

Formulation and Stability Optimization of a Choline-Enriched DHA/ARA Emulsion for Preterm Infants

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Examiner: Lars Nilsson

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Abbreviations

4-HHE: 4-Hydroxy-2-hexenal

ACN: Acetonitrile

AOCS: American Oil Chemists' Society

ARA: Arachidonic acid

AUC: Area under the curve

CC: Choline chloride

CD: Conjugated dienes

D[4,3]: Volume-weighted mean diameter

DHA: Docosahexaenoic acid

D_v (0.5): Volume median diameter

E475: Polyglycerol esters of fatty acids

E476: Polyglycerol polyricinoleate

EDTA: Ethylenediaminetetraacetic acid

EOC: EOC emulsifier commercial product from AquaCelle®

EPA: Eicosapentaenoic acid

GC: Gas chromatography

GP1: GP1 emulsifier commercial product from AquaCelle®

GPC: α -glycerophosphorylcholine

HPLC: High-performance liquid chromatography

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

INFOGEST: Lipid Digestion and Nutraceutical Bioaccessibility Assessed by Standardized Gastrointestinal Model(a standardized static in vitro gastrointestinal digestion method)

IR: Infrared spectroscopy

L·: Lipid alkyl radical

LCPUFA: Long-chain polyunsaturated fatty acid

LH: Unsaturated lipid

LO $\cdot$: Lipid alkoxyl radical

LOO $\cdot$: Lipid peroxy radical

LOOH: Lipid hydroperoxide

MeOH: Methanol

Mⁿ⁺: Transition-metal ion in oxidation state n⁺

MS: Mass spectrometry

NMR: Nuclear magnetic resonance spectroscopy

NOAEL: No observed adverse effect level

p-AV: p-Anisidine value

PC: Phosphatidylcholine

PV: Peroxide value

PVDF: Polyvinylidene fluoride

R₁ and R₂: Residual lipid groups

ROP: Retinopathy of prematurity

RT: Retention time

SD: Standard deviation

RSD: Relative standard deviation

SPH: Sphingomyelin

TBARS: Thiobarbituric acid-reactive substances

TOTOX: Total oxidation value

UV–Vis: Ultraviolet–visible spectroscopy

Baby Nutrition of Omega-3,6 Oil Formulation Stability Analysis

Some nutrients are especially important for very premature infants, but they are also difficult to formulate safely. This project studied how to make an oil-in-water emulsion containing omega-3 and omega-6 and choline, while reducing the risk of oil oxidation. Very premature infants miss part of the nutrient transfer that normally happens during late pregnancy. Among these nutrients, omega-3 and omega-6 are important fatty acids for the developing brain and retina, while choline is needed for cell membranes and may help transport these fatty acids in the body. A practical supplement should therefore be able to deliver oil-soluble omega-3 and 6 together with water-soluble choline in a small and stable dose. One possible solution is an oil-in-water emulsion. This is similar in principle to many everyday products, such as milk or salad dressing: tiny oil droplets are dispersed in water with the help of emulsifiers. Smaller droplets can be good because they may improve stability and digestion. However, there is a challenge. Omega-3 and omega-6 are sensitive to oxidation. When oils oxidize, they can lose nutritional quality and form unwanted breakdown products. For preterm infants, who are already vulnerable to oxidative stress, controlling this process is especially important. In this project, different emulsion formulations were prepared by high-pressure homogenization. The formulations differed in emulsifier type (called GP1 and EOC), emulsifier amount and choline form (called CC and GPC). Their droplet size was measured to evaluate stability, and several chemical tests were used to follow oxidation over 15 days. The most interesting finding was that the smallest droplets were not always the best choice. Formulations made with the EOC emulsifier produced smaller droplets, but they also showed more secondary oxidation products. These products appear later in the oxidation process and are more complex than the first oxidation products. In contrast, formulations made with the GP1 emulsifier had slightly larger droplets but better control of secondary oxidation. The choline form also mattered. GPC performed better than choline chloride in reducing secondary oxidation. Based on the overall screening, the most promising formulation was GP1 with GPC combination. This formulation did not have the smallest droplet size, but it gave the best overall balance for stability. The project shows that formulation development is not only about making droplets as small as possible. For sensitive nutritional emulsions, the stability of the oil is just as important. The selected formulation is not yet a final product, but it provides a useful starting point for further validation, longer storage studies and future bioavailability testing

Abstract

Preterm infants benefit from combined supplementation of arachidonic acid (ARA), docosahexaenoic acid (DHA) and choline, but ARA/DHA-rich oils are highly susceptible to lipid oxidation. This project aimed to develop and evaluate choline-enriched DHA/ARA oil-in-water emulsions with suitable physical and oxidative stability. Emulsions were prepared by high-pressure homogenization using two AquaCelle® emulsifier systems which are EOC and GP1, choline chloride and α -glycerophosphocholine, and two emulsifier levels. Droplet size was used to evaluate physical stability, while peroxide value, conjugated dienes, para-anisidine value and TOTOX were used to assess oxidation during accelerated storage. AUC-based responses were mainly used for formulation comparison. The results showed that emulsifier type was the main factor affecting droplet size. EOC produced smaller droplets than GP1 but also showed higher accumulation of secondary oxidation products. In contrast, GP1-containing formulations showed better control of p-AV and overall oxidation burden. Choline form mainly influenced secondary oxidation, with α -glycerophosphocholine performing better than choline chloride. Based on Design-Expert screening, GP1 with GPC combination was selected as the most promising formulation, with the highest desirability value.

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1 Background:

Certain nutrients are particularly important for very preterm infants but are challenging to formulate in a stable and safe manner. This project investigated the development of an oil-in-water oral emulsion containing arachidonic acid (ARA) and docosahexaenoic acid (DHA) fatty acids together with choline, with particular emphasis on minimizing lipid oxidation.

Retinopathy of prematurity (ROP) arises after preterm birth disrupts normal retinal vascular and neural development, and prevention in early life is essential^[1]. Extremely preterm infants miss the third-trimester surge of arachidonic acid (ARA) and docosahexaenoic acid (DHA) accretion, while postnatal nutrition often provides relatively high linoleic acid but insufficient ARA, DHA and choline^{[2][3]}. Balanced supplementation with ARA plus DHA (ARA: DHA $\approx$ 2:1) has been associated with reduced severe ROP, suggesting that restoring a physiological balance may support retinal development^[4]. Co-supplementation with choline may further improve DHA (and ARA) delivery by promoting phosphatidylcholine (PC) synthesis (including via methyl-donor-dependent pathways), shifting DHA from neutral lipids toward DHA-PC and thereby recreating a more fetal-like lipid transport milieu^[3].

Therefore, it is necessary to develop oral supplements that can deliver ARA/DHA at clinically meaningful doses while also containing choline. For practical use, the available products on the market are quite scarce. One of them is the pure oil from NeoMega36® and the other is the emulsion from Enfamil®. But they all lacked the crucial choline supplementation. To enable accurate dosage delivery, acceptable sensory properties, and improved bioavailability, the emulsion formulation is preferable^[5]. In addition, studies showed that emulsification of omega-3 long chain polyunsaturated fatty acid (LCPUFA) ethyl esters increases their surface area, this can facilitate digestion and absorption, ultimately improving the bioavailability^[6]. Furthermore, the commercial emulsifier AquaCelle® significantly improved bioavailability in an animal model compared with standard fish oil^[7]. However, The ARA and DHA, as well as the oils present in the emulsifier Aquacell, contain many unsaturated fatty acids, they are highly susceptible to oxidation. Oxidation could lead to loss of nutrition and negatively affect quality and safety, and it may also shorten shelf life^[8].

Preterm infants are exposed to a state of high oxidative stress combined with limited antioxidant capacity. The transition from a relatively hypoxic intrauterine environment to a higher oxygen extrauterine environment, together with clinical interventions, can promote the generation of reactive oxygen and nitrogen species. Due to the immaturity of their antioxidant defense systems, preterm infants are therefore more susceptible to oxidative damage. Among the diseases associated with oxidative injury, ROP is one of the most prominent^[9]. Consequently, ensuring the quality and safety of emulsions containing ARA/DHA is of particular importance.

Unlike bulk oils, lipid oxidation in oil in water (O/W) emulsion is often controlled by reaction occurring on the oil-water interface^[10]. Therefore, even when the oil and aqueous phases are nominally similar, changes in emulsifier composition and concentration may lead to large differences in oxidation behavior. Many factors can affect oxidation, such as droplet size, interfacial composition, emulsifier type, and the distribution and reactivity of antioxidants and peroxide^[10]. For example, a common approach to improve physical stability is to increase the emulsifier level, but this may unintentionally accelerate or slow oxidative process because the added emulsifier changes the interfacial or aggregation environment^[11]. A comprehensive

understanding and control of these reactions is challenging, which also makes it difficult to develop rational formulation strategies.

Therefore, in order to develop an emulsion that contains both choline and ARA/DHA components and has physical and chemical stability, while ensuring safety. Find the most similar stability testing method to the pharmacopoeia or official recommendations, and select the best formulation of the emulsion. This project was carried out in collaboration with CR Competence AB and under the joint guidance of Lund University and Gothenburg University.

2 Aim of the thesis:

This project aims to develop an evaluation and formulation strategy for DHA/ARA oil-in-water emulsions.

1. To establish a method for measuring the oxidation degree of emulsions under limited oil sample availability, while keeping the method as close as possible to pharmacopeial and American oil chemists' society (AOCS) standards.
2. To design emulsion formulations within a composition range that is expected to be tolerable for infants.
3. To design experiments to compare the physicochemical stability of different formulations, including particle size, visual stability, and oxidative stability, in order to identify the most stable emulsion formulation.

3 Theoretical background:

3.1 Retinopathy of Prematurity

ROP is one of the major complications of extremely preterm birth and remains an important cause of visual impairment in surviving preterm infants. ROP is now understood not simply as an isolated eye disease, but as a disorder of disrupted neurovascular development. After preterm birth, the immature retina is exposed to an extrauterine environment in which oxygen tension, nutrient supply, inflammation, and growth-factor regulation differ markedly from the intrauterine condition. Hellström et al. described ROP as a two-phase disease: in the first phase, hyperoxia and the loss of maternally supplied factors suppress normal retinal vascularisation; in the second phase, the increasingly metabolically active but incompletely vascularised retina becomes hypoxic, leading to pathological vasoproliferation and, in severe cases, retinal detachment^[1]. Importantly, the same review emphasized that prevention of ROP requires not only oxygen control, but also optimization of nutrition and normalization of essential developmental factors, including insulin-like growth factor-1 and omega-3 polyunsaturated fatty acids.

3.2 Preterm nutrition

Nutrition is therefore central to the prevention of prematurity-related morbidity. Preterm infants have higher nutrient requirements than term infants because they must continue rapid fetal-like growth outside the uterus while facing gastrointestinal immaturity, limited nutrient stores, feeding intolerance, and frequent illness. Joosten and Vermeulen noted that very low birth weight infants are prone to postnatal growth retardation and altered body composition, and that early parenteral amino acid and lipid administration is often needed to prevent protein loss and support early growth^[12]. Similarly, recent guideline reviews have emphasized that preterm infants are at risk of nutrient deficits due to immature gastrointestinal function, low breast-milk supply, and the difficulty of providing adequate nutrition; adequate nutrition during this period is crucial for growth and neurodevelopment, while both undernutrition and excessive catch-up growth may have long-term consequences^[13].

Among nutrients, lipids are particularly important because they serve not only as an energy source but also as structural and functional components of the brain, retina, liver, and cell membranes. More than 90% of fetal fat mass is deposited during the third trimester, and long-chain polyunsaturated fatty acids (LCPUFAs) are necessary for central nervous system development^[12]. ARA and DHA are especially relevant because they are critical constituents of neural and retinal membranes. Kamino et al. found that, in preterm neonates, higher postnatal DHA levels were associated with larger cortical gray matter, deep gray matter, cerebellar and brainstem volumes, and that larger brain volumes at term-equivalent age were associated with better language and motor outcomes at preschool age^[14].

3.3 Supplement of DHA, ARA

However, extremely preterm infants miss the placental transfer of ARA and DHA that normally occurs during the third trimester. Lapillonne et al. summarized that although DHA supplementation had historically been designed to match average breast-milk levels, this may be insufficient for preterm infants, whose requirements may be closer to fetal accretion rates. The review further suggested that DHA supplementation may be more promising when combined with ARA, with potential benefits for brain maturation, long-term cognitive function,

and prevention of ROP^[2]. This is supported by the randomized clinical trial, in which enteral supplementation with ARA 100 mg/kg/day and DHA 50 mg/kg/day from early life to 40 weeks' postmenstrual age reduced severe ROP from 33.3% in the control group to 15.8% in the supplemented group, corresponding to an adjusted relative risk of 0.50^[4].

At the same time, the formulation of ARA/DHA-containing products presents technological challenges. ARA and DHA are highly unsaturated fatty acids and are therefore susceptible to lipid oxidation. Preterm infants themselves are also vulnerable to oxidative stress because their antioxidant systems are immature. Lembo et al. reported that oxidative stress contributes to multiple prematurity-related diseases, including ROP, bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, and periventricular leukomalacia; lipid peroxidation of polyunsaturated fatty acids is one important mechanism of cellular injury^[9].

3.4 Supplement of Choline

3.4.1 Physiological Nutrition

Choline is an essential nutrient with increased requirements during fetal and neonatal development. It is required for the synthesis of phosphatidylcholine (PC) and sphingomyelin (SPH), two major choline-containing phospholipids in cell membranes and biological secretions. In addition, choline is a precursor of acetylcholine, which is important for neurodevelopment^{[15][16]}.

Preterm infants may be especially vulnerable to insufficient choline supply. During the fetal growth spurt at 24–34 weeks postmenstrual age, plasma choline concentrations are markedly higher than later in gestation and approximately threefold higher than those in pregnant women. After premature birth, this continuous placental supply is interrupted, and plasma choline rapidly decreases. Bernhard et al. therefore suggested that choline deficiency may contribute to impaired lean body mass growth, pulmonary development and neurocognitive development in preterm infants, even when macronutrient intake and weight gain appear adequate^[15].

Recent metabolic evidence further supports this concern. Böckmann et al. noted that choline supply is not guaranteed in current preterm infant nutrition; breast milk, fortifiers and formulas may provide variable amounts and forms of choline, and total postnatal choline intake may remain lower than prenatal supply. In their discussion, they emphasized that preterm birth interrupts active transplacental choline transport during a period of rapid brain and parenchymal growth, while low plasma and hepatic PC pools may lead to redistribution of choline from other organs such as the lung^[17].

3.4.2 The Combined Effect with DHA/ARA

The interest in choline supplementation is also related to its metabolic connection with LCPUFAs, especially DHA and ARA. PC is not only a membrane phospholipid, but also a major plasma transport form of DHA and ARA. Bernhard et al. described PC as the major carrier of arachidonic acid and docosahexaenoic acid in plasma, suggesting that choline status may influence the transport and tissue delivery of these LCPUFAs^[3]. This is particularly important for preterm infants because DHA and ARA are critical for brain and retinal development, while preterm birth interrupts the placental transfer of these fatty acids during the third trimester^[12].

Clinical and mechanistic studies support the importance of this pathway, a study found enteral supplementation with ARA 100 mg/kg/day and DHA 50 mg/kg/day reduced severe ROP in

extremely preterm infants^[4]. In another small randomized controlled trial, combined choline and DHA supplementation increased DHA-containing PC more strongly than DHA alone. Specifically, DHA alone increased DHA-PC by 35%, whereas combined DHA and choline increased DHA-PC by 63%, suggesting that adequate choline availability may support DHA incorporation into PC and improve DHA homeostasis in preterm infants^{[17][3]}.

Therefore, choline may not simply act as an independent micronutrient but may also support DHA/ARA delivery through PC-mediated lipid transport. This provides a mechanistic rationale for combining choline with ARA/DHA in a nutritional formulation, especially when the aim is to recreate a more fetal-like lipid transport environment for the developing brain and retina.

3.4.3 In Formulation

Different choline forms have different structures and physicochemical properties, which may influence their suitability in emulsion formulation. Common supplemental forms include choline chloride (CC), choline bitartrate and α -glycerophosphorylcholine (GPC). CC is a small water-soluble quaternary ammonium salt, while GPC is a water-soluble organic choline ester and one of the physiological choline-containing forms found in milk. Previous metabolic evidence in preterm infants showed that water-soluble choline forms, including choline chloride and GPC, can increase plasma choline and betaine, indicating that both forms are nutritionally relevant choline sources^[18].

In this project, CC and GPC were selected as two representative water-soluble choline sources. Since ARA/DHA-rich oils are incorporated into the oil phase, while CC and GPC are dissolved in the aqueous phase, an oil in water (O/W) emulsion allows hydrophobic lipids and hydrophilic choline to be delivered in the same formulation. However, because CC and GPC differ in molecular structure, polarity and possible interaction with the aqueous phase or oil–water interface, they may affect the physicochemical stability of the emulsion differently. Therefore, comparing CC- and GPC-containing emulsions is necessary to identify which choline form provides better formulation stability.

The supplementation level was set at 20 mg/kg/day choline-equivalent. This dose was designed to complement, rather than replace, the background choline intake from routine formula feeding. Bernhard et al. reported that choline concentrations were comparable in preterm and term infant formulas for infants ≤ 6 months, with median values of 31.9 and 33.3 mg/100 kcal, respectively, which can be roughly estimated as approximately 38 mg/kg/day under full formula feeding^[19]. Therefore, adding 20 mg/kg/day would bring the estimated total intake close to the 50–60 mg/kg/day range proposed to approach fetal plasma choline concentrations in preterm infants^[20]. This level is also consistent with available infant nutrition reference values: the NIH lists an adequate intake of 125 mg/day for infants aged 0–6 months, and no tolerable upper intake level has been established for this age group because of insufficient evidence on adverse effects from excess intake. Thus, the selected dose was considered a conservative supplemental level within a reasonable nutritional range^[16].

3.5 Marketed ARA/DHA supplements and formulation rationale

ARA and DHA supplementation for preterm infants has already been translated into commercial products, but the available options remain limited. For example, Enfamil® DHA & ARA Supplement is a commercially sterile emulsified liquid for oral or enteral feeding, providing 40 mg ARA and 20 mg DHA per 0.5 mL^[21]. NeoMega36® is an European food for special medical purposes designed for extremely preterm infants, providing 100 mg/kg/day

ARA and 50 mg/kg/day DHA at 0.4 mL/kg/day [22]. These products demonstrate the clinical and market feasibility of ARA/DHA supplementation in early-life nutrition, but they mainly focus on lipid delivery and do not include choline as a co-supplemented nutrient.

This represents a formulation gap because choline is metabolically linked with ARA/DHA through phosphatidylcholine-mediated transport. If ARA/DHA and choline are to be delivered in one oral supplement, an oil-in-water emulsion is a rational strategy: the oil phase can solubilize ARA/DHA-rich oils, while the aqueous phase can contain water-soluble choline. Such a system may also support accurate small-volume dosing and compatibility with oral or enteral administration, which are important for preterm infant use.

Emulsification is also relevant for lipid bioavailability. Garaiova et al. reported that pre-emulsification significantly improved postprandial absorption of long-chain highly unsaturated fatty acids, especially EPA and DHA, compared with non-emulsified oil^[5]. A recent comprehensive review similarly noted that emulsification disperses oil-soluble EPA/DHA in an aqueous medium, increases surface area, and may facilitate digestion and absorption^[6].

Therefore, developing an ARA/DHA–choline O/W emulsion could address both the nutritional gap in current products and the formulation need for co-delivering hydrophobic lipids with water-soluble choline.

3.6 Emulsion System

O/W emulsions are widely used for the delivery of lipophilic nutrients such as ARA and DHA. In an O/W emulsion, the oil phase is dispersed as small droplets in a continuous aqueous phase, and emulsifiers adsorbed at the oil–water interface reduce interfacial tension and prevent droplet coalescence.

Several food and nutritional emulsification strategies have been reported, including conventional emulsions, multilayer emulsions, gelled emulsions, Pickering emulsions, and self-emulsifying delivery systems. Conventional emulsions are usually stabilized by a single interfacial layer of emulsifier, while multilayer emulsions and gelled emulsions improve stability by increasing interfacial thickness or aqueous phase viscosity. Pickering emulsions use solid particles to form a more rigid interfacial layer. These approaches can improve physical and oxidative stability, but they may also increase formulation complexity. For an oral nutritional product intended for small volume dosing, a relatively simple and reproducible emulsification strategy is therefore preferable^[23].

However, emulsifier concentration must be carefully controlled. Too little emulsifier may lead to coalescence and phase separation, whereas excessive surfactant may increase excipient burden or affect gastrointestinal tolerability, causing health effects for infants.

3.7 Mechanism of Lipid Oxidation in Emulsions

3.7.1 Emulsion Structure and Interfacial Oxidation

Oil-in-water emulsions are multiphase systems in which oil droplets are dispersed in a continuous aqueous phase and stabilized by emulsifiers at the oil-water interface. Compared with bulk oils, emulsions contain a large oil-water interfacial area. This allows unsaturated lipids in the oil phase to come into contact more easily with oxygen, metal ions, and other pro-oxidants in the aqueous phase. Therefore, lipid oxidation in emulsions is usually more difficult

to control than that in bulk oils. Hennebelle et al. pointed out that the oil-water interface is the key region where lipids, oxygen, and pro-oxidants meet. Droplet size, interfacial composition, emulsifier type, and the partitioning of pro-oxidants and antioxidants can all affect lipid oxidation in emulsified systems^[10].

Thus, lipid oxidation in emulsions should not be regarded only as oxidation inside oil droplets. Instead, it should be understood as a reaction involving the oil phase, aqueous phase, and oil-water interface. In ARA/DHA-containing emulsions, the oil phase provides oxidation-sensitive lipid substrates, the aqueous phase may contain oxygen, metal ions, salts, and buffer components, while the interfacial layer controls how these components interact with each other.

3.7.2 Basic Process of Lipid Oxidation

Lipid oxidation in emulsions mainly follows a free radical chain reaction, including initiation, propagation, and termination. During initiation, unsaturated fatty acids lose hydrogen atoms under the influence of heat, light, metal ions, or pre-existing hydroperoxides, forming lipid radicals. These radicals then react rapidly with oxygen to form lipid peroxy radicals. The peroxy radicals can attack other unsaturated fatty acids and produce lipid hydroperoxides. Lipid hydroperoxides are the primary oxidation products, but they are unstable and can further break down into secondary oxidation products, such as aldehydes, ketones, and alcohols. These secondary products are often related to rancid, fishy, and other unpleasant flavors^[24]. Figure 3.1, Figure 3.2 and Figure 3.3 illustrate the free radical chain reaction mechanism of lipid auto-oxidation, from initiation to propagation, and finally to the termination of the chain reaction. The amount of the primary oxidation product LOOH serves as the main indicator for measuring the primary oxidation. Then LOOH undergoes further decomposition, resulting in products such as aldehydes, ketones, alcohols, acids, and hydrocarbons.

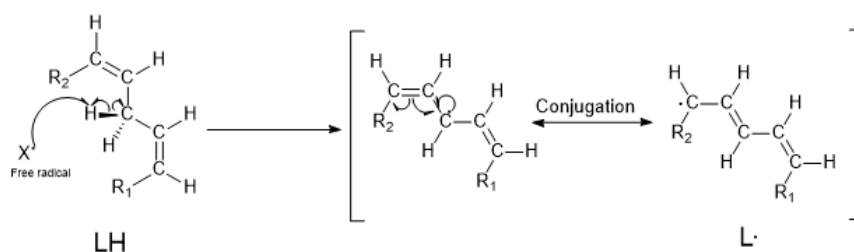


Figure 3.1. Initiation of Free Radical Chain Reaction (LH, unsaturated lipid; L·, alkyl radical; LOO·, peroxy radical; LOOH, lipid hydroperoxide; LO·, alkoxyl radical; Mⁿ, metal ion; R₁ and R₂, residual group of lipids.)

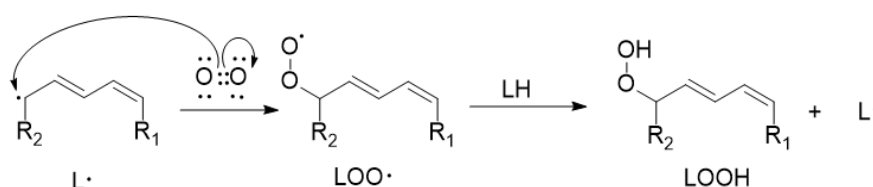


Figure 3.2. Propagation of Free Radical Chain Reaction (LH, unsaturated lipid; L·, alkyl radical; LOO·, peroxy radical; LOOH, lipid hydroperoxide; LO·, alkoxyl radical; Mⁿ, metal ion; R₁ and R₂, residual group of lipids.)

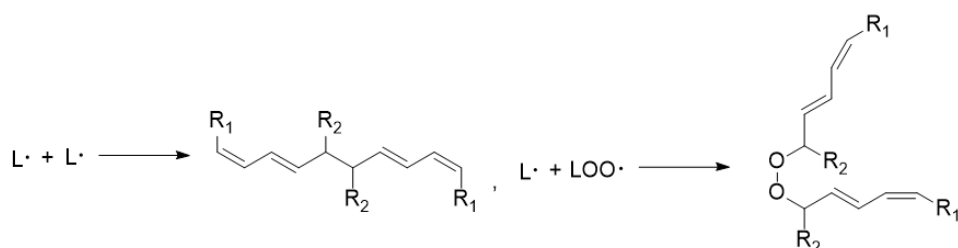


Figure 3.3. Termination of Some Free Radical Chain Reaction (LH, unsaturated lipid; L, alkyl radical; LOO, peroxy radical; LOOH, lipid hydroperoxide; LO, alkoxyl radical; M^n , metal ion; R_1 and R_2 , residual group of lipids.)

Transition metal ions, especially iron and copper, are important pro-oxidants in emulsions, the mechanism is shown in Figure 3.4. They can promote the decomposition of lipid hydroperoxides and generate new radicals, which further accelerate the oxidation reaction. Liu et al. found that even low levels of endogenous iron and copper at the ppm level could promote lipid oxidation in oil-in-water emulsions. The addition of chelating agents such as EDTA or sodium citrate effectively inhibited oxidation, showing the practical importance of metal-catalyzed oxidation in emulsions^{[10][25]}.

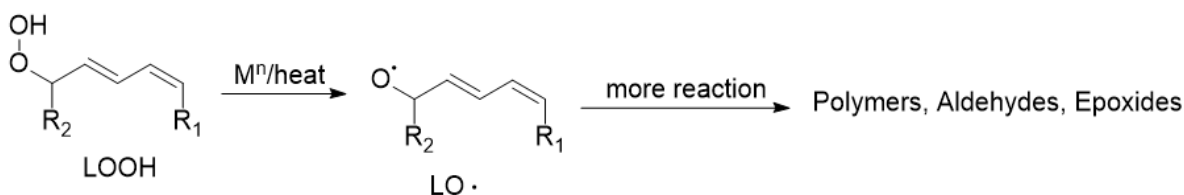


Figure 3.4. The Mechanism of Metal-catalysed Decomposition of LOOH and Secondary Oxidation (LH, unsaturated lipid; L, alkyl radical; LOO, peroxy radical; LOOH, lipid hydroperoxide; LO, alkoxyl radical; M^n , metal ion act as catalyst; R_1 and R_2 , residual group of lipids.)

3.7.3 Migration of Oxidation Products

Recent studies suggest that some lipid oxidation products may move between different regions of emulsion. For example, Klooster et al. found that triglyceride-bound hydroperoxides did not easily transfer between oil droplets, while smaller and more hydrophilic oxidation products, such as 4-hydroperoxy-2-nonenal and 2,4-decadienal, could redistribute between droplets^[26]. This indicates that oxidation in emulsions may not be limited to one single oil droplet.

However, the importance of this transfer depends on the emulsion system. Excess emulsifiers may form micelles in the aqueous phase, and these micelles can act as carriers or small reaction sites for oxidation-related molecules^{[10][11]}. Therefore, migration of oxidation products is one possible factor that makes emulsion oxidation more complex than oxidation in bulk oils, but its actual contribution needs to be considered together with emulsifier type and formulation composition.

3.7.4 Effects of Formulation and Interfacial Structure on Oxidation

The oxidative stability of emulsions is closely related to their formulation and interfacial structure. Droplet size is one important factor. Smaller droplets usually have a larger total oil-water interfacial area, which may increase the contact between lipids and aqueous pro-oxidants. However, this relationship is not always simple, because changes in droplet size may also change emulsifier distribution and interfacial composition. Aslam and Schroën noted that smaller droplets may oxidize faster, but this effect can be reduced by protective components located at the interface or in the continuous phase^[27].

The interfacial layer is also important because it controls the contact between lipid substrates, oxygen, metal ions, and antioxidants. Different emulsifiers can form interfacial layers with different thickness, mobility, and charge. Therefore, emulsifiers influence not only physical stability, but also oxidative stability. Hennebelle et al. also emphasized that emulsifier type, interfacial composition, and the distribution of pro-oxidants and antioxidants are key factors in emulsion oxidation^[10].

The aqueous phase can further affect oxidation through pH, ionic strength, salts, metal complexation, and viscosity. For example, citrate and phosphate buffers may form complexes with Fe(II), and their charge and interaction with the oil-water interface can change the pro-oxidant activity of iron^[28]. Liu et al. also reported that NaCl or KCl slightly increased lipid oxidation in Tween 60-stabilized emulsions, possibly by changing the surface potential and structure of the surfactant layer^[25]. In addition, higher aqueous phase viscosity may limit the diffusion of oxygen and metal ions, but this effect should be considered together with emulsion structure.

Finally, antioxidants should be evaluated according to their location in the emulsion. Their effect depends not only on radical-scavenging ability, but also on whether they can reach the oil-water interface, where oxidation mainly occurs. Therefore, antioxidant use should be considered together with emulsifier type, interfacial structure, and metal ion environment.

3.7.5 Quality and Health Implications

The safety concern of ARA/DHA-containing formulations is mainly related to lipid oxidation rather than the unoxidized oils themselves. Lewis et al. evaluated ARA-rich oil and DHA-rich oil using genotoxicity tests and 28-day and 90-day dietary toxicity studies in Wistar rats. The results showed no treatment-related adverse effects on clinical observations, body weight, food consumption, hematology, clinical chemistry, urinalysis, organ pathology, or genotoxicity endpoints, and no observed adverse effect level (NOAEL) for both oils was 5000 mg/kg bw, the highest dose tested. This suggests that ARA- and DHA-rich oils themselves have a favorable toxicological profile for food use^{[29][30]}.

However, these oils are highly unsaturated and therefore sensitive to oxidation. Awada et al. showed that oxidized n-3 PUFA can generate reactive aldehydes such as 4-hydroxy-2-hexenal (4-HHE), which may be absorbed in the intestine and was associated with increased plasma 4-HHE, oxidative stress, and inflammatory markers in mice. Therefore, the key formulation challenge is not only to include ARA and DHA as nutritional lipids, but also to protect them from oxidation during preparation and storage. In this project, controlling lipid oxidation is important for maintaining nutritional value and product quality, and also for reducing the potential health risks caused by oxidized lipid product^[31].

3.8 AquaCelle®

Based on the discussion above, the stability of ARA/DHA emulsions is strongly related to the emulsifier system and the oil–water interface. In this study, AquaCelle® was used as a compound emulsifier, and the emulsions were prepared by high-pressure homogenization.

AquaCelle® is a mixed emulsifier system containing oil-based components. These components can help reduce the oil–water interfacial tension and support the formation of dispersed oil droplets. The study on AquaCelle® omega-3 formulation improved the dispersion of EPA/DHA oil in water [7].

In this project, high-pressure homogenization was used to further break the oil phase into smaller droplets and promote emulsifier adsorption at the newly formed interface. This is important because lipid oxidation in emulsions is closely related to the oil–water interface, where lipids, oxygen, and pro-oxidants can interact^[10]. Fernandez-Avila and Trujillo also reported that ultra-high pressure homogenization could improve the interfacial properties and oxidative stability of oil-in-water emulsions under suitable conditions^[32].

Table 3.1 lists the material contained in each emulsifier. The two AquaCelle® types used in this study also differ in their tocopherol-related components. AquaCelle® GP1 contains tocopherol, while AquaCelle® EOC contains tocopherol acetate. Tocopherol can directly act as a lipid-soluble antioxidant by donating hydrogen to lipid radicals, while tocopherol acetate is more stable but has weaker direct radical-scavenging activity because its phenolic hydroxyl group is esterified.

In GP1 emulsifier, E475 (polyglycerol esters of fatty acids) has a relatively broad HLB range and can be used in both O/W and W/O emulsions depending on its composition and degree of esterification. In contrast, E476 (polyglycerol polyricinoleate) as one of component in EOC emulsifier, is more lipophilic and is mainly used as a strong W/O emulsifier. Therefore, E476 may improve emulsification and droplet-size reduction, but it also introduces a more complex interfacial composition^{[33][34]}.

Therefore, AquaCelle® was selected because it is suitable for emulsifying hydrophobic ARA/DHA oils and can work with high-pressure homogenization to form oil-in-water emulsions. The difference between tocopherol and tocopherol acetate also provides a basis for comparing how the two emulsifier systems may affect the oxidative stability of ARA/DHA emulsions. The ingredients are mainly lipids and lipid-derived emulsifiers. However, safety evidence for the complete formulation in the target infant population remains limited, and further evaluation is required.

Table 3.1. Material list of AquaCelle® Emulsifier GP1 and EOC

Material	GP1	EOC
Medium Chain Triglycerides(coconut)	✓	-
Olive Oil	✓	✓
Citrus Oil	✓	✓
Coconut Oil Fractionated	-	✓
Lecithin from Sunflower	✓	✓
Polyglycerol Esters of Fatty Acids(E475)	✓	-

Polyglycerol Polyricinoleate(E476)	-	✓
Mono- and Diglycerides of Fatty Acids	✓	-
Tocopherol	✓	-
Tocopherol Acetate	-	✓

3.9 Formulation Design

The emulsion contained an ARA- and DHA-rich oil at a ratio of 2:1, supplied by NeoMega36®. The oil consisted of ARA-rich oil derived from the fungus *Mortierella alpina*, DHA-rich oil derived from the microalga *Schizochytrium*, and antioxidants. The recommended daily dose of the original oil product was 0.4 mL/kg once daily, corresponding to 100 mg/kg/day of ARA and 50 mg/kg/day of DHA. Further information is provided in the product specification^[22].

Bernhard et al. reported similar choline concentrations in preterm and term infant formulas for infants below 6 months of age, with values of 31.9 and 33.3 mg/100 kcal, respectively. Based on full formula feeding, this corresponds to an estimated choline intake of approximately 38 mg/kg/day^[15]. Another study reported that enteral supplementation of 50–60 mg/kg/day choline-equivalent in preterm infants produced plasma choline concentrations similar to those measured in umbilical cord plasma^[18].

Therefore, the supplementation level in this study was set at 20 mg/kg/day choline-equivalent. This dose was intended to complement, rather than replace, the background choline intake provided by routine formula feeding. The oil-to-water ratio of the emulsion was set at 4:6. Therefore, 1 mL of emulsion contained approximately 0.4 mL of oil and 0.6 mL of aqueous phase. The proposed daily dose for preterm infants was 1 mL/kg once daily, providing 100 mg/kg/day of ARA, 50 mg/kg/day of DHA, and 20 mg/kg/day choline-equivalent. Based on previous studies conducted by CR Competence, the emulsion was homogenized for three passes at 750 bar. The amount of emulsifier was also selected based on previous internal studies, as described in the Appendix.

3.10 Evaluation of Physical Stability

Emulsions are thermodynamically unstable systems, and may show physical instability during storage, such as creaming, sedimentation, flocculation, coalescence, Ostwald ripening and phase separation. For oil-in-water emulsions, physical stability mainly refers to whether oil droplets can remain uniformly dispersed in the aqueous phase, and whether visible separation or oil layer formation occurs. Zhu et al. reported that emulsifiers can improve emulsion stability by reducing interfacial free energy and forming a protective layer around oil droplets. They also used droplet size, zeta potential, microscopy and visual appearance to evaluate the physical stability of emulsions during storage^[35]. Particle size and particle size distribution are therefore important indicators. Smaller droplets with a narrower distribution usually suggest better physical stability, while an obvious increase in particle size may indicate flocculation or coalescence.

In this study, the physical stability of ARA/DHA emulsions was mainly evaluated by particle size/particle size distribution. Particle size analysis was used to compare the droplet size and uniformity of emulsions prepared with different AquaCelle® emulsifiers after high-pressure homogenization, while visual observation was used to check whether phase separation, creaming, sedimentation, oil layer formation or abnormal color changes occurred. Hebishy et

al. showed that high-pressure homogenization can reduce droplet size and improve emulsion stability against creaming and coalescence. They also suggested that physical stability is related to oxidative stability, because a stable interfacial layer and good droplet dispersion may help reduce the contact between oil droplets and prooxidants^[36]. Therefore, the physical stability evaluation in this study also provides useful background for understanding the oxidative stability of ARA/DHA emulsions.

3.11 Evaluation methods for lipid oxidative stability

A relatively complete system has been developed for evaluating lipid oxidative stability. peroxide value (PV) and conjugated dienes (CD) hydroperoxides are mainly used to evaluate primary oxidation products; para-anisidine value (p-AV) and thiobarbituric acid reactive substances (TBARS) are used to evaluate secondary oxidation products; totoal oxidation (TOTOX) combines PV and p-AV to describe the overall oxidation status. Other methods, such as infrared Spectroscopy (IR), ultraviolet–visible spectroscopy (UV–vis), raman spectroscopy, gas chromatography (GC), High-performace liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), electronic nose, and electrochemical methods, can provide additional information from the perspective of different oxidation products or physicochemical properties^[37].

For this study, the oil phase contains highly unsaturated DHA/ARA oils. The oil samples are also relatively expensive, and the emulsion system is complex. Therefore, the selection of analytical methods should consider not only the reliability of the detection principle, but also sample consumption, similarity to pharmacopoeial methods, and possible interference from the complex matrix.

3.11.1 Peroxide Value

Peroxide value is a classic indicator for evaluating primary lipid oxidation products^[38], and hydroperoxides are formed during lipid oxidation. The review also points out that iodometric titration is the most commonly used traditional method for PV determination^[39]. Its principle is similar to European Pharmacopoeia 2.5.5. PEROXIDE VALUE^[40]. Lipid hydroperoxides oxidize iodide ions (I^-) to iodine (I_2), and the released iodine is then titrated with sodium thiosulfate. After blank correction, the result is calculated as mmol LOOH/kg oil. The priciples are shown in equation 3.1 and 3.2.



Therefore, iodometric titration was selected as the main method for evaluating primary oxidation in this study, because it is closest to the pharmacopoeial method, and PV itself is a widely used indicator of early lipid oxidation.

3.11.2 Conjugated Dienes hydroperoxides

The presence of antioxidants may interfere with iodometric titration, because this method is based on a redox reaction. In addition, the oxidation analysis of ARA/DHA-containing complex formulations may be affected by matrix effects and method reliability. Therefore, CD measurement was used as an independent primary oxidation indicator that does not rely on iodometric titration, in order to cross-check the PV results.

CD measurement is another method for evaluating primary oxidation products but more easily to handle. In the study by Schröder et al.^{[41][42]}, CD hydroperoxides were clearly measured as primary oxidation products. The absorbance was measured at 233 nm.

3.11.3 Para-anisidine value

European Pharmacopoeia 2.5.36.ANISIDINE VALUE^[43] and relevant literature^{[37][39][41]} show that p-AV is mainly used to evaluate secondary oxidation products. In these studies, p-AV is used as a method for measuring total aldehydes and as an indicator of secondary lipid oxidation products. The substrate reacts with p-anisidine, and the absorbance is then measured at 350 nm.

However, p-AV also has clear methodological limitations. Bannenberg et al. pointed out that p-AV is suitable for measuring secondary oxidation in refined marine triglyceride oils or ethyl ester concentrates, but it is not robust for many additives^[44]. Flavored or colored oils may produce false-positive signals, so p-AV may not reliably evaluate secondary oxidation in these products. The same study also noted that emulsion samples and phospholipid-rich samples may have problems such as poor dissolution, phase separation, and sample preparation difficulties. Therefore, a suitable method should be selected according to the specific oil type and formulation complexity.

3.11.4 TOTOX

TOTOX is a combined indicator based on PV and p-AV. In the study by Bannenberg et al.^{[37][44]}, TOTOX was calculated using the following equation (3.3):

$$TOTOX = 2 \times PV + p - AV \quad (3.3)$$

The same study stated that AOCS standards usually include PV, p-AV, and TOTOX at the same time, because PV may decrease when primary oxidation products are converted into secondary oxidation products.

3.11.5 Oxidation Kinetics Model

Lipid oxidation is not a static process, but a reaction process that changes over time. Pinchuk and Lichtenberg pointed out that, to avoid misjudgement caused by single-time-point measurements, lipid peroxidation should preferably be evaluated based on its time-dependent changes^[45]. The oxidation curve can be described by several characteristic time points and rate parameters.

In general, the early stage of lipid oxidation is mainly shown by the formation of primary oxidation products such as hydroperoxides. Therefore, PV or CD values usually increase at this stage. However, hydroperoxides are not stable and can further decompose into secondary oxidation products, such as aldehydes, ketones, and alcohols. Therefore, PV may not keep increasing in the later stage of oxidation. Zhang et al. also stated that hydroperoxides are primary oxidation products, but they can further degrade into secondary oxidative products. Therefore, comparing PV at only one time point may not fully describe the oxidation degree. Secondary oxidation indicators should also be included in the analysis^[38].

3.11.6 Methodology of Tocopherol and Tocopherol acetate

To further explain the changes in oxidation degree, tocopherol and tocopherol acetate were considered because it acts as an antioxidant and can reduce the accumulation of peroxides

during the early stage of oxidation. Tocopherol compounds are fat-soluble substances and are commonly found in oil matrices such as vegetable oils.

Several methods have been reported for the determination of tocopherols and tocopherol acetate, including HPLC-UV, HPLC-FLD, LC-MS/MS, SFC-MS, GC-MS, spectroscopic methods, and electrochemical methods^{[46][48]}. Among these methods, HPLC is commonly used for the determination of tocopherol and tocopherol acetate compounds in vegetable oils because of its good separation ability and relatively mature operation^[47]. Liu et al. reviewed that oil samples can be pretreated by direct dilution, saponification, liquid–liquid extraction, or solid-phase extraction^[46]. Direct dilution is simple to operate, but it may suffer from matrix interference. Saponification can be used to determine total tocopherols, but inappropriate treatment conditions may lead to degradation or structural changes of the target compounds.

In this project, the changes in tocopherol and tocopherol acetate contents in the emulsion during storage or oxidation are the main focus. Therefore, HPLC was selected for quantitative analysis. This method can directly reflect the remaining amounts of the target compounds. Since saponification may change the chemical form of the target compounds, extraction or dilution followed by direct HPLC analysis is more suitable for this study. The quantitative results can then be confirmed using standard calibration curves.

4 Material and Methods:

4.1 Material

Table 4.1. Material Information

Reagent	Apparatus
ARA- and DHA- rich vegetable oil (2:1)	Centrifuge Machine
GP1 and EOC Emulsifier	
Choline cholride	
α -glycerophosphocholine	
FeSO ₄ • 7H ₂ O	Rotary Evaporator
Na ₂ EDTA • 2H ₂ O	
NaCl	Shaking Incubator
Hexane	
Isopropanol	UV spectrophotometer
Glacial Acetic Acid	
Isooctane	HPLC
Potassium Iodide	
Potato starch	Centrifuge Machine
Sodium Thiosulfate	
α -tocopherol	-40°C Freezer
α -tocopherol Acetate	
p-anisidine	Mastersizer
Methanol	
Acetone Nitrile	
Ultrapure Water	

4.2 Methods

4.2.1 Preparation of the O/W Emulsions

The Emulsion contains 16 mL ARA- and DHA- rich vegetable oil (2:1); 24 mL Ultrapure water; 2.5/3 mL GP1 or EOC emulsifier; 850/860 mg Choline-equivalent (correspond to amount of emulsifier). The Equation (4.1) shows the calculation of Choline-equivalent.

$$\text{Choline equicalent}(mg) = \text{Compound}(mg) \times \frac{M_{\text{choline}}}{M_{\text{compound}}} \times \text{purity} \quad (4.1)$$

Where $M_{\text{choline}}=104.17$ g/mol, $M_{\text{choline chloride}}=139.62$ g/mol, $M_{\alpha\text{-glycerophosphocholine}}=257.22$ g/mol.

A total of emulsifiers was weighed directly into a 50 mL centrifuge tube, 16 mL ARA- and DHA- rich vegetable oil (2:1) was measured in a measuring cylinder. The oil was added to 50 mL centrifuge tube. 24 mL ultrapure water was measured in a measuring cylinder, transfered to 50 mL centrifuge tube. The mixture of water, emulsifiers and oil was stirred for approximately 30 min with magnetic stirring until a homogeneous dispersion was obtained. The coarse emulsion was finally processed in the LM10 Microfluidizer® Processor, equipped with a Y-Chamber geometry, to create a very finely dispersed emulsion. The instrument was operated at 750 bar, the procedure was repeated until the emulsion had passed the Y-Chamber geometry 3 times. The final emulsion was collected, and samples were stored in new 50 mL centrifuge tube.

The main process parameters selection was included in the appendix.

4.2.1.1 Incubation of O/W Emulsion, for accelerated study

The Fe–EDTA pro-oxidant was prepared according to the literature method ^[41], FeSO₄ solution and EDTA solution were separately prepared using ultrapure water at the same concentration of 84 mM. The FeSO₄ and EDTA solutions were then mixed at an equal volume ratio of 1:1 (v/v) and stirred at moderate speed for 1 h under dark conditions to form the iron–EDTA complex.

For the oxidation initiator experiment, 2 mL of emulsion was transferred into 3 mL polypropylene tubes. Then, 100 µL of freshly prepared iron–EDTA solution was added to each sample, giving equal final concentrations of Fe and EDTA in the system (2 mM diluted from the 84 mM stock solution). The samples were then used for subsequent incubation and analysis of oxidation-related indicators. Samples were stored at room temperature on a shaking platform at 20 rpm, the oxygen source is from headspace (1 mL, 1/3 of the container volume) of the vial and the oxygen dissolved in liquid, store at 25 °C, collected 7 times over a period of 15 days (0, 1, 3, 5, 7, 10 and 15).

4.2.2 Determination of Physicochemical Index in Emulsion During aging

4.2.2.1 Extraction of The Lipid Phase From O/W Emulsion

All samples were maintained in an ice bath throughout the extraction procedure. For each assay, 2 mL of the O/W emulsion was transferred into a 10 mL centrifuge tube. Saturated NaCl solution (1.0 mL) was added to facilitate demulsification, and the mixture was shaken twice. Subsequently, 7.0 mL of hexane/isopropanol (1:1, v/v) was added as the extraction solvent. The mixture was vortexed for exactly 1 min and centrifuged at 2000 × g for 8 min at 4 °C.

The supernatant was collected and kept on ice for 10 min. When phase separation was not clearly observed, the centrifugation step was repeated. The organic phase was then transferred into a pear-shaped rotary evaporation flask and evaporated at 40 °C for exactly 10 min. The recovered oil was weighed, divided into five 1.5 mL microcentrifuge tubes, and stored at –40 °C until further analysis. The oil recovery was subsequently calculated.

4.2.2.2 Determination of Peroxides Value

Approximately 0.10 g of the extracted oil sample was weighed and dissolved in 25 mL of a glacial acetic acid/isooctane mixture at a ratio of 3:2 (v/v). The solution was then transferred into a conical flask. Subsequently, 1 mL of freshly prepared saturated potassium iodide solution, prepared with ultrapure water, was added to each flask. The mixture was gently shaken by hand for 1 min and then kept in the dark for 5 min to allow the reaction to proceed.

After incubation, 75 mL of ultrapure water and 1 mL of potato starch indicator solution were added. The starch solution was prepared by dissolving 1 g of potato starch in 100 mL of boiling water. The solution became dark blue after the addition of starch. The released iodine was titrated with 0.001 mol/L sodium thiosulfate solution until the blue color disappeared and the mixture turned white or nearly colorless. The volume of sodium thiosulfate consumed by the oil sample was recorded as V_{eq}. A blank test was carried out using the same procedure but without the oil sample, and the consumed sodium thiosulfate volume was recorded as V_b.

The amount of lipid hydroperoxides, expressed as LOOH, was calculated based on the difference between V_{eq} and V_b as shown in equation 4.2 and 4.3.

$$n_{LOOH} = \frac{(V_{eq} - V_b) \times C_{S_2O_3^{2-}}}{2} \quad (4.2)$$

Where the unit of V is L, C is mol/L, n is mol

To calculate the PV, the mass of oil is needed.

$$V = C_{LOOH \text{ in oil}} \left(\frac{mmol}{kg} \right) = \frac{n_{LOOH} \times 1000}{m_{oil}(kg)} \quad (4.3)$$

Where the n_{LOOH} is the amount of lipid hydroperoxides, m_{oil} is the mass of oil.

4.2.2.3 Determination of Conjugated Diene

Approximately 0.03 g of the extracted oil sample was accurately weighed and diluted with hexane. The dilution factor was initially set at 100-fold and was adjusted when necessary to ensure that the final absorbance was within the range of 0.2–0.5. Pure hexane was used as the reference solution

The absorbance of the diluted oil sample was measured at 233 nm using a UV–visible spectrophotometer. The concentration of conjugated dienes was then calculated according to the Beer–Lambert law with 27,000M⁻¹cm⁻¹ as the molar extinction coefficient of CD hydroperoxides, as shown in equation 4.4

$$n(CD) = \frac{A_{233}}{\epsilon \cdot l} \times DF \times V \quad (4.4)$$

Where A_{233} is the absorbance at 233 nm, DF is the dilution factor, ϵ is the molar extinction coefficient of conjugated dienes, and l is the optical path length of the cuvette.

To calculate the CD, the mass of oil is needed and shown in equation (4.5).

$$CD_{mmol/kg \text{ oil}} = \frac{n(CD)}{m_{oil}} \times 1000 \quad (4.5)$$

Where $n(CD)$ is the concentration of conjugated dienes, m_{oil} is the mass of oil.

4.2.2.4 Determination of Para-anisidine Value

Approximately 0.03 g of the extracted oil sample was dissolved in 1 mL of hexane. Pure hexane was used as the blank, and the absorbance of the oil solution was measured at 350 nm. This absorbance was recorded as Ab .

For the reagent blank, 1.0 mL of hexane was mixed with 0.2 mL of p-anisidine solution, which was prepared by dissolving p-anisidine in acetic acid at a concentration of 2.5 g/L. The mixture was incubated for exactly 10 min at room temperature.

For the sample reaction, 0.2 mL of the p-anisidine solution was added to the oil solution. The mixture was mixed well and incubated in the dark at room temperature for exactly 10 min. After incubation, the absorbance was measured at 350 nm and recorded as As .

The p-anisidine value was calculated using the following equation 4.6:

$$pAV = \frac{(1.2A_s - A_b)}{m} \quad (4.6)$$

Where m_{oil} is the mass of oil.

4.2.2.5 HPLC-DAD Analysis of α -tocopherol and α -tocopherol Acetate

Approximately 0.1 g of the extracted oil sample was weighed and diluted with 400 μ L isopropanol. The mixture was vortexed for 1 min, then centrifuged at 8000 rpm for 10 mins, the upper layer was collect and filter through 0.22 μ m PVDF syringe filters and injected in the chromatographic instrument.

The chromatographic analysis was performed using an Agilent 1260 Infinity II HPLC-DAD system, all opeations were carried out from the OpenLAB software. All samples were filtered through 0.22 μ m PVDF syringe filters

For the separation of the analytes, a reversed-phase HALO 90 Å C18(2.1 $\times$ 100 mm, 2.7 μ m) analytical column was used. A mobile phase consisting of methanol (MeOH)(A) and acetonitril (ACN)(B) was used for the separation of tocopherol and tocopherol acetate. The temperature of the column was maintained at 28 °C. The elution program lasted for 15 min for one sample. The flow rate was set to 0.4 mL/min and the absorbance was measured at 294 nm for tocopherol and 284 nm for tocopherol acetate.[47]

Linearity was evaluated using standard solutions of tocopherol and tocopherol acetate at seven concentration levels. The concentration range was 0.278–286.2 μ g/mL for tocopherol and 0.198–50.9 μ g/mL for tocopherol acetate. Calibration curves were constructed by plotting the peak area against the concentration of each standard compound. The coefficient of determination, r^2 , was calculated to assess the linearity of the method

4.2.2.6 Particle size measurement

The oil droplet size distribution of the emulsions were measured by laser diffraction using a Malvern Mastersizer. A few drops of emulsion was added into the sample unit containing water as the dispersant until the laser obscuration reached 10–20%. The refractive index of sunflower oil was set as 1.464, and water was used as the dispersant in the calculation. The stirring speed was set at 1200 rpm. The measurement was performed on the same day after emulsion preparation.

4.2.3 Experimental Design

To systematically compare the effects of choline form, emulsifier system, in order to identify the most promising formulation candidates. A full factorial screening design was applied. The experimental matrix was generated using Design-Expert 12.0 software. The matrix is shown in Table 4.2 below.

Table 4.2. Run Sequence of Each Emulsion(GPC refers to α -glycerophosphocholine, and CC refers to choline chloride.)

Run	Factor 1 Emulsifier amount (mL)	Factor 2 Emulsifier Type	Factor 3 Choline Form
1	3	GPC	EOC

2	2.5	GPC	EOC
3	3	CCC	EOC
4	2.5	GPC	GP1
5	3	CC	GP1
6	2.5	GPC	EOC
7	3	GPC	GP1
8	2.5	CC	EOC
9	2.5	CC	GP1

Responses: Droplet Size, Oxidation kinetics index (oxidation accumulation, Δ PV-AUC, Δ p-AV-AUC, Δ TOTOX-AUC)

4.2.4 Statistical Analysis

The data will be analyzed using Design-Expert 12.0. Main effects plots will be generated, and ANOVA will be used to assess statistical significance and rank the magnitude of factor effects. For desirability and emulsion selection, the first priority is that the emulsion must be physically stable with a reasonable droplet size distributio; Second should be good oxidation stability, and third would be to have low emulsifier amount.

4.2.5 Overall Workflow

Figure 4.1 shows the workflow of all procedures in experiments.

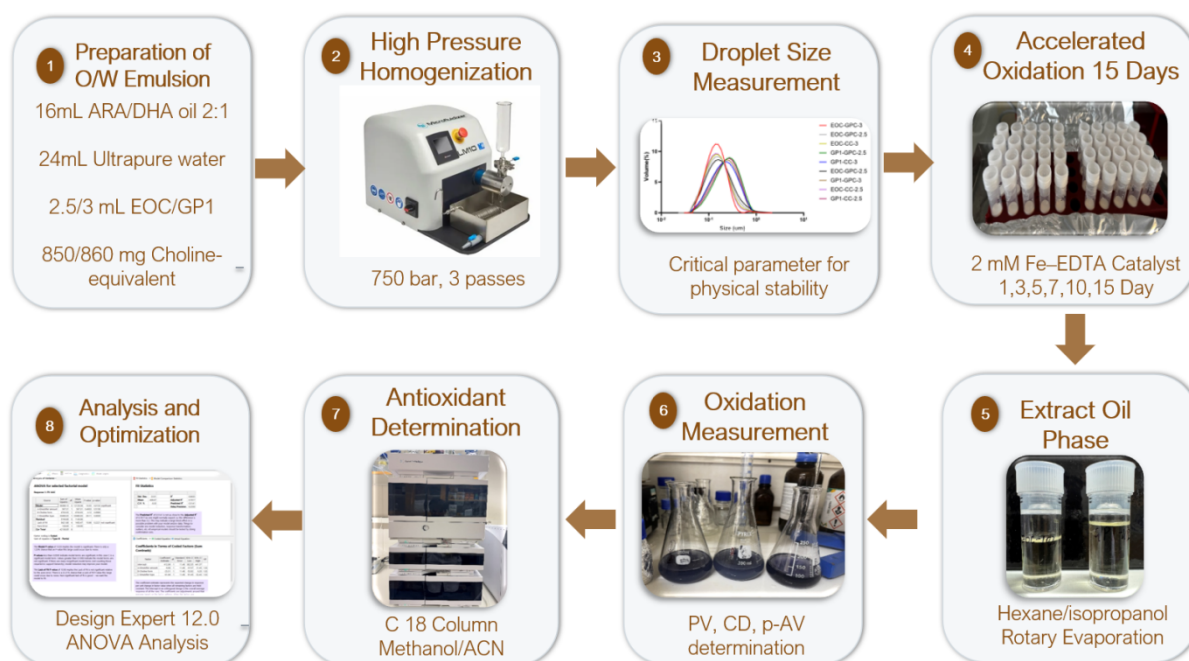


Figure 4.1. Workflow of Experiment.

5 Results and Discussion

5.1 Extraction

Table 5.1 shows the mean oil recovery rate and standard deviation of each sample at different time points.

Table 5.1. Oil Recovery of each Run (mean $\pm$ SD, $n = 7$)

Run	Oil Recovery(mean $\pm$ SD)
1	60.66 $\pm$ 14.97
2	67.5 $\pm$ 3.11
3	76.09 $\pm$ 4.12
4	65.10 $\pm$ 4.42
5	58.28 $\pm$ 3.20
6	63.32 $\pm$ 4.39
7	63.67 $\pm$ 4.94
8	59.22 $\pm$ 3.64
9	66.06 $\pm$ 2.90

5.2 Droplet Size

The droplet-size results from Figure 5.1 and Figure 5.2 showed that emulsifier type was the dominant factor controlling emulsion droplet size. Under the same formulation conditions, the EOC system produced smaller droplets than the GP1 system. In the factor-effect model graphs in Figure 5.3, changing from GP1 to EOC significantly reduced $D_v(0.5)$ ($***p < 0.0001$), whereas choline form had little effect. Increasing the emulsifier amount from 2.5 to 3 mL slightly reduced droplet size ($*p=0.0407$), but this effect was weaker than the effect of emulsifier type.

This result is consistent with the compositional difference between the two emulsifier systems. EOC contains E476/PGPR, whereas GP1 contains E475/polyglycerol esters of fatty acids. PGPR/E476 is described as polyglycerol polyricinoleate and is widely known as a highly effective emulsifier and viscosity modifier in lipid-rich systems[33]. In contrast, E475 is a broader class of polyglycerol fatty acid esters; its hydrophilic-lipophilic balance depends on the length of the polyglycerol chain and the degree of esterification, so it may behave as either an O/W or W/O emulsifier depending on composition [33][34]. Therefore, the smaller droplets in EOC may be related to stronger interfacial activity or more efficient droplet disruption.

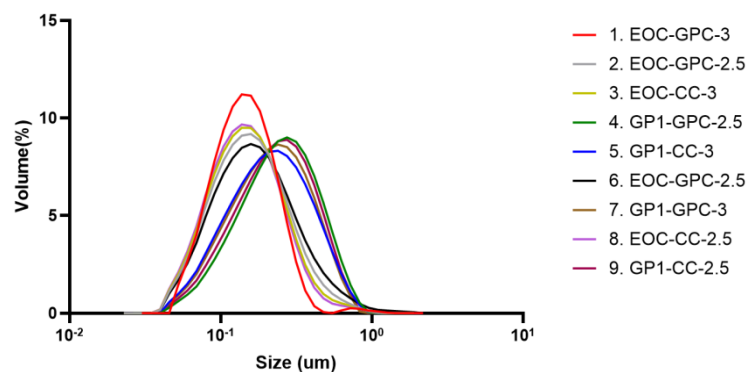


Figure 5.1. Droplet size distribution of all formulations

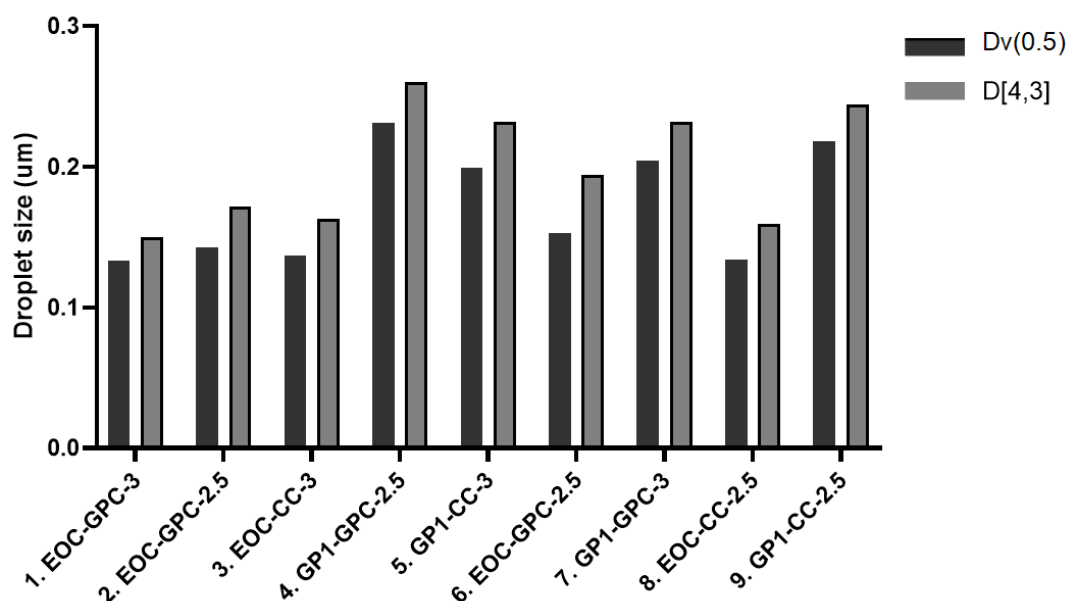


Figure 5.2. Dv(0.5) and D[4,3] of all formulations

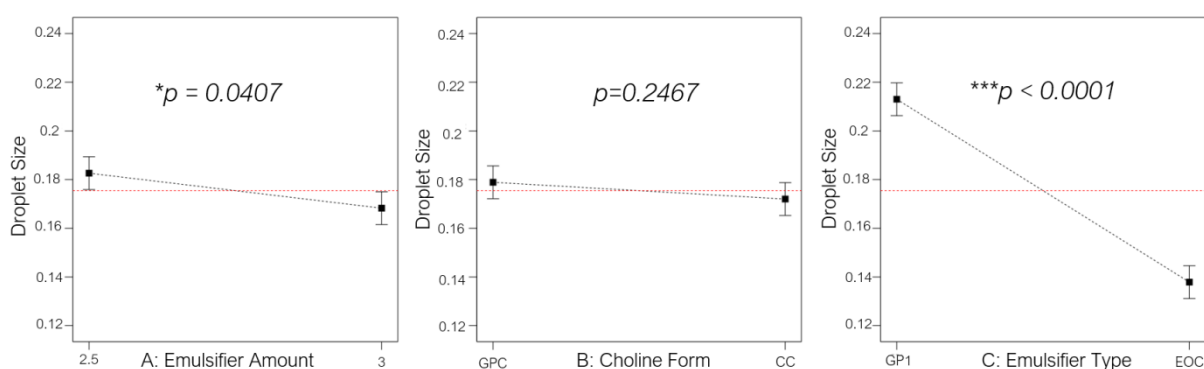


Figure 5.3. All Factor Model Graphs on droplet size Dv(0.5) (significant model, $p = 0.0002$; A: emulsifier amount, $*p = 0.0407$; C: emulsifier type, $***p < 0.0001$; B: choline form, $p = 0.2467$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All p -value were calculated by ANOVA analysis through Design Expert 12.0).

5.3 Repeatability of Each Quantitative Method

Table 5.2 shows the repeatability of each method, the medium oxidative sample were used for each determination, all repeatability are below 5% and acceptable.

Table 5.2. Repeatability of Each Quantitative Method ($n=6$)

Method	Mean	SD(RSD)
PV	46.37 mmol/kg	2.22mmol/kg (4.79%)
CD	79.36 mmol/kg	1.54 mmol/kg (1.94%)
p-AV	7.23	0.31(4.29%)

5.4 Oxidation Index Measurement

Because the determination of oxidation indicators required demulsification and extraction of the oil phase, formulation-dependent differences in oil extraction efficiency, droplet size and residual emulsifier may have influenced the measured values. All pretreatment steps were kept as consistent as possible, but small differences introduced by demulsification and extraction could not be fully excluded. Therefore, time-course interpretation and AUC-based comparison were used instead of relying only on a single time point.

PV was used as the main indicator of primary oxidation products and was measured in duplicate by iodometric titration. Since the oil phase contained VE and VE acetate, early-stage PV values may have been underestimated due to possible redox interference. CD hydroperoxides were therefore used as a supporting primary-oxidation indicator. p-AV was used to describe secondary oxidation products, mainly aldehydic products. TOTOX was used as a comprehensive oxidation index calculated from PV and p-AV. Since CD and p-AV were measured only once, Vmax calculated from adjacent time points was considered less robust than AUC. Therefore, AUC-based parameters were used as the main responses in Design-Expert.

The Δ PV curves which were shown in Figure 5.4 showed a common early increase in most samples, indicating accumulation of primary oxidation products during the first storage period. Around Day 7 to Day 10, the curves started to diverge. Some samples showed a decrease in PV, whereas others continued to accumulate hydroperoxides. The subsequent decrease in PV in several formulations is consistent with the instability of lipid hydroperoxides and their further conversion into secondary oxidation products. All curve conforms to the experimental expectations and accurately corresponds to the description in the literature^[37].

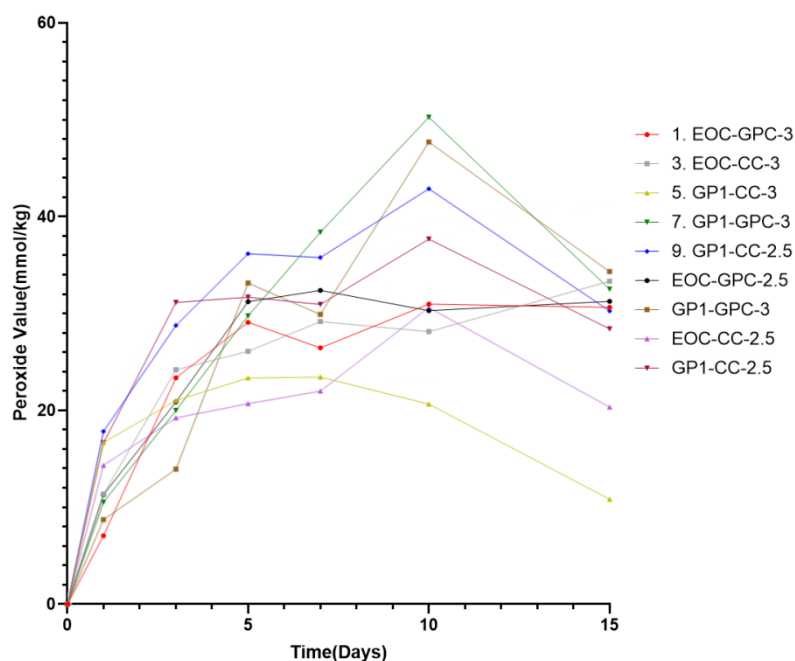


Figure 5.4. Δ PV curves of all samples during accelerated oxidation (PV measured in duplicate)

Based on the kinetic index from appendix, samples 1, 3, 4 and 7 were selected as representative candidate formulations for detailed curve comparison. These formulations were not selected to

represent the worst-performing samples, but because they showed favourable performance in at least one oxidation-related parameter. As shown in Figure 5.5 and kinetics index in appendix, sample 7. GP1-GPC-3 showed the lowest Δ TOTOX-AUC and TOTOX Vmax, indicating the lowest overall oxidation burden. sample 4. GP1-GPC-2.5 showed the lowest Δ p-AV-AUC, suggesting effective suppression of secondary oxidation products. sample 3. EOC-CC-3 had the lowest Δ PV-AUC and PVmax, but its Δ p-AV-AUC was relatively high, indicating that evaluation based only on PV may underestimate later-stage oxidation. sample 1. EOC-GPC-3 showed a relatively long PV lag time and low PV Vmax, although its secondary oxidation accumulation was less favourable.

In the selected curves shown in Figure 5.5, PV and CD were used as complementary indicators of primary oxidation, while p-AV was used to describe secondary oxidation. A temporary decrease in CD values was observed around Day 3 in several samples. This may reflect the ARA and DHA oil special dynamic balance between the formation and consumption of conjugated diene hydroperoxides during oxidation. The comparison also showed that PV and CD did not always change in parallel, this is rational result, since conjugated dienes structure not only presented in LOOH (primary oxidation product), but also in L $\cdot$, LO $\cdot$, LOO $\cdot$ or other intermedia structure; therefore, this observation was interpreted only as a trend rather than definitive mechanistic evidence.

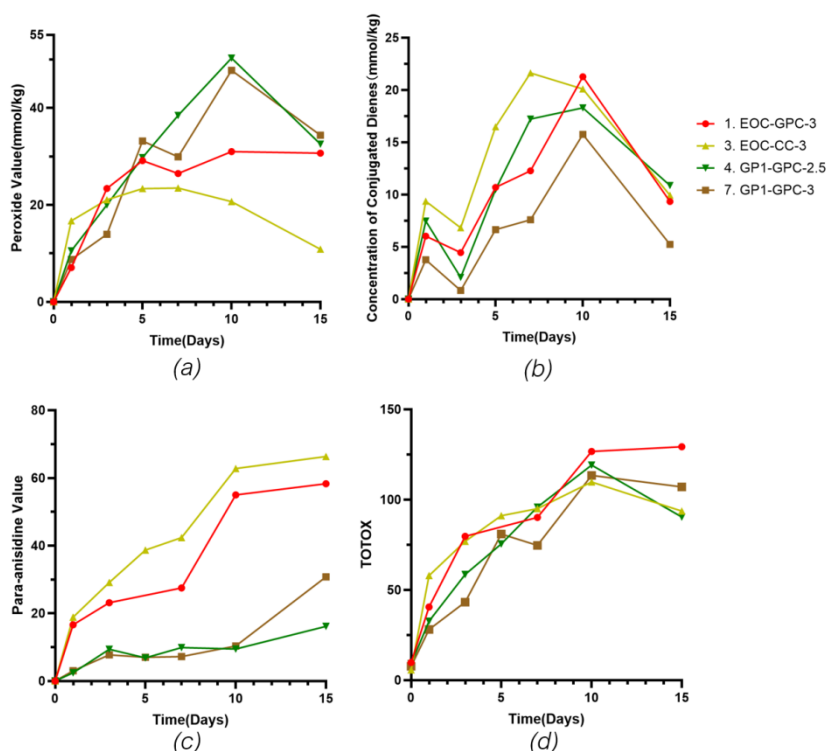


Figure 5.5. PV (a), CD (b), p-AV (c) and TOTOX (d) curves of selected formulations.

Figure 5.6 shows Δ PV-AUC, which represents the cumulative burden of primary oxidation products over the storage period. Figure 5.6 shows Δ p-AV-AUC, and it represents the cumulative burden of secondary oxidation products. Compared with Vmax, AUC is less sensitive to one abnormal time point and is therefore more suitable for formulation screening when some indicators are measured only once. The Δ PV-AUC bar plot suggested that several

EOC-containing formulations had lower primary oxidation accumulation than GP1-containing formulations. However, the Δp -AV-AUC bar plot showed the opposite trend: EOC-containing formulations, especially EOC-CC formulations, accumulated more secondary oxidation products

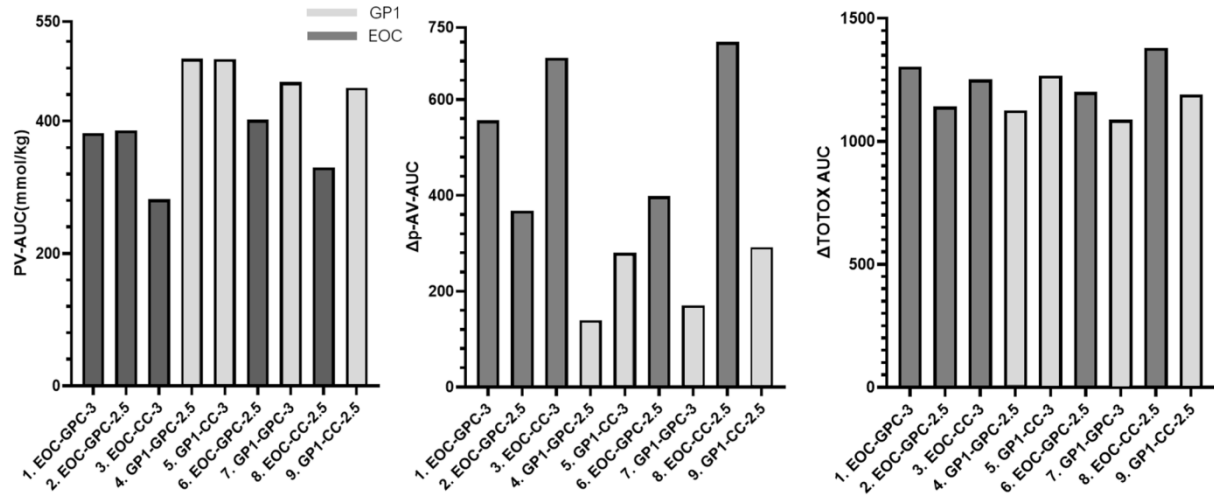


Figure 5.6. ΔPV -AUC (left), Δp -AV-AUC (middle) and $\Delta TOTOX$ -AUC (right) of all formulations

5.5 ANOVA analysis

A main-effects factorial model was fitted for ΔPV -AUC, Δp -AV-AUC, $\Delta TOTOX$ -AUC and $Dv(0.5)$. P-values between 0.05 and 0.10 were described as trends only. The analysis showed that ΔPV -AUC and Δp -AV-AUC were suitable oxidation responses for explaining formulation effects, whereas $\Delta TOTOX$ -AUC was more appropriate as a criteria for formulation optimization.

A significant model p-value indicates that the model can explain the variation of the response, while a significant factor p-value indicates that this factor has a measurable effect on the response. The lack of fit test evaluates the suitability of the model. In general, a non-significant lack of fit is preferred, as it suggests that the model adequately fits the experimental data.

Table 5.3. Summary of ANOVA results for selected responses (Factor A: emulsifier amount, Factor B: Choline form, Factor C: emulsifier type; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; All p-value were calculated by ANOVA analysis through Design Expert 12.0)

Response	Model p-value	Factor A p-value	Factor B p-value	Factor C p-value	Lack of fit
ΔPV -AUC	0.0133*	0.5138	0.0980	0.0030**	0.2221
Δp -AV-AUC	0.0024**	0.2953	0.0085**	0.0007***	0.2030
$\Delta TOTOX$ -AUC	0.1361	0.7115	0.0727	0.0905	0.3894
$Dv(0.5)$	0.0002***	0.0407*	0.2467	< 0.0001***	0.5785

The analysis result is shown in Table 5.3. For ΔPV -AUC, the model was significant (* $p = 0.0133$) and the lack of fit was not significant ($p = 0.2221$). Only emulsifier type was significant (** $p = 0.0030$), while choline form showed a slight trend to be significant ($p = 0.0980$). This

supports the observation that EOC reduced $\Delta PV-AUC$ compared to GP1. For $\Delta p-AV-AUC$, the model showed higher significance (** $p=0.0024$), and both choline form (** $p=0.0085$) and emulsifier type (** $p=0.0007$) were significant. GPC and GP1 were associated with lower $\Delta p-AV-AUC$, suggesting lower accumulation of secondary oxidation products. Because the $\Delta TOTOX-AUC$ model was not significant ($p=0.1361$), it was not used to explain factor effects. It was only used to descriptively compare the overall oxidation level of different formulations. For $Dv(0.5)$, the model was highly significant (** $p=0.0002$), mainly due to emulsifier type, confirming that EOC formed smaller droplets than GP1.

The most significant result shown in Figure 5.7 and Figure 5.8. is that EOC and GP1 have opposite effects on primary and secondary oxidation. EOC reduces $\Delta PV-AUC$ but increases $\Delta p-AV-AUC$; while GP1 shows a higher $\Delta PV-AUC$ but a lower $\Delta p-AV-AUC$. This implies that EOC may reduce the accumulation of hydroperoxides, but this does not necessarily mean that the overall oxidation level has decreased. A reasonable explanation is that the hydroperoxides produced in the EOC system decomposes more quickly into aldehyde substances, thereby causing an increase in $\Delta p-AV-AUC$.

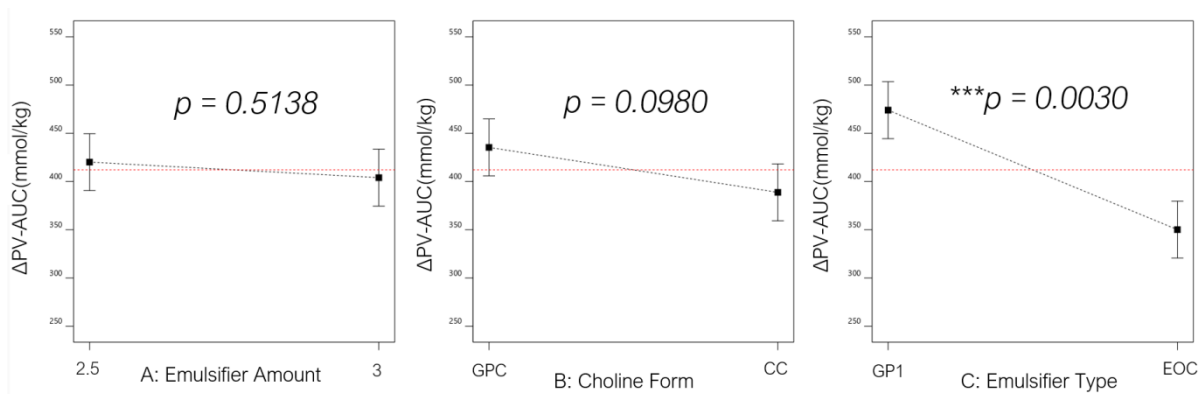


Figure 5.7. All Factor Model Graphs of ANOVA on $\Delta PV-AUC$ (significant model, * $p=0.0133$; ** $p<0.05$, ** $p<0.01$, *** $p<0.001$; Lack of Fit=0.2221).

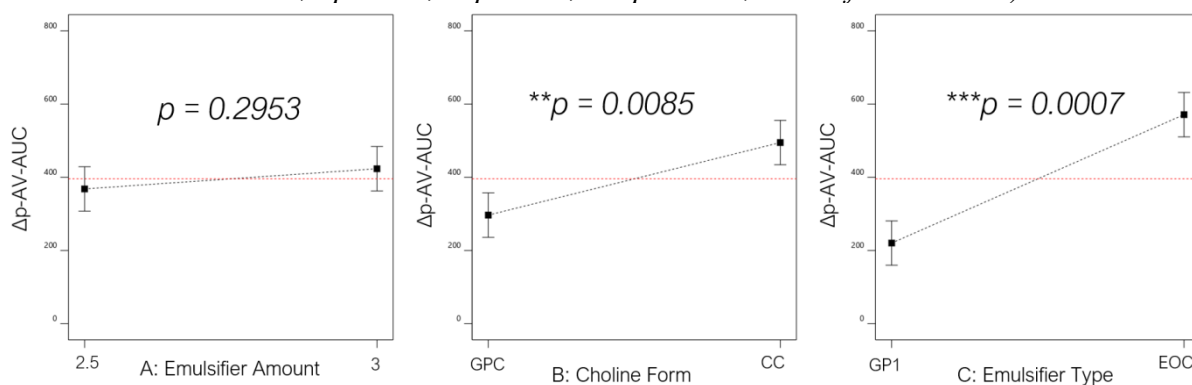


Figure 5.8. All Factor Model Graphs of ANOVA on $\Delta p-AV-AUC$ (significant model, ** $p=0.0024$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Lack of Fit=0.2030).

This interpretation is consistent with current understanding of lipid oxidation in emulsions. Hennebelle et al. emphasized that droplet size, interfacial composition, emulsifier type and the partitioning of pro-oxidants and antioxidants jointly control oxidation in O/W emulsions. Smaller EOC droplets provide larger interfacial area, which may increase the contact between DHA/ARA lipids, oxygen, iron and other aqueous pro-oxidants. Meanwhile, E476 may produce

a different interfacial layer and different non-adsorbed emulsifier structures compared with E475.

Ten Klooster et al. showed that TAG-bound hydroperoxides hardly transfer between droplets, whereas smaller and more hydrophilic secondary oxidation products or small hydroperoxide-containing molecules can transfer more rapidly^[26]. Therefore, the smaller droplet size in the EOC system which contains E476 may have promoted the redistribution of some small oxidation products or antioxidants between droplets, leading to a faster formation of secondary oxidation products at the early stage.

The emulsion oxidation was catalyzed by metals, as the aqueous phase contains metal ion catalysts. The metal ion catalysts may be affected by the combined effects of different emulsifiers and choline ion concentrations, resulting in different distributions of the catalysts on the emulsion surface. The metal ions mainly catalyze the formation of secondary oxidation products, which is one possible reason of why the choline form and emulsifier type have significant effects in the model. A possible explanation is that the smaller droplets produced by EOC provide a larger total oil–water interfacial area. This may increase the opportunity for metal ions in the aqueous phase to interact with lipid hydroperoxides located near the droplet interface, thereby accelerating hydroperoxide decomposition and the formation of secondary oxidation products. In addition, the smaller droplets may provide a larger interfacial area and shorter diffusion distances, thereby facilitating the migration of small and relatively polar oxidation products from the oil droplets and promoting their further conversion into secondary oxidation products.

Choline form mainly influenced Δp -AV-AUC. GPC produced lower Δp -AV-AUC than CC, while its effect on ΔPV -AUC was only a trend. This suggests that GPC did not mainly act by changing droplet formation, but may have influenced the interfacial or aqueous-phase environment involved in the conversion of hydroperoxides to secondary aldehydes. Compared with choline chloride, α -glycerophosphocholine is a larger zwitterionic molecule containing a glycerophosphate group and several hydrophilic functional groups. A possible explanation is that GPC may modify the hydration, polarity, and ionic environment near the oil–water interface. This could reduce the contact between lipid hydroperoxides inside the droplets and metal ions in the aqueous phase, or limit the transfer of oxidation products from the oil droplets to the surrounding aqueous phase. However, this mechanism remains hypothetical because the interfacial distribution of GPC was not directly measured in this study.

This remains a hypothesis because metal binding, antioxidant partitioning and interfacial composition were not directly measured.

Based on the corrected AUC responses from Table 5.4, sample 4.GP1-GPC-2.5 was selected as the most promising formulation in this experimental design. It showed the best desirability value of 0.942825 and had the lowest Δp -AV-AUC and $\Delta TOTOX$ -AUC in the model-based ranking. Although its PV-AUC and droplet size were not the lowest, this formulation showed the best balance for controlling secondary oxidation and overall oxidation accumulation.

EOC-containing formulations showed lower ΔPV -AUC and smaller droplet size, but they also had much higher Δp -AV-AUC, suggesting stronger formation of secondary oxidation products. For DHA/ARA emulsions, this is particularly important because secondary oxidation products are more complex and may be less desirable for sensitive users such as preterm infants. Therefore, sample 4.GP1-GPC-2.5 was considered the model-based optimized formulation.

Table 5.4. Model-based optimization ranking of formulation combinations generated by Design Expert 12.0 (Desirability was calculated by using Δ TOTOX-AUC as Criteria)

Solution	Emulsifier amount (mL)	Emulsifier Type	Choline Form	Δ TOTOX-AUC	Desirability
1	2.5	GPC	GP1	1104.43	0.942825
2	3	GPC	GP1	1123.148	0.87874
3	2.5	GPC	EOC	1204.492	0.600241
4	2.5	CC	GP1	1212.813	0.571753
5	3	GPC	EOC	1223.21	0.536156
6	3	CC	GP1	1231.53	0.507668
7	2.5	CC	EOC	1312.875	0.229168
8	3	CC	EOC	1331.593	0.165083

5.6 Antioxidant Consumption

Overall, all samples collected at different time points were analysed in duplicate to determine the total tocopherol content and the tocopherol acetate content. As shown in Figure 5.9, the characteristic chromatogram of the tocopherol standard mixture was monitored at 294 nm. The tocopherol peaks were separated within 3.5 min and were quantified as the sum of α -tocopherol, ($\beta + \gamma$)-tocopherol and δ -tocopherol. The analysed compounds, molecular structures and retention times (RT) are labelled in Figure 5.9. The three peaks corresponded to δ -tocopherol (RT = 2.183 min), ($\beta + \gamma$)-tocopherol (RT = 2.509 min) and α -tocopherol (RT = 2.933 min), respectively.

As shown in Figure 5.10, the tocopherol acetate standard was monitored at 284 nm and was separated within 4 min, with a retention time of 3.558 min. The analysed compound, molecular structure and retention time are also labelled in Figure 5.10. The standard curve for total tocopherol was established using the sum of the three tocopherol peak areas, as shown in Equation 5.1. The standard curve for tocopherol acetate is shown in Equation 5.2.

$$y = 3.14020x + 5.82947, R^2 = 0.99955 \quad (5.1)$$

Where x is sum tocopherol concentration[ug/mL] , y is the peek area.

$$y = 3.20067x + 0.500641, R^2 = 0.99967 \quad (5.2)$$

Where x is tocopherol acetate concentration[ug/mL] , y is the peek area.

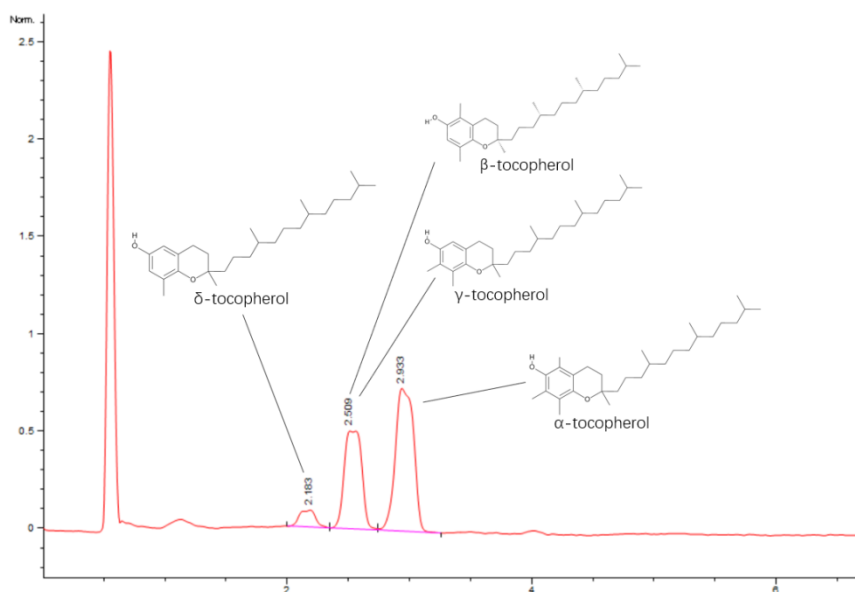


Figure 5.9. Characteristic Chromatogram of a Standard Mixture of Tocopherol Monitored at 294 nm.

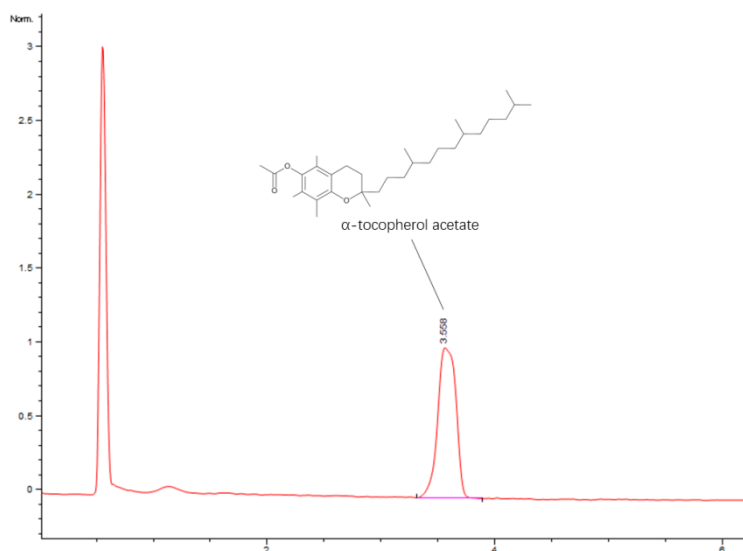


Figure 5.10. Characteristic Chromatogram of a Standard Tocopherol Acetate Monitored at 294 nm.

Figure 5.11 shows the antioxidant content in the fresh oil sample and in the emulsion samples immediately after high-pressure homogenization. The fresh oil sample, dissolved in 400 μ L of isopropanol, contained approximately 213.66 mg/mL of tocopherol. However, except for Samples 1, 6 and 8, no clear tocopherol or tocopherol acetate signals were detected in the other emulsion samples. This may be related to antioxidant loss during sample preparation and processing. Since the emulsions were prepared by high-pressure homogenization, tocopherol and tocopherol acetate may have partially degraded during emulsification. In addition, long rotary evaporation time, and storage at -40°C for 15 days may also have contributed to antioxidant loss or degradation. As no reliable antioxidant signals were detected in the oil samples collected at later time points, these data were not further shown in the figure but in the appendix. It should also be noted that the chromatograms of the extracted oil samples became

more complex after oxidation. Several impurity peaks appeared at different retention times, and some samples showed apparent false-positive signals.

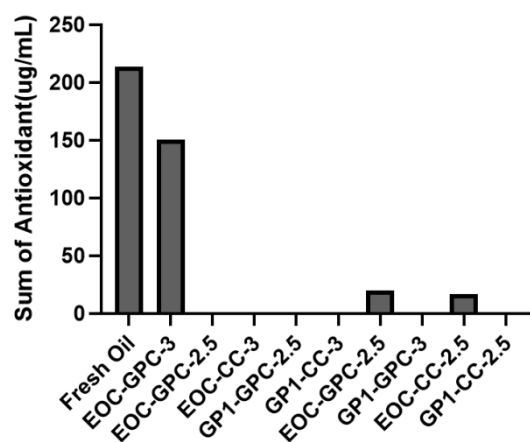


Figure 5.11. Antioxidant Concentrations in Oil Extracted from Emulsion

6 Conclusion:

In this project, choline-enriched DHA/ARA oil-in-water emulsions were prepared and evaluated for physical and oxidative stability. The study focused on the effects of emulsifier amount, emulsifier type and choline form. The oil extraction method gave moderate recovery, but the recovery was generally acceptable for further oxidation analysis. PV, CD, p-AV and TOTOX were used together to describe different stages of lipid oxidation. Since single time-point values may not fully reflect the oxidation process, AUC-based responses were mainly used for formulation comparison.

The droplet-size results showed that emulsifier type was the main factor affecting physical properties. EOC-containing formulations produced smaller droplets than GP1-containing formulations. However, smaller droplet size did not directly mean better oxidative stability. EOC formulations generally showed lower Δ PV-AUC, suggesting lower accumulation of primary oxidation products, but they also showed much higher Δ p-AV-AUC, indicating stronger formation of secondary oxidation products. This suggests that hydroperoxides in EOC systems may have decomposed more rapidly into aldehydic products.

The ANOVA results further supported this interpretation. The Δ TOTOX-AUC model was not significant for factor-level interpretation, but it was still useful as a descriptive indicator for overall oxidation burden.

Based on the Design-Expert screening, GP1 and GPC combination was selected as the most promising formulation. It showed the highest desirability value and the lowest Δ p-AV-AUC and Δ TOTOX-AUC in the model-based ranking. Although its Δ PV-AUC and droplet size were not the lowest, it provided the best balance between physical stability and oxidative stability, especially in controlling secondary oxidation. Therefore, GP1 with GPC emulsion was considered the optimized formulation.

7 Future work:

7.1 Validation

To validate this prediction, the optimized formulation should be prepared again as independent batches and evaluated for particle size, visual stability, PV, CD, p-AV and TOTOX. The observed values should then be compared with the predicted values from the model. If the experimental results are close to the predicted responses, Sample 4 or 7 can be considered as the optimized formulation for further stability evaluation.

The long-term stability of this emulsion is also a potential area for future research, exploring its physical and chemical stability under both normal temperature and refrigerated conditions, without added metal ions for acceleration

At the same time, to meet the truly established industry release standards, through the methodological regulations of ICH, more methodological validations are required to determine whether the various indicators of the emulsion meet the requirements. The only method validation that have done is repeatability.

7.2 Evaluation of Bioavailability

In addition, the bioavailability of this emulsion is also one of the research topics of this project. However, due to time constraints, we were unable to complete the subsequent work of this project. However, based on the literature screening conducted earlier, it has been found that there are already relatively mature evaluation systems for emulsion bioavailability. The model is called "Lipid Digestion and Nutraceutical Bioaccessibility Assessed by Standardized Gastrointestinal Model" (INFOGEST). At the same time, SOP related to simulating gastrointestinal digestion based on the literature were drafted earlier and will be listed in the appendix.

7.3 Safety Issue

Although the research in the literature indicates that this product has certain guarantees in terms of safety and its oxidation products do not pose any biological hazards^{[29][30]}, but the reactive aldehyde substances generated through oxidation may be absorbed by the intestinal tract, and are associated with increased oxidative stress and inflammatory markers in mice^[31]. A more thorough clinical biological safety evaluation might still need to be conducted.

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9 Appendices

9.1 Pre-experiment study

9.1.1 Emulsifier amount and type:

Figure 9.1 shows the droplet distribution of different amount of GP1 emulsifier from CR

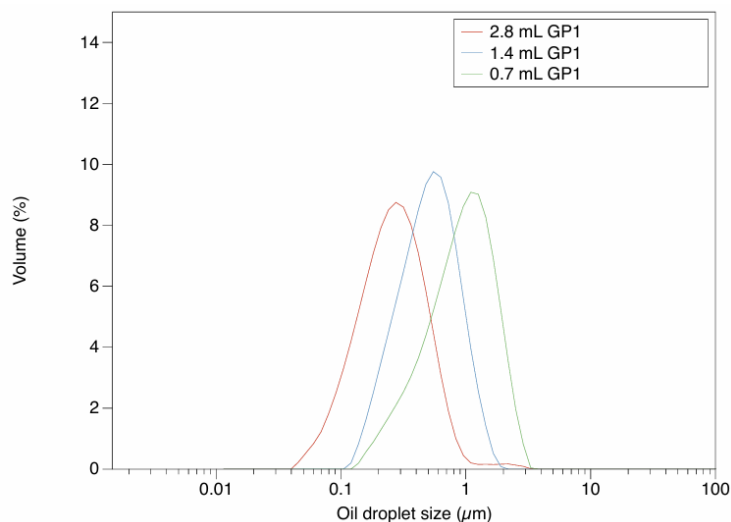


Figure 9.1. Droplet Size Distribution of GP1 from CR

In Figure 9.1 shows, at 1.4 mL (3.4 wt%), the droplet size distribution was around 0.5 μm , and some of those are above 1 μm . At 2.8 mL (6.5 wt%), the droplet size could be reduced to approximately 0.2 μm , and most of those are below 1 μm ; however, an excessive amount of emulsifier may increase the risk of adverse effects in infants. By contrast, at 0.7 mL (1.7 wt%), the most droplets size exceeded 1 μm . In general, all droplet size below 1 μm is better for physical stability, but in practice, the stability systems are often affected by several factor like property of interface, zeta potential, viscosity etc.

Therefore, this study will further optimize the emulsifier concentration from 2.5 mL to 33 mL in order to achieve the best overall performance.

9.1.2 Choline form:

As choline is introduced as a new ingredient, its presence will inevitably affect the interfacial behavior of the emulsion droplets. Accordingly, the specific form of choline used in the formulation is also an important factor that deserves further study. Finally, two types of choline with higher biological relevance were selected. The combinations of the remaining choline with emulsifiers may also possess certain antioxidant potential. CC and GPC are used in this case.

9.1.3 Extraction Process:

The oil recovery in pre-experiment is $59.89\% \pm 5.36\%$ (RSD, n=6), the recovery is quite low, more extraction process is needed.

9.1.4 Oxidation Acceleration

In pre-experiment, the oxidation acceleration condition is under 2 mM Fe-EDTA level at rotator, incubate tubes in the dark at 25 °C, oxygen source is from headspace and solved in liquid, total incubation duration: 3 days.

Figure 9.2 and Figure 9.3 shows the Peroxide Value (iodide titration) and Conjugated Diene Value in 3 days oxidation under catalyst acceleration. As shown in Fig.3 and Fig.4, the emulsion already had a measurable peroxide value at 1h. This is likely because the oil used in the pre-experiment had been stored for a long time. The oil sample was first opened at the end of 2024, so partial oxidation had probably already occurred before use. Under the influence of different emulsifiers, the initial peroxide values were slightly different, which may be related to the type and amount of antioxidants present in the emulsifiers. However, since the antioxidant content in the emulsifiers is unknown, and antioxidants may interfere with determination, a detailed comparison of the initial values could not be made. Therefore, the differences in the initial values were simply ignored in this study.

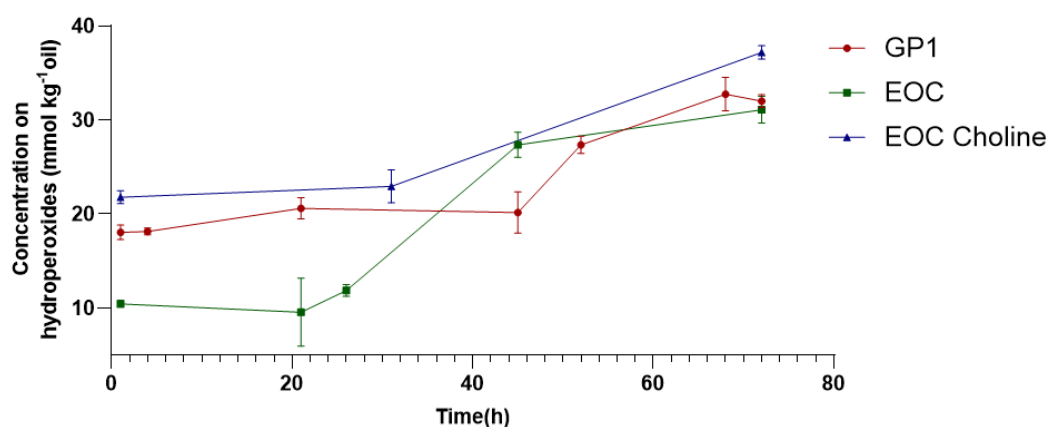


Figure 9.2. Peroxide Value of Different Emulsion (GP1: GP1 as emulsifier. EOC: EOC as emulsifier. EOC Choline: EOC as emulsifier, 0.2g choline chloride were added in emulsion)

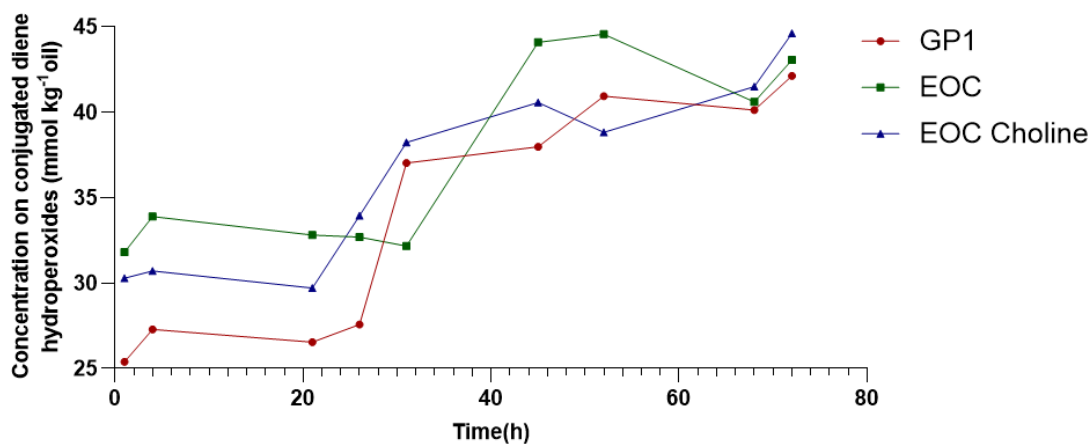


Figure 9.3. Conjugated Diene Value of Different Emulsion (GP1: GP1 as emulsifier. EOC: EOC as emulsifier. EOC Choline: EOC as emulsifier, 0.2g choline chloride were added in emulsion)

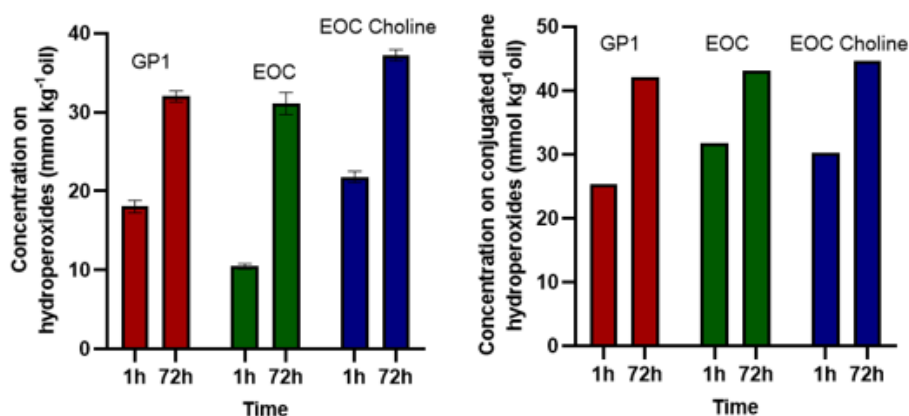


Figure 9.4. Peroxide Value (left) and Conjugated Diene Value (right) from 1h and 72h

As shown in Figure 9.4, by comparing the peroxide value in 1h and 72h, the increase in peroxide value indicates that oxidation did occur in the iron/EDTA catalyst system. However, because only one sample was made without duplicate, no significant analysis could be performed.

However, compared with the literature^{[39] [41]}, the oxidation level observed in this experiment was relatively low. In reference ^[41], the CD value of the oil could reach 300 mmol/kg after 72 h under accelerated conditions, whereas under the same 72 h accelerated oxidation in this experiment, the highest CD value was only 45 mmol/kg. In addition, according to reference ^[39], the PV value could reach 100 mmol/kg after 15 days at room temperature without catalyst addition and with the container left open, while in this experiment, the maximum PV was only 35 mmol/kg after 72 h of accelerated oxidation.

In addition, due to this insufficient extent of oxidation, the kinetic analysis was unable to be carried out to support a comparison of the chemical stability among different emulsions. Future studies may require a longer oxidation time or more severe conditions to better compare the chemical stability of different emulsions.

Moreover, for the oxidation results at 72 h, the sample size was only n=1, so a significance analysis of peroxide value at 1h and 72 h could not be performed. In future experiments, two additional groups of samples at 1 h and 72 h will be reserved for statistical analysis. In subsequent studies, the oxidation conditions will be adjusted by keeping the same accelerated conditions but extending the oxidation period to 15 days, with sample collected at 1,3,5,7,10,15 day for analysis.

9.2 Evaluation of Bioavailability

All information is according to literature^{[49][50][51][52][53]}.

9.2.1 Special Solution:

simulated gastric fluid:

gastric enzyme solution: 400 mg rabbit gastric lipase (15U/mg, 6000U), 24 mg porcine pepsin, dissolved in 2.5 mL distilled water

Biliary secretion stimulation solution: 500 mg bile salts, 200 mg egg yolk phosphatidylcholine, 40 mg cholesterol, 1 mL of 325 mM CaCl₂ solution, 3 mL of 3.25 mM NaCl solution, 20 mL 0.1 M trizma-maleate buffer, pH 7.5. Homogenized for 2 min at 3500 rpm.

Fresh pig pancreatin extraction: 100 mg pancreatin in 4 mL of trizma-maleate buffer 0.1 M, pH 7.5, stirred at 900 rpm for 10 min, and centrifuged at 1600 G, at 5 degrees for 15 min. Collect supernatant as extraction.

9.2.2 In vitro gastrointestinal digestion

Prepare new batches of emulsion, filled with nitrogen, stored in the fridge.

Mix the emulsion with donated breast milk, ratio 1:1. The clinical dosage ratio is not 1:1.

For fat sample that puts to the simulated gastric fluid, 1.86 g fat sample is needed.

Calculation: For breast milk, 3.5-4.5 g/100 mL, 0.04 g/mL, so 1 mL breast milk provides 0.04 g fat, 1 mL emulsion provides 0.4 g fat. The fat concentration is 0.22 g/mL, 1.86 g fat sample for digestion, $1.86/0.22=8.45$ mL mixture is needed.

Add 7.44 mL simulated gastric fluid, mix under 37 degrees, 900 rpm, 10 mins. Then sonicate for 1 min, for pre-emulsification.

Add 1.16 mL **gastric enzyme solution**, stimulate under 37 degree, 900 rpm, 60 mins.

Collect the sample in 10, 20, 30, 40, 60 mins, 400 uL per sample.

Collect 5.883 mL solution, to start with 1 g of fat in intestinal digestion.

Calculation: All solution volumes are $8.45+7.44+1.16=17.05$ mL, for intestinal digestion, the solution volume should be $17.05*100/186=9.167$ mL, so for gastric digestion, the solution volume should be $17.05-9.167=7.883$ mL.

9.167 mL residue sample after gastric digestion, 26 mL of trizma-maleate buffer (0.1 M, pH 7.5) was added to the stirred mixture.

Add all biliary secretion stimulation solution. And homogenize for 2 more minutes at 3500 rpm.

The digestion intestine begins by adding a solution of fresh pig pancreatin extraction, together with 2.5 mL of phospholipase A2 food grade. The intestinal phase was carried out for 60 mins at 37 degrees and 900 rpm.

Collect the sample 1 mL at minutes 0, 2, 5, 10, 20, 30, 45 and 60.

Take half of residue as final product, another half of residue is centrifuge at 4500 rpm and 37 degrees for 45 mins, three phases could be obtained: upper oily phase, middle micellar phase and lower precipitate phase. Separate and weight each phase.

For the sample oily phase, micellar phase, each aliquot and final product, extract with solvent/sample 3:1(v/v). For the sample precipitate phase, extract with 6:1(v/v). The extraction solvent is: ①n-hexane: methyl-tert-butyl-ether (MTBE) (50:50, v: v). ②MTBE: Petroleum ether (50:50, v: v). ③ PE: ethanol (1:0.6, v: v).

Collect each supernatant in an empty vial. Combine all supernatant to another previously weighed vial and evaporated under a stream of nitrogen to contain the weight of residue.

Redissolve the sample with acidified chloroform(0.1 % formic acid) to a final concentration of 6 mg/mL before injection in the GC system.

9.2.3 Gas Chromatography Quantitation

After simulated gastrointestinal digestion, the emulsion samples were centrifuged, and the lipid fraction was extracted with an organic solvent. The solvent was then evaporated to obtain the recovered oil for GC analysis.

The degree of lipolysis was calculated as in equation 9.1

$$\text{Degree of lipolysis} = \frac{\text{Released fatty acids(TMS - diazomethane selective methylation)}}{\text{Total fatty acids(methanolic HCl non - selective methylation)}} \quad (9.1)$$

For quantification, C17:0 free fatty acid was used as the internal standard for free fatty acid analysis, while tri-C17:0 glyceride was used as the internal standard for total fatty acid analysis. External calibration curves were prepared for ARA (C20:4) and DHA (C22:6), with a fixed concentration of the internal standard, equation in 9.2 and 9.3.

$$\frac{A_{DHA/ARA}}{A_{FFA}} = k \frac{C_{DHA/ARA}}{C_{FFA}} + b(\text{Determine FFA}), 2 \text{ standard curve(DHA and ARA)} \quad (9.2)$$

$$\frac{A_{DHA/ARA}}{A_{TAG}} = k \frac{C_{DHA/ARA}}{C_{TAG}} + b(\text{Determine TAG}) 2 \text{ standard curve(DHA and ARA)} \quad (9.3)$$

For quantification, C17:0 free fatty acid was used as the internal standard for free fatty acid analysis, while tri-C17:0 glyceride was used as the internal standard for total fatty acid analysis. External calibration curves were prepared for ARA (C20:4) and DHA (C22:6), with a fixed concentration of the internal standard.

After digestion, each sample was divided into two parts. One part was used to determine released free ARA and DHA, and the other part was used to determine total ARA and DHA. The lipolysis degree was then calculated by comparing the released fatty acid content with the total fatty acid content.

9.3 Raw Data

In a paper on algal oil emulsion, the "time required for hydroperoxides to reach 15 meq/kg" was directly used as the oxidation lag time, which was employed to compare the antioxidant stability[53], lag time was calculated through the same way when the PV reach 15 mmol/kg.

Table 9.1. PV Kinetic Index

Run	Lag time	Vmax	max	ΔPV-AUC
1	2.24	8.16	28.8	382

2	1.67	11.35	32.7	386
3	1.04	16.68	21.7	282
4	2.05	10.53	49.7	494
5	0.98	17.84	40.4	493
6	2.19	11.3	30.4	402
7	2.44	9.6	50.2	459
8	1.86	14.34	29.2	330
9	0.92	16.64	37.4	450

Table 9.2. CD Kinetic Index

Run	Vmax	max	Δ CD-AUC
1	6.03	46.7	178
2	10.46	47.1	214
3	9.37	42.5	220
4	7.47	36.5	180
5	11.75	41.7	194
6	10.77	47.5	218
7	3.77	36.7	116
8	9.47	42.7	212
9	12.1	41.4	244

Table 9.3. p-AV Kinetic Index

Run	Vmax	max	Δ p-AV-AUC
1	16.64	72.4	556
2	13.32	49.6	368
3	18.87	75.5	687
4	3.49	26.3	139
5	15.64	34.9	280
6	15.31	48.8	398
7	4.08	33.4	170
8	28.4	93.6	720
9	14.48	39.8	292

Table 9.4. TOTOX Kinetic Index

Run	Vmax	max	Δ TOTOX-AUC
1	30.77	129.35	1303.97
2	36.03	114.93	1141.19

3	52.24	109.81	1251.13
4	23.49	119.14	1126.32
5	51.32	108.37	1266.65
6	37.9	107.34	1200.56
7	20.46	113.42	1087.73
8	57.08	131.37	1379.81
9	47.76	97.76	1191.22

Table 9.5. Oxidation Index of EOC-GPC-3 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	-1.860465116	-2.427184466	25.46903859	14.0641
1	6.292352372	3.553299492	31.49467758	30.70022
3	24.17794971	18.29268293	29.92311864	37.20572
5	26.34099617	27.56286267	36.15520282	-
7	25.56237219	23.06182532	37.74655882	41.55331
10	27.05627706	30.6122449	46.74897119	69.05357
15	27.75024777	29.23976608	34.8095292	72.35929

Table 9.6. Oxidation Index of EOC-GPC-2.5 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	3.198294243	-4.491017964	22.53625196	14.81777778
1	13.77952756	7.637017071	33.00094967	28.13641364
3	24.37810945	22.74975272	28.24495298	35.45551983
5	22.0657277	28.84615385	33.85921989	35.95777778
7	26.31578947	30.71833648	36.76268861	36.53025303
10	25.35885167	29.62206333	47.09391039	45.7249725
15	33.44917463	31.91489362	34.71220138	49.57058824

Table 9.7. Oxidation Index of EOC-CC-3 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	0.469043152	-4	20.82937638	9.226927253
1	16.563147	13.27433628	30.19614131	28.09652076
3	21.2305026	17.28499157	27.64540312	38.36323367
5	21.44351464	21.71664943	37.31138546	47.92168675
7	18.33976834	25.02360718	42.46057233	51.61303891
10	21.10091743	16.68302257	40.93251828	72.02373247
15	8.955223881	9.18762089	30.76165867	75.54972678

Table 9.8. Oxidation Index of GPI-GPC-2.5 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	4.504504505	-5.511022044	18.22174739	10.17957351
1	11.78781925	8.273009307	25.69077014	12.59978425

3	22.55985267	16.42710472	20.31151625	19.57377049
5	29.6692607	28.83675464	28.71347969	17.00112233
7	35.78528827	40.01976285	35.44609902	20.07335491
10	50.55611729	48.93410853	36.51831103	19.65333333
15	32.22557905	31.80661578	29.09537218	26.33954451

Table 9.9. Oxidation Index of GPI-CC-3 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	-0.950570342	-3.986332574	22.99301065	7.011494253
1	16.27670397	14.46945338	34.73926082	22.65337763
3	25.95494613	26.65995976	30.33151595	24.73777778
5	31.60040775	35.82554517	35.14009391	20.58991228
7	35.99605523	30.64202335	37.99159985	23.8658147
10	42.41516966	38.42105263	41.74562159	27.53514739
15	26.10837438	29.5	32.82234904	34.85185185

Table 9.10. Oxidation Index of EOC-GPC-2.5 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	-1.527494908	-2.470355731	21.44427533	15.60699588
1	8.100810081	10.49618321	32.21288515	30.91638796
3	19.86754967	17.91044776	27.41510495	41.96742671
5	28.79841112	29.64824121	34.77244503	40.81284607
7	26.05703048	34.71596032	37.33682174	41.53898305
10	28.66242038	27.95899348	47.46637184	46.4954955
15	29.16274694	29.35514918	29.17401529	48.82683983

Table 9.11. Oxidation Index of GPI-GPC-3 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	0.897666068	4.127966976	20.9200555	2.627192982
1	13.05220884	9.415262636	24.69135802	5.640852974
3	17.56097561	15.35087719	21.74886458	10.36101883
5	38.65461847	32.66331658	27.57310803	9.629139073
7	35.32875368	29.49438202	28.50494474	9.842873176
10	50.74626866	49.67502321	36.67273831	13.00112994
15	33.93213573	39.75095785	26.15817137	33.41202186

Table 9.12. Oxidation Index of EOC-CC-2.5 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	-0.973709834	-1.912045889	23.7967436	17.85016287
1	13.80368098	11.99261993	33.26191205	46.25027685

3	15.40616246	20.16546019	38.42988287	52.725
5	19.75540922	18.76172608	40.27991125	57.74410774
7	21.82347236	19.30320151	42.71253876	67.95862069
10	31.88259109	26.47058824	40.10475122	71.26621924
15	17.87487587	19.94017946	33.14276245	93.5508982

Table 9.13. Oxidation Index of GP1-CC-2.5 (PV1 done by Ruilin Xue, PV2 done by Ziyi Dong)

Day	PV1	PV2	CD	p-AV
0	-2.982107356	2.427184466	18.62940785	3.04510451
1	15.54404145	17.1875	30.72702332	17.52035204
3	32.42009132	29.32692308	32.27203812	14.55208333
5	33.26904532	29.57486137	32.9393223	16.73754153
7	33.81893861	27.50491159	35.2303523	22.29753915
10	38.5786802	36.23898139	41.43786268	22.94117647
15	27.17391304	29.09270217	33.38861249	39.77462121

Table 9.14. Oxidation Index of Fresh oil

Sample	PV1	PV2	CD	p-AV
Fresh oil	-1.008064516	-0.502008032	7.555399774	1.666667

Table 9.15. HPLC analysis of Ve and Ve acetate of EOC-GPC-3

Day	Vit e sum	Vit E acetate
0	85.82073975	26.65693419
1	0	0
3	0	0
5	0	0
7	0	0
10	0	0
15	85.82073975	26.65693419

Table 9.16. HPLC analysis of Ve and Ve acetate of EOC-GPC-2.5

Day	Vit e sum	Vit E acetate
0	0	0
1	0	0
3	0	0
5	0	0
7	0	9.374762582
10	0	0
15	24.27704304	0

Table 9.17. HPLC analysis of Ve and Ve acetate of EOC-CC-3

Day	Vit e sum	Vit E acetate
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0	0	0
1	0	0
3	0	0
5	0	0.70463172
7	0	0.801206433
10	0	0
15	4.967330065	0

Table 9.18. HPLC analysis of Ve and Ve acetate of GPI-GPC-2.5

Day	Vit e sum	Vit E acetate
0	0	0
1	0	30.57435657
3	0	0
5	0	0
7	0	0
10	0	0
15	42.40351873	0

Table 9.19. HPLC analysis of Ve and Ve acetate of GPI-CC-3

Day	Vit e sum	Vit E acetate
0	0	0
1	0	0
3	0	0
5	0	18.29369194
7	0	0
10	1.382162599	0
15	0	0

Table 9.20. HPLC analysis of Ve and Ve acetate of EOC-GPC-2.5

Day	Vit e sum	Vit E acetate
0	19.82029829	0
1	0	0
3	0	0
5	0	0
7	0	0
10	0	0

15	29.56139974	1.939978332
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Table 9.21. HPLC analysis of Ve and Ve acetate of GP1-GPC-3

Day	Vit e sum	Vit E acetate
0	0	6.523821879
1	0	0
3	0	40.35237447
5	0	39.73107404
7	0	0
10	0	40.0817213
15	0	0

Table 9.22. HPLC analysis of Ve and Ve acetate of EOC-CC-2.5

Day	Vit e sum	Vit E acetate
0	16.74883669	0
1	0	0
3	0	0
5	0.79255999	0
7	4.67802517	0
10	0	0
15	5.185984625	4.645586399

Table 9.23. HPLC analysis of Ve and Ve acetate of GP1-CC-2.5

Day	Vit e sum	Vit E acetate
0	0	0
1	0	0
3	0	0
5	0	9.03833102
7	0	16.0072991
10	0	0
15	0	0

Table 9.24. Droplet size distribution

Size(u m)	EOC- GPC- 3	EOC- GPC- 2.5	EOC- CC-3	GP1- GPC- 2.5	GP1- CC-3	EOC- GPC- 2.5	GP1- GPC- 3	EOC- CC- 2.5	GP1- CC- 2.5	EOC- GPC- 3
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0.02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.04	0.000	0.161	0.179	0.000	0.006	0.132	0.000	0.204	0.000	0.000
0.05	0.000	1.115	1.189	0.288	0.574	1.000	0.523	1.249	0.401	0.000
0.05	1.458	1.885	2.009	0.612	0.993	1.694	0.918	2.106	0.757	1.458
0.06	2.665	2.843	3.028	0.945	1.513	2.557	1.401	3.171	1.161	2.665
0.07	4.017	4.006	4.264	1.373	2.162	3.608	2.006	4.460	1.673	4.017
0.08	5.928	5.439	5.778	2.014	3.041	4.914	2.838	6.025	2.403	5.928
0.09	7.793	6.750	7.154	2.755	3.956	6.128	3.719	7.428	3.208	7.793
0.10	9.402	7.835	8.273	3.590	4.871	7.159	4.621	8.546	4.073	9.402
	10.58	8.633	9.072	4.508	5.761	7.953	5.525	9.310	4.983	10.58
0.12	5									5
	11.20	9.097	9.496	5.493	6.599	8.471	6.409	9.672	5.924	11.20
0.14	6									6
	11.14	9.179	9.492	6.496	7.336	8.665	7.228	9.585	6.848	11.14
0.16	7									7
	10.35	8.848	9.031	7.446	7.912	8.504	7.922	9.028	7.696	10.35
0.18	2									2
0.21	8.860	8.103	8.118	8.253	8.262	7.978	8.419	8.019	8.384	8.860
0.24	6.854	7.003	6.827	8.805	8.322	7.123	8.638	6.650	8.813	6.854
0.28	4.634	5.663	5.301	9.004	8.055	6.023	8.508	5.068	8.887	4.634
0.32	2.634	4.287	3.783	8.776	7.468	4.841	7.993	3.521	8.536	2.634
0.36	1.164	3.055	2.484	8.097	6.598	3.722	7.094	2.216	7.734	1.164
0.42	0.361	2.112	1.563	7.027	5.536	2.797	5.898	1.307	6.552	0.361
0.48	0.059	1.440	0.986	5.652	4.342	2.065	4.501	0.760	5.095	0.059
0.55	0.000	0.996	0.674	4.158	3.142	1.514	3.098	0.494	3.577	0.000
0.63	0.141	0.696	0.512	2.677	2.009	1.090	1.795	0.388	2.121	0.141
0.72	0.257	0.467	0.391	1.441	1.085	0.759	0.860	0.324	1.062	0.257
0.83	0.231	0.266	0.252	0.537	0.413	0.479	0.087	0.233	0.112	0.231
0.95	0.137	0.096	0.111	0.052	0.042	0.277	0.000	0.123	0.000	0.137
1.10	0.086	0.025	0.034	0.000	0.000	0.168	0.000	0.080	0.000	0.086
1.26	0.030	0.000	0.000	0.000	0.000	0.133	0.000	0.033	0.000	0.030
1.45	0.000	0.000	0.000	0.000	0.000	0.110	0.000	0.000	0.000	0.000
1.66	0.000	0.000	0.000	0.000	0.000	0.082	0.000	0.000	0.000	0.000
1.91	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000
2.19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.51	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.88	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Figure 9.5. *p*-AV of each sample from each time point

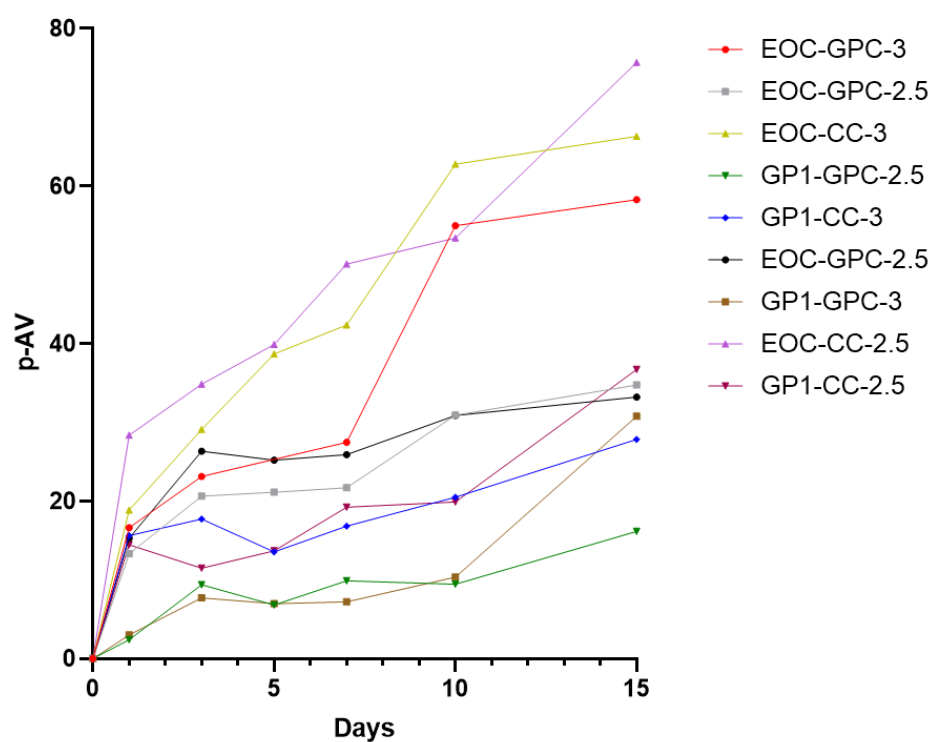
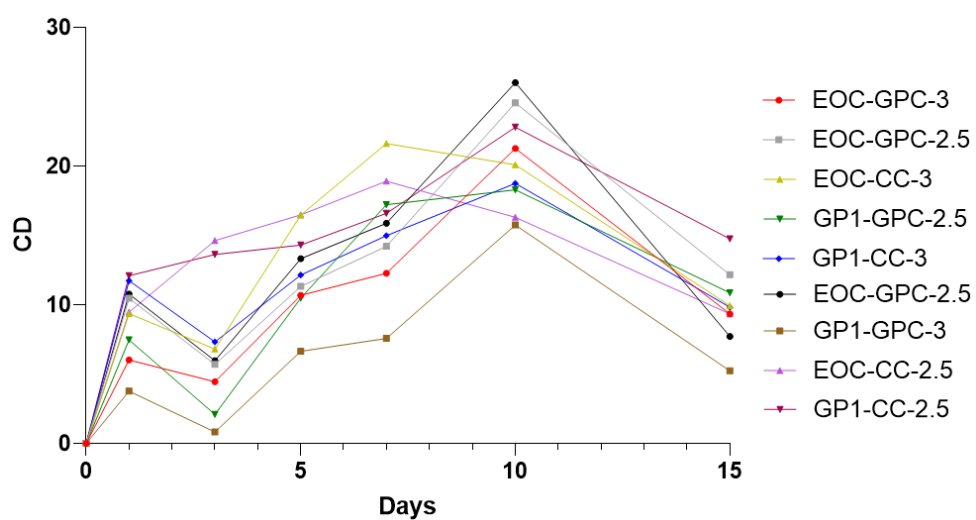


Figure 9.6. CD of each sample from each time point



10 Use of AI Tools

During the preparation of this thesis, ChatGPT was used only as a language-support tool. Its use was limited to English translation, when necessary, grammar checking, and improving the clarity, readability, and flow of the writing. All AI-assisted suggestions were carefully checked and revised by the author to make sure that the final text accurately expressed the intended meaning.