media that hinders triglyceride solubility. The physical-chemical properties of triglycerides are therefore a critical issue to assure their availability in hydrophilic media and their ingestion by *C. elegans* in order to achieve digestion and absorption. A reported method to map the pH of the *C. elegans* intestine (Chauhan 2013) using nanoparticles suggests a potential application of nanoemulsions for the delivery of triglycerides. In addition, reported research suggests that triglycerides may be absorbed by *C. elegans* (Reisner 2011).

Based on these arguments, we hypothesized that *C. elegans* can ingest triglycerides in the form of nanoemulsions, and the nanoemulsion particle size may influence the level of ingestion. In such a way, high energy methods were used for preparing nanoemulsions with specific properties; triglyceride concentration, particle size, surfactant type and surfactant-to-oil ratio for use in *C. elegans*. The ingestion of nanoemulsions was validated by triglyceride staining, spectrometry and microscopy. In addition, fatty acid composition of *C. elegans* was determined by gas chromatography / mass spectrometry (GC/MS) and flame ignition detection (FID) in order to evaluate potential changes in fatty acid profile as a result of nanoemulsion ingestion. Life span evaluations were also carried out to evaluate potential effects of dietary triglyceride in the form of nanoemulsions.

As a result, triglyceride nanoemulsions with different particle size were ingested by *C. elegans*. The level of ingestion was dependent on both the triglyceride concentration in the growth media and the particle size of the nanoemulsion. The fatty acid composition of *C. elegans* showed no significant change after ingestion of triglyceride nanoemulsions and no significant changes were detected in life span of *C. elegans* after nanoemulsion ingestion.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioactive lipids

"Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds." (Christie 2013). Lipids can be classified in different functional categories (Akoh 2008): fatty acids, acylglycerols, sterols and sterol esters, waxes, phospholipids, ether (phospho) glycerides, glyceroglycolipids, sphingolipids, fat soluble vitamins, and hydrocarbons.

Fatty acids mostly occur in triglyceride form. Triglycerides are hydrophobic molecules comprised by three fatty acid moieties bound to one glycerol backbone. Biologically, humans may biosynthesize triglycerides or ingest them as dietary compounds that are further digested by enzymes. As a result of biochemical processes, triglycerides are stored as energy source mostly in adipose tissue.

Sterols, diacylglycerol, phospholipids and certain fatty acids are lipids that have proved to confer bioactive properties (Aluko 2012). Their effect is due to two basic properties; either their ability to modify tissue fatty acid compositions or the induction of cellular biochemical pathways (Aluko 2012). The best known bioactive fatty acids are the group of *cis*-polyunsaturated fatty acids generically named omega-3 and omega-6, which may reduce hypercholesterolemia and inflammatory conditions that lead to chronic diseases like cancer (Aluko 2012). Medium chain fatty acids (MCFAs) and their triglyceride form, medium chain triglycerides (MCTs), help reduce body weight in

humans (Tsuji 2001); and conjugated linoleic acid (CLA) have demonstrated potential benefits in energy expenditure and obesity prevention (Kim 2012).

2.1.1 MCFAs and MCTs

Due to their smaller size and relatively higher polarity, MCFAs are more hydrophilic and may be more rapidly absorbed than long chain fatty acids. MCFAs may be transported within the cell by three possible pathways (Naupert 1975); the hydrophilic route, the carrier-mediated route, and the lipophilic route. As a consequence, MCFAs and MCTs show higher metabolic rate and increased thermogenesis (Baba 1982) than long chain fatty acids or long chain triglycerides. As a matter of fact, diets rich in MCTs help may reduce fat stores (Lavau 1978) and may reduce body weight and fat compared to long chain fatty acids in humans (Tsuji 2001).

2.1.2 Conjugated linoleic acid - CLA

The CLA family includes various isomers of the conjugated octa-decadienoic acid (C18:2). They are naturally present in ruminant fat, meat and derived dairy products. CLA has been widely studied and found to be an anti-obesity bioactive compound; specifically, *trans*-10, *cis*-12 is the most bioactive isomer (Kim 2012) and enhances fatty acid beta-oxidation in skeletal muscle. This effect suggests that fat is preferentially used over glucose as an energy source in muscle during exercise (Kim 2012).

2.1.3 Octadecatrienoic fatty acid (C18:3) conjugated isomers

C18:3 conjugated isomers exhibit bioactive properties (Henessy 2011) due to the double-bond conjugation in its hydrocarbon chain. They mainly influence the promotion of apoptosis (Yasui 2006, Dulf 2013, Li 2013) and the reduction of adiposity (Nagao 2005, Chardigny 2003). These compounds are present at high concentrations in certain vegetable oils; pomegranate oil contains 9c,11t,13c-C18:3 (punicic acid); bitter melon oil and tung oil contain 9c,11t,13t-C18:3 (alpha-eleostearic acid); catalpa oil contains 9t,11t,13c-C18:3 (catalpic acid) (Nagao 2005); and calendula seed oil contains 8t,10t,12c-C18:3 (alpha-calendic acid).

Calendula seed oil is extracted from the core seed of *Calendula officinalis* and may contain 30 - 55% of calendic acid (Dulf 2013). Calendula is a well-known ornamental plant which flowers and leaf extracts are used for home treatment of digestive disorders and skin affectation. Its flower extract is also used as ingredient in cosmetics that claim skin hydration and softness; however, little is known about the bioactivity of calendic acid or calendula seed oil. A few studies on anti-carcinogenic and anti-obesity effects of alpha-calendic acid in rats (Li 2013, Shinohara 2012), and the hepatoprotective effects of calendula seed oil in human liver (Pintea 2004) have been reported. Therefore, calendula seed oil may be a potential bioactive component with, similar to CLA, energy expenditure and other effects.

2.2 *Caenorhabditis elegans* (*C. elegans*)

2.2.1 Introduction

“*C. elegans* is a self-reproducing hermaphrodite, each animal producing both sperm and eggs. The adults are about 1mm in length and the life cycle for worms grown on *Escherichia coli* is 3.5 days at 20°C” (Brenner 1973). *C. elegans* is an emerging model that presents important advantages for research in comparison to other animal models (Pellettieri 2014); practical methodology and convenience of use, large brood size, transparency, lower research costs and less complexity. Most importantly, *C. elegans* genome has approximately 72% of similarity (Table 2.1) to humans (Zhang 2013). This gene correspondence is originated in a common descending evolutionary line that shares common ancestors in the animal phylogeny between *C. elegans* and humans (ortholog genes). In particular, some lipid genes and lipid metabolic pathways are also conserved; thus, rendering *C. elegans* a suitable model for lipid evaluation.

Table 2.1: Conservation of Lipid Metabolic genes in 5 model organisms (Zhang 2013)

Organisms (lipid genes)	<i>C. elegans</i> (471) Orthologs	Human (581) Orthologs
<i>C. elegans</i> (471)	-	419(72.12%)
Human (581)	370(78.56%)	-
Mouse (585)	389(82.59%)	539(92.77%)
Rat (563)	375(79.62%)	517(88.98%)
<i>Drosophila</i> (428)	352(74.73%)	340(58.52%)

2.2.2 Developmental stages

The developmental life cycle of *C. elegans* has four stages and lasts approximately 55 hours at 20°C from the moment of egg-laying until the worms have

reached adulthood (Figure 2.1). In the initial stage, laid eggs take 9 hours for developing and hatching, giving birth to new L1 larvae. The following four development stages occur during 12 hours (L1 larvae grow to L2 stage), 8 hours (L2 larvae grow to L3 stage), 8 hours (L3 worms grow to L4 stage), and 18 hours for reaching adulthood, which is defined as the ability of egg laying.

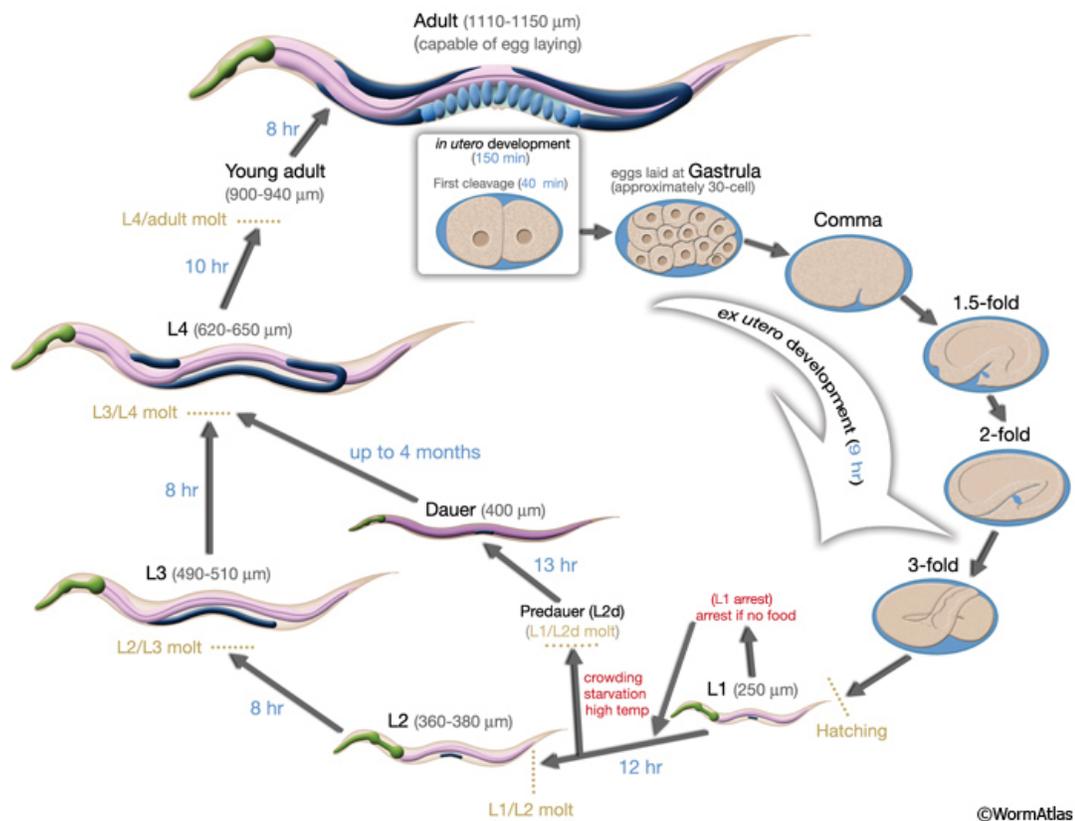


Figure 2.1: *C. elegans* life cycle. (Z. F. Altun 2002 - 2006)

The complete life cycle of *C. elegans* from laid egg to gravid adult takes 55 hours at 20°C, and the life span from adulthood can reach up to 6 weeks in the presence of food. Pre-dauer (L2d) stage can be developed as a consequence of scarce food or unfavorable environmental conditions. dauer larvae can survive up to 4 months without food by reducing their metabolic rate, and return back to L3 regular stage under favorable food and environmental conditions.

L1 Larvae is the first stage of the worms outside the eggs. During the transitions from L1 larvae to adulthood most differential changes occur in the neuronal and reproductive systems. The development from L1 larvae to L2 larvae stage occurs normally in the presence of food; however, if food or pheromone is not available or if the temperature is inadequate, L2 larvae may enter an arrested state called the *dauer* larvae, a morphologically distinct L2 stage larvae designated L2d (Z. F. Altun 2002 - 2006). L2d larvae stage is a non-aging state in which feeding is arrested and locomotion is reduced, and does not affect post-*dauer* life span. Depending on the environmental factors and availability of food, L2d larvae may remain as L2d or grow to a L3 stage.

2.2.3 Digestive system and feeding behavior

The digestive system of *C. elegans* is comprised by three main parts (Figure 2.2) (McGhee 2007): Foregut (buccal cavity and pharynx), Midgut (intestine) and Hindgut (rectum and anus). The mouth of adult *C. elegans* has six symmetrical lips surrounding its opening (McGhee 2007) and forming a circular cavity from where food is transported to the pharynx. The mouth size is between 1 and 3 μ m. *C. elegans* ingests dietary bacteria by pumping them in and concentrating them in the pharynx (Ashrafi 2007). At the exit section of the pharynx, a teeth-like structure – the grinder- breaks food particles that are then pushed into the lumen by peristaltic pharynx action. This pumping action occurs regardless of food presence; however, pumping rate is modulated by food availability (Ashrafi 2007). The pharynx pumps approximately once per second when food is scarce, but the rate increases to about four pumps per second when food is present (McGhee 2007).

In laboratory settings, *C. elegans* is fed *Escherichia coli* OP50 -*E. coli* OP50- (Solis G.M. 2011) dispersed in hydrophilic growth media. In the *C. elegans* eating mechanism, bacteria are selectively taken, concentrated, ground and transported to the intestine by the pharynx, where the liquid is expelled outside by the function of the corpus and anterior isthmus (McGhee 2007). The selectivity of *C. elegans* for *E. coli* OP50 represents a barrier for the intake of other dietary substrates like triglycerides. However, the permanent pumping-in reflex in the *C. elegans* pharynx (Ashrafi 2007) may overcome that limitation. The further step in food ingestion is the chemical metabolism in the intestinal lumen, where digestive hydrolases can be found, which activity is optimal in mildly acidic conditions (Chauhan 2013).

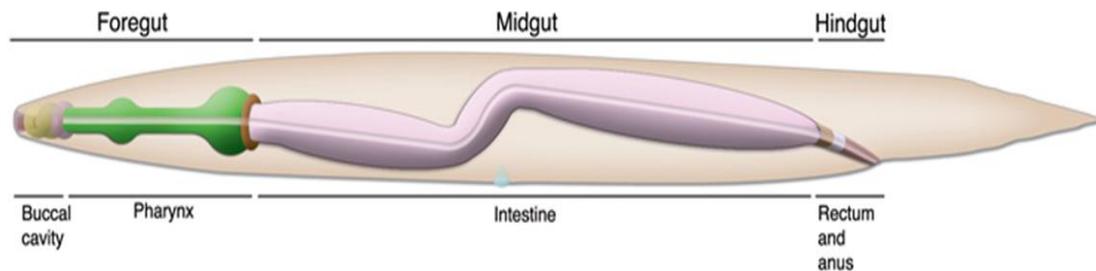


Figure 2.2: Illustration of the digestive system in *C. elegans* (Z. H. Altun 2009).

A depiction of the *C. elegans* digestive system showing the location and relative size of the Foregut, the Midgut and the Hindgut, as well as their sub-divisions. The pharynx is a major organ responsible for grinding and transporting food, as well as the disposal of non-useful nutrients to the exterior before entering the intestine. The pharynx pumps in food permanently and its pumping frequency depends on the availability of foods.

The pH of the *C. elegans* intestine is carefully regulated so that the digestive enzymes are able to breakdown proteins and carbohydrates before their rapid ejection

from the lumen. Proton pumps, exchangers and transporters, are actual regulators of *C. elegans* lumen pH located on the apical membrane of the intestinal lumen. The average pH in the *C. elegans* pharynx is reported as pH 5.35 ± 0.26 , and the average pH in their intestine is pH 3.92 ± 0.22 (Chauhan 2013).

2.2.4 Lipid metabolism in *C. elegans*

Lipids in *C. elegans* perform as energy storage, biological membrane structuring molecules, signaling molecules, and directly participate in biological processes like growth, development, reproduction, stress resistance, aging and longevity (Zhang 2013).

2.2.4.1 Lipid metabolic pathways

C. elegans conserves several human metabolic processes including fat synthesis, fat breakdown and neuroendocrine regulators of metabolism, and has 471 identified genes which seem to be involved in 16 conserved lipid metabolic pathways (Zhang 2013), some of which are listed in Table 2.2.

The adequate adjustment of energy flux through catabolic pathways (breakdown of molecules into smaller units to release energy) and anabolic pathways (construction of complex molecules from smaller units) as a response to changes in nutritional status is critical for cellular survival (Ashrafi 2007). Metabolic sensors respond to changing concentrations of fatty acids and metabolites. Three important fat metabolism sensors in *C. elegans* are *sbp-1*, *nhr-49* and *aak-1*. *sbp-1* is extensively expressed in the intestine and regulates expression of *elo-5* and *elo-6*, required for synthesis of mono methyl-branched-chain fatty acids (M. S. Kniazeva 2003). Similar to

mammalian PPAR-alpha, *nhr-49* regulates expression of fatty acid desaturation and lipid binding proteins (Ashrafi 2007), and *aak-1* is an AMPK homologue.

A group of mammalian transport protein analogues are encoded in the *C. elegans* genome (McGhee 2007). These include fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP), fatty acid binding proteins (FABPs), acyl-CoA binding proteins (ACBPs), carnitine palmitoyl transferases (CPTs) and ATP-binding-cassette (ABC). All these proteins have sequence homology to conserved components of lipid synthesis and breakdown as in mammals.

Table 2.2: Partial listing of metabolic pathways in *C. elegans* (Ashrafi 2007)

Carbohydrate metabolism	Lipid metabolism
Glycolysis/Gluconeogenesis	Lipolysis (hormone sensitive lipase)
Glycogen synthesis/Glycogen breakdown	Carnitine shuttle (fatty acid uptake)
Trehalose synthesis/Trehalose breakdown	Mitochondrial β -oxidation
Galactose metabolism	Peroxisomal β -oxidation
Fructose and mannose metabolism	Glycerol catabolism
Glyoxylate pathway	Fatty acid synthesis
Citric acid (TCA) cycle	Fatty acid elongation and desaturation
	Triacylglyceride synthesis
Energy metabolism	Phospholipids biosynthesis
Oxidative phosphorylation	Synthesis and utilization of ketone bodies
ATP synthesis	Sphingolipid and ceramide synthesis

Few studies on exogenous triglyceride metabolism in *C. elegans* are available. The effect of dietary *cis* and *trans* triglycerides in *C. elegans* was addressed by Reisner, *et.al.* (Reisner 2011). The study reported genes *acs-2*, *fat-7*, and *mdt-15*, as significantly increased by *cis*- or *trans*-C18:1n9. These results also confirmed that dietary triglycerides were metabolized by *C. elegans*.

2.2.4.2 Lipogenesis

C. elegans may endogenously synthesize fatty acids *de novo* from acetyl CoA (Figure 2.3), or may obtain dietary fatty acids from exogenous diet (J. Watts 2008). In the endogenous source the synthesis of palmitic acid (C16:0) is controlled by two multifunctional enzymes: acetyl CoA carboxylase and fatty acid synthase. Palmitic acid can be further integrated into phospholipids or be modified by elongases or desaturases to form polyunsaturated fatty acids – PUFA- (J. Watts 2008), like linoleic acid (C18:2n-6) or omega-3 (n-3) fatty acids. These PUFAs are essential and are precursors for longer-chain polyunsaturated fatty acids such as arachidonic acid (C20:4n-6) and docosahexaenoic acid (C22:6n-3).

Mammals cannot synthesize C18:2n-6 from oleic acid (C18:1n-9) nor can they produce omega-3 from omega-6(n-6) fatty acid because they lack the delta-12 and omega-3 fatty acid desaturases. On the contrary, *C. elegans* is capable of synthesizing all of its PUFAs from dietary or *de novo* palmitic acid.

Seven fatty acid desaturases (FAT-1 to FAT-7) are present in *C. elegans* for fatty acid biosynthesis (Figure 2.3) (J. Watts 2008). FAT-2 (delta12-desaturase) and FAT-3 (delta6-desaturase) are required in the hermaphrodite *C. elegans* for the generation of C18 and C20 PUFAs (Branicky 2010). In addition, one 3-ketoacyl-CoA reductase (LET-767) and fatty acid elongases (ELO-1 and ELO-2) are necessary for long-chain PUFA biosynthesis in *C. elegans* (J. Watts 2008).

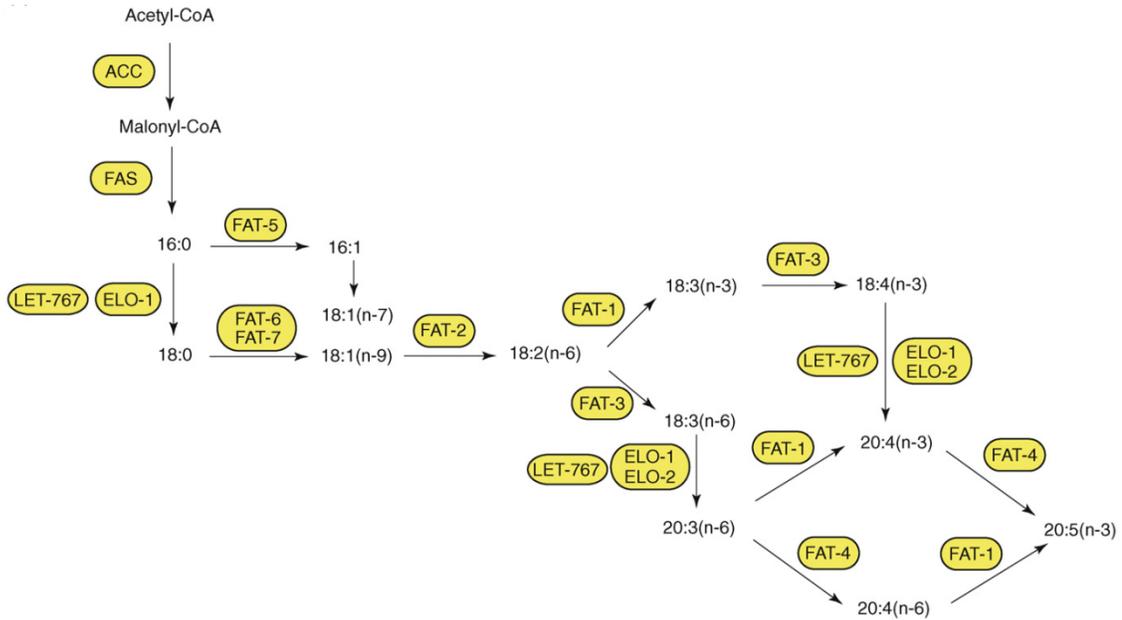


Figure 2.3: Biosynthesis of polyunsaturated fatty acids in *C. elegans* (J. Watts 2008)

Several proteins and enzymes (indicated in capital letters inside the yellow ovals) are responsible for the synthesis of polyunsaturated long chain fatty acids (C18:2, C18:3, C18:4, C20:3, C20:4 and C20:5) in *C. elegans*. This large family of proteins enables *C. elegans* to synthesize a wide range of essential fatty acids from Acetyl-CoA.

The synthesis of mono methyl-branched fatty acids (mmBCFAs) in *C. elegans* (Figure 1.4) is done by elongation enzymes *elo-5* and *elo-6*. Enzyme *elo-5* is required for production of both C15-ISO and C17-ISO, while *elo-6* is required for production of C17-ISO (Branicky 2010). Mono methyl-branched-chain fatty acids synthesized by *C. elegans* (Zhang 2013) include 11-methyl dodecanoic acid (C13:iso), 13-methyl tetradecanoic acid (C15:iso) and 15-methyl hexadecanoic acid (C17:iso). These fatty acids are essential for *C. elegans* growth (M. C. Kniazeva 2004), and have a vital role in structuring membrane-components sphingolipids, which influence signal transduction

and cell recognition. In bacteria, mmBCFAs is synthesized starting from a primer derived from leucine to form mmBCFAs acyl-CoA primers that can be elongated by fatty acid synthase (J. Watts 2008). In the case of *C. elegans*, mmBCFAs are also synthesized from a branched-chain CoA primer (Figure 2.4). In addition to mmBCFAs, two methyl substituted fatty acids are found in *C. elegans*; cis-9,10-methylene hexadecanoic acid (C17 delta), and cis-11,12-methylene octadecanoic acid (C19 delta), (Brooks 2009).

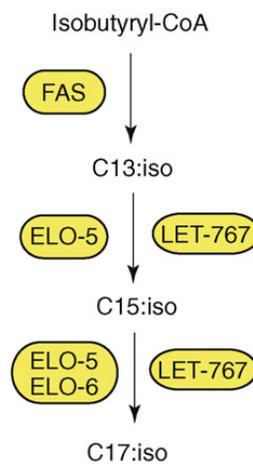


Figure 2.4: Synthesis of mmBCFAs in *C. elegans* (J. Watts 2008)

C. elegans enzymes ELO-5, ELO-6 and LET-767 synthesize mono methyl- branched fatty acids C13:iso, C15:iso and C17:iso from isobutyryl-CoA. mmBCFAs are essential for *C. elegans* growth.

2.2.5 Current methods for bioactive fatty acid evaluation using *C. elegans*

C. elegans is grown in hydrophilic media with hydrophilic components intended to assure healthy growth under controlled conditions. The election of either solid or liquid media depends on the specific interest and type of study to be carried out (Solis G.M.

2011). The hydrophilicity of the growth media hinders the evaluation of hydrophobic compounds. Therefore, *C. elegans* studies have focused on evaluating the effect of water-soluble compounds. Unlike triglycerides, free fatty acids have been extensively studied in *C. elegans* to evaluate their bioactive effects on obesity, diabetes, insulin resistance, and adiposity. These studies are usually performed by converting the fatty acids into water-soluble sodium salts (Horikawaa 2010).

The study of free fatty acids may provide valuable information about their digestion and bioactivity. However, fatty acid digestion and absorption may be different when the fatty acid is part of a triglyceride molecule. Factors like fatty acid position within the triglyceride molecule, the properties of the two neighboring fatty acids within the triglyceride, the different types of triglycerides present into the complete mix, and the hydrolysis action of digestive enzymes may influence the digestion and absorption of a bioactive fatty acid.

2.3 Nanoemulsion-based delivery systems

2.3.1 Definition

An oil-in-water (O/W) nanoemulsion is a system consisting of two immiscible liquids, with the oil (dispersed phase) being dispersed as small spherical droplets with particle sizes usually below 100 nanometers in the water as continuous phase (McClements 2004). Nanoemulsions are thermodynamically unstable (will separate over time), optically transparent below 100nm, with an intermediate surfactant-to-oil ratio (1:1) and high surface area (30m²/g).

Despite thermodynamic instability and limited scope for controlling delivery mechanisms, nanoemulsions offer several advantages (McClements 2004).

Nanoemulsions are simple and inexpensive to prepare, already used to existing technology, confer a high loading capacity (oil-to-emulsifier ratio), can be made transparent or opaque by controlling particle size, can use wide variety of ingredients to prepare and can allow control interfacial properties.

2.3.2 Stability

Physical stability of a nanoemulsion is its ability to resist changes in spacial distribution of ingredients (droplet size, droplet dispersion) over time. On the other hand, kinetic stability refers to the ability of nanoemulsions to resist changes in their properties during storage or when exposed to specific environmental conditions (McClements 2004). Since nanoemulsions are thermodynamically unstable, their physical stability principle is governed by the Stokes law; therefore, they are susceptible to gravitational separation. In order to retard gravitational separation, three key factors may be manipulated: reduction of density difference between phases, reduction of droplet size, and increase continuous phase viscosity (McClements 2004).

2.3.3 Preparation methods

Two basic methods are used for the preparation of nanoemulsions. High energy methods break liquids into smaller parts using high intensity mechanical energy (McClements 2004). Low energy methods are based on spontaneously formation of droplets due to changes in physicochemical properties of phases (McClements 2004), and

include solvent mixing and phase inversion methods. Examples of high energy methods include ultrasonic disruption (sonication), high pressure valve homogenization and micro-fluidizing.

2.3.4 Factors affecting droplet size

Homogenizer properties, component phases and emulsifier efficiency are key factors affecting droplet size. Homogenizer properties include energy Input (pressure, rotation speed, time) and maximum energy input. Component phases influence interfacial tension and viscosity, and emulsifiers influence adsorption kinetics, interfacial tension reduction and stabilization of droplets against aggregation (McClements 2004).

2.3.5 Emulsifiers

Emulsifiers play a role in both emulsion preparation and stabilization. During preparation, emulsifiers are rapidly adsorbed to the oil surface while the oil phase is disrupted into tiny droplets within the continuous phase. In addition, emulsifiers lower the interfacial tension between immiscible phases and facilitate breakup of the dispersed phase (McClements 2004). During stabilization, emulsifiers generate repulsive forces, form resistant membranes and prevent coalescence (McClements 2004).

A typical surfactant molecule is comprised by a hydrophobic tail attached to a hydrophilic head (Figure 2.5). According to the nature of the hydrophilic head, the surfactant may be classified as anionic, cationic or non-ionic. On the other hand, the hydrophobic tail may contain a single or a double hydrocarbon chain, may be saturated or unsaturated, linear or branched, and aliphatic or aromatic (McClements 2004).

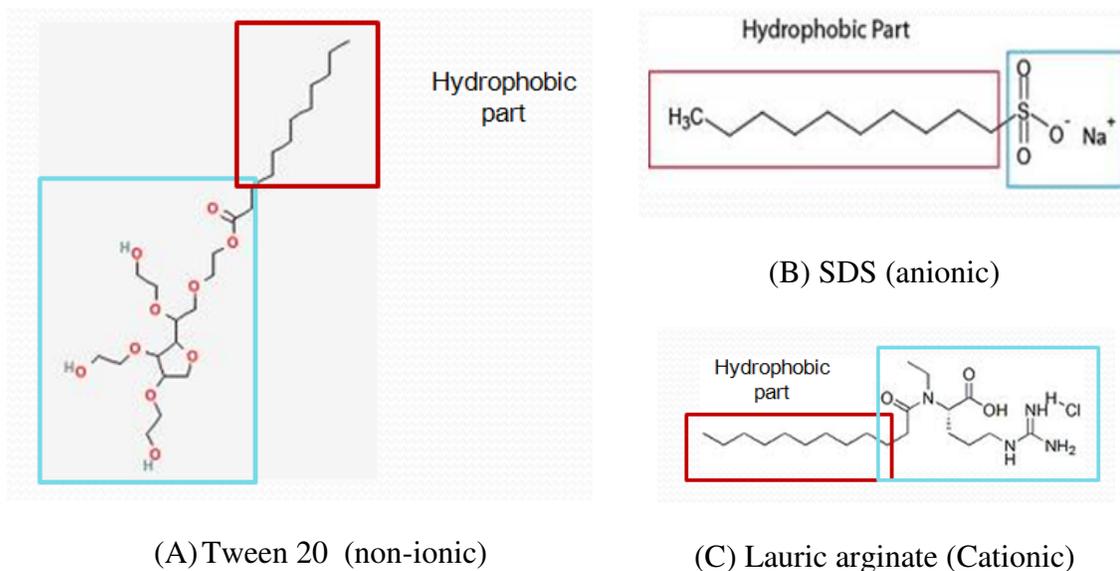


Figure 2.5: Examples of small molecule surfactants (McClements 2004)

The presence of a hydrophilic part (blue squares) and a hydrophobic part (red squares) in a small surfactant molecule enables the formation of an emulsion. Surfactants may be non-ionic (A) which do not ionize in water, or electrically charged: anionic (B) or cationic charge (C).

The properties that determine the final effectiveness of a surfactant (McClements 2004) include: solubility characteristics (solubility in oil and/or water), critical micelle concentration (CMC, Surfactant concentration where micelles first form), Cloud Point (TCP, temperature above which solution goes cloudy), Kraft Point (TKP, Temperature below which micelles do not form), solubilizing ability (ability to incorporate non-polar molecules within micelle structure) and surface activity (tendency to adsorb to

interfaces). Surfactant Properties are important to assure a stable and fine nanoemulsion (McClements 2004). The emulsifier concentration should be sufficient to cover all droplet interfaces formed. The adsorption kinetics should be sufficiently fast to prevent re-coalescence due to collisions, and should have enough protection capacity to protect against droplet coalescence (Hydrodynamic, Colloidal). In addition, emulsifiers should have low surface pressure to facilitate breakup.

CHAPTER 3

PRELIMINARY DESIGN OF NANOEMULSIONS FOR *C. elegans*

3.1 Introduction

Hydrophobicity of triglycerides represents a limitation for being used as dietary source for *C. elegans*. Potential methods to overcome that limitation are: simple mechanical dispersion of triglycerides, partial solubility of triglycerides in dimethyl sulfoxide (DMSO), triglycerides suspended into an *E. coli OP50* broth, and dispersion of triglyceride nanoemulsions in growth media. Mechanical dispersions of triglycerides into hydrophilic media, without surfactant added, may result in triglyceride separation (Reisner 2011). On the other hand, hydrophobicity of triglycerides hinders their solubility in DMSO or other hydrophilic solvents; and dispersions of triglycerides into *E. coli OP50* suspension may enzymatically modify them by the action of *E. coli* membrane lipases (McGhee 2007) before being ingested. Therefore, nanoemulsions are technically better options to deliver unmodified triglycerides to the digestive system of *C. elegans*.

Nanoemulsions are biologically suitable for delivery of triglycerides into the *C. elegans* body. Nanoemulsions have been successfully used to deliver particles into *C. elegans* to map their digestive system pH (Chauhan 2013). *C. elegans* may voluntarily ingest easy-to-eat substrates (Borisovich Shtonda 2006); therefore, easy-to-eat nanoemulsion with appropriate particle size could be ingested by voluntary action. Alternatively, the continuous pharynx pumping action of *C. elegans* may enable nanoemulsion particles to be ingested simultaneously with *E. coli OP50*. The selectivity

of *C. elegans* for *E. coli* OP50 (McGhee 2007) suggests that nanoparticle ingestion may be involuntary rather than voluntary, meaning that the particle size of nanoemulsions for *C. elegans* should be smaller enough to be involuntarily ingested by the worms. In addition, the presence of hydrolases in the *C. elegans* digestive system (Ashrafi 2007) and the conservation of fat metabolic pathways (Brooks 2009) suggest that triglycerides may undergo digestion by hydrolysis and may be absorbed (Reisner 2011).

A nanoemulsion to feed *C. elegans* should, therefore, have a particle size smaller than 3 μ m (size of the mouth of *C. elegans*), mildly neutral pH, low toxicity, and be physically stable at ambient temperature for 6 weeks, which is the time for a complete life span study. With that in mind, a study for determination of nanoemulsion properties was done by comparison of high energy methods, oil concentration, non-ionic surfactants, and SOR; and the results are reported in the present chapter.

3.2 Materials

3.2.1 Triglyceride source

Refined olive oil and crude cold pressed flax seed oil were purchased in a local store. Calendula seed oil was extracted and neutralized to final free fatty acid content 0.26% and density 0.92g/cm³, from raw seed purchased from Eden Brothers Inc (Asheville, NC).

3.2.2 Surfactants

Tween 20 (polyoxyethylene-sorbitan monolaurate) and Tween 80 (Polyoxyethylene-sorbitan monooleate) are non-ionic surfactants (Figure 3.1). Tween 20

is miscible in water (100 mg/ml) yielding a clear yellow solution, and is also miscible with alcohol. Tween 80 is a polyethylene sorbitol ester (also known as Polysorbate 80, PEG (80) sorbitan monooleate, or polyoxyethylenesorbitan monooleate), miscible in water (approx. 0.1 ml/ml) and with HLB (hydrophile-lipophile balance) value of 15.

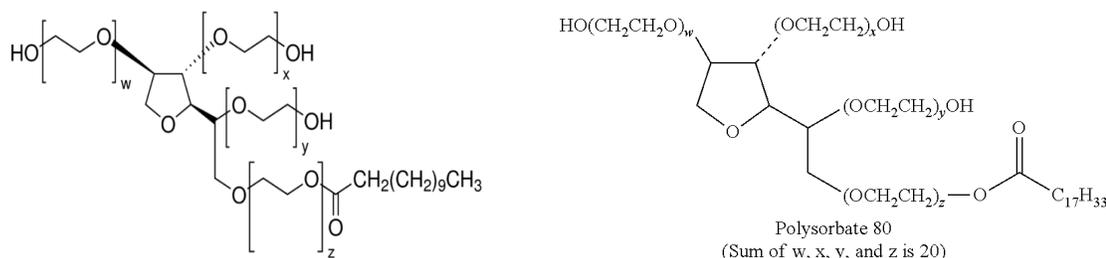


Figure 3.1: Molecular structure of Tween 20 and Tween 80

Tween 20 (left), polyoxyethylene sorbitol ester, has a molecular weight of 1,225 daltons (assuming 20 ethylene oxide units, 1 sorbitol, and 1 lauric acid as the primary fatty acid). Tween 80 (right), polyethylene sorbitol ester, has a calculated molecular weight of 1,310 daltons, assuming 20 ethylene oxide units, 1 sorbitol, and 1 oleic acid as the primary fatty acid. Source: Sigma-Aldrich product catalog.

3.2.3 Continuous phase

S-complete buffer (pH 6.4) is used for *C. elegans* growth and is composed by 98.5% water, a mix of salts (sodium chloride, potassium phosphate, potassium citrate, magnesium sulphate, calcium chloride, sodium EDTA, iron sulphate, manganese chloride, zinc sulphate, copper sulphate), and supplementary cholesterol (Stiernagle 2006).

3.3 Methods

3.3.1 Nanoemulsion preparation by high energy methods

Ultrasonic disruption (sonication) and homogenization by micro-fluidizing are high energy methods for production of stable emulsions (Yang 2012) in short times, using small amounts of surfactant, and at laboratory scale (Komaiko, 2015).

3.3.1.1 Sonication

Nanoemulsions were prepared by mixing triglycerides (oil), 200mg/ml emulsifier solution, and S-complete, in a 1.5ml tube to a final volume of 1 ml. The mix is then sonicated in a Fischer Scientific sonicator 505 with pulse 10/5 seconds ON/OFF, amplitude 30%, and variable sonication time. The samples were prepared and stored at room temperature for particle size measurement, stability determination and further use in *C. elegans*.

3.3.1.2 Homogenization by micro-fluidizing

A microfluidizer M 110L with capacity for individual batches of 70ml was used for nanoemulsion preparation. Seventy milliliter (70ml) nanoemulsion batches were prepared by initially mixing S-complete and 200mg/ml emulsifier solution to conform the continuous phase, to which triglycerides were then incorporated. The blend was then homogenized using an immersion blender and then introduced to the microfluidizer. Nanoemulsion samples were taken for different values of pressure and pass number.

3.3.2 Droplet size measurement

Particle size was determined with a Malvern® Zetasizer Nano ZS equipment. The Zetasizer Nano system measures zeta potential and electrophoretic mobility in aqueous and non-aqueous dispersions using Laser Doppler Micro-Electrophoresis. An electric field is applied to the liquid sample, which particles then move with a velocity related to their zeta potential. This velocity is measured using a laser interferometric technique (Phase analysis Light Scattering). This enables the calculation of electrophoretic mobility, the zeta potential and zeta potential distribution.

3.4 Results

3.4.1 Comparison between high energy methods

Micro-fluidizing was used for preparation of calendula seed oil nanoemulsions containing different SOR, at different homogenizing pressures in 1, 2 or 3 homogenizing passes through homogenizer. The particle size of nanoemulsions was highly dependent on a change in SOR (Figure 3.2); however, process conditions (homogenizing pressure, number of passes) did not affect significantly particle size of the nanoemulsions.

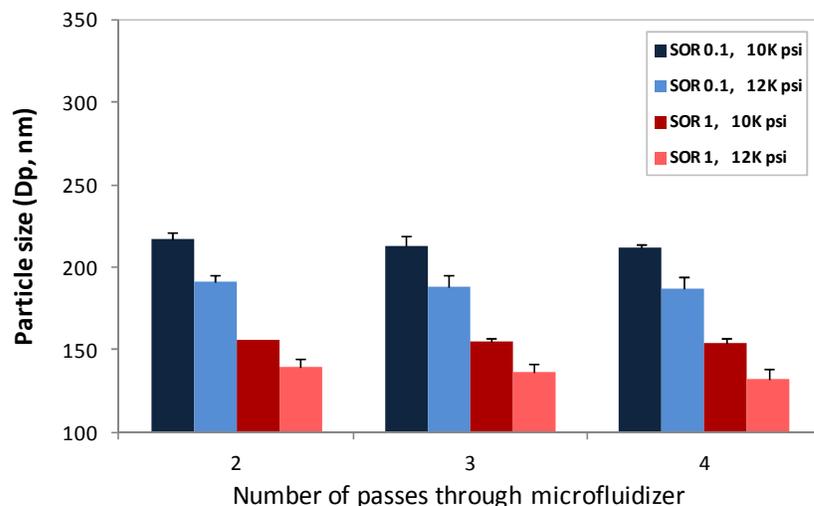


Figure 3.2: Particle size of nanoemulsions prepared by micro-fluidizing

100mM nanoemulsions were formulated with surfactant-to-oil ratio 0.1:1 and 1:1 and then micro-fluidized using two homogenizing pressures: 10,000 (10K) psi and 12,000 (12K psi). Samples were collected after 2, 3 and 4 passes of the mix through micro-fluidizer and their particle size further analyzed. Particle size was highly dependent on surfactant-to-oil ratio (SOR); the higher the SOR, the smaller the particle size. Process conditions showed a less dependent effect on particle size. Values are mean \pm standard error (n=2.)

A second group of 100mM calendula seed oil nanoemulsions with different values of SOR (0.1, 1 and 2) was prepared by sonication at different sonication times (3, 6 and 9 minutes). The particle size was highly influenced by SOR (Figure 3.3), but not significantly influenced by a change in sonication time.

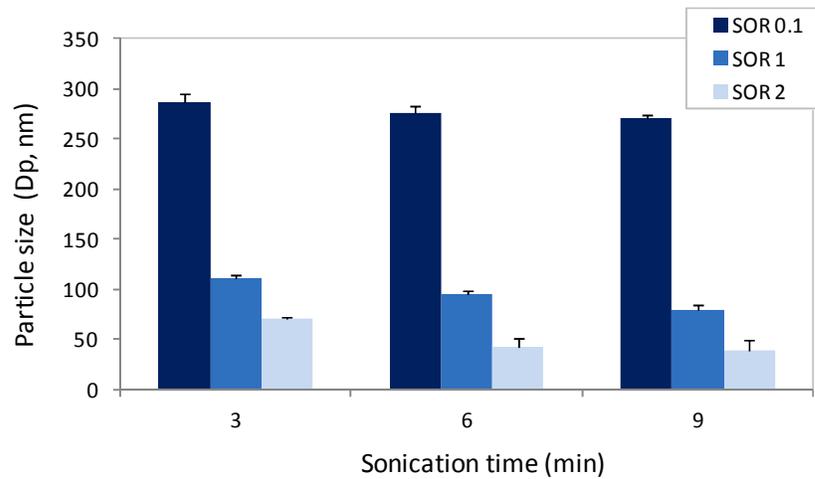


Figure 3.3: Particle size of nanoemulsions prepared by sonication

100mM nanoemulsions were prepared by sonication using three process times (3min, 6min, 9min). Nanoemulsions were formulated with three different surfactant-to-oil ratios (SOR): 0.1:1, 1:1 and 2:1. Smaller particle size was achieved with higher values of SOR in a highly dependent ratio. The influence of SOR in particle size is higher than the effect of time increase. Values are mean \pm standard error (n=2).

These results indicate that particle size is highly dependent on SOR for both methods. Conversely, process conditions (homogenizing pressure in micro-fluidizing, and time for sonication) show a less significant influence on particle size. Even though Dp is approximately 40% bigger in sonication nanoemulsions, it is still within the required range for *C. elegans*. Sonication requires smaller volumes, shorter time and generates less waste. Therefore, sonication may be a preferred method for preparing nanoemulsions with appropriate particle size for use in *C.elegans* at laboratory level.

3.4.2 Determination of nanoemulsion properties

The effect of sonication time, type of emulsifier, SOR, and oil concentration in nanoemulsion particle size was addressed in this section. Smaller particle size was

obtained using Tween 80 in comparison to Tween 20 (Figure 3.4). Smaller Dp was obtained with longer sonication time. However, when time was longer than 6 minutes no additional effect was observed. Dp was also SOR dependent (Figure 3.5) and the use of oil concentrations above 100mM rapidly increased the particle size (Figure 3.6).

These results show that Dp in the range 50 - 150nm may be achieved by sonication (6 minutes) using Tween 80 as emulsifier at SOR between 1 and 2. These conditions were used for preparing nanoemulsions for evaluation of physical stability (gravitation or agglomeration). Visual properties and Dp were monitored for six weeks (Figure 3.7) without relevant changes.

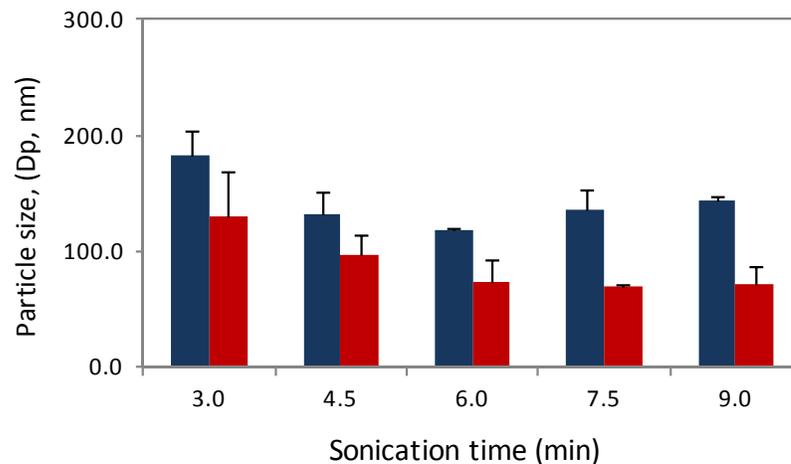


Figure 3.4: Effect of emulsifier and sonication time on particle size of nanoemulsions

Particle size (Dp) of 100mM calendula seed oil nanoemulsions prepared with Tween 20 (blue columns) or Tween 80 (red columns), surfactant-to-oil ratio 1:1, and different sonication times. Tween 80 consistently delivered nanoemulsions with smaller particle size. Sonication time longer than 6 minutes does not reduce particle size of the nanoemulsions significantly. Values are mean \pm standard deviation (n=3).

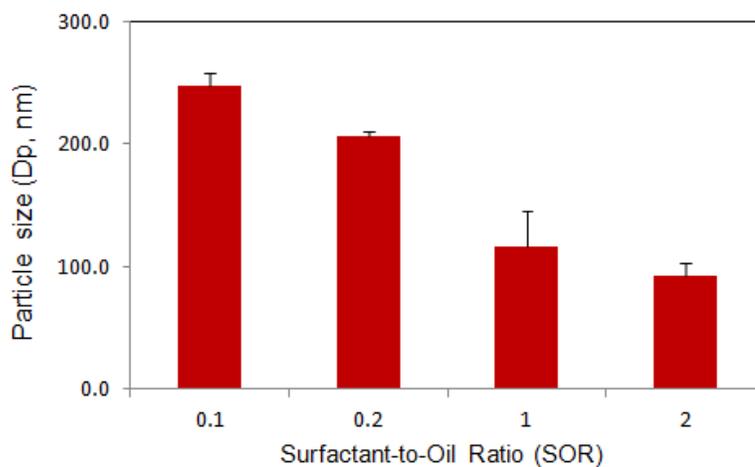


Figure 3.5: Effect of SOR on nanoemulsion particle size

100mM calendula seed oil nanoemulsions were formulated with Tween 80 at different values of SOR, and sonicated for 6 minutes. Numbers are mean \pm standard deviation (n=3).

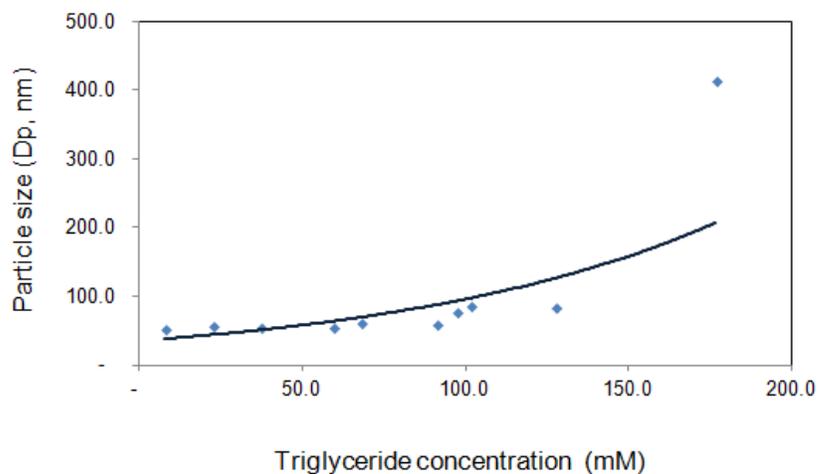


Figure 3.6: Effect of oil concentration on particle size of nanoemulsions

Nanoemulsions of calendula seed oil (blue dots) were prepared with different oil concentrations (10mM to 180mM) and Tween 80 at surfactant-to-oil ratio 1:1. The continuous line represents the fitted model for particle size of nanoemulsions as a function of oil concentration.

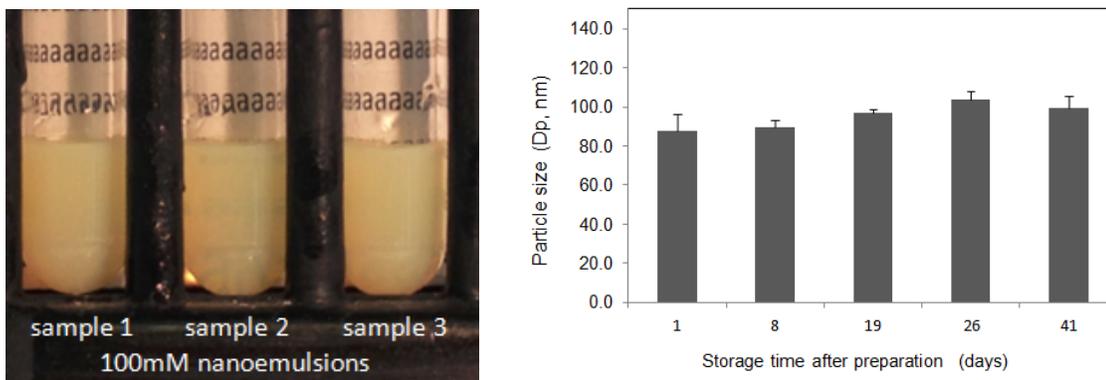


Figure 3.7: Nanoemulsion stability

Samples of 100mM calendula seed oil nanoemulsions were stored for 6 weeks at room temperature after preparation with Tween 80 at surfactant-to-oil ratio 1:1, and sonication time 6 minutes. No detectable changes were observed in physical instability (left), or major changes in particle size (right) after 6 weeks. Values are mean \pm standard deviation (n=3).

3.4.3 Use of different types of oil

The reproducibility of particle size for triglyceride nanoemulsions prepared by sonication was validated by using three different triglyceride sources (neutral calendula seed oil, crude flax seed oil, deodorized olive oil) (Figure 3.8). No significant differences were observed in particle size, which range for the three oils was 75 - 125nm. This indicates that particle size is not dependent of the triglyceride source.

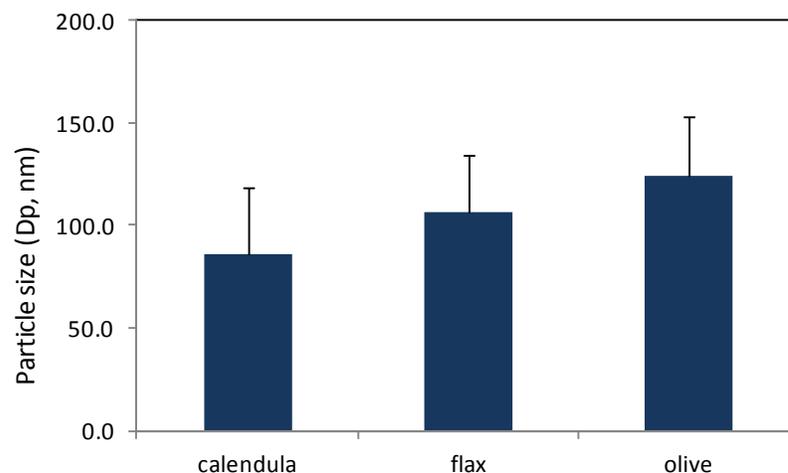


Figure 3.8: Particle size of nanoemulsions prepared with different oil types

Particle size of nanoemulsions prepared with different oils. 100mM nanoemulsions of calendula seed oil, flax seed oil and olive oil were prepared with Tween 80, surfactant-to-oil ratio 1:1 by sonication for 6 minutes. Under identical preparation conditions, no significant difference was observed in particle size between oil nanoemulsions. Numbers are mean \pm standard deviation (n=3).

3.5 Discussion

Nanoemulsions initially prepared by high energy methods (sonication and microfluidizing) showed particle size smaller than 300nm, which is less than one tenth of the mouth size of *C. elegans*. Both methods exhibited technical feasibility for the production of appropriate nanoemulsions for *C. elegans*; however, sonication implied lower cost, less complexity and less waste generation, which were more convenient factors for a practical method to prepare nanoemulsions for feeding *C. elegans* in laboratory settings. Therefore, sonication was preferred for further preparation of nanoemulsions for ingestion and fatty acid composition assays. In addition, 100mM nanoemulsions obtained by sonication under defined conditions were physically stable to aggregation or

gravitation for 6 weeks at room temperature, and their properties (particle size, stability) were achieved using different types of oils and are adequate for use in *C. elegans*.

Tween 80 and Tween 20 are surfactants widely used in research and food applications (Rao, 2012). Tween 20 at a concentration of 1% has been reported toxic for *C. elegans* (Katiki 2011). Tween 80 offers a wider range of use showing no toxicity at concentrations below 2% (Chitwood 1995) in *C. elegans*. In the present study, smaller particle size was obtained for nanoemulsions with Tween 80, which represents a lower toxicity risk in further applications.

Nanoemulsions with particle size smaller than 200nm seemed adequate for use in *C. elegans*. However, particle size maybe a critical parameter in the favorability of nanoemulsion ingestion. Therefore, the effect of particle size of nanoemulsions in the level of ingestion by *C. elegans* is evaluated in following chapters.

CHAPTER 4

INGESTION OF NANOEMULSIONS BY *C. elegans*

4.1 Introduction

The selectivity of *C. elegans* for *OP50* suggests that nanoemulsion intake would occur as a simultaneous involuntary event with *E. coli* ingestion. In such a way, particle size of dietary nanoemulsions may be critical to favor their ingestion. Nanoparticles with Dp 40nm have been used to map the pH of the digestive system of *C. elegans* (Chauhan 2013). However, the influence of particle size in nanoemulsion ingestion has not been reported. We hypothesize that the ingestion of triglyceride nanoemulsions may be favored under specific particle size of the nanoemulsions and specific oil concentrations in the growth media.

The above-mentioned hypotheses were addressed by preparing nanoemulsions with particle size within four different ranges. The oil was previously stained with oil soluble dyes and *C. elegans* worms were exposed to the four nanoemulsions at different oil concentrations on agar. The transparent body of *C. elegans* made possible to observe ingested nanoemulsion by microscopy and quantify it by spectrometry.

4.2 Materials

4.2.1 *C. elegans*

N2 Wild type *C. elegans* were purchased from the Caenorhabditis Genetics center (CGC), University of Minnesota. The worms were grown, cultured and maintained according to standard protocols (Stiernagle 2006).

4.2.2 Escherichia coli OP50

Escherichia coli are rod-shaped organisms with cell volume of $0.6\text{-}0.7\mu\text{m}^3$, $2\mu\text{m}$ long and up to $1\mu\text{m}$ diameter (Kubitschek 1990). *E. coli OP50* was purchased from the CGC, University of Minnesota, and prepared as a 100mg/ml suspension in S-complete buffer according to CGC recommendations (Stiernagle 2006) and preparation protocols (Solis G.M. 2011).

4.2.3 Nematode growth media (NGM solid agar)

Petri dishes containing agar gel were prepared under sterile conditions (Stiernagle 2006) and kept at room temperature for 24 hours. The agar gel contained 98% water, 2% of a mix of salts (sodium chloride, potassium phosphate, magnesium sulphate, calcium chloride and potassium phosphate), peptone, agar powder and cholesterol.

4.2.4 C. elegans stock solutions

Stock solutions were required to synchronize, grow and culture *C. elegans* worms: bleach solution (2.5 parts of 5% hypochlorite solution, 1 part of 10N NaOH and 6.5 parts of sterile water); M9 buffer, S-complete; and LB broth (mix of Bacto-tryptone, Bacto-yeast, NaCl, water, and 1.0N NaOH (pH 7.3)).

4.2.5 Triglyceride source

Crude cold pressed flax seed oil was purchased in a local store.

4.2.6 Surfactant

Tween 80 (previously described in chapter 2) was used as nanoemulsion surfactant. The emulsifier is prepared as a 200mg/ml solution of S-complete buffer.

4.2.7 Dyes

Sudan red (amethoxybenzenazo- β -naphthol) is a non-fluorescent lipid dye (Figure 4.1) used in histological visualization of neutral fat. In this study Sudan red was used for staining of flax seed oil and further optical microscopy for observation of stained oil inside *C. elegans*. Nile red (9-diethylamino-5-benzo[α]phenoxazinone) is a fluorescent dye commonly used to localize and quantitate lipids. Nile red is almost non-fluorescent in water, but is fluorescent with large absorption and emission in non-polar environments (excitation at wavelength 520nm, maximum emission at wavelength 600nm).

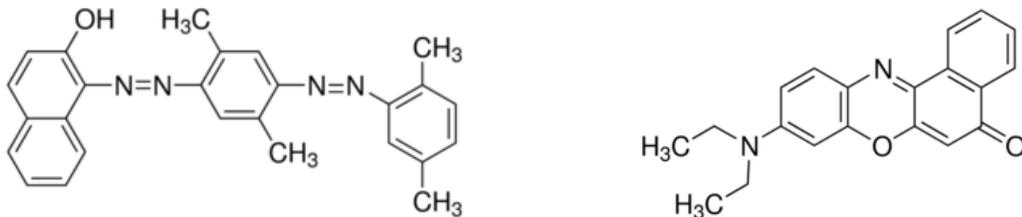


Figure 4.1: Sudan red and Nile red chemical structures

Sudan red (left) and Nile red (right) were used to validate the ingestion of nanoemulsions by *C. elegans* based on the transparency of the worm body, and the fluorescence of Nile red. The dyes were used at a concentration of 1 mg/ml respectively to stain oil, prepare nanoemulsions and observe the red color and measure fluorescence through the *C. elegans* bodies.

4.2.8 Anesthetizing solution

Sodium azide (NaN_3) is prepared as stock solution in M9 buffer, at a concentration of 20mM. The solution was used for worm immobilization for fluorescence and microscopy.

4.3 Methods

4.3.1 Oil staining method

10mg of dye were dissolved in 1ml of methanol in a 1.5ml tube and vortexed until complete dissolution. The solution was diluted in 9ml of water, homogenized, and then mixed with 10 ml of oil. The mix was vortexed and shaken for 5 minutes in order to transfer the dye from the aqueous phase to the oil phase. The mix was centrifuged at 3000rpm for 3 minutes and the oil phase was recovered. The colorless water phase indicated that all the dye had been transferred to the oil phase, which contained a final dye concentration of 1mg / ml.

4.3.2 Nanoemulsion preparation

A group of four 100mM flax seed oil nanoemulsions, with different particle size in the range of 40nm to 800nm, were prepared by sonication. A Fischer Scientific sonicator model 505 with a probe for 1ml was used. SOR and sonication time were variables in order to obtain a group of nanoemulsions with different particle size. The nanoemulsions were kept in dark conditions at room temperature to prevent dyes from degradation. The four nanoemulsions were diluted in S-complete by serial dilution (1:10) in order to evaluate a set of 4 concentrations of oil on the agar: 100mM, 10mM, 1mM,

0.1mM. Nanoemulsions containing Sudan red were used for optical microscopy while nanoemulsions containing Nile red were used for evaluation of nanoemulsion ingestion by fluorescence and confocal microscopy.

4.3.3 Droplet size measurement

Particle size was determined with a Malvern® Zetasizer Nano ZS equipment, as previously described in chapter 3.

4.3.4 Nanoemulsion ingestion assay on solid media

200 µl of 100mM nanoemulsion were homogeneously dispersed onto the agar surface of individual NGM plates and air-dried overnight. A layer of 150µl of *E. coli* *OP50* solution was then dispersed over the surface of these treatment plates and air-dried for 24 hours. L1 starved larvae worms were then transferred to treatment and control plates according to existing protocols. Worms were grown to adulthood for 72 hours.

4.3.5 Determination of nanoemulsion ingestion by spectrometry (fluorescence)

Adult worms from treatment and control plates were washed off and transferred to individual 1.5 ml tubes containing 1mL of NaN₃ 20mM solution. The mix was centrifuged (3000 rpm for 3 minutes), supernatants were discarded and the pellets were rinsed five times with NaN₃ solution and centrifuged until the supernatants were clear. A final volume of 400µl of NaN₃ solution was added to the pellets. The concentration of adult worms per tube was determined by counting the worms present in a 10µl droplet sample, and then adjusted to 0.3 worms per µl by addition of NaN₃ solution to each tube.

Tubes were centrifuged and 300µl of supernatant were individually measured as blank in triplicates (100µl each). The remaining samples in the tubes were homogenized and measured in triplicates (100µl each) in the same plate.

Fluorescence was read from 100µl samples in individual wells of a black-wall 96-well plate using a SpectraMax spectrophotometer. The equipment was set up for top-reading well-scan full mode fluorescence, and wavelengths 520nm (excitation), and 600nm (emission) according to absorbance maxima of Nile red. The final value of RFU /worm was calculated as:

$$\text{RFU / worm} = [(\text{rfu of sample}) - (\text{rfu of blank}) / \text{number of worms}]$$

The average of the triplicates for each particle size and oil concentration was reported as RFU/worm.

4.3.6 Microscopy

Microscopy observations were done on immobilized adult *C. elegans* worms from Sudan red treatment plates (optical) and Nile red plates (confocal) at different oil concentrations on the agar and for different particle size of nanoemulsions. The transparency of the body of *C. elegans* allowed to confirm the presence of stained triglycerides within their digestive system and internal organs. Confocal microscopy was done using a Nikon Eclipse 80i SOP confocal microscope, and optical microscope using an Olympus 40SZ optical microscope.

4.3.7 Synchronizing of *C. elegans* populations in liquid media

L1 starved larvae were chunked from a *C. elegans* stock plate and transferred to fresh NGM plates containing a lawn of *E. coli OP50* (Solis G.M. 2011). The fresh plates were incubated for 65 hours at 20 °C until the majority of the worms were gravid adults and enough eggs had been laid on the agar. Eggs and worms were then collected by washing them off the NGM plates with sterile water. The worm/water solution was transferred into 15ml conical tubes and the worms were washed twice with water by centrifuging for 2 min at 3500 rpm. In order to destroy the worm bodies and recover the eggs, freshly prepared bleach solution was added to the pellets. The mix was vortexed for 4 minutes and then centrifuged at 3500 rpm for 2 minutes. The pellets were then washed three times with M9 buffer and once with S- complete by centrifuging for 2 minutes at 3500 rpm. 10ml of S-complete were added to each pellet and the solution was gently agitated for 24 hours. When the eggs had hatched, the concentration of worms/ml was adjusted to 500worms/ml by addition of S- complete. Concentrated *E. coli OP50* dispersion was added to each tube and the solution was stored for 48 hours until worms reached adulthood.

4.3.8 Nanoemulsion ingestion assay in liquid media

A population of synchronized *C. elegans* worms at a concentration of 500 worms/ml in S-complete buffer was distributed in a 12-well transparent plate (1ml of worm solution per well). Six wells contained control worms (fed *E. coli OP50*) and the other six wells contained treatment worms (fed *E. coli OP50* and 100mM flax seed oil

nanoemulsion at a final concentration of 10mM oil in the medium). Sterilizing reagents and antibiotics were also added to each well according to standard protocols (Solis G.M. 2011). The plate was stored at room temperature and monitored for six days. Then, the worms were transferred to 1.5ml individual tubes and centrifuged. The pellets were washed four times with distilled water to remove nanoemulsion and *E. coli* and were observed under the optical microscope.

4.4 Results

4.4.1 Determination of level of ingestion by fluorescence

Figure 4.2 shows the results of intensity of fluorescence, expressed as RFU/worm, and calculated as the mean of four independent experiments. The values for each treatment, (one concentration and one particle size) were obtained from triplicates. RFU/worm results showed a direct correlation between RFU/worm and oil concentration of the nanoemulsion, which is more evident at oil concentrations above 10mM. In addition, a correlation between RFU/worm and particle size of the nanoemulsion was also found. These results suggest that ingestion of smaller droplets do not provide enough oil, thus, enough Nile red, to be detected. Alternatively, *C. elegans* were more selective for bigger particles for ingestion. Figure 4.2 shows the results of fluorescence (expressed as RFU / worm) calculated as the mean of results of four independent experiments, and each result was obtained from three samples. Worms were treated with nanoemulsions at four oil concentrations on the growth media, and four different particle sizes per experiment.

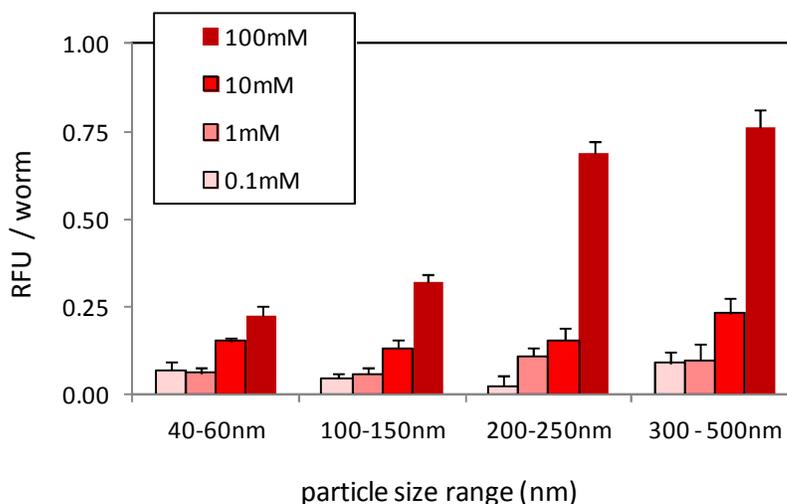


Figure 4.2: Fluorescence (RFU/worm) as a function of particle size

Intensity of fluorescence is expressed as relative fluorescence units (RFU) divided by the number of worms (RFU/worm). Fluorescence was determined by spectrometry and each sample in triplicates. Wavelength set at 520nm (excitation) and 600nm (emission) with Nile red as fluorescent dye. Highest values at concentrations 10mM - 100mM and Dp 200 - 500nm indicate highest ingestion of nanoemulsion by *C. elegans* in those intervals. Values are mean \pm standard error (n=4).

The same values of RFU/worm expressed referred to control (worms fed only OP50 without nanoemulsions) was calculated as:

$$[(\text{RFU/worm}) \text{ for treatment worms}] / [(\text{RFU/ worm}) \text{ for control worms}],$$

where RFU/worm for control was 0.09. Data are shown in Table 4.1. Worms grown on oil concentration 100mM and Dp 200 - 500nm exhibit 8 times higher fluorescence than control worms. However, at the same concentration, the intensity reduces around 50% in

worms grown on agar containing nanoemulsions with Dp 40 – 150nm. Worms grown on 10mM treatment plates were two-fold more fluorescent than control, and samples from 1mM and 0.1mM show similar fluorescence as the control.

Table 4.1: Values of relative intensity of fluorescence RFU referred to control

oil concentration on agar	Particle size range (Dp, nm)			
	40 to 60	100 to 150	200 to 250	300 to 500
100	2.5	3.5	7.7	8.5
10	1.7	1.5	1.7	2.6
1	0.0	0.0	1.2	1.1
0.1	0.8	0.0	0.0	1.0

4.4.2 Validation by microscopy

4.4.2.1 Confocal microscopy

Worms from treatment plates containing Nile red were transferred to agar pads, immobilized and recorded by confocal microscopy. The worms revealed different fluorescence intensity in their bodies due to differences in the amount of Nile red ingested in each treatment. Results showed a direct dependence of Nile red intensity with oil concentration and particle size (Figure 4.3), which confirms the findings from fluorescence spectrometry.

Figure 4.3 shows representative samples of immobilized worms from treatments plates at different concentrations of oil (keeping particle size constant), or different particle size (keeping oil concentration constant). The worms were immobilized after

more than 90% of the initial L1 population reached adulthood. The more intense Nile red areas are observed at the exit of the pharynx and entrance to the intestine.

The results of confocal microscopy confirm that at concentrations above 10mM the ingestion of nanoemulsion by *C. elegans* is higher. Similarly, ingestion increases at particle size between 200 and 500nm.

4.4.2.2 Optical microscopy

Worms from Sudan red treated plates were transferred to agar pads, immobilized and observed by optical microscopy after 60 hours of L1 transference, and 90% of the population having reached adulthood. A worm sample from the 100mM treatment plate (Figure 4.4) suggests that nanoemulsion is ingested and that the oil is delivered at the entrance of the intestine. The red colored areas aside the intestine suggests that the oil was digested and absorbed.

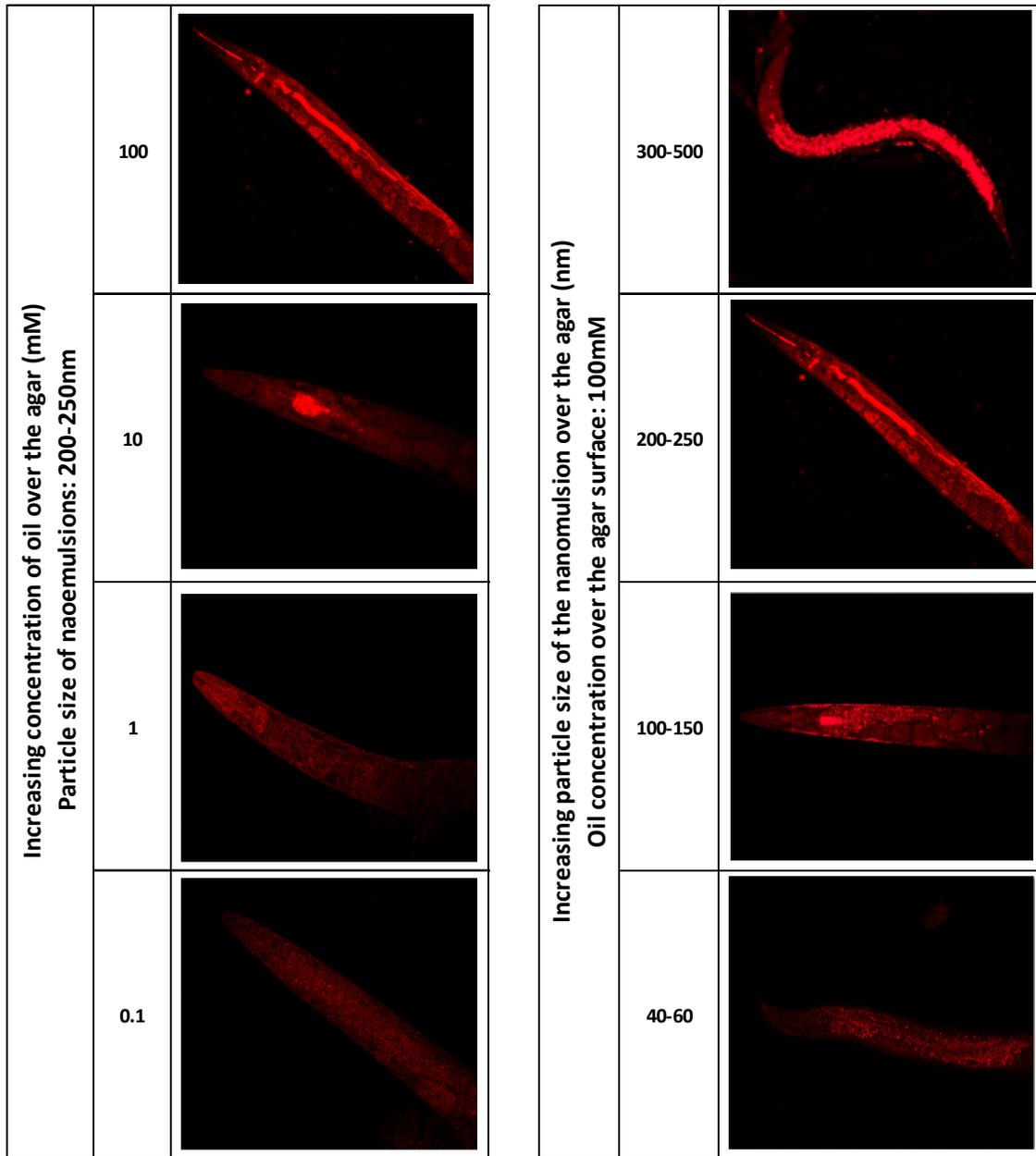


Figure 4.3: Confocal microscopy worm images

Worms, grown on agar plates containing nanoemulsions with different oil concentrations and different particle size, were collected after 72 hours. Nile red had been previously added to oil at a concentration of 1mg/ml. A direct correlation between oil concentration and ingestion of nanoemulsion (left) was determined by the intensity of fluorescence of Nile red inside the worms. There was also a direct correlation between nanoemulsion ingestion and particle size (right).

Representative worm samples from treatment plates containing different oil concentrations and different particle size of the nanoemulsions (Figure 4.4) were observed under the optical microscope. The reddish areas indicate the presence of Sudan red, initially present in the oil delivered to *C. elegans* as nanoemulsion. Different intensities of red color indicate different amount of nanoemulsion ingested by the worms. Similar to the results form confocal microscopy, the intensity of red (ingestion of nanoemulsion) is directly correlated with oil concentration and particle size.

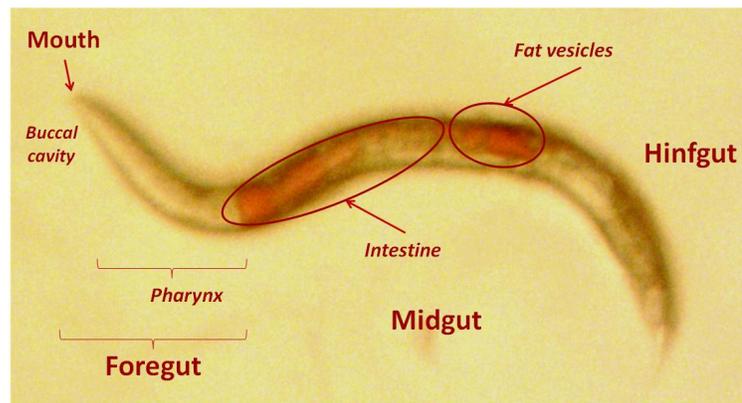


Figure 4.4: Stained triglycerides delivered and ingested within *C. elegans* digestive system

Optical image of an adult *C. elegans* worm from an NGM plate containing 100mM flax seed oil as nanoemulsion. The internal red color suggests that the oil is released at the entrance of the intestine and is then absorbed.

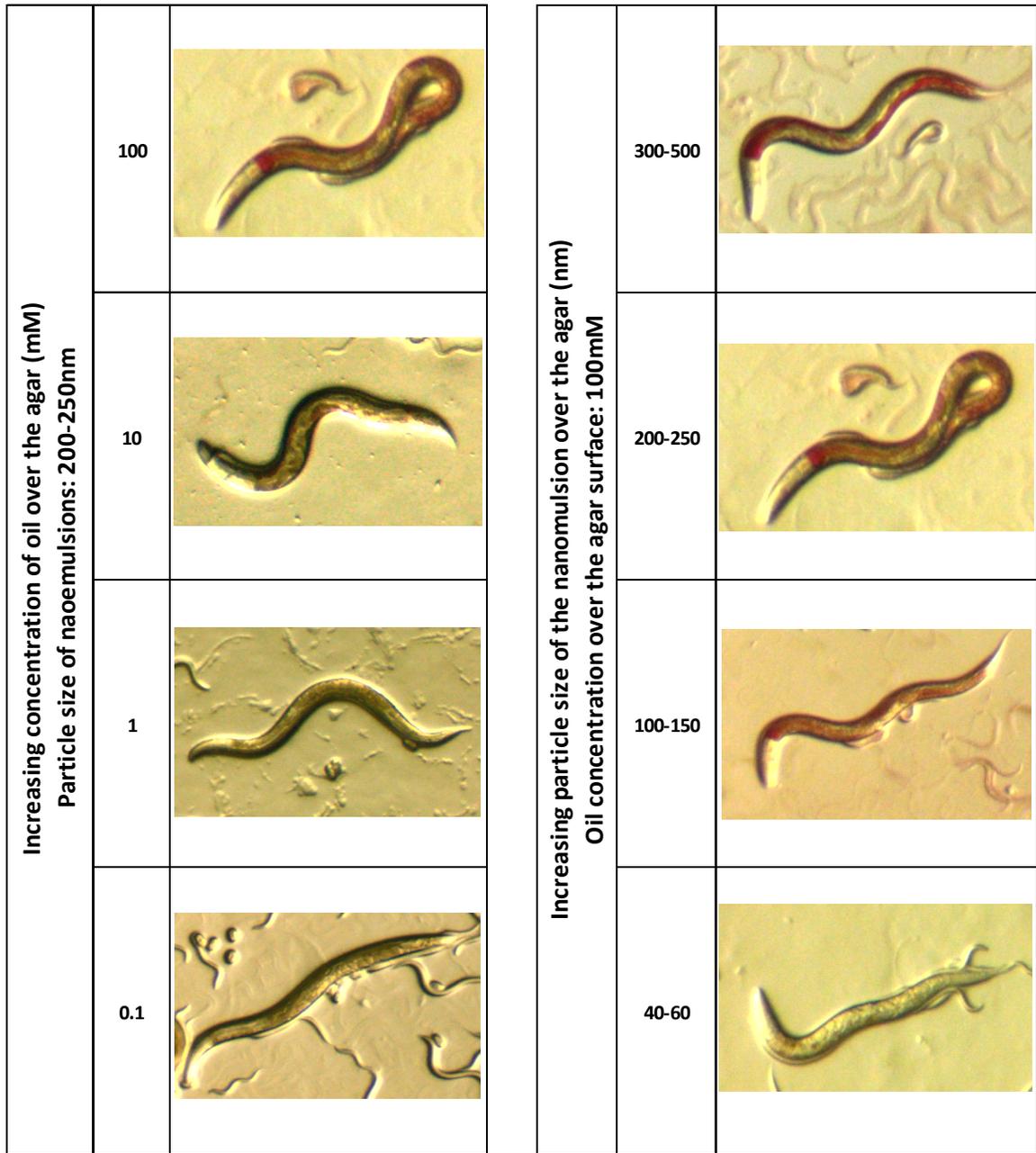


Figure 4.5: Optical microscopy worm images

Worms, grown on agar plates containing nanoemulsions with different oil concentrations and different particle size, were collected after 72 hours. Sudan red (non-fluorescent dye) had been previously added to oil at a concentration of 1mg/ml. A direct correlation between oil concentration and ingestion of nanoemulsion (left) was determined by the intensity of red color due to Sudan red inside the worms. There was also a direct correlation between nanoemulsion ingestion and particle size (right).

4.4.3 Nanoemulsion ingestion in liquid media

Worms were treated with 100mM nanoemulsion at a final concentration of 10mM of oil into the liquid media and compared with a no fat control. The oil was previously stained with Sudan red for further optical microscope observation. After three days of treatment, the worms were collected and washed off. Optical microscope observations did not show the presence of Sudan red inside the *C. elegans* bodies. Unlike control worms, the pellets of treated worms after centrifugation exhibited a pink color. This suggests that, alternatively, the pink color may be due to dye diffusion through the worm bodies without ingestion of the nanoemulsion or is not enough to detect.

4.5 Discussion

The indirect determination of triglyceride ingestion, through the measurement of Nile red fluorescence by spectrometry, indicated that triglyceride nanoemulsions were ingested in a wide range of particle size and at different levels of oil concentration on the growth media; specifically, more ingestion was observed oil concentrations 10 - 100mM and particle size 200 - 500nm. This is an indication that under similar pharynx pumping rate conditions, *C. elegans* ingested bigger amounts of triglyceride from nanoemulsions with bigger particle size. In addition, higher concentrations of oil rendered nanoparticles more available for ingestion.

These nanoemulsions were prepared using SOR 0.5:1.0 and sonication time 6 minutes. The particle size limit was found above 500nm, where the emulsions became unstable, separated on the agar and increased worm mortality by asphyxia was observed (Figure 4.6)



Figure 4.6: nanoemulsion separation for $D_p > 500\text{nm}$

Mortality of worms by asphyxia was evidenced after a few minutes of exposure to nanoemulsion ($D_p > 500\text{nm}$) on the growth media. The grey areas show separated aqueous phase, the pink areas are separated oil containing Nile red. Nile red stained oil can also be seen inside the intestine of this *C. elegans* sample, which evidences nanoemulsion ingestion.

CHAPTER 5

EFFECT OF TRIGLYCERIDE NANOEMULSIONS IN FATTY ACID COMPOSITION

5.1 Introduction

C. elegans synthesizes all of its unsaturated fatty acids *de novo* from acetyl CoA (Figure 2.3), or may obtain dietary fatty acids from exogenous diet (J. Watts 2008). Synthesized or exogenous palmitic acid can be modified by elongases and/or desaturases to form C18:2 and C18:3 fatty acids (J. Watts 2008) which are essential and precursors for longer-chain polyunsaturated fatty acids. The reported basic fatty acid composition of *C. elegans*, fed *E. coli* OP50, contains primarily 27% to 36% of carbocyclic and monomethyl branched-chain (mmBCFAs) fatty acids (Brooks 2009, Watts 2008), 21% vaccenic acid (C18:1n-7) (Brooks 2009) or 24% of total unsaturated C20 fatty acids (C20:n) (J. Watts 2008). The remaining FA content is comprised by 8 - 9% oleic acid (C18:1), 5 - 7% linoleic acid (C18:2), 4 - 7% stearic acid (C18:0) and 4 - 7% palmitic acid (C16:0) (Brooks 2009, Watts 2008).

This study was aimed to determine whether dietary triglyceride delivered as nanoemulsions were absorbed by *C. elegans*. We used calendula seed oil to determine if ingested emulsion can modify fatty acid composition in *C. elegans* by determining the presence of conjugated C18:3 (8t,10t,12c-Octadecatrienoic acid, calendic acid).

5.2 Materials

5.2.1 Triglyceride source

Olive oil (70%, C18:1n9) was purchased in a local store. Calendula seed oil was extracted and neutralized (free fatty acid content 0.26%, density 0.92g/cm³) from raw seed purchased from Eden Brothers Inc. (Asheville, NC). Calendula seed oil contained 50% of alpha calendic acid determined by GC MS.

5.2.2 *C. elegans* fat extraction and methylation reagents

Chloroform, methanol and potassium chloride were purchase from Sigma Aldrich, and were used for fat extraction from *C. elegans* according to the Folch method (Folch 1957). Hexane and 3N Methanolic HCl were purchased form Sigma-Aldrich (Saint Louis, MO) and used for methylation of fatty acids.

5.3 Methods

5.3.1 Nanoemulsion ingestion on solid media

100mM nanoemulsions of calendula seed oil and olive oil were prepared by sonication (6 minutes) and Tween 80 at SOR 0.5 as surfactant ($D_p = 220 \pm 40$ nm) and used in treatment groups in comparison to a no fat control group. The oil percentage in a 100mM nanoemulsion is 8.7%, and the concentration of Tween 80 in the same nanoemulsion is 0.043mg/ml (SOR 0.5). Therefore, A Tween 80 treatment group was included as a blank by dispersing 0.043g/ml Tween 80 solution over each agar plate.

A layer of 150µl of *E. coli* OP50 solution was dispersed on the surface of individual agar plates and dried overnight. 200µl of nanoemulsion or Tween 80 solution

alone were then dispersed over the OP50 layer and air dried. After 24 hours, *C. elegans* L1 larvae were transferred to the prepared plates and left for 60-72 hours until they reached adulthood. A total number of 5 plates per treatment or control were prepared in order to obtain enough adult worms for further fat extraction.

5.3.2 Fat extraction

Worms were transferred from Petri plates to individual 1.5 ml tubes and washed five times with distilled water. Centrifugation at 2500 RPM for 3 minutes was applied after each rinsing step. The supernatants were discarded and worm pellets suspended in 800 μ l of distilled water were sonicated (4-5 minutes, amplitude 30%) prior to extraction. The sonicated mix was transferred to glass tubes and 2ml of extraction solution (2 parts of chloroform per part of methanol) were added to each sample (Folch 1957). The samples were vortexed and shaken thoroughly for 20 minutes and then centrifuged at 3000 RPM for 5 minutes. The bottom layer containing chloroform and lipids was recovered in a glass vial and washed once with 1ml 5% KCl solution and then with 1ml distilled water. The chloroform-lipid bottom layer was recovered and dried out with nitrogen.

5.3.3 Methylation

Fatty acid methyl esters (FAME) were prepared by addition of 1 ml of 3N methanolic HCl to lipids in glass tubes. The solution was kept at 55-60°C for 20 minutes with thorough shaking and vortexing every 5 minutes. Then, 1 ml of water and 0.5 ml of hexane were added to the mix, and the mix was gently vortexed for 10 seconds and then

centrifuged at 3000 RPM for 5min. The upper layer containing hexane and lipids was transferred to GC glass vials and kept at -20°C for further GC analysis.

5.3.4 Fatty acid determination

The fatty acid composition was determined by FAME analysis using a Supelcowax 10 (fused silica) 100m x 0.25mm x 0.25µm column. The analysis was carried out in Shimadzu GC-17A equipment coupled to an FID detector. Oven conditions: initial temperature 50°C, 20°C/min to 190°C, holding 30 minutes, 10°C/min to 220°C, and 60 minutes holding, for a total run time of 180 minutes. Injector temperature 250°C, detector temperature 250°C, carrier gas: Helium, and split ratio: 3:1. The identification of fatty acids was carried out either comparing with fatty acid standards or by GC/MS (6890N Agilent GC equipment coupled to a 5973 Agilent mass selective detector). The mass spectra of fatty acid methyl esters were further compared with the AOCS mass spectra data files (AOCS lipid library).

5.4 Results

5.4.1 *C. elegans* fatty acid composition

Twenty six fatty acids were identified within the fatty acid composition of wild type *C. elegans*, fed *E. coli* OP50. Table 5.1 summarizes the mean values for each fatty acid and group from 6 independent experiments. The major components of the fatty acid composition are: 23.8% carbocyclic fatty acids (C17:delta and C:19 delta), 23.2% vaccenic acid (C18:1c11), 13.54% of unsaturated C20:n isomers, 8.5% mmBCFAs (C15:iso and C17:iso), linoleic, oleic, palmitic and stearic acids.

Table 5.1: Total fatty acid composition of wild type *C. elegans*

	<i>Fatty acid (IUPAC name)</i>	<i>abbreviated formula</i>	<i>Fatty acid content (%) *</i>		
Saturated fatty acids:					
a	Dodecanoic & short chain F.A.	<C12:0	0.05	±	0.02
b	Tetradecanoic acid	C14:0	1.48	±	0.10
e	Hexadecanoic acid	C16:0	5.01	±	0.36
	Heptadecanoic acid	C17:0	0.22	±	0.04
i	Octadecanoic acid	C18:0	4.50	±	0.17
	Nonadecanoic acid	C19:0	0.77	±	0.12
m	Eicosanoic acid	C20:0	0.10	±	0.03
	<i>total SAFA:</i>		<i>12.13</i>		
Monomethyl branched-chain fatty acids.					
c	9-Methyltetradecanoic acid	C15:iso	4.21	±	0.19
g	Hexadecanoic acid - 15-methyl	C17:iso	4.30	±	0.13
	<i>total mmBFAs:</i>		<i>8.52</i>		
Carbocyclic fatty acids:					
h	Cyclopropaneoctanoic acid, 2-hexyl	C17:delta	16.95	±	0.75
l	Cyclopropaneoctanoic acid, 2-octyl	C19:delta	6.85	±	0.50
	<i>total carbocyclic FAs:</i>		<i>23.80</i>		
Unsaturated fatty acids (except C20:n):					
	11-Tetradecenoic acid (Z)	C14:1	0.30	±	0.05
f	11-Hexadecenoic acid	C16:1	3.12	±	0.15
	9-Octadecenoic acid (Z)	C18:1 (9c)	4.99	±	0.42
j	11-Octadecenoic acid (Z)	C18:1 (11c)	23.26	±	0.74
	8-Octadecenoic acid (E)	C18:1 (8t)	0.12	±	0.03
k	9,12-Octadecanoic acid	C18:2 (9,12)	6.99	±	0.20
	10,13-Octadecadienoic acid	C18:2(10,13)	0.14	±	0.03
	9,12,15-Octadecatrienoic acid (Z)	C18:3 (9c,12c,15c)	0.33	±	0.13
	<i>total unsaturated FAs:</i>		<i>39.26</i>		
n	Unsaturated C20:n isomers:				
	11-Eicosenoic acid	C20:1(11c)	0.68	±	0.12
	11,14-Eicosadienoic acid (Z)	C20:2 (11c,14c)	1.54	±	0.27
	8,11,14-Eicosatrienoic acid (Z)	C20:3 (8c,11c,14c)	1.81	±	0.05
	5,8,11,14-Eicosatetraenoic acid (Z)	C20:4 (5c,8c,11c,14c)	0.41	±	0.10
	11,14,17-Eicosatrienoic acid (Z)	C20:3 (11c,14c,17c)	0.32	±	0.15
	8,11,14,17-Eicosatetraenoic acid (Z)	C20:4(8c,11c,14c,17c)	2.16	±	0.22
	5,8,11,14,17-eicosapentaenoic acid (Z), EPA	C20:5 (5c,8c,11c,14c,17c)	6.63	±	0.15
	<i>total C20:n isomers:</i>		<i>13.54</i>		
Un-identified fatty acids:					
	<i>total un-identified FAs:</i>		<i>2.76</i>		

* values are mean ± standard error, (n=6)

A representative chromatogram of total *C. elegans* fatty acid methyl esters is shown in Figure 5.1, indicating the elution order and abundance for each methyl ester.

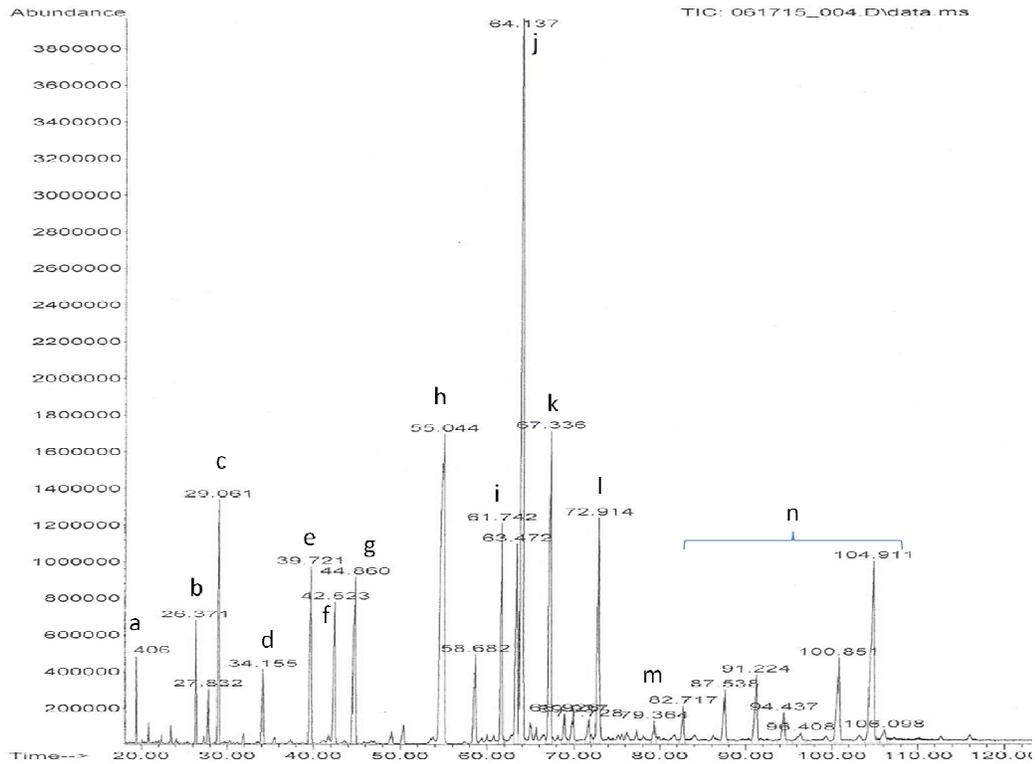


Figure 5.1: Gas chromatogram of *C. elegans* fat

The fatty acid composition of *C. elegans* is mainly comprised by vaccenic acid (j), Carbocyclic fatty acids C17: δ (h) and C19: δ (l), mono methyl-branched fatty acids C15:iso (c) and C17:iso (g), a group of unsaturated C20:n fatty acid isomers (n), Palmitic acid (e), oleic acid and stearic acid (i). The Figure is representative of 6 independent experiments. The analysis was carried out in 6890N Agilent GC equipment coupled to a 5973 Agilent mass selective detector. Column: Supelcowax 10 (fused silica), 100m x 0.25mm x 0.25 μ m. Oven conditions: initial temperature: 50°C; 20°C/min to 190°C, 30 min holding, 10°C/min to 220°C, 60 min holding. Injector: 250°C, detector: 250°C, carrier gas Helium, split ratio 3:1.

5.4.2 Effect of dietary triglyceride nanoemulsions in fatty acid composition

5.4.2.1 Effect of Tween 80

Tween 80 is a polyethylene sorbitol ester which molecule contains 1 unit of oleic acid per 20 units of ethylene oxide. Its fat composition is comprised by approximately 60% of oleic acid, and the remaining percentage linoleic, palmitic and stearic acids. Tween 80 has been reported to significantly increase C16:0, C16:1, C18:0 and C18:1 fatty acids in *Thraustochytrium aureum* when used at concentrations of 1% (Taoka 2011). However, no evidence was found regarding the effect of Tween 80 in fatty acid composition of *C. elegans* in this study as shown in Figure 5.2.

5.4.2.2 Effect of triglycerides

Figure 5.2 shows the fatty acid composition of *C. elegans* worms after a 3 day treatment with calendula seed oil or olive oil. There were no significant differences between different oil treatments and controls.

5.5 Discussion

Despite visual and quantitative confirmative data for triglyceride ingestion by *C. elegans* referred in chapter 4, the above mentioned-results do not provide conclusive evidence that *C. elegans* may also absorb triglycerides, particularly calendula seed oil. Calendula oil may have not been actually absorbed. It is possible that when *C. elegans* ingested nanoemulsions with dye, dye may have diffused into the body, rather than absorbed with triglycerides.

However, the absence of calendic acid in the fatty acid composition may be due to fast metabolism of this fatty acid, or too low concentration to be detected by GC. Thus, further investigation is needed with a marker that is known to be not metabolized by *C. elegans* to confirm if fat in nanoemulsion can be absorbed by *C. elegans*.

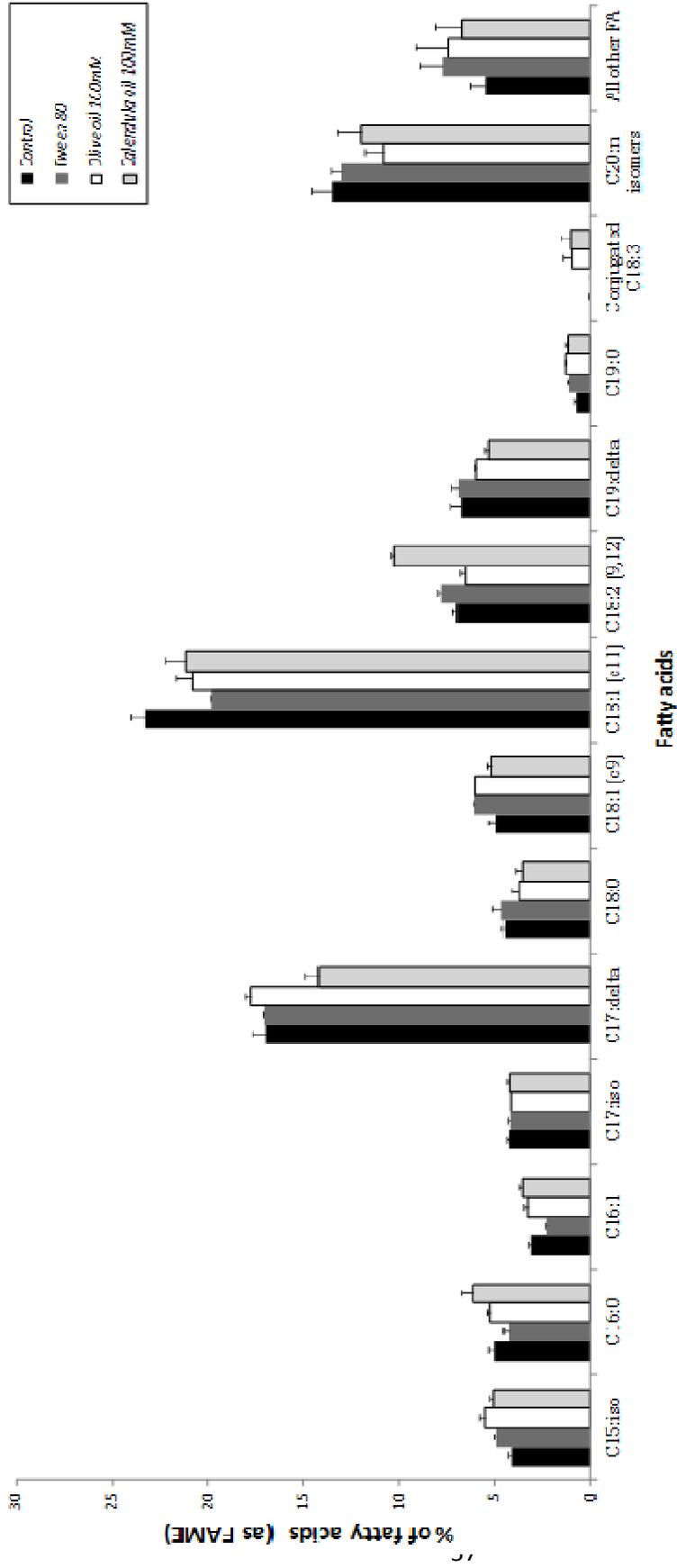


Figure 5.2: Fatty acid composition of *C. elegans* after triglyceride ingestion

The analysis was carried out in a Shimadzu GC-17A chromatograph coupled to a flame ionization detector (FID). Column: Supelcowax 10 (fused silica), 100m x 0.25mm x 0.25µm. Oven conditions: initial T: 50°C, 20°C/min to 190°C, 30 min holding, 10°C/min to 220°C, 60 min holding. Injector: 250°C, detector: 250°C, carrier gas Helium, split ratio 3:1. Values are mean +/- standard error (n=3).

CHAPTER 6

FUTURE RESEARCH

In this current research triglyceride nanoemulsions with particle size between 200 and 500nm were ingested by *C. elegans* in a concentration and particle size dependent manner. This was quantitatively confirmed by spectrometry fluorescence and evidenced by optical and confocal microscopy. However, no conclusive support was found from fatty acid composition analysis or life span study to support that triglycerides were absorbed.

In order to compliment this current research, future work should be addressed from three standpoints: new and alternative methods to optimize and detect absorption of triglyceride nanoemulsions, including the use of non lipid-based surfactants, non digestible markers and genetic techniques; evaluation of already assessed nanoemulsions for evaluation of bioactives different from triglycerides; and a study of the effect of emulsifiers on fatty acid composition and health indicators of *C. elegans*. In fact, recent research has linked emulsifiers and metabolic syndrome (Chassaing 2015).

Even though this research is not conclusive regarding the absorption of triglyceride nanoemulsions by *C. elegans*, nanoemulsions proved to be ingested in a large extent under specific conditions of particle size and concentration on growth media. These findings enable *C. elegans* to evaluate bioactive absorption of other hydrophobic bioactives different from triglycerides.

APPENDIX

EFFECT OF DIETARY TRIGLYCERIDES IN LIFE SPAN

It is known that *C. elegans* synthesizes polyunsaturated C20:n fatty acids from palmitic acid (C16:0) through a biochemical pathway that includes intermediate C18:n fatty acids (J. Watts 2008). Polyunsaturated C20:n fatty acids play an important role in neuromuscular development, brood size and regulation of biorhythm in *C. elegans* (J. P. Watts 2003). Previous research (Reisner 2011) has reported no significant effect of C18:1 cis9 and C18:1 trans9 dietary fatty acids in triglyceride form in life span of *C. elegans*. However, provided that dietary triglycerides are absorbed, we hypothesized that triglycerides containing calendic acid might influence life span. Therefore, we evaluated the effect of calendula seed oil nanoemulsions in life span in *C. elegans*.

The protocol for life span determination was adapted from the standard method (Solis G.M. 2011) and carried out by exposure of synchronized worms to dispersed 100mM nanoemulsions at different oil concentrations in liquid media.

C. elegans eggs were collected according to protocols and incubated overnight at 37°C until the eggs hatch. The following day, the concentration of worms (synchronized L1 larvae) was adjusted to 150-160 worms/ml. In order to prevent microbial growth in the medium, Carbenicillin B solution was added to a final concentration of 50µg/ml and the worm solution was shaken on a Nutator for 3 hours. *E. coli* OP50 concentrated suspension (100mg/ml) was added to the worm solution to a final concentration of 12mg/ml and the solution was transferred to individual wells in a 96 well plate. The final number of worms per well accounted for 10 and 12.

Forty eight hours later, the worms had reached the L4 stage. Then, they were sterilized by adding 60 μ l of a 0.6mM FUdR (Fluorodeoxyuridine) solution to each 300 μ l plate well. The worms were stored at 20°C in an incubator.

After 24 hours, nanoemulsions at the desired final oil concentration were added to each well. The plate was sealed and shaken for 5min and returned to the 20°C incubator. The worms were counted and the reports for the following 6 weeks were carried out twice or three times a week, with addition or change for fresh media twice a week. Results are shown in Figure A.1

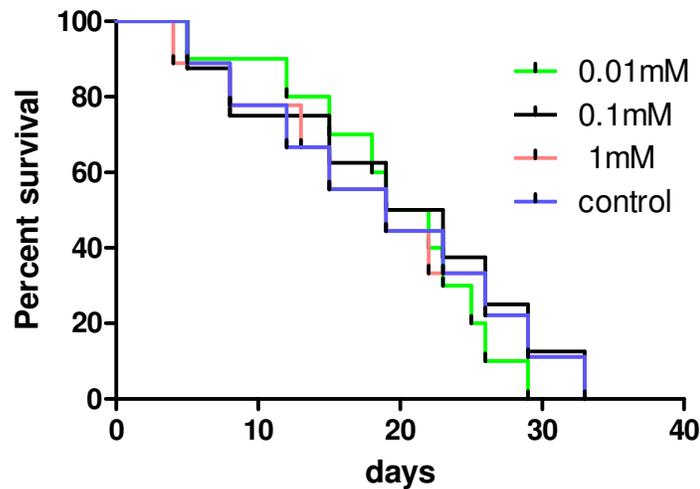


Figure A.1: Life span assay results

Mean life span curves for liquid media treatments (0.01mM, 0.1mM, 1mM) of calendula seed oil nanoemulsions compared to control (n=3). Populations of approximately 80 worms per treatment were used in a 96-well plate. Fresh media was replenished twice a week. Worms that exhibited motion as a response to light beam was accounted for survival. The Log-rank (Mantel-Cox) test showed no significant difference between treatments and control (p=0.9420, n=3). Median survival rate: 20 days at 20°C. Log-rank test done with Graph Pad Prism-5 software.

Life span was not influenced by 0.01mM, 0.1mM or 1mM calendula seed oil nanoemulsions with particle size 200-250nm, in liquid media. The medium survival rate in each case is shown in Table A.1.

Table A.1: Medium survival rate, life span assay

Treatment	Medium survival rate (days), n=3
Control	22
0.01mM	21.5
0.1mM	22.5
1mM	22

Life span was not affected compared to control, indicating either low concentration of oil, or insufficient absorption by *C. elegans*, or low bioactivity of the fatty acids.

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