IMPROVE BIOACCESSIBILITY OF QUERCETIN USING PSEUDO-ORGANOGEL BASED NANOEMULSIONS

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Food Science

written under the direction of

Dr. Qingrong Huang

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New Brunswick, New Jersey

January 2014

ABSTRACT OF THE THESIS

Improve Bioaccessibility of Quercetin Using Pseudo-Organogel Based Nanoemulsions

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Flavonoids which are widely distributed in plants can be categorized as flavonols, flavanols, flavanones, flavones, anthocyanidin and isoflavones. Quercetin (3, 3', 4', 5, 7-pentahydroxylflavone) accounts for the largest percentage of flavonol intake in the diet. Like many other flavonoids, quercetin has many health-promotion and disease-prevention benefits. However, its low solubility in water and gastric fluid leads to a low bioavailability *in vivo*. Lipid-based formulations, such as O/W nanoemulsions and solid lipid nanoparticles have been developed to increase the bioaccessibility of flavonoids. Modern delivery systems such as organogel or pseudo-organogel based emulsions have not yet been studied as quercetin delivery vehicles.

In this study, pseudo-organogel and pseudo-organogel based emulsion systems have been developed for oral delivery of quercetin to improve its bioaccessibility. The formation of pseudo-organogel inhibited quercetin crystallization in the oil phase. Using mixture of limonene and medium chain triglyceride (MCT) at the ratio of 2:8 (w/w), solubility of quercetin was increased by 40 times compared to quercetin in MCT at room temperature. Bioaccessibility and the extent of lipolysis were determined for the pseudo-organogel based emulsion system. After the first 30 min of digestion, the extent of lipolysis of the pseudo-organogel based emulsion (71.9 %±1.2 %)

was significantly higher ($p<0.001$) than that of the quercetin loaded oil mixture (same oil mixture as that used to form pseudo-organogel based emulsion) (54.0 % ±0.6 %). The percentages of bioaccessibility of quercetin in oil mixture and in pseudo-organogel based emulsion were determined to be (35.6 % ± 0.6 %) and (49.7% ±5.3%) respectively, which were significantly higher than that of quercetin in water suspension (less than 3 %). With a higher and stable loading of quercetin, and the improved bioaccessibility, this pseudo-organogel based emulsion system of quercetin may have potential applications in food, cosmetic and pharmaceutical industries.

ACKNOWLEDGEMENT

First I would like to thank my adviser Dr. Qingrong Huang, for taking me into the field of food nanotechnology. During two years of study, Dr. Huang had given me valuable advice and suggestions not only in the field of research but also about life and career, guiding me toward the bright sides.

I would also like to thank my committee members Dr. Chi-Tang Ho and Dr. Michael A. Rogers, for their times and advices through the years of my study in the department, and especially during the time I was finishing the research project and writing the thesis.

The other precious part of my two years of study in food science department is all my dear friends and lab mates, for their companion their helps and most importantly, their supports.

My thanks also goes to the kindly and most helpful department staffs, Karen Ratzan, Deborah Koch, Irene Weston, Yakov Uchitel and David Petrenka, it was them that made the life of everyone in the department easier and happier.

Most importantly, I would thank the most loving parents of mine. Thanks for always being there when I need you the most, and always make me feel loved every time I am hurted. And also thanks for not spoiling me, give me the chance to be independent and responsible.

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Chapter 1 Introduction and Literature Review

1.1 General information of quercetin

As one important kind of nutraceuticals, flavonoids have been given a lot of attentions over the years, majorly because of the beneficial effects they have regarding human health. Flavonoids can be further classified as flavonols, flavones, flavanones, flavan-3-ols, etc. **Table 1** summarized the major subgroups of flavonoids and the represent compound in each group. A study based on USDA Database for the Flavonoid Content of Selected Foods and 24-h dietary recall of the NHANES concluded that the total flavonoid intake of US adults was 189.7 mg/d [1].

Table 1 Subgroups of flavonoids

Group	Examples
Flavonol	Isorhamnetin, Kaempferol, Myricetin, quercetin
Flavone	Apigenin, Luteolin
Flavanone	Eriodictyol, Hesperetin, Naringenin
Flavan-3-ol	Catechin, EGCG
Theaflavin	Theaflavin,
Anthocyanidin	Cyanidin, Delphinidin, Malvidin

Flavonol, a subgroup of flavonoid, is most ubiquitous in food, and has been more extensively studied[2, 3]. Among flavonols, quercetin is one of the most abundant ones presents in fruits and vegetables[4]. Several examples of rich sources of quercetin from common foods are red onions (39.21 mg/100 g edible portion), fennel leaves (48.80 mg/100 g edible portion), cilantro leaves (52.90 mg/100 g edible portion), apple skin (19.36 mg/100 g edible portion)[5]. In US health

professionals, quercetin was reported to account for 73 % in women and 76 % in men of the flavonol and flavone intake [6].

Quercetin, 3, 3', 4', 5, 7-pentahydroxylflavone, has molecular formula of $C_{15}H_{10}O_7$, molecular weight of 302.24 g/mol, and melting point of 316 ℃. The chemical structure of quercetin is shown in **Figure 1**. Quercetin is hydrophobic in nature, having an octanol-water partition coefficient (log P) of 1.82 [7].

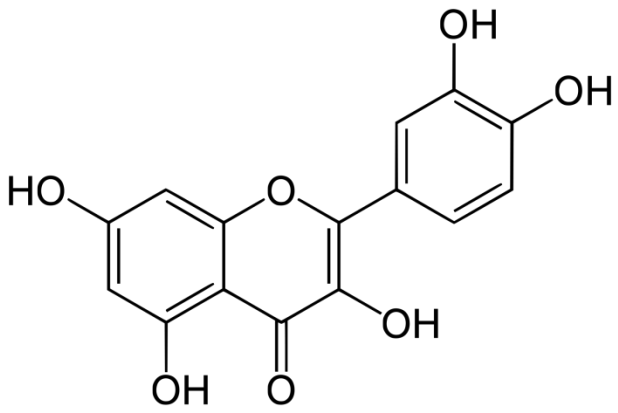


Figure 1 Chemical structure of quercetin

In November 2010, quercetin with a purity of 99.5%, marked as QU995^{TM} received the GRAS (General Recognized as Safe) notified status from FDA, with an intended use listed as quoted "Ingredient in beverages and beverage bases, grain products and pastas, processed fruits and fruit juices, and soft candies at levels up to 500 milligrams per serving"[8] . The fact that quercetin having GRAS status as an ingredient in a relatively wide range of food products gives it more possibility in functional foods, and thus draws more attentions of scientists in the field.

1.2 Biological properties of quercetin

Quercetin has been reported to have a lot of health beneficial properties, such as antioxidant, anti-inflammatory properties, prevention of cancer and obesity, etc.

1.2.1 Cardiovascular protection properties

Early in the 80's, in the Zutphen Elderly Study, a cohort study about risk factors of chronic diseases among elderly men, researchers had found a connection between a larger amount of flavonoids intake and a reduced mortality from coronary heart disease[9]. In a more recently study with a much larger cohort: 38,180 men (with an average age of 70) and 60,289 women (with an average age of 69), similar conclusion were made, that is, a greater flavonoid consumption was associate with a lower cardiovascular diseases mortality risk[10].

The effects of individual flavonoids on reducing the risk for cardiovascular disease had also been studied. The protective effect of quercetin was confirmed on studies that were performed on hypertensive patients and a population that were already at-risk of cardiovascular diseases. Quercetin as supplement at 730 mg/day were given to 22 subjects had stage 1 hypertension for 28 days; systolic, diastolic, and mean arterial pressure reduced after the quercetin treatment.[11] Similarly, in another study, 93 subjects that were overweight and with a high risk of cardiovascular disease received 150 mg/day of quercetin for 6-week periods; results shown that quercetin reduced not only the systolic blood pressure but also the oxidized low-density lipoprotein (LDL) concentrations.[12]

1.2.2 Physical fitness improvement properties

VO_{2max}, stands for maximum oxygen uptake, is the highest rate of oxygen can be uptake and utilized by the body during physical exercise. An increase in VO_{2max} is often associated with an improvement of physical fitness.[13] Similarly as the result found in studies about the preventive

property of quercetin toward cardiovascular disease, significant effects only shown on population that already at-risk; in the study regarding physical performance, positive results were only found in the studies performed on subjects that had not been trained for physical exercise.

In the study, 12 untrained participants randomly assigned to two groups. One group received the quercetin treatment of 500mg twice daily and the other group treated with placebo only, result showed that 7 days of quercetin treatment associated with an increase in VO_{2max} by 3.9 % compared to the placebo group, and a 13.2 % increase in ride time to fatigue[14].

1.2.3 Skin protection properties

Quercetin is more abundant in plants skin comparing to other tissues, which naturally serves as the mechanism defending against UV light[4]. Inspired by that, quercetin has also been explored in for treatment of skin conditions, such as, treatment and prevention for skin cancers[15], UV radiation damage[16], and wound healing[17].

Quercetin has been used as chemotherapeutic drugs for treatment of various cancer types, especially skin cancer. Paliwal et al. combined low frequency ultrasound and quercetin to selectively induce cytotoxicity in skin and prostate cancer cells, while having minimal effect on corresponding normal cell lines. Within 48 hours after the ultrasound-Quercetin treatment, 90% of the viable skin cancer cell population was lost[15].

UV rays from the sun are known to deplete the antioxidant systems of the skin and lead to a marked increase of reactive oxygen species (ROS). A combination of quercetin and titanium dioxide showed synergistic effect when used in cosmetic products, and the sun protection factor (SPF) of the product was about 30, which was capable of providing protection effect over the whole range of UV[16].

A study in rats also demonstrated the beneficial effect of quercetin in dermal wound healing[17]. Quercetin was incorporated to the collagen matrix, and the group of animals treated by quercetin incorporated collagen matrix showed a better healing compared to the control group, since there was an increase in proliferation of cells in the treated group.

1.3 Bioavailability

It is important to keep in mind that the nutraceuticals that have a large amount among common diet, such as quercetin in this study, are not necessarily as active as we thought it would be within the body. The reasons are they have low *in vivo* activity, they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated and hardly made to the systemic circulation[18, 19].

From the nutritional point of view, the term bioavailability is defined as the fraction of nutraceutical or bioactive compound ingested that is eventually available for use in physiologic functions or storage[20]. As shown in the graphic representation in **Figure 2**[21], the overall oral bioavailability (F) is influenced by three processes: bioaccessibility, intestinal transport and metabolism[22]. As shown in the equation below:

$$F = F_B \times F_A \times F_M \qquad (1)$$

F_B is the fraction of lipophilic component released from the food matrix into the juices of the gastrointestinal tract to become bioaccessible, F_A is the fraction of the released component that is transported across the intestinal epithelia, and F_M is the fraction of the absorbed component that reaches the systemic circulation without being metabolized. The major purpose of using delivery systems for nutraceuticals is to increase the overall bioavailability by increasing the bioaccessibility.

Quercetin, as a representative poorly water-soluble lipophilic component, it must be released from the containing matrix (the delivery systems) and then solubilized within mixed micelles present in the small intestine before it can be absorbed. These mixed micelles are containing bile salts and phospholipids secreted by the body, and also lipid digestion products: monoacylglycerides (MAGs) and free fatty acids. The solubilization step therefore determines the fraction of lipophilic component that is bioaccessible (F_B).

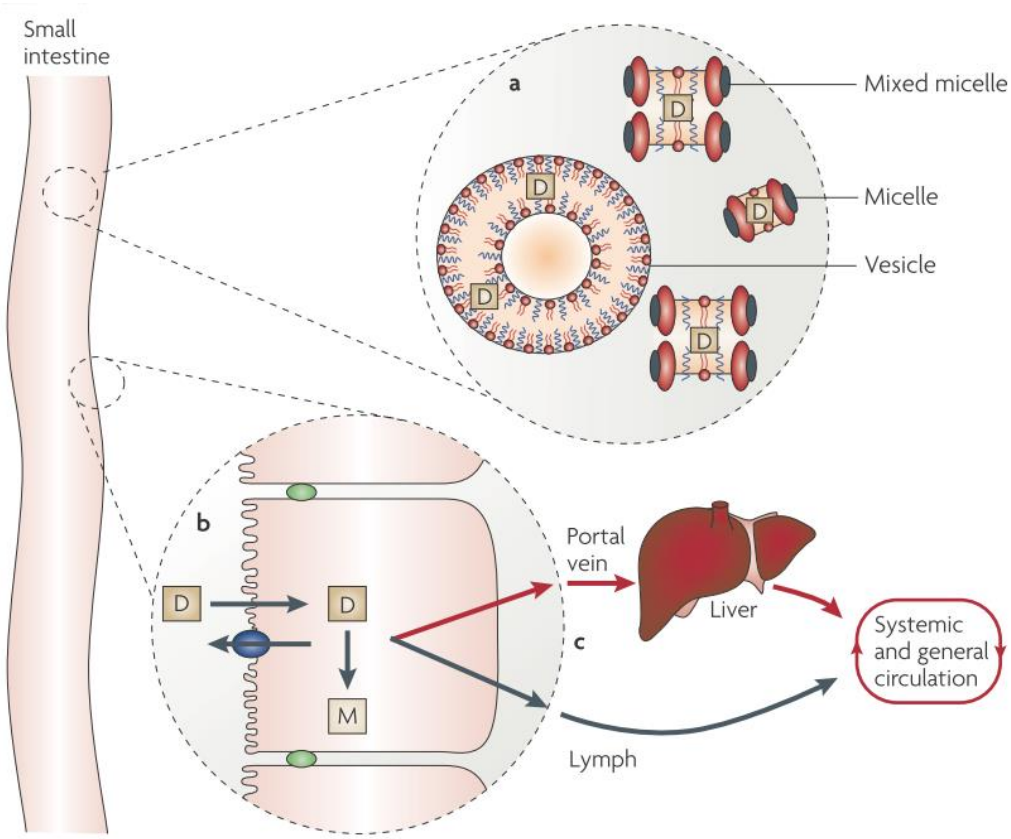


Figure 2 Potential effects of lipids and lipidic excipients on drug absorption.
Lipids can affect drug absorption in three ways: by enhancing drug (D) solubilization in the intestinal milieu through alterations to the composition and character of the colloidal environment — for example, vesicles, mixed micelles and micelles (a); by interacting with enterocyte-based transport and metabolic processes, thereby potentially changing drug uptake, efflux, disposition and the formation of metabolites (M) within the enterocyte (b); or by altering the pathway (portal vein versus intestinal lymphatic system) of drug transport to the systemic circulation — which in turn can reduce first-pass drug metabolism as intestinal lymph travels directly to the systemic circulation without first passing through the liver (c). Cellular junctions are represented by green ovals, and a representative transport protein is depicted by a blue oval[21].

1.4 Current approach of quercetin delivery

As mentioned before, because of the low bioavailability of quercetin, the circulating concentrations of quercetin generated from a regular diet cannot provide adequate amounts of quercetin that may show any described health beneficial effects , such as chemopreventive and/or cardioprotective[23]. Consequently, it is necessary to develop delivery systems for quercetin. Several approaches have been taken for the delivery of quercetin through oral administration systems such as liposomal[24], liquid crystal[25], nano particles[26], nano crystals[27], solid lipid nanoparticles[28]; or through topical delivery system such as lipid based nano systems[29], water in oil microemulsion[30] and collagen matrices[17].

1.4.1 Oral administration

Wang and Chiu[24] developed a liposome delivery system for quercetin and vincristine, another cancer chemotherapy drug. Quercetin and Vincristine both have effect against ER^- (Estrogen receptor negative) breast cancers. Their synergistic effect can only be achieved at certain ratios. However the low water solubility of quercetin has hampered these suggested concentrations from being achieved. The liposome systems were prepared with quercetin by film hydration method and then followed by extrusion, subsequently vincristine was loaded into the core of the liposomes. The optimized formulation was formed by egg sphingomyelin/cholesterol/PEG2000/ceramide/quercetin (72.5:17.5:5:5 mol ratio), this formulation could enhance quercetin solubility by 8.6 times.

Vicentini et al.[25] developed a liquid crystalline formulation for quercetin delivery, and they also studied the antioxidant activity of quercetin and whether the formulation would affect its antioxidant activity. Three antioxidant assay methods were performed and validated: inhibition of lipid peroxidation induced by Fe^{2+} assay, inhibition of chemiluminescence of H_2O_2/luminol/HRP system assay, and antioxidant activity by DPPH assay. Based on all assays results, liquid

crystalline formulation containing vitamin E TPGS, IPM, and PG-H_2O (63.75:21.25:15 w/w/w), considered to have potential for pharmaceutical or cosmetic use since the antioxidant effect of quercetin was persevered.

Kumari et al.[26] encapsulated quercetin on nanoparticles formed by poly-D, L-lactide (PLA), using solvent evaporation method. Quercetin loaded nanoparticles had diameter of about 130 ±30nm. Not only were the antioxidant activities of quercetin retained after encapsulation, but also *in vitro* release profile showed an initial burst of quercetin release followed by sustained release. These properties of the quercetin loaded PLA nanoparticles made it a promising delivery system for nanomedicine.

Another study[27] of quercetin delivery system was also focused on the *in vitro* dissolution rate; however it was aimed to increase the dissolution rate in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8). Quercetin nanosuspensions with quercetin loading of 2 % to 10 % were produced using high pressure homogenization with Tween 80 as stabilizer, and then dried by lyophlization. The mean particles size of the nanocrystal obtained was 483 nm. Because of the increase of the effective surface area, the dissolution rate of quercetin was dramatically increased compared with the original quercetin, and there was also an increase of the antioxidant activity.

1.4.2 Topical delivery

Gomathi et al.[17] developed a system of collagen matrices incorporated with quercetin for dermal wound healing, as a novel biomaterial. Quercetin incorporated into collagen and control collagen were both given to rats to evaluate the wound healing effect. A better wound healing was observed in the group treated with quercetin incorporated collagen as there was an increase in the rate of cell proliferation. Antioxidant study also showed that the quercetin incorporated collagen

quenched radicals more efficiently. Results showed that the quercetin incorporated collagen could be a novel topical delivery system with dermal wound healing effect.

Bose and Michniak-Kohn[29] manufactured and evaluated the lipid nanosystem of quercetin for topical delivery. Lipid systems such a solid lipid nanoparticles and nanostructured lipid carrier systems were formed and optimized. Mean particles size of the optimize system was around 282 nm, and a zeta protential around -37. Full thickness human skin were used in the *in vitro* permeation study, superior topical delivery of quercetin was observed, and nanostructured lipid carrier showed significant improvement judging based on the amount of quercetin retained in the skin.

1.5 Sugar ester

Gelator is a key component of the gel (or gel-like colloid system) formation. Because of the percentage amount of gelator used in this study (20%) was higher than normally defined amount for organogel (less than 5%), the gel-like colloid systems formed in this study were therefore referred as pseudo-organogel. There is a lot of gelator available in the market. In this study, sugar esters were chosen for the formulation of pseudo-organogel. Basically, sugar ester is made by esterification of sugar with fatty acid. Take sucrose for example, as the structure showed below in **Figure 3**, it has eight hydroxyl group, three of them are most reactive, and can be easily substitute with fatty acid, so theoretically, mono- to octa- esters are all possible to form. Other than that, the property of sucrose ester also influenced by different type of acyl chains, provided by the fatty acid.

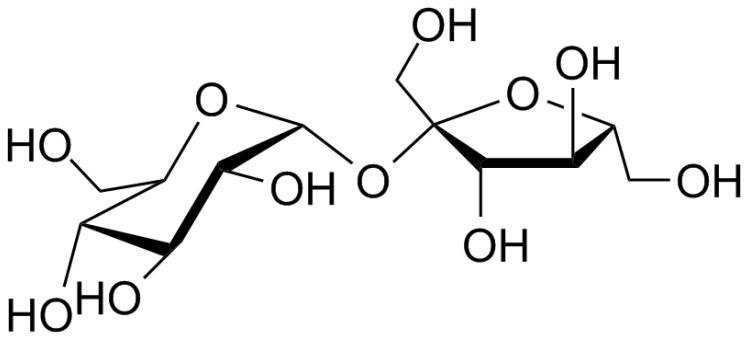


Figure 3 Chemical structure of sucrose

In summary, by varying the degree of esterification and type of the fatty acid, it will results in a vast amount of possible combinations, and thus a large amount of different sugar esters[31], ranging from having a HLB value of 1 to HLB value of 18, as shown in **Figure 4.** This gives them a lot of potentials in food application.

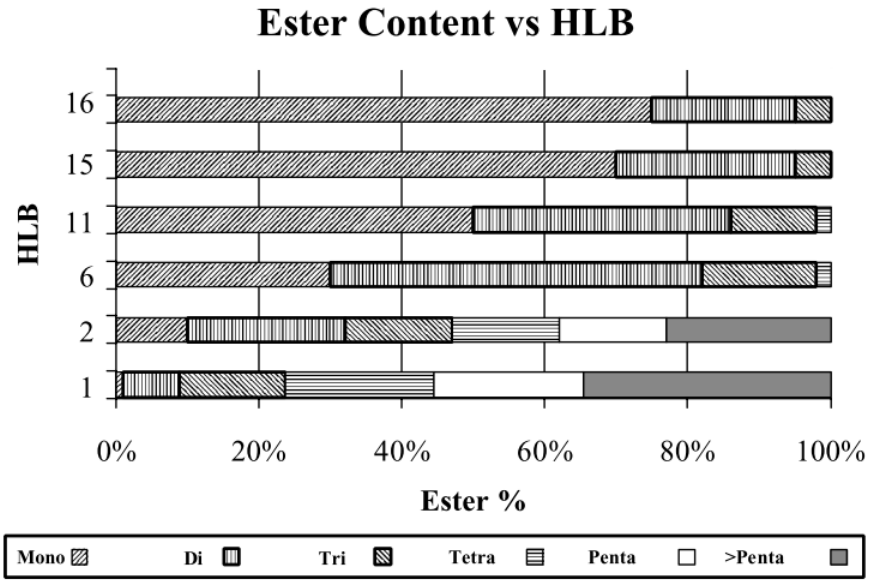


Figure 4 Ester substitution and its effect on HLB

Other than that, sugar ester is tasteless, odorless, nontoxic and non-irritant to eye and skin, which give them potentials not only in food application but also applications on topical delivery systems.

The sugar ester used in this particularly study is the sucrose stearate, example of sucrose stearate showing in **Figure 5**. Depending on different degree of esterification, they have been divided to different types (See **Table 3** in **3.1 Selection of sugar ester**).

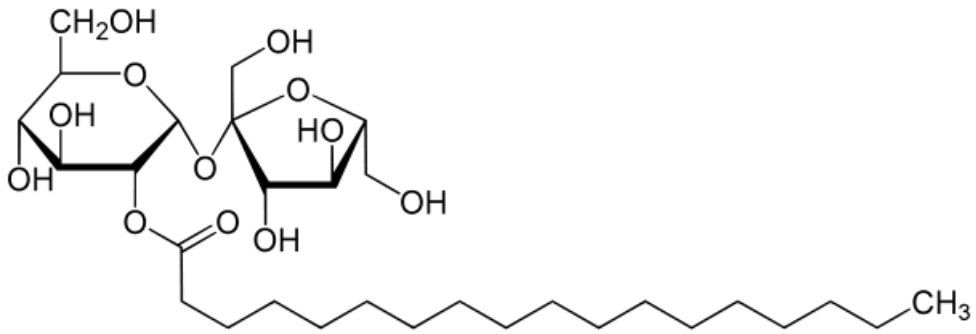


Figure 5 Chemical structure of sucrose monostearate

1.6 *In vitro* digestion model – Lipolysis model

As mentioned before, the importance of using a delivery system is to increase the solubilization of the bioactive compound into the micelles or mixed micelles in the juice of GI tract, thus to increase the bioaccessibility. To mimic the process, and be able to measure the quantity of bioactive compound solubilized, *in vitro* digestion model should be established to assess the bioaccessibility, and then used as the screening tool for formulation optimization of lipid based delivery systems. *In vitro* digestion model – Lipolysis model[21] was chosen in this study to validate the efficacy of the delivery system before further application in animal studies. **Figure 6**[21] showed the schematic diagram for this model.

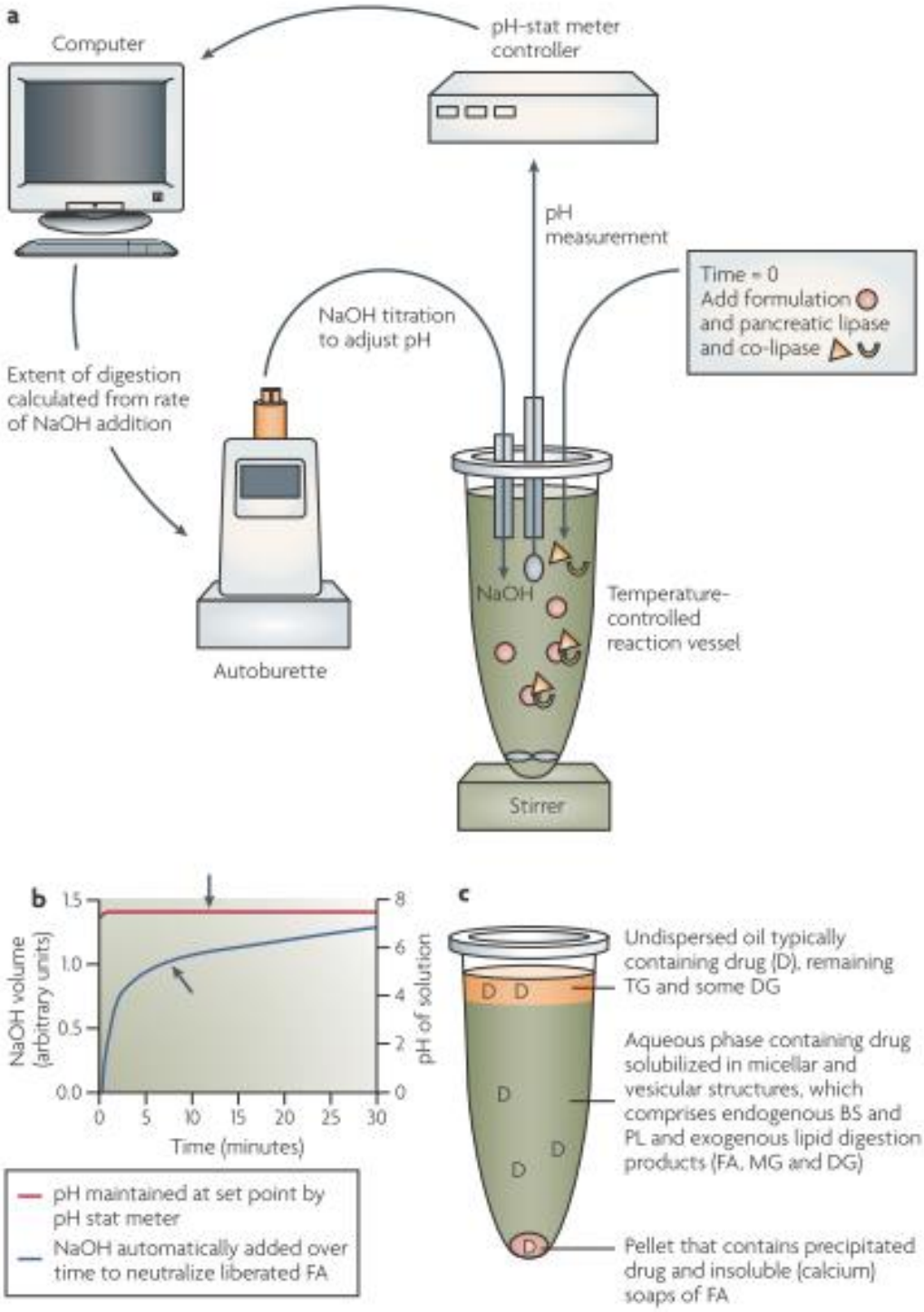


Figure 6 Lipid digestion models for *in vitro* assessment of lipidic formulations

Chapter 2 Materials and Methods

2.1 Materials

Quercetin Dihydrate (purity >90 %) was obtained from Merck Chemicals. Food-grade limonene was kindly provided by Florida Chemical Co. (Winter Haven, FL). Medium Chain Triacylglycerol (MCT) was provided by Stepan Co. (Northfield, IL). Sugar Esters were provided by Mitsubishi-Kagaku Foods Corporation (White Plains, NY). Ethyl alcohol (Ethanol) was purchased from Pharmoco-Aaper (Brookfield, CT). Tween 20 [polyoxyethylene (20) sorbitan monolaurate] was purchased from Sigma-Aldrich (St. Louis, MO). Pancreatin with 8X USP specification and Tris maleate were purchased from Sigma-Aldrich (St. Louis, MO), Sodium taurodeoxycholate (NaTDC) was purchased from CalBiochem (La Jolla, CA). PC75 rapeseed lecithin containing 75% phosphatidylcholine was provided by American Lecithin Co. (Oxford, CT).

2.2 Methods

2.2.1 Preparation of pseudo-organogel

2.2.1.1 Preparation of blank pseudo-organogel

To prepare the blank pseudo-organogel, the original oil (MCT, limonene or mixture of MCT and limonene) was first weighted in a glass vial, and then heated on a hot plate to the designate temperature (120 ℃ or 90 ℃). Sugar esters (S170, S270, S370, S570, S770 or S970) of specific weight (weight %=15 %, 20 % or 25 %) were added into the pre-heated oil, stirred using magnetic stirring until fully dissolved. The mixture of sugar ester and oil was then cooled down at ambient environment and stood overnight for stabilization.

2.2.1.2 Preparation of quercetin loaded pseudo-organogel

To prepare the quercetin loaded pseudo-organogel, an oil mixture of limonene: MCT (w/w) =2:8 was first heated to 90 ℃ under N_2, then excess amount of quercetin was added to the oil mixture and kept at 90 ℃ while stirring for 10 min, cooled down to room temperature and centrifuged the solution at 10k RPM for 30 min. The supernatant was collected and heated to 90 ℃. Then 20 % (w/w) of S370 sugar ester was added, held at 90 ℃ and stirred until the sugar ester was fully dissolved. The mixture of sugar ester and quercetin loaded oil was then cooled down to ambient temperature, gel-like structure usually formed after few hours, samples were stored overnight for stabilization.

2.2.2 Rheological measurement

The rheological measurements of the pseudo-organogel were performed using ARES strain controlled rheometer (Rheometric Scientific, Piscataway, NJ). A parallel plate geometry (25 mm diameter) was used for all measurements. Pseudo-organogel samples were directly deposited on to the plate for each measurement. The linear viscoelastic region was determined by dynamic strain sweep test performed at range 10^{-1} to 10^{2}, according to the strain sweep result, strain value of 0.15% was adopted. A frequency sweep test was performed at frequency ω ranging from 1 to 100 rad/s with 10 data points per decade. The gap between two parallel plates had been adjusted to prevent sample slip.

2.2.3 Quercetin concentration measurement by UV-visible spectrometer

The concentration of quercetin was determined from UV absorbance data. UV absorbance data of quercetin-containing samples were obtained by measuring the UV absorbance at 375 nm, using a Cary UV–Visible spectrophotometer (Varian Instruments, Walnut Creek, CA), with a 1cm path length quartz cuvette. Samples were diluted using ethanol prior to measurement. Aqueous phase samples obtained after lipolysis assay were diluted with water. A calibration curve was obtained

by dissolving quercetin standard in ethanol. The calibration curve had an equation of y = 0.0757x - 0.0155, and R^2 of 0.9995, the linear range was between 1-15 ppm.

2.2.4 Determination of the solubility of quercetin

To determine the solubility of quercetin, excess amount of quercetin was added to the preheated oil, kept at the same temperature while stirring for 30 min or 10 min. The heating processes were conducted under open air or under protection of N_2 (detailed conditions were shown in section 3.4.2 Processing condition affect the final concentration of quercetin). The oil and quercetin mixtures were then cooled to room temperature and centrifuged at 10k RPM for 30 min. The supernatant was collected and diluted with ethanol before analysis.

2.2.5 Polarized light microscopy

The microscopy images of the samples were acquired using a Linkham Imagining Station (Linkham, Surrey, England) equipped with a Q imaging 1280×960 pixel CCD camera (Micropublisher, Surrey, Canada) and a 50X Olympus lens and a polarized condenser. Samples were placed on glass slides and a covered with a cover slip.

2.2.6 Preparation of pseudo-organogel based nanoemulsion

To prepare quercetin loaded pseudo-organogel nanoemulsion, deionized water was used as water phase and Tween20 was used as the emulsifier as it has been reported to have a better *in vitro* bioaccessibility as emulsifier in similar delivery systems[32]. Samples were prepared with different combination ratios of water: oil: emulsifier, in summary, water concentrations were from 55 % to 85 %, pseudo-organogel was from 5 % to 35 %, and Tween20 were from 40 % to 10%. Tween20 was first mixed with water, the mixture were heated to about 70 ℃, then the melted pseudo-organogel were poured into the aqueous phase, mixed using magnetic stirring for about 2 min, then homogenized using a high speed homogenizer (Ultra-turrax T25 digital, IKA Works Inc.,

Wilmington, NC) for accumulatively about 5min at 17500rpm. Samples were then stored at room temperature for 24h, then were visually examined and classified as stable or unstable systems.

2.2.7 Particle size measurement

Emulsion samples or aqueous phase of the samples after lipolysis were diluted 100 times with deionized water and mixed well. The average particle sizes (hydrodynamic diameters) of the lipid droplets were determined with a dynamic light scattering method using a BIC 90Plus particle size analyzer (Brookhaven Instrument, New York, NY) at a fixed scattering angle of 90° at ambient temperature, with a solid-state laser operating at 658 nm with 30 mW power. The scattering signals were detected by a high-sensitivity avalanche photodiode detector.

2.2.8 Stability study of nanoemulsion formulation

Stability study of the sample was carried out in two parts. The first was to visually examine the physical stability of the sample, and the presence of quercetin crystals in the sample. The second was particle size analysis described in section 2.2.7 Particle size measurement.

2.2.9 *In vitro* lipolysis of quercetin loaded formulations

The test method used in this study to monitor the lipid digestion is the *in vitro* lipolysis assay using a pH stat method. It was used to simulating lipid digestion in the small intestine. Fed state *in vitro* lipolysis assays were carried out in this study. Fed state buffer was prepared as list in **Table 2**. At the beginning, 0.25 g pseudo-organogel or 1.0 g emulsion were added to 9 mL of fed state lipolysis buffer, then the pH of the mixture were adjusted to 7.5±0.2 by 0.25 M NaOH, and kept at 37 ℃. 1 g of pancreatin was added to 5mL of fed state lipolysis buffer, well mixed under magnetic stirring, and then centrifuged at 2000 rpm for 15 min, supernatant was collected afterwards. At the beginning of lipolysis assay, 1mL of pancreatin solution was added to the sample mixture that was kept at 37 ℃. Maintained the pH of the lipolysis sample at 7.5±0.2 by

adding 0.25 M NaOH manually, the time and volume of NaOH added were recorded during the 2h period of the lipolysis assay. After 2h, the sample mixture after digestion was ultracentrifuged at 50K rpm for 40 min, aqueous phase was collected and filtered through a 0.22 μm filter, first 1mL of filtrate was discarded and the remaining was collected for further analysis.

Table 2 Recipe of lipolysis buffer in fed states

Fed State Buffer	
Tris Maleate (mM)	50
NaCl (mM)	150
CaCl2·2H2O (mM)	5
NaTDC (mM)	5
Phosphatidylcholine (mM)	1.25

2.2.10 Determination of bioaccessilibity

The density of all quercetin loaded system was estimated as 1 g/mL. Concentration of quercetin was measured and calculated as stated before. The %Bioaccessility was calculated as followed, in which the mass of quercetin was calculated from the concentration of quercetin, density of the sample and the mass of the oil or the emulsion.

$$\%\text{Bioaccessibility} = \frac{mass\ of\ solubilised\ quercetin}{mass\ of\ quercetin\ loaded\ in\ the\ system\ initially} \times 100\%$$

2.2.11 Determination of the extent of lipolysis

The extent of lipolysis was determined using the amount of NaOH consumed and the theoretical NaOH amount needed for completed lipolysis. As follows:

$$\%\text{Extent of Lipolysis} = \frac{\text{NaOH amount consummed}}{\text{Theoretical NaOH amount needed for completed lipolysis}} \times 100\%$$

To calculate the theoretical NaOH amount needed for completed lipolysis, it was assumed 1 molecule of MCT needs 2 molecules of NaOH for degestion. The theoretical amount of NaOH needed to digest sugar ester and Tween20 were determined by test result for the blank sample (only sugar ester and Tween20), and were subtracted in the calculation.

2.2.12 Statistical analysis

OriginPro9.0 was used to perform the statistical analysis including t-test and one-way ANOVA (One-way ANOVA test were followed by Holm-Sidak method).

Chapter 3 Results and Discussions

3.1 Selection of sugar ester

In this study sucrose stearate was used to modified the oil forming the pseudo-organogel, **Table 3**[33] showed all of the sucrose stearate that were available from the vender (Mitsubishi-Kagaku Foods Corporation), depending on the extent of esterification and the type of the acyl chains, sucrose stearates have different HLB values. Based on the fact that, in this study, it was the oil phase that needed sucrose stearate to modify, only sucrose stearates with HLB value in between 1 to 9 were tested, and the ability to form MCT into pseudo-organogel were examined. Sucrose stearates at specific amount can form a pseudo-organogel system were selected (marked as √ in **Table 4**). To examine the formation of pseudo-organogel system, vials were put upside down on the table, and if there was no gravitative flow of the substance could be observed at ambient temperature in the time frame of the experiment, as the third example showed in **Figure 7**, then the pseudo-organogel system were considered to be formed. From the results show in **Table 4**, when up to 25 % of sucrose stearates were used, almost all of them could form the pseudo-organogel system. However when the concentration of sucrose stearate decreased, pseudo-organogel system could only be formed at level of 15 % and 20 % of S370 and S770. Upon three months of storage at ambient environment, pseudo-organogel system formed by 20 % S370 were more stable (no phase separate were visually observed), therefore sucrose stearate S370 had been chosen for further formulations.

Table 3 Sucrose stearate available from vender, with different types, HLB value and percentage of mono ester content

1)S-370F is ultrafine powdered product of S-370.[33]

PRODUCT	TYPE	HLB value	Mono ester content	Form
Sucrose stearate	S-070	<1	<1%	Powder
	S-170	1	1%	Powder
	S-270	2	10%	Powder
	S-370	3	20%	Powder
	S-370F[1)]	3	20%	Powder
	S-570	5	30%	Powder
	S-770	7	40%	Powder
	S-970	9	50%	Powder
	S-1170	11	55%	Powder
	S-1570	15	70%	Powder
	S-1670	16	75%	Powder

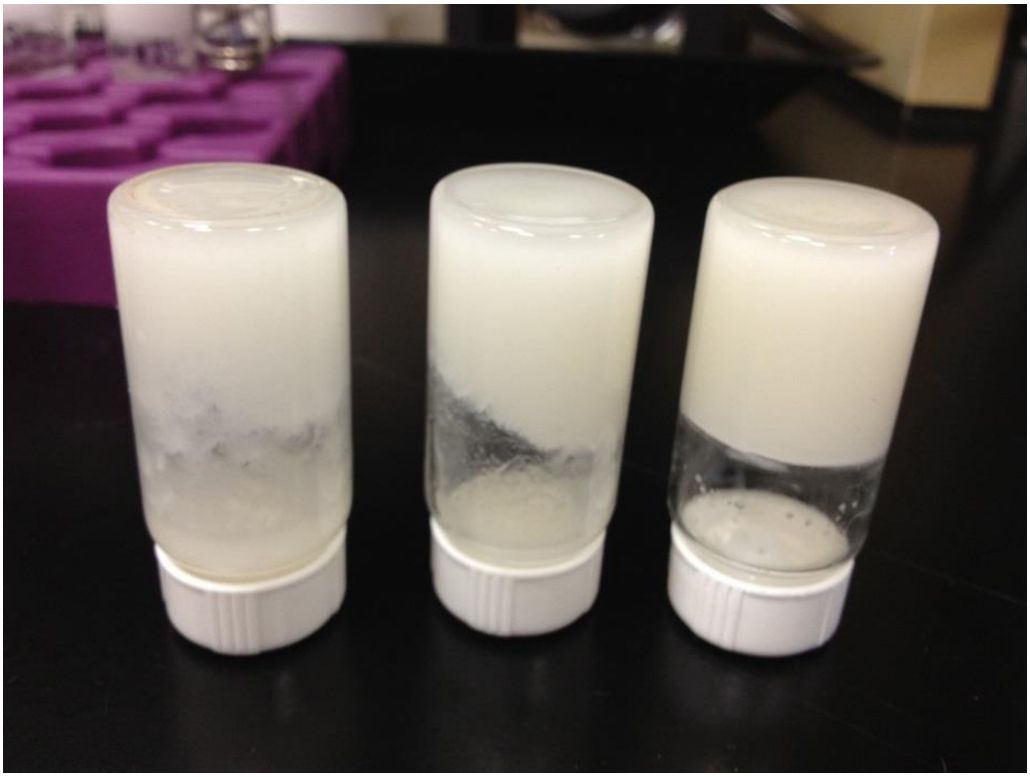


Figure 7 Photographs of vials examining the formation of pseudo-organogel.

Table 4 Results of pseudo-organogel system formation using different sucrose stearate in different concentrations
(√,pseudo-organogel system formed; ×, pseudo-organogel system not formed; -, experiment was not carried out)

Weight ratio / Sucrose Stearate	15%	20%	25%
S170	×	×	×
S270	-	×	√
S370	√	√	√
S570	×	×	√
S770	√	√	√
S970	×	×	-

3.2 Rheology results

Figure 8 shows the rheological test result of the pseudo-organogel sample forming from MCT and 20 % S370. From the rheological results, storage modulus G' is higher than loss modulus G", suggesting a viscoelastic property of the sample, and also from the complex viscosity data, sample exhibited shear thinning behavior.

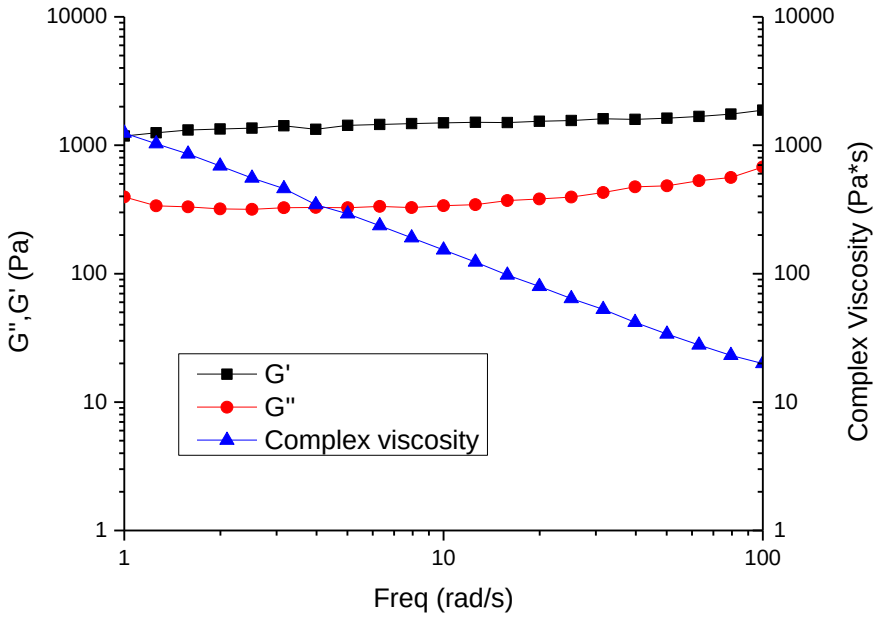


Figure 8 Rheological test result of pseudo-organogel soft material sample

3.3 UV calibration curve

Calibration curve was obtained by dissolving quercetin standard in ethanol, and dilute to specific concentrations by ethanol. Standard samples at each linearity levels were measured triplicate. Calibration Curve has an equation of y = 0.0757x - 0.0155, R^2= 0.9995 and a linear range between 1-15 ppm, as showed in **Figure 9.**

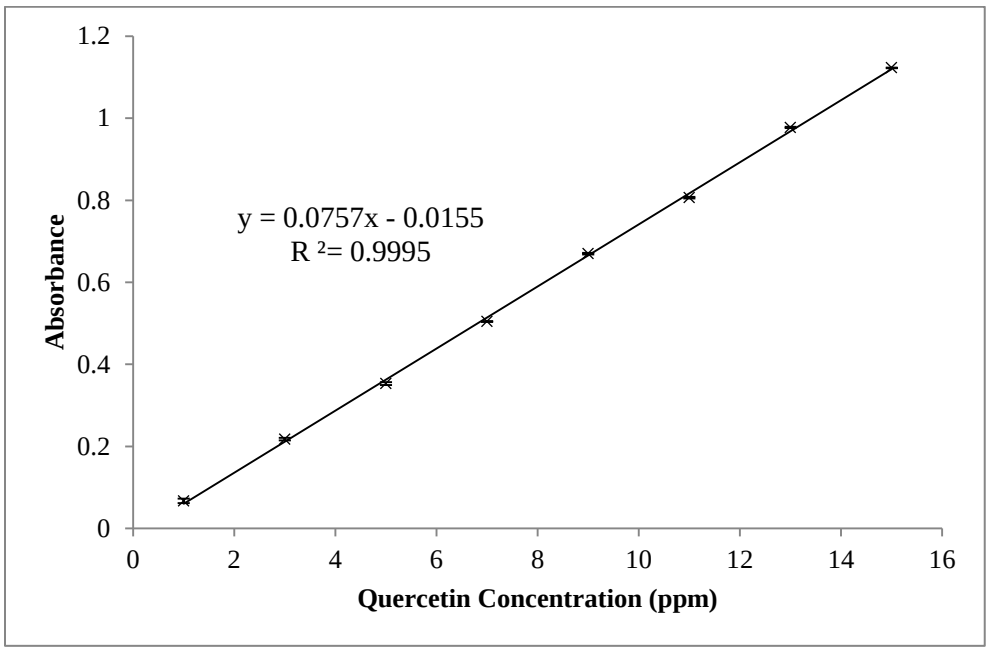


Figure 9 Standard calibration curve of quercetin

3.4 Solubility of quercetin

As a delivery system, one of the most important criteria to determine the efficacy is the loading capacity of the bioactive compound in the system. Different approaches had been evolved in order to increase the loading of the delivery system. Some study managed to increase the loading by dissolve the bioactive compound at the elevated temperature to achieve supersaturation, hence to increase the solubility[34]. Other studies added modifier with different polarity to change the overall polarity of the oil in order to increase the metastable solubility[35, 36]. In this study, a combination of several approaches has been evolved to achieve a stable high loading in the oil phase.

3.4.1 Quercetin solubility in MCT, limonene and 20%limonene and MCT mixture

According to a similar study[36], oil was first heated to 120 ℃ on a hot plate in open air, then quercetin was added in oil, kept at 120 ℃ and open air for 30 min while stirring to achieve a maximum dissolving of quercetin, then cooled to room temperature. The mixture was then centrifuged at 10k RPM for 30 min. The supernatant was collected and diluted with ethanol before analysis. As the results showed in **Figure 10**, at 120 ℃, under open air 30 min condition, solubility of quercetin in MCT was 1.77±0.29 mg/g, and even lower in limonene 1.39±0.03 mg/g. However, when dissolved quercetin in a mixture of MCT and limonene, the solubility of quercetin was 2.23±0.27mg/g, it was not in between the amount of two individual solubility but higher than each one of them. Statistical analysis (t-test between groups of MCT and 20 % limonene) showed that there was no significant difference between the two group at significant level of 0.05 ($p=0.12$), however the trend of increasing solubility sill worth of further investigation (section 3.4.3 Quercetin solubility in MCT, limonene and 20% limonene and MCT mixture under optimized processing condition).

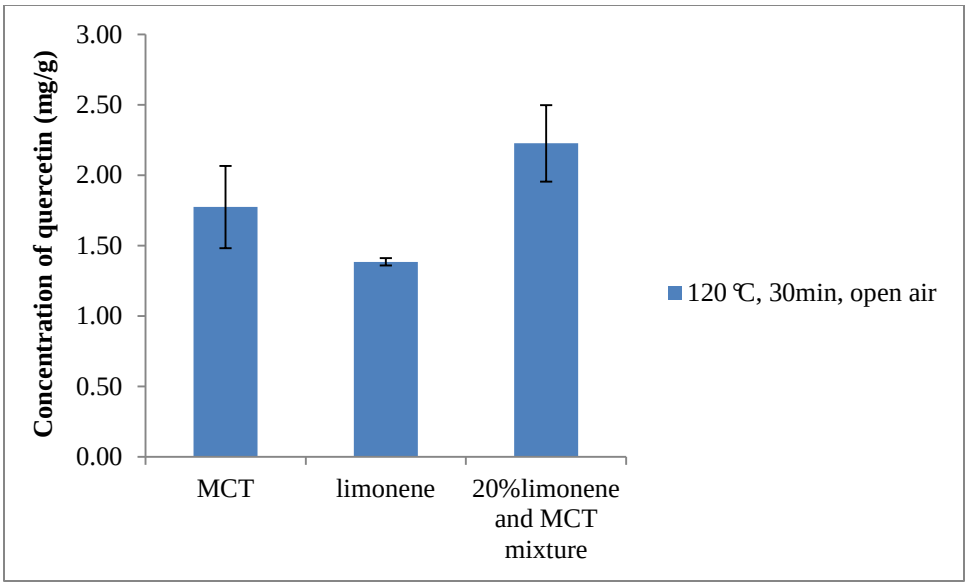


Figure 10 Solubility of quercetin in MCT, limonene, and 20 % limonene and MCT mixture, heated at 120 ℃ for 30 min at open air condition.

3.4.2 Processing condition affect the final concentration of quercetin

Now that we know adding limonene to MCT may increase the overall metastable solubility of quercetin in the oil phase, but we cannot say that for sure since the significant lever between two groups of data, MCT group and 20% limonene group, was not lower than 0.05. The processing conditions we used when heated the oil and dissolved quercetin were heated at 120 ℃ for 30min under open air. Quercetin as an antioxidative compound, there was possibility that oxidation or other forms of degradations of quercetin could occur under intense heating conditions. So when attempting to achieve a supersaturation of quercetin at elevated temperature, it was also necessary to control the processing condition in a range that could prevent quercetin from extensive oxidization. Processes under several less intense heating conditions were carried out: lower the heating temperature, shorten the heating time or protect quercetin from oxygen when heating.

Results of final concentrations of quercetin in MCT under different process condition were shown in **Figure 11**. The shortened heating time combined with the addition of N_2 could significantly

($p<0.001$) increase the final concentration of quercetin in MCT, which partially proven the hypothesis made earlier: that there was some degree of oxidation or other forms of degradation of quercetin could have occurred under previous heating condition (120 ℃ for 30min in open air). Similar trend was found when dissolved quercetin in 20% limonene and MCT mixture, as showed in **Figure 12**.

For both MCT or 20% limonene and MCT mixture as solvent, only heated to 90 ℃ and kept at 90 ℃ for 10 min and under protection of N_2 was enough to achieve a significant ($p<0.001$) higher final concentration of quercetin comparing to the initial condition (120 ℃ for 30 min in open air), there was no need to use a more intense condition, from both the point of protect quercetin from oxidation and the point of energy saving. Heating to 90 ℃ and keeping at 90 ℃ for 10min and under protection of N_2 were chosen as the optimized processing conditions.

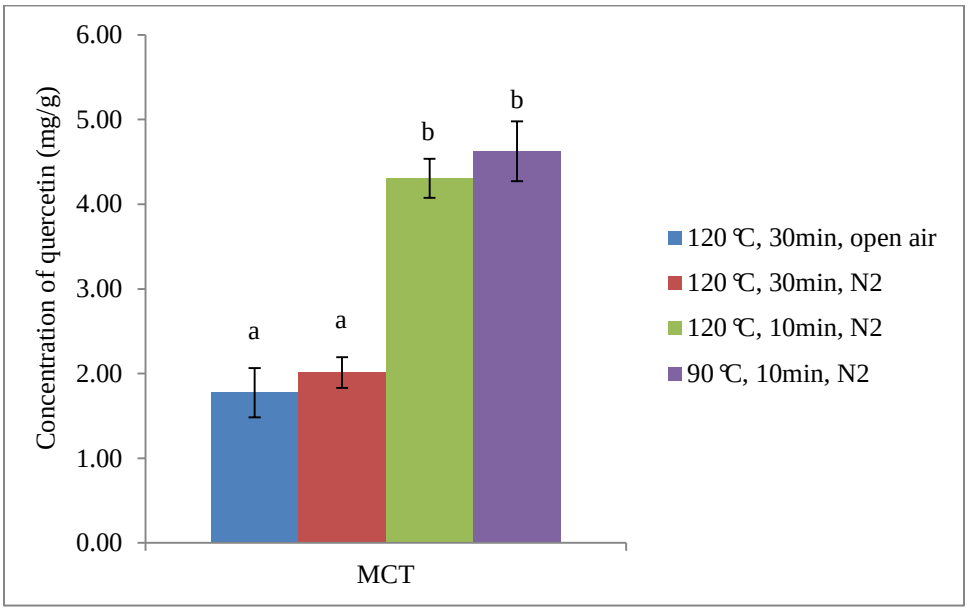


Figure 11 Final concentration of quercetin in MCT using different processing conditions. Different letters (a or b) indicate significant difference from one-way ANOVA at level of $p<0.001$.

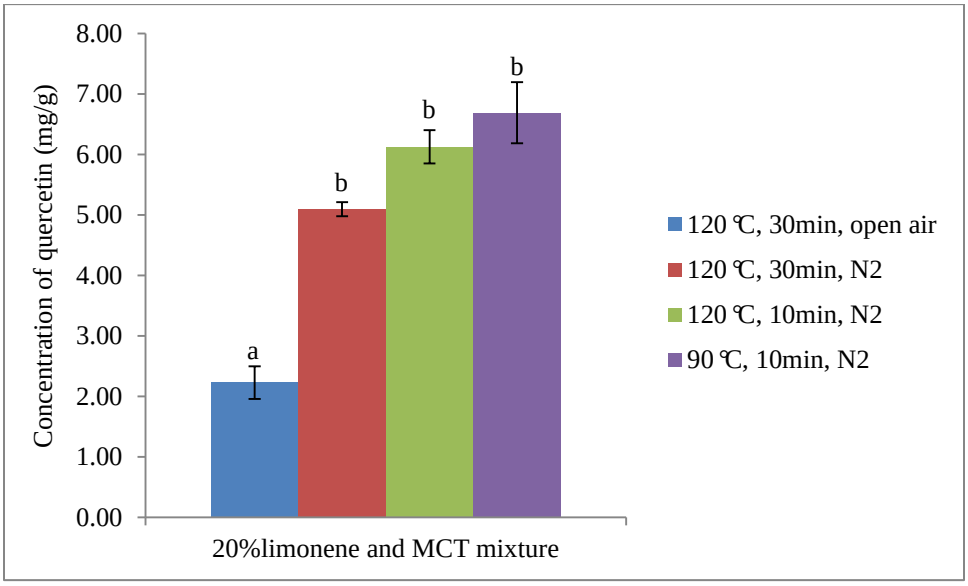


Figure 12 Final concentration of quercetin in 20%limonen and MCT mixture using different processing conditions. Different letters (a or b) indicate significant difference from one-way ANOVA at level of p<0.001.

3.4.3 Quercetin solubility in MCT, limonene and 20% limonene and MCT mixture under optimized processing conditions

It had got proven that for a higher final concentration of quercetin, we could use a less intense heating condition to achieve the maximum solubility and protect quercetin form oxidation or other forms of degradations. We knew that from section 3.4.1 Quercetin solubility in MCT, limonene and 20%limonene and MCT mixture, that there was a trend that when using limonene and MCT mixture, the solubility of quercetin was higher than that in each one of the oil alone. The trend was examined again under the optimized processing conditions. As showed in **Figure 13**, when used the optimized processing conditions: heated at 90 ℃ for 10min under N_2 protection to dissolve quercetin, solubility of quercetin was significant higher in the limonene and MCT mixture than that of MCT, at level of $p<0.01$. This finding confirmed that the addition of limonene had effect on increasing the solubility of quercetin in the mixture with MCT.

This increasing of solubility may cause by the co-solvent effect of the addition of limonene to MCT. Even though the solubility of quercetin in limonene itself is much lower, adding limonene introduce carbon ring structure into the oil mixture, which gives more similarity to the three rings structure of quercetin, thus increase the overall similarity to the solute comparing to the chains structures of triglycerides itself.

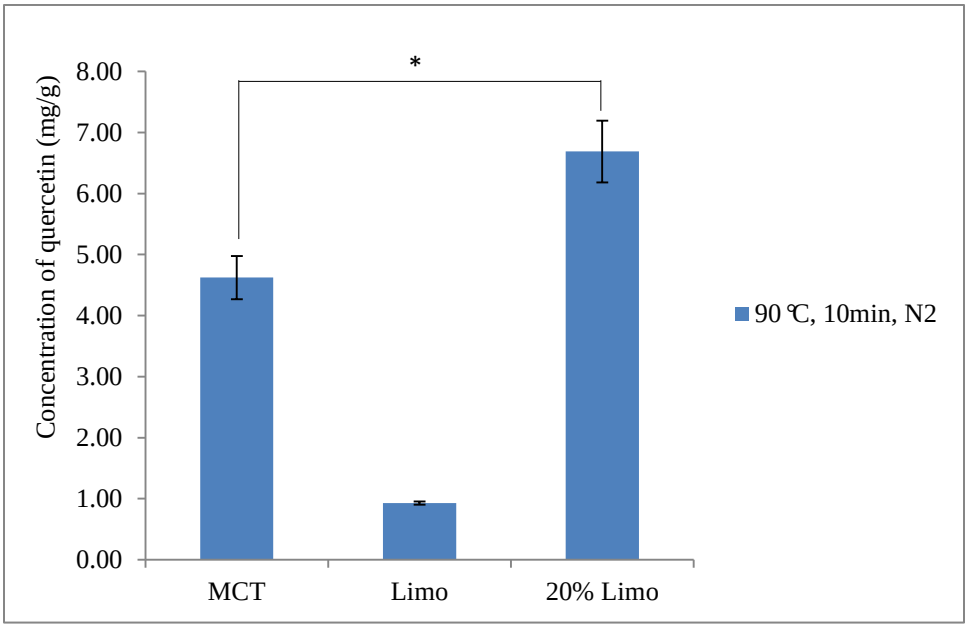


Figure 13 Solubility of quercetin in MCT, limonene, and 20 %limonene and MCT mixture, heated at 90 ℃ for 10 min under N_2 protection. * indicate a significant level of p<0.01.

3.4.4 Solubility of quercetin increased 40 times in the modified oil

If we compared all the data regarding quercetin concentration under different processing conditions, as showed in **Figure 14** and **Table 5**, the concentration of quercetin was as high as 6.69±0.51 mg/mL (20 % limonene MCT mixture, 90 ℃, 10min, and under N_2 protection), and concentration of quercetin at initial condition (MCT, 120 ℃, 30 min, and open air) was 1.77±0.29 mg/mL, that is, by optimizing the solvent and the processing condition, the concentration of quercetin was increased by about 3 times. And it was 40 times higher compared to the maximum concentration of quercetin in MCT at room temperature[37].

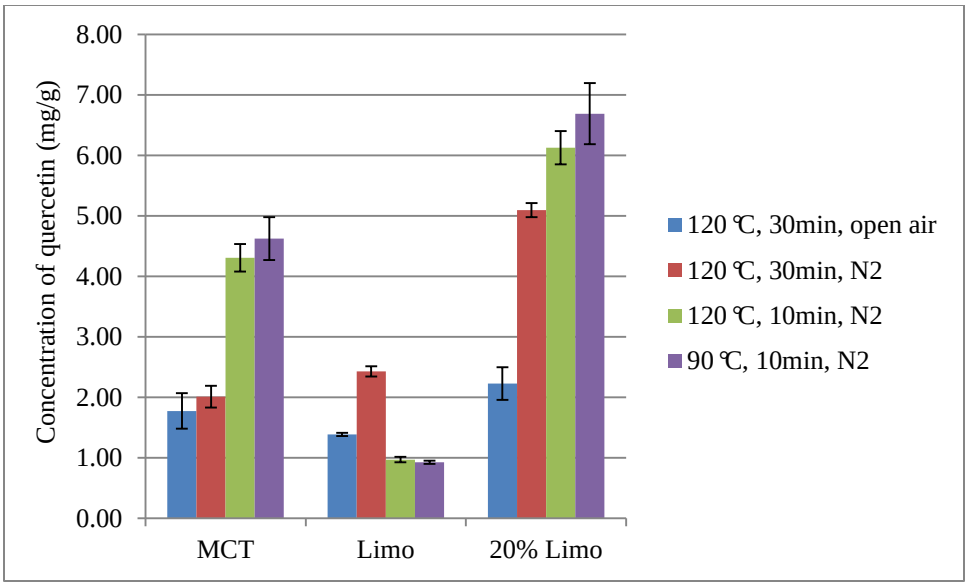


Figure 14 Quercetin concentrations under different processing conditions in different solvents

Table 5 Quercetin concentrations under different processing conditions in different solvents

Concentration of quercetin(mg/g)	120 ℃, 30 min, open air	120 ℃, 30 min, N_2	120 ℃, 10 min, N_2	90 ℃, 10 min, N_2
MCT	1.77±0.29	2.01±0.18	4.31±0.23	4.62±0.35
Limonene	1.39±0.03	2.43±0.08	0.97±0.04	0.93±0.03
20%Limonene+MCT	2.23±0.27	5.09±0.11	6.13±0.28	6.69±0.51

3.5 Effect of limonene on pseudo-organogel system formation

Adding limonene to MCT can increase the overall solubility of quercetin in the mixture, we also needed to know if the adding of limonene has any effects on the formation of pseudo-organogel system. Different ratios of limonene and MCT were mixed and then heated to 90 ℃, added 20 % of S370, heated and stirred to fully dissolved, cool to room temperature and store at room temperature overnight. Then the formations of pseudo-organogel system were examined by put the vials upside down and visually observed the flow. When increase the limonene: MCT ratio to 3:5 (37.5% of limonene in the whole mixture), the pseudo-organogel system could no longer form,

shown in **Figure 15**. So the higher limit of limonene that could be used to modify MCT oil in this system was around 25%.

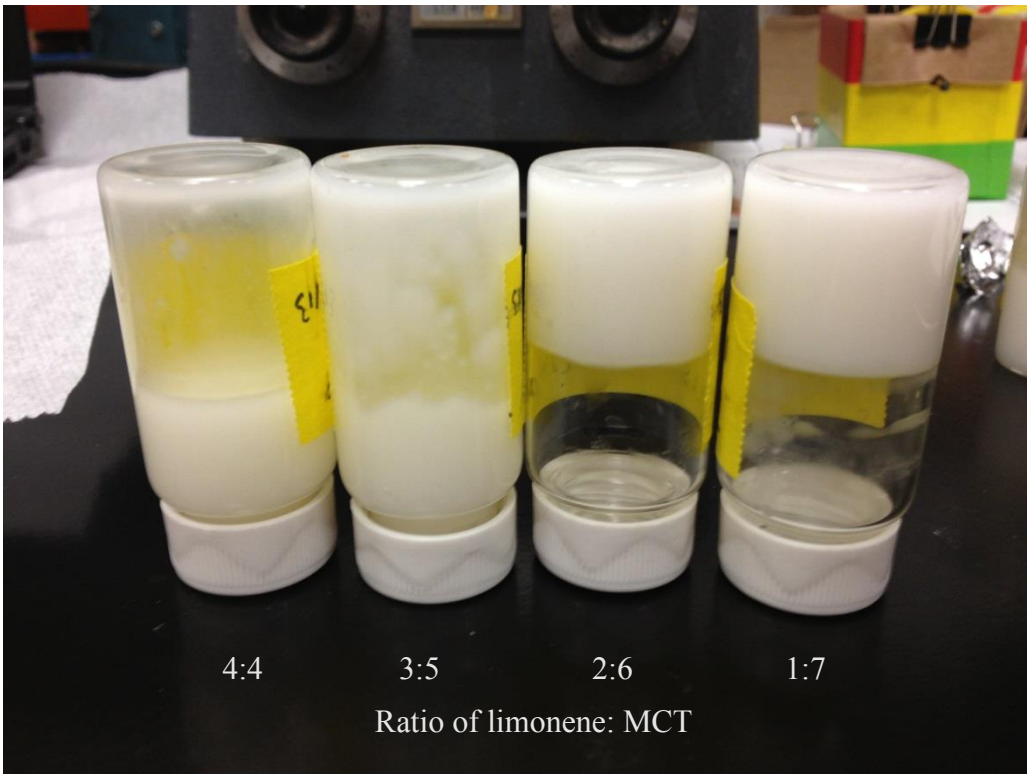


Figure 15 Effect of limonene ratio in the mixture on the formation of pseudo-organogel system

3.6 Effect of limonene on quercetin solubility

To investigate the effect of limonene ratio in the oil mixture on the solubility of quercetin, oil mixtures containing 0-25% of limonene were prepared. Quercetin was dissolved in the oil mixtures following the optimized condition described in section 3.4 Solubility of quercetin. Briefly, the oil mixture was first heat to 90 ℃ under nitrogen protection, and then excess amount of quercetin was added into the oil and kept at 90 ℃ for 10 min. centrifuged the mixture after cooled to room temperature, then filter through 0.22 μm filter, diluted with ethanol before analysis. From the results shown in **Figure 16** we could conclude that among the range defined in section 3.5 Effect of limonene on pseudo-organogel system formation, 20% of limonene addition

resulted in the best overall solubility of quercetin in the oil mixture. So the oil mixture of limonene and MCT ratio (w/w) at 2:8 was used for further formulations. Statistically there were significant difference ($P<0.05$) between 20 % limonene and MCT mixture and other 0 %-15 % of limonene and MCT mixture. When further increase the ratio of limonene from 20 % to 25 %, there were no significant increase on quercetin solubility could be observed.

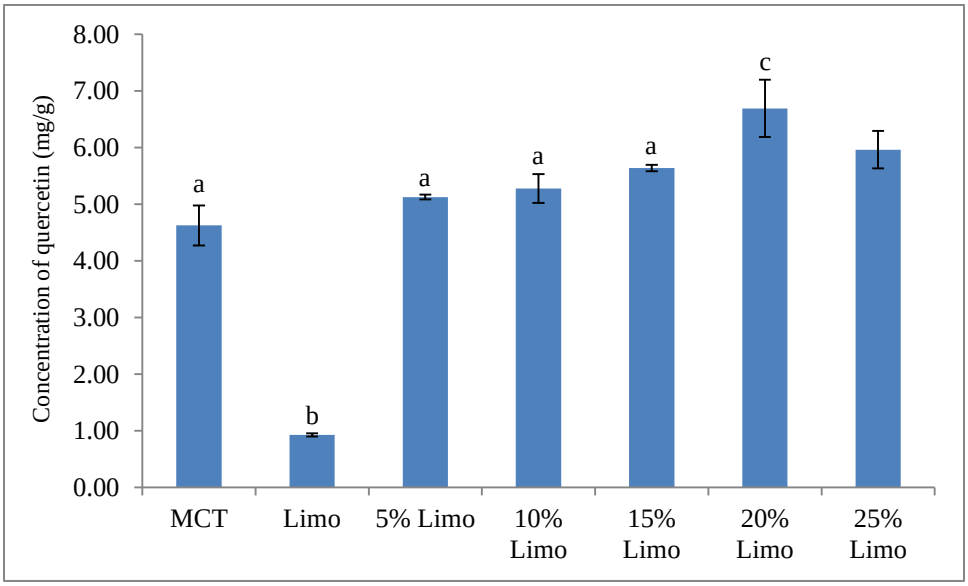


Figure 16 Metastable solubility of quercetin in deferent oil mixture with different ratio of limonene. Different letters (a, b or c) indicate significant difference from one-way ANOVA at level of $P<0.05$.

3.7 Stability of quercetin

As mentioned before, the loading of bioactive compound in the delivery system is a crucial factor that defines the efficacy of the system. By heating when dissolving can increase the amount of bioactive compound loaded, but to make this metastable solubility last longer and be kinetically stable was the other part of the problem.

3.7.1 Stability of quercetin in oil

The stability of quercetin in oil was defined as the concentration of quercetin at the time of testing. Sample used for stability test was obtained at the optimized condition as mentioned earlier: excess amount of quercetin was dissolved at 90 ℃ for 10 min under nitrogen. Concentrations of quercetin were measured by UV at the time of sample initially prepared (T0), 36 hours after (T36h), and 1 week after (T1week). As shown in the **Figure 17**, after 1 week of storage at room temperature, the concentration of quercetin dropped significantly (more than 20 %), and yellow crystals could be visually observed at the bottom of the bottle. The lack of stability of quercetin in the oil phase was a non-desirable factor for being used as a delivery system in food or cosmetic applications, so further modification of the oil phase was needed to overcome this drawback.

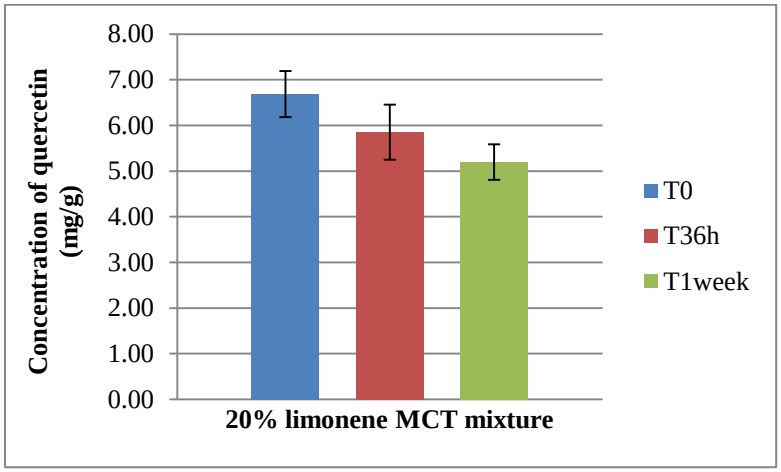


Figure 17 Concentration of quercetin in 20% limonene and MCT mixture at T0, T36h, and T1week.

3.7.2 Stability of quercetin in pseudo-organogel

To further modify the oil phase in this study, the pseudo-organogel system was formed. The stability of quercetin in pseudo-organogel was determined by observing the crystal formation using polarized microscope. **Figure 18** (A) showed quercetin crystal dispersed in the oil phase, under polarized microscope condition, needle like crystals can be observed. This was used as the indicator of the presence of quercetin crystal. **Figure 18 (**B) showed the blank pseudo-organogel under polarized microscope tiny bright spots was the sugar ester crystal.

The quercetin loaded pseudo-organogel sample after 6 months of storage at room temperature was used as the sample for stability testing. Sample was first visually examined, and there were no obvious crystals presence from the bottom view of the sample vial. **Figure 18** (C) was the polarized microscope picture showing that there was only tiny bright spots could be observed, which had the same scale as the bright spots in (B), which contributed to the sugar ester; no needle like crystal could be found, indicating the absence of quercetin crystal.

Another study[32] of curcumin loaded in Span20-MCT oil mixture using similar method as in this study showed that, if there were no further modification of the oil mixture, not only the concentration of the bioactive compound will decrease over the time, when tried to incorporate this un-modified oil phase into the nanoemulsion systems, significant amount of precipitation of the bioactive compound was observed, and hence resulted in a significantly decreased bioaccessibility. Together with the stability test result stated above, further modification of the oil phase into the gel-like soft material, as referred to be pseudo-organogel in this thesis, was necessary and could increase the kinetic stability of the delivery system.

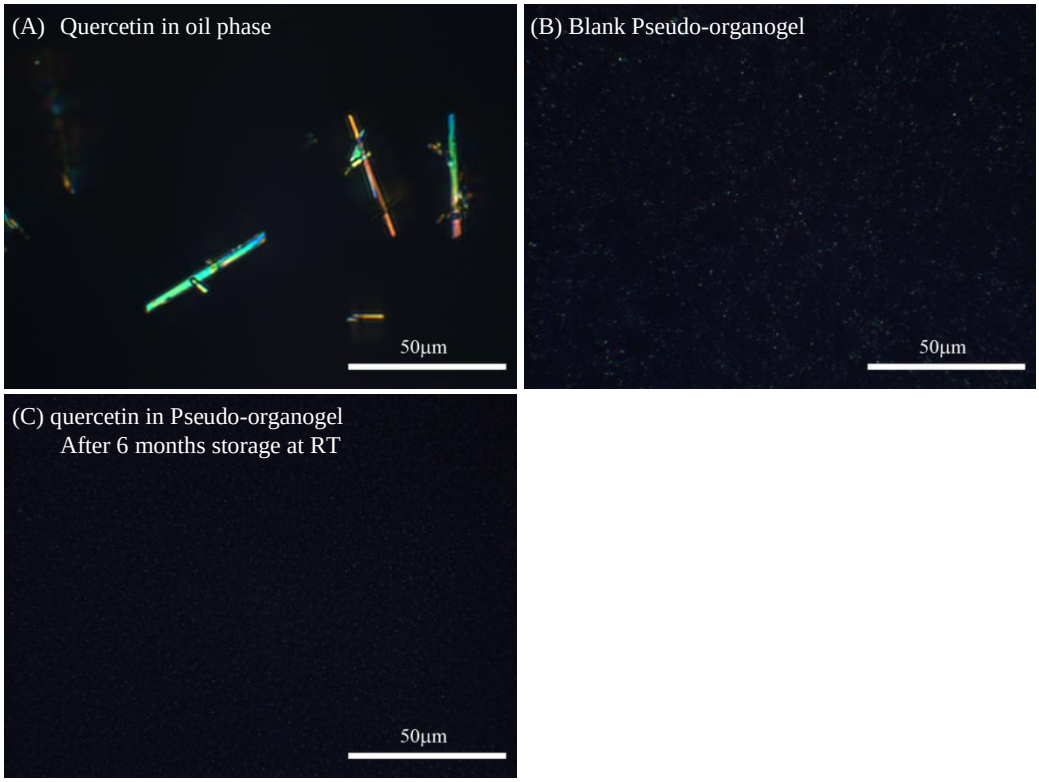


Figure 18 Polarized microscopy images of (A) quercetin crystal dispersed in the oil phase; (B) blank pseudo-organogel; (C) quercetin loaded pseudo-organogel sample after 6months storage at room temperature

3.8 Selection of stable systems

After 24h of storage since initially prepared, emulsion samples were visually examined, systems that appeared as homogeneous as showed in **Figure 19** (B) were classified as stable systems, while phase separation were observed in other samples as showed in **Figure 19** (A). Stable systems were marked in the circle as shown in ternary phase diagram in **Figure 20**. Emulsion with 25% of oil phase, 55% of water and 20% of emulsifier was chosen as the representative of the stable system and reserved for further particle size analysis, stability study and lipolysis study, sample were kept at room temperature for storage.

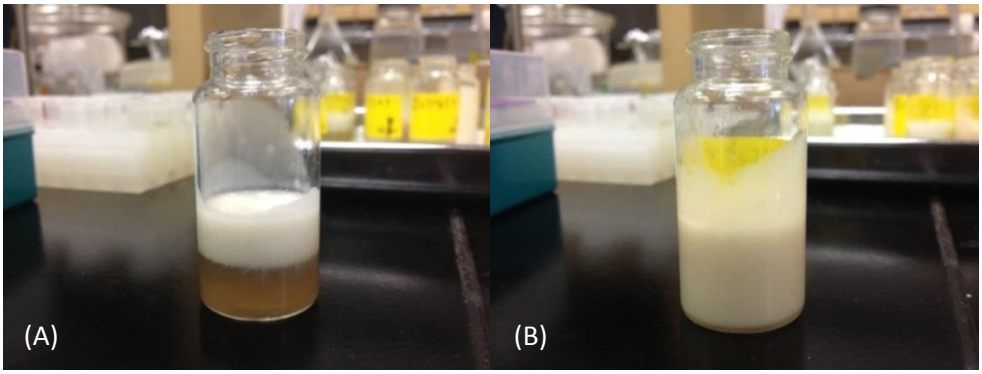


Figure 19 Photograph of emulsion samples: (A) phase separated; (B) stable emulsion

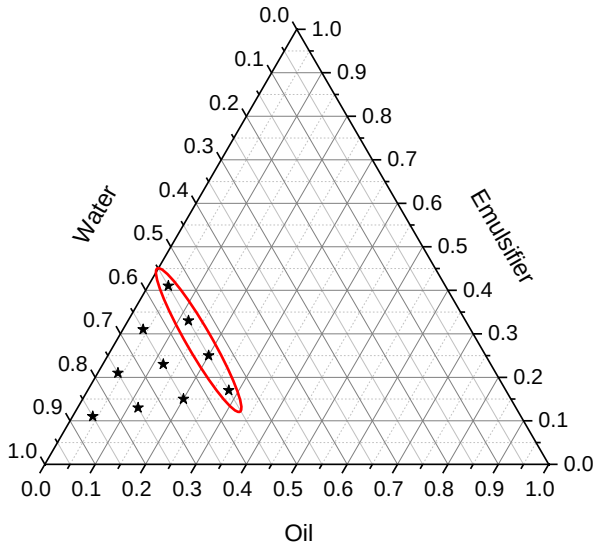


Figure 20 Ternary phase diagram and stability of samples prepared by pseudo-organogel, water and Tween20 using high speed homogenizer. Sugar ester counted as emulsifier in this diagram.

3.9 Stability of nanoemulsion

Stability of nanoemulsion in this section was determined by the growth of the particle size. Emulsion with 25 % of oil phase, 55 % of water and 20 %of emulsifier prepared from previous steps was used for particle size measurements. The initial particle size of the nanoemulsion was 182.1 nm±7.3 nm, and after 4 months of storage under room temperature the particle size increased to 310.7 nm±23.0 nm, which still could be considered as stable nanoemulsion.

3.10 Lipolysis profile

During the *in vitro* lipolysis assay, the extent of lipolysis over time was recorded and drafted below. From the lipolysis profile of the first 30 min of digestion we can see that the digestion of emulsion is much faster than of the oil, it have been digested over 60% in about 2 min for emulsion, which taken over 40 min for oil. This was because of the small droplet size of emulsion and the large surface area for lipase-catalyzed lipid hydrolysis.

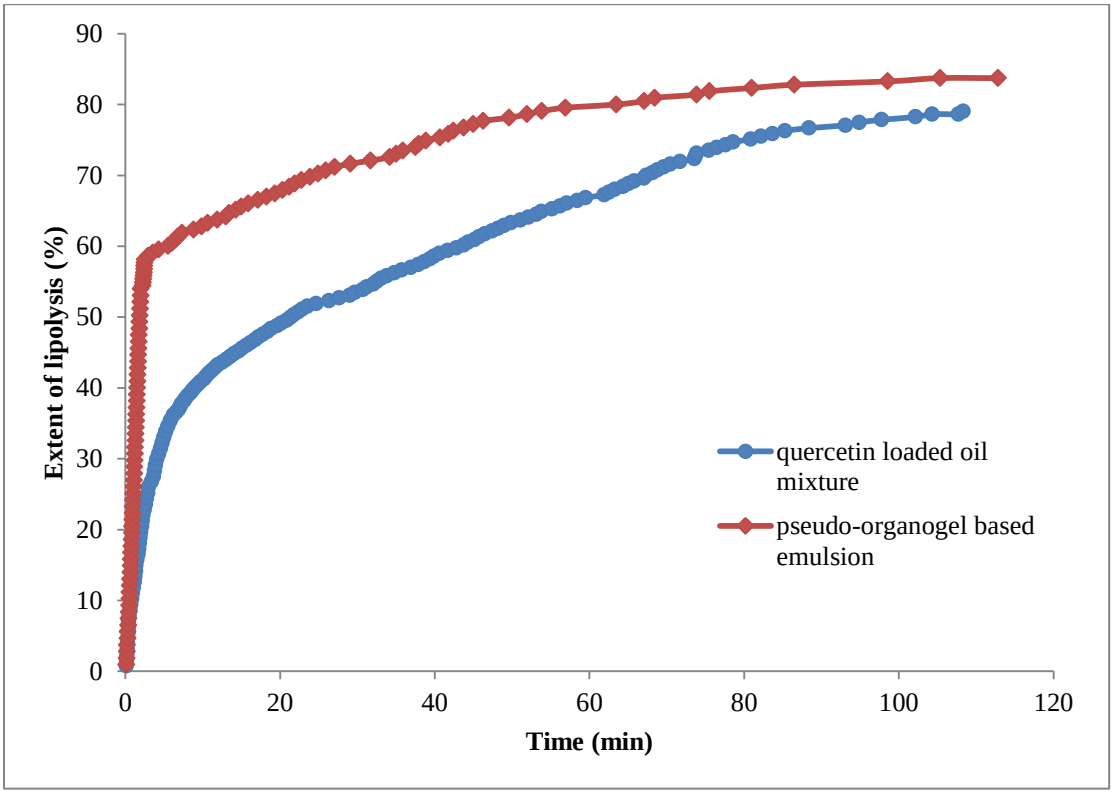


Figure 21 Lipolysis profile of quercetin loaded oil mixture and pseudo-organogel based emulsion samples at fed stage

3.11 Extent of lipolysis

Not only the lipolysis profile showed that the digestion of emulsion was faster than oil, it also showed that after first 30 min of digestion, the extent of lipolysis of emulsion (71.9 %±1.2 %) was significantly higher ($p<0.001$) than that of oil (54.0 %±0.6 %).

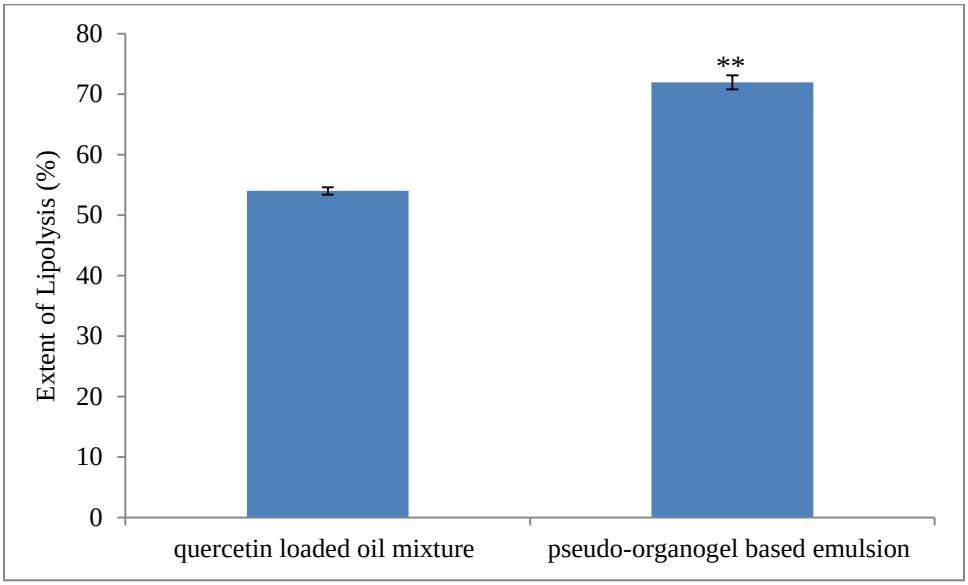


Figure 22 Extent of Lipolysis of quercetin loaded oil mixture and pseudo-organogel based emulsion at fed stage, ** indicate a significant level at p<0.001

3.12 Bioaccessibility

The percentage of bioaccessibility of quercetin loaded oil mixture was measured to be 35.6 %±0.6 %, and the percentage of bioaccessibility for pseudo-organogel based emulsion was 49.7 %±5.3 % which is significantly higher ($p<0.01$) than the unformulated oil. The bioaccessibility of quercetin suspended in water is extremely low (less than 3 %), result not showed in the comparison chart. The formulation did have effect on increase the bioaccessibility of quercetin.

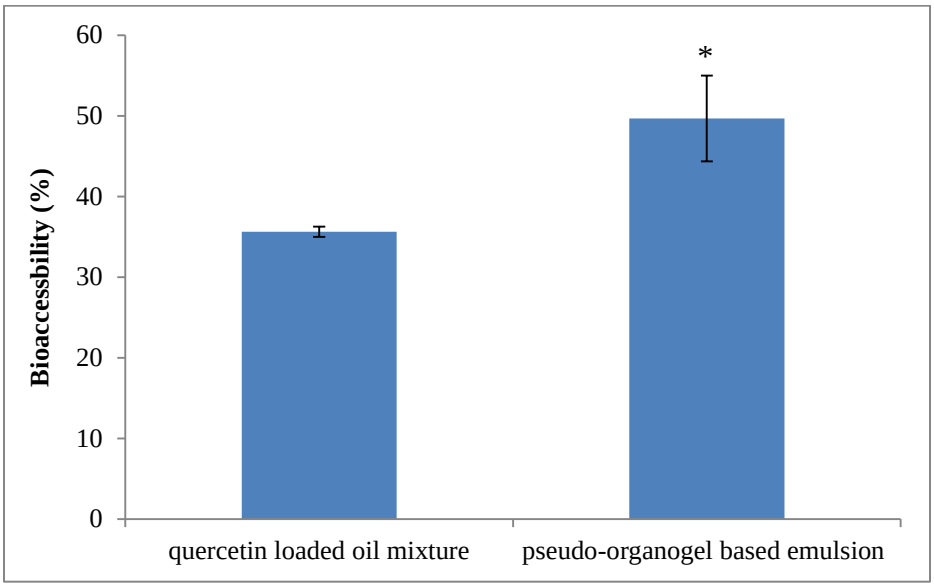


Figure 23 %Bioaccessibility of quercetin loaded oil mixture and pseudo-organogel based emulsion at fed stage, * indicate a significant level at p<0.01

3.13 Particle sizes of quercetin micelles after lipolysis

The particle sizes of quercetin micelles after lipolysis for emulsion is 165.9 nm±62.9 nm which indicate an aggregation of the micelles. Which might because of the complexity of the system, since all Tween20 sugar ester and monoacylglycerin could stay at the interface and form traction force of each other. However, as long as the bioaccessibility successfully increased, the structure of the *in vitro* formed micelles may not have effect on solubilisation of the quercetin.

Chapter 4 Conclusions and Future Work

In this study, pseudo-organogel and pseudo-organogel based emulsion systems are being developed for oral delivery of quercetin to improve bioaccessibility. The formation of pseudo-organogel inhibited quercetin crystallization in the oil phase. Using mixture of limonene and medium chain triglyceride (MCT) at ratio of 2:8 (w/w), solubility of quercetin increased 40 times compared to quercetin in MCT at room temperature. Bioaccessibility and the extent of lipolysis were determined for the pseudo-organogel based emulsion system. After the first 30min of digestion, the extent of lipolysis of the pseudo-organogel based emulsion (71.9 % ±1.2 %) was significantly higher ($p<0.001$) than that of the quercetin loaded oil mixture (same oil mixture as used to form pseudo-organogel based emulsion) (54.0 % ±0.6 %). The percentage of bioaccessibility of oil mixture was measured to be 35.6 % ± 0.6 %, and the percentage of bioaccessibility for pseudo-organogel based emulsion was 49.7% ±5.3% which was significantly higher ($p<0.01$), and they all remarkably higher than the extremely low bioaccessibility of quercetin suspended in water(less than 3 %). With the high and stable loading of quercetin, and the improved bioaccessibility, this pseudo-organogel based emulsion system of quercetin may have potential in pharmaceutical applications or cosmetic products.

Further works will include antioxidant activity study of the formulated system, further optimization of the formulation by using different mixtures of food grade oils, *in vitro* and *in vivo* studies of the formulations.

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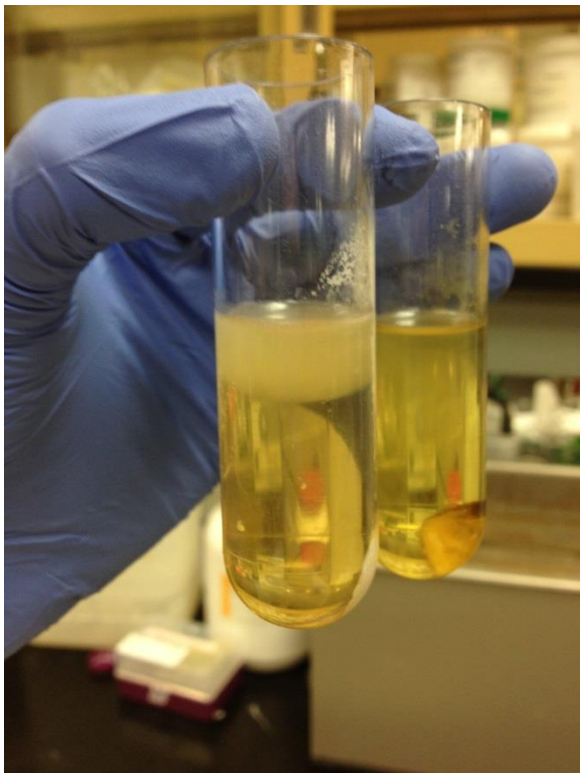
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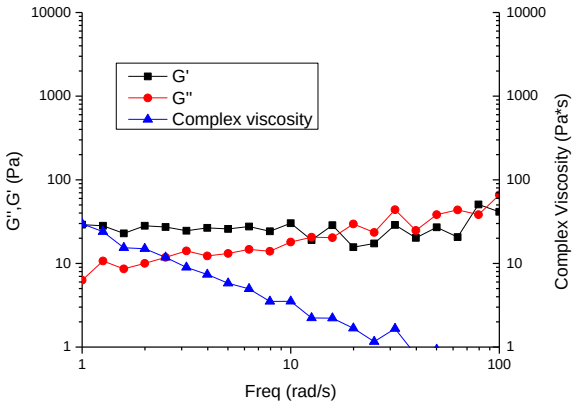
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Appendix

Appendix 1 Photograph of Lipid formulations after *in vitro* degistion



Appendix 2 Rheological test result of pseudo-organogel soft material sample (15 % of S770)



Appendix 3 Rheological test result of pseudo-organogel soft material sample (15 % of S370)

