University of Massachusetts Amherst

ScholarWorks@UMass Amherst

Masters Theses

Dissertations and Theses

August 2014

Influence of Physical States (Crystalized Versus Solubilized) of Bioactive Components And Oil Composition on Bioaccessibility And Bioavailability

Ziyuan Xia
University of Massachusetts Amherst

Follow this and additional works at: https://scholarworks.umass.edu/masters_theses_2

Part of the Food Chemistry Commons

Recommended Citation

Xia, Ziyuan, "Influence of Physical States (Crystalized Versus Solubilized) of Bioactive Components And Oil Composition on Bioaccessibility And Bioavailability" (2014). *Masters Theses*. 53.
https://scholarworks.umass.edu/masters_theses_2/53

This Open Access Thesis is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UMass Amherst. It has been accepted for inclusion in Masters Theses by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

INFLUENCE OF PHYSICAL STATES (CRYSTALIZED *VERSUS* SOLUBILIZED) OF BIOACTIVE COMPONENTS AND OIL COMPOSITION ON BIOACCESSIBILITY AND BIOAVAILABILITY

A Thesis Presented

By

ZIYUAN XIA

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

MASTER OF SCIENCE

May 2014
Food Science

INFLUENCE OF PHYSICAL STATES (CRYSTALIZED *VERSUS* SOLUBILIZED) OF BIOACTIVE COMPONENTS AND OIL COMPOSITION ON BIOACCESSIBILITY AND BIOAVAILABILIT

A Thesis Presented

by

ZIYUAN XIA

Approved as to style and content by:

____

H. Xiao, Chair

____

D. J. McClements, Member

____

E. A. Decker, Member

____

E. A. Decker, Department Head
Department of Food Science

DEDICATION

I would like to dedicate this work to my lovely and supportive parents. I am very appreciative of their love and support. Without their guidance, I would not be able to achieve this.

ACKNOWLEDGMENTS

I would like to acknowledge all of those associated with the Food Science Department at the University of Massachusetts Amherst. I would like to give my special thanks to Dr. Xiao and Dr. McClements for the instructions and knowledge they gave to me.

ABSTRACT

INFLUENCE OF PHYSICAL STATES OF BIOACTIVE COMPONENTS (CRYSTALIZED *VERSUS* SOLUBILIZED) AND OIL COMPOSITION ON BIOACCESSIBILITY AND BIOAVAILABILITY

MAY 2014

ZIYUAN XIA, B.A., FLORIDA INSTITUTE OF TECHNOLOGY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor H. Xiao

β-carotene, a precursor to Vitamin A, is considered as a potent nutraceutical in foods and beverages due to its ability of reducing the incidences of certain chronic diseases. However, because of its lipophilicity, its use is certainly limited. In this study, we investigated the bioaccessibilities of solubilized β-carotene and β-carotene crystals through a simulated gastrointestinal digestion model. Three systems were compared in the first case study: (1). pre-dissolved β-carotene nanoemulsion (*d*< 200nm); (2). corn oil emulsion (*d*< 200nm) with β-carotene crystals being added before digestion; (3). phosphate buffer saline with β-carotene being added before digestion. Oil-in-water nanoemulsions were formed by high-pressure homogenization using Tween 20 as emulsifier and corn oil as carrier oil and then they were subjected to a simulated mouth, stomach and small intestine digestion. The rate and extent of free fatty acid production in small intestine decreased in the order (2)>(1)>(3); whereas the β-carotene bioaccessibility decreased in the order (1)>>(2)>(3). In system (3), even without any fat content, there is still noticeable consumption of NaOH, which is due to

the ester bonds existing in the non-ionic surfactant (Tween 20). In the second case study, we developed two comparing groups by differentiating their oil concentration (20%, 4% respectively). The bioaccessibility of the high fat group is only half of the low fat group due to the insufficient digestion of fat in the former group. In the third case study, the bioaccessibility of nobiletin with different physical states (crystalized *vs* solubilized) and in different delivery system (conventional emulsion *vs* nanoemulsion) was compared. Not like β-carotene, the bioaccessibility of nobiletin as crystals in slightly lower than it is as solubilized state. Meanwhile, in conventional emulsion, the bioaccessibility is slightly lower than in nanoemulsion. This study provides important information for developing effective delivery systems for lipophilic bioactive components in food and beverage applications.

Keywords: nanoemulsions; delivery systems; β-carotene crystals; solubilized β-carotene ; bioaccessibility; digestion.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iv

ABSTRACT v

LIST OF FIGURES ix

CHAPTER

1. LITERATURE REVIEW 1

1.1 General Introduction of Bioactive Components 1

1.2 Study of β-carotene 1

1.3 Study of Nobiletin 3

1.4 *In Vitro* Digestion Model 5

2. INFLUENCE OF PHYSICAL STATES OF β-CAROTENE (CRYSTALIZED *VERSUS* SOLIBILIZED) ON BIOACCESSIBILITY..6

2.1 Introduction 6

2.2 Materials and Methods 7

2.2.1 Materials 7

2.2.2 Preparation of Beta-carotene Enriched Emulsion 7

2.2.3 Particle Characterization 8

2.2.4 Microstructural Analysis 8

2.2.5 *In vitro* Digestion 8

2.2.6 Bioaccessibility Determination..10

2.2.7 Statistical analysis..11

2.3 Results and Discussion 11

2.3.1 Nanoemulsion stability in digestion model 11

2.3.2 Lipid Digestion via *In Vitro* Digestion 17

2.3.3 In Vitro Digestion Bioaccessibility...18

2.4 Conclusions 20

3. THE INFLUENCE OF OIL CONCENTRATION ON BETA-CAROTENE BIOACCESSIBILITY..22

3.1 Introduction 22

3.2 Materials and Methods 22

3.2.1 Materials 22

3.2.2 Preparation of Beta-carotene Enriched Emulsion.....................................23

3.2.3 Particle Characterization........23
3.2.4 *In vitro* Digestion........24
3.2.5 Bioaccessibility Determination........25
3.2.6 Statistical analysis........26
3.3 Results and Discussion........27
3.3.1 Nanoemulsion stability in digestion model........27
3.3.2 Lipid Digestion via *In Vitro* Digestion........31
3.3.3 In Vitro Digestion Bioaccessibility........32
3.4 Conclusions........34

4. INFLUENCE OF PHYSICAL STATES AND SIZE OF EMULSION ON NOBILETIN BIOACCESSIBILITY........35
4.1 Introduction........35
4.2 .Materials and Methods........36
4.2.1 Preparation of Nobiletin Enriched Emulsion and Corn Oil Emulsion.....36
4.2.2 Particle Characterization........36
4.2.3 *In vitro* Digestion........37
4.2.4 Bioaccessibility Determination........38
4.2.5 Statistical analysis........39
4.3 Results and Discussion........40
4.3.1 Nanoemulsion stability in digestion model........40
4.3.2 Lipid Digestion via *In Vitro* Digestion........45
4.3.3 In Vitro Digestion Bioaccessibility........47
4.4 Conclusions........48

BIBLIOGRAPHY........50

LIST OF FIGURES

Figure Page

1a.Influence of β-carotene on mean particle size..11

1b. Particle size distribution of β-carotene solubilized group.................................... 12

1c. Particle size distribution of β-carotene crystals group 12

2. The changes of morphologies of lipid 13

3. The change of microstructure of β-carotene crystals. 13

4. Charge of the particles 15

5. Volume of NaOH.. 17

6. Free fatty acids released percentage.. 17

7. Beta-carotene bioaccessibility.. 19

8a.Influence of oil concentration on mean particle size...27

8b. Particle size distribution of 20% fat group.. 27

8c. Particle size distribution of 4% fat group 28

9. Influence of oil concentration on particle charge.. 29

10. Influence of oil concentration on micelles size 30

11. Volume of NaOH... 31

12. Free fatty acids released percentage..31

13.Influence of oil concentration on β-carotene bioaccessibility. 33

14a.Influence of nobiletin on mean particle size..40

14b. Particle size distribution of nobiletin nanoemulsion group................................ 41

14c. Particle size distribution of nobiletin coarse emulsion group 41

14d. Particle size distribution of nobiletin crystal nanoemulsion group 42

14e. Particle size distribution of nobiletin cryastal coarse emulsion group 42

15. Influence of nobiletin on particle charge.. 43

16. Influence of nobiletin on micelles size 44

17. Volume of NaOH.. 46

18. Free fatty acids released percentage... 46

19. Nobiletin bioaccessibility. 47

CHAPTER 1

LITERATURE REVIEW

1.1 General Introduction of Bioactive Components

There is currently great interest in developing functional foods within the food industries. A functional food refers food that in addition to providing a short-term nutritional function has positive effects on the long-term maintenance of health. There is also a perceived commercial advantage in replacing existing synthetic additives with new high performance natural additives (Wiseman 1999). However, most of the bioactive components of these functional foods are hydrophobic which are hard to dissolve in water and then less bioaccessible to human gastrointestinal tract. Researchers are trying to find some ways to modify their physical states, in order to fortify their overall benefits to human health. Many nutraceutical and functional food components would benefit from being encapsulated in appropriate edible delivery systems, including vitamins, bioactive peptides, antimicrobials, antioxidants, flavors, colors, minerals, and preservatives(Ubbink 2002, Shefer 2003, Chen 2006, Ubbink 2006). Some bioactive lipids are in crystalline state at room temperature in their pure form, e.g., carotenoids. The crystalline nature of these lipids may provide challenges in the manufacture of certain types of food products, or they may adversely affect the long-term stability or organoleptic properties of the final product (McClements, Decker et al. 2009).

1.2 Study of β-carotene

It has been reported that β-carotene is the most important pro-vitamin A

carotenoid found in foods and beverages (Rao and Rao 2007, Boon, McClements et al. 2010). The results of epidemiological studies suggest that β-carotene has biological activities that may reduce the risk of certain chronic diseases, such as cancers, cardiovascular disease, age-related macular degeneration, and cataracts (Omenn, Goodman et al. 1996, Karppi, Laukkanen et al. 2012, Miller and Snyder 2012). The bioavailability of β-carotene could be affected by dietary and physiological factors such as dietary triglycerides, protein-energy malnutrition, food matrix, D-carotene dietary history and B-carotene intake (Mittal 1983, Brubacher and Weiser 1985, Alam, Brown et al. 1990, Poor, Bierer et al. 1993). A number of physicochemical and physiological mechanisms have been proposed to account for low bioavailability and high variability of β-carotene: β-carotene is trapped within the organic matrix of many natural foods, which inhibits its release within the gastrointestinal tract (GIT); β-carotene is highly insoluble in water and only slightly soluble in oil at ambient temperatures, which limits its incorporation into many foods and may cause its precipitation within the GIT; β-carotene must be released from the food matrix and incorporated into mixed micelles before it can be absorbed; β-carotene is highly susceptible to oxidation due to its conjugated double bond structure, which means that it may degrade during storage (Castenmiller and West 1998, Elliott 1999, Huo, Ferruzzi et al. 2007, Boon, McClements et al. 2010). It has been shown that the bioavailability of β-carotene and other carotenoids can be improved by cooperating them with digestible lipids (van Het Hof, West et al. 2000, Borel 2003, Thakkar, Maziya-Dixon et al. 2007). When the lipid is digested by gastric and pancreatic

lipases it forms free fatty acids that are incorporated along with bile acids and phospholipids into mixed micelles that can solubilize and transport carotenoids to epithelium cells (Tyssandier, Lyan et al. 2001).

Crystalline materials are often difficult to incorporate into liquid products because of their tendency to aggregate and sediment, which causes problems with consistent dosing since some of the bioactive component may not be ingested. The presence of crystalline materials may also adversely affect the appearance, texture, and mouth feel of commercial products so that consumers are unwilling to consume them, leading to poor compliance with daily recommended intakes. However, β-carotene exists as crystalline in ambient temperature. In order to investigate its bioavailability in its natural state and mimic the way people take nutracuetical tablets, we co-administered β-carotene crystals with oil-in-water emulsion delivery system to increase its bioavailability in GIT.

1.3 Study of Nobiletin

Accumulating evidence has suggested that flavonoids found in fruits and vegetables may have various health-promoting effects. Among those flavonoids, polymethoxyflavones (PMFs) are a unique class of flavonoids and almost exclusively exist in the citrus genus, particularly in the peels of sweet oranges (Citrus sinensis L. Osbeck) and mandarin oranges(C. reticulate Blanco) (Li, Lo et al. 2006). PMFs have been reported to have a series of biological activity, such as anti-inflammatory, anti-carcinogenic, antioxidant, antiviral, and anti-atherogenic properties (Li, Pan et al. 2009). It is reported that citrus flavonoids including nobiletin exert an anti-invasive

activity in mouse(Sato, Koike et al. 2002). However, as same as many a bioactive agent, nobiletin is a non-polar compound with high melting point, low water-solubility, and poor oral bioavailability (Brouwers, Brewster et al. 2009, Bevernage, Brouwers et al. 2010, Kleberg, Jacobsen et al. 2010). Because of their tendency to precipitate and sediment, it is difficult to incorporate many of these bioactive compounds into commercial products, such as functional foods and beverages. The poor absorption of crystalline compounds in the gastrointestinal tract (GIT) compared to soluble ones reduces the amount of bioactive compound that reaches the blood circulation and target tissues (Brouwers, Brewster et al. 2009, Kawabata, Wada et al. 2011, Wang and Hou 2011). Many of these problems can be overcome by ensuring that the bioactive component remains in a soluble state within the product during storage, as well as during its passage throughout the GIT after ingestion.

The food industry may be able to overcome some of these potential problems by using strategies developed within the pharmaceutical industry to increase the aqueous solubility of crystalline hydrophobic drugs so as to improve their biopharmaceutical properties (Brewster and Loftsson 2007, Brouwers, Brewster et al. 2009, Warren, Benameur et al. 2010). A number of technological approaches have been investigated by pharmaceutical researchers to achieve this goal such as converting the drug into nanocrystals (Muller and Keck 2004), solubilizing the drug within colloidal delivery systems (Hauss 2007, Ziani, Fang et al. 2012), and complexing the drug with various polymers (Brewster and Loftsson 2007, Miller and Dahan 2012).

1.4 *In Vitro* Digestion Model

In these three studies, we used a gastrointestinal model that simulates the mouth, stomach and small intestine to test the stability of the delivery systems and bioaccessibilities of bioactive components. The bioaccessibility is defined as the fraction of an ingested component that is successfully transferred from the food matrix into the mixed micelles that are formed by bile salts, phospholipids, and lipid digestion products (*i.e.,* free fatty acids and monoacylglycerols) (Huo, Ferruzzi et al. 2007, McClements and Xiao 2012). The pH-stat method is designed to simulate lipid digestion within the small intestine (the place where the majority of lipid digestions normally occur), and is based on measurements of the amount of free fatty acids (FFA) released from lipids (usually triacylglcyerols) after lipase addition at pH values close to 7.0. The sample to be analyzed is placed in a reaction chamber containing appropriate concentrations of digestive components, such as lipase, bile salts, and minerals. The concentration of alkali that must be titrated into the reaction cell to neutralize any free fatty acids produced by lipid digestion, and thereby maintain the pH at the initial value, is recorded versus time. In principle, the pH-stat method is relatively simple and rapid to carry out and enables quantitative comparison of different lipid formulations under similar conditions(Li, Hu et al. 2011).

CHAPTER 2

INFLUENCE OF PHYSICAL STATES OF β-CAROTENE (CRYSTALIZED *VERSUS* SOLIBILIZED) ON BIOACCESSIBILITY

2.1 Introduction

Beta-carotene, the most important pro-vitamin A carotenoid, are widely used in beverages and food as natural colorants and nutraceuticals. Consuming β-carotene may reduce the risk of certain chronic diseases, age-related macular degeneration and cataracts(Gonnet, Lethuaut et al. 2010) However, the absorption of β-carotene from many natural and processed foods is often highly variable and not efficient, which are attributed to a number of causes: entrapment within food matrices; low water-solubility; high melting point; poor chemical stability (Castenmiller and West 1998, Yonekura and Nagao 2007, Boon, McClements et al. 2010) Researchers already showed that dissolved β–carotene in digestible oil and made into emulsion could significantly increase its bioaccessibility (Hörter and Dressman 2001, Qian, Decker et al. 2012, Rao, Decker et al. 2013) Meanwhile, a recent study showed consumed carotenoids enriched vegetables with meal triacylglycerol could end up with increased bioavailability (Goltz, Campbell et al. 2012). We proposed the hypothesis that mix raw β–carotene crystals with lipid-based emulsion systems would also facilitate the absorption of β–carotene human GIT.

The objective of this Chapter is to compare the bioaccessibilites between the solubilized β-carotene and the crystalized β-carotene with emulsion systems through *in vitro* digestion.

2.2 Materials and Methods

2.2.1 Materials

Corn oil was purchased from a local supermarket. Beta-carotene (Type I, C9750) was purchased from Sigma Chemical Company (St. Louis, MO) and Tween 20 was purchased from Fisher Scientific Company (Fair Lawn, NJ). Lipase (from porcine pancreas pancreatin) and bile extract (porcine) and Nile Red used for confocal microscopy work were also purchased from Sigma. All other chemicals used were of analytical grade. Double distilled water was used to prepare all solutions and emulsions.

2.2.2 Preparation Of Beta-Carotene Enriched Nanomulsion

An oil phase was prepared by solubilizing 0.5% (w/w) of crystalline β-carotene into corn oil, and then mild heated at 50 ºC for 5 min and stirred at room temperature for about 1 hour to make sure crystals fully dissolve. An aqueous phase was formed by mixing 0.5% (w/w) Tween 20 with aqueous buffer solution (10.0 mM phosphate buffer saline (PBS), pH 7.0). Coarse emulsion was formed by mixing oil phase with aqueous phase in 1:16 ratio, then use a high-speed blender (Tissue-Tearor, Biospec Products, Bartlesville, OK) blending for 2 minutes at room temperature to make the coarse emulsion, the coarse emulsion was then passed through a high pressure microfluidizer (Model 101, Microfluidics, Newton, MA) three times at 9,000psi. The corn oil emulsion was made by mixing corn oil with the aqueous phase described above in 1:16 ratio followed by coarse blending and fine homogenizing through microfluidizer. The freshly made nanoemulsion was stored at 20 ºC.

2.2.3 Particle Characterization

The mean particle diameters (z-average) and particle size distribution of the original emulsion and digesta after each digestion step were measured through a dynamic light scattering instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples of each step were diluted 20 times by using the buffer of the same pH as the samples. Then samples were placed to a capillary test tube and were loaded into the instrument. After loading the sample, it was equilibrated about 60s before data was collected over 10 sequential readings.

The electrical charge (ζ-potential) of the original emulsion and the digesta after each digestion step were monitored using a micro-electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). The same diluted samples used above were used for measuring electrical charge..

2.2.4 Microstructural Analysis

The microstructural changes of fat through digestion were observed by using confocal scanning fluorescence microscopy. The physical changes of β-carotene crystals were observed through a cross-polarized lens equipped in a Nikon optical microscope (C1 Digital Eclipse, Tokyo, Japan). In the set of experiments, samples from each step were dyed with Nile Red (0.1%), while the oil phases of emulsion and digesta were stained. All images were taken by using a 10x eyepiece and a 60x objective lens (oil immersion).

2.2.5 *In vitro* Digestion

Three comparative groups were prepared, which were β-carotene-enriched

nanoemulsion, β-carotene crystals in corn oil nanoemulsion and β-carotene crystals in phosphate buffer saline (PBS). The concentrations of β-carotene in the three groups were the same. Beta-carotene crystals were put into corn oil emulsion and PBS respectively just before digestion started. Each sample was passed through a three-step simulated GI model that consisted of a mouth, gastric, and small intestine phase. This model was established and described in previous study (Mun and McClements 2006).

Mouth phase: Simulated saliva fluid (SSF), containing 3% mucin and various salts, was prepared as described in previous studies(Sarkar, Goh et al. 2009, Mao and McClements 2012). Ten ml of sample was placed in a beaker and mixed with 10 ml of SSF to make the oil concentration to be 2%. The mixture was then adjusted to pH 6.8 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic oral conditions (Innova Incubator Shaker, 132 Model 4080, New Brunswick Scientific, New Jersey, USA).

Gastric phase: Simulated gastric fluid (SGF) was prepared according to a protocol described before (Sarkar, Goh et al. 2009). The “bolus” sample from the mouth phase was mixed with SGF at a 1:1 mass ratio to make the final oil concentration to be 1%. The mixture was then adjusted to pH 2.5 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic gastric conditions.

Small Intestine phase: Gastric digesta (30 ml) was incubated in a water bath (37 ºC) for 10 min and then used NaOH solution to adjust the pH to 7.0. The 1.0 ml of $CaCl_2$ solution, containing 110 mg of $CaCl_2$ (pH 7.0, PBS) was added. Then 4 ml bile extract, containing 187.5 mg bile extract (pH 7.0, PBS), was first added into the 30 ml digesta

with stirring and the resulting system was adjusted to pH 7.0. Finally, 2.5 ml of pancreatic suspension, containing 60 mg of lipase (pH 7.0, PBS) was added to the solution. At this point, titration was started automatically. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7.0) of the digestion solution. As the triacylglycerol of fat was digested into free fatty acids by lipolysis, the NaOH solution (0.25 M) was added in to neutralize the pH (7.0). With the volume of NaOH, the amount of free fatty acids released in the system can be calculated. The whole digestion lasted for 2 hours. The calculation of free fatty acids released was described in the previous study (Li and McClements 2010).

2.2.6 Bioaccessibility Determination

After *in vitro* digestion, digesta of each sample group was collected and centrifuged (14000 rpm, Thermo Scientific, CL10 centrifuge) at 25 ºC for 40 min. Centrifuged samples separated into three phases: sediment phase at the bottom, a relatively clear water phase contained micelles in the middle, and sometimes an undigested oil phase at top. The mixed micelles in the water phase were considered that entrapped the bioactive components. The β-carotene was extracted using organic solvent isopropanol-isooctane solution (1:1 volume ratio). Micelle phase or total digesta (0.2 ml) from the small intestine was added into 4.8 ml isopropanol-isooctane solution and centrifuged at 4000 rpm at 25ºC for 10 min. Then the middle micelle phase was passed through a syringe filter with the pore size of 0.45 um. Both the filtered and unfiltered β-carotene concentration was analyzed using a UV-visible spectrophotometer (Ultrospec 3000 pro, GE Health Sciences, USA) at the spectrum of

450 nm. The concentration of β-carotene was converted from the absorbance by the standard curve. The bioaccessibility was calculated using the following equation:

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{Digesta}} \right)$$

In this equation, $C_{Micelle}$ and $C_{Digesta}$ are the concentrations of β-carotene in the filtered and unfiltered micelle fraction and in the overall sample (total digesta) after the pH-stat experiment, respectively.

2.2.7 Statistical analysis

All measurements were performed at least twice on freshly prepared samples and were reported as calculated means and standard deviations.

2.3 Results and Discussion

2.3.1 Nanoemulsion stability in digestion model

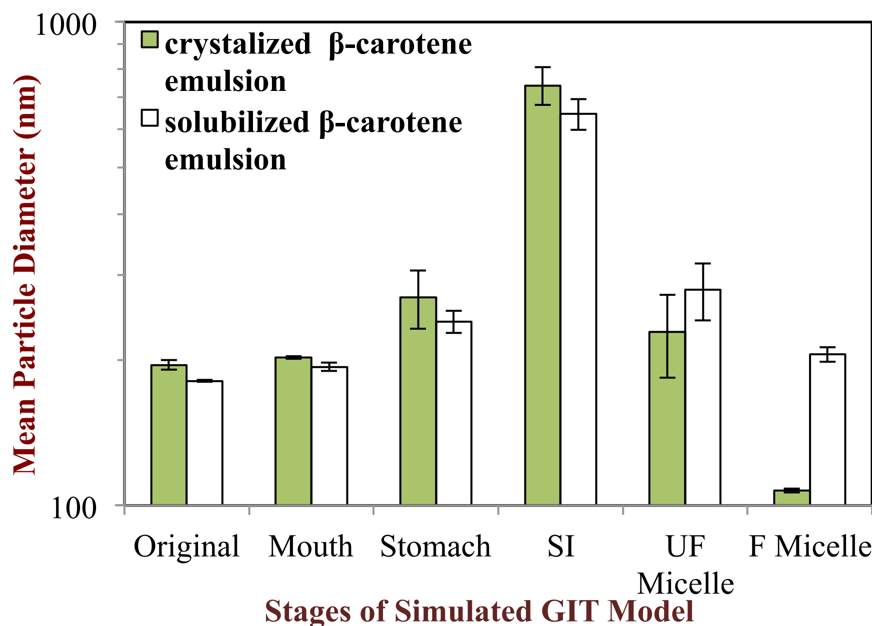

Figure 1a. Influence of β-carotene on mean particle size. Influence of physical state of β-carotene and *in vitro* digestion on the mean diameter of the particles in oil-in-water nanoemulsions subjected to a simulated gastrointestinal model. Small Intestine (SI), Unfiltered Micelle (UF Micelle), Filtered Micelle (F Micelle).

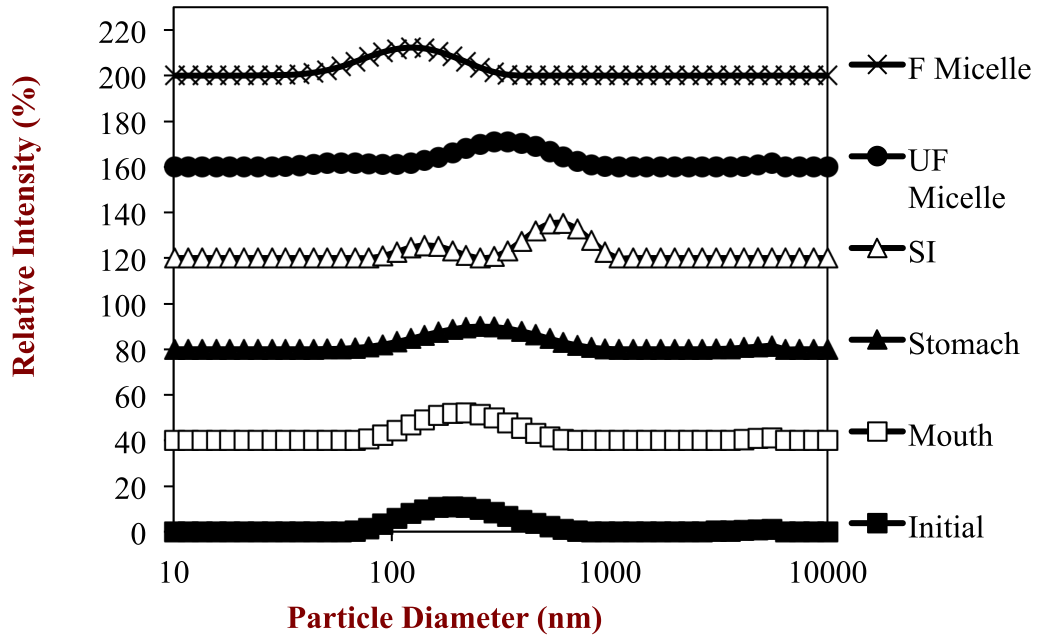

Figure 1b. Particle size distribution of β-carotene solubilized group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. The physical state of β-carotene is crystal.

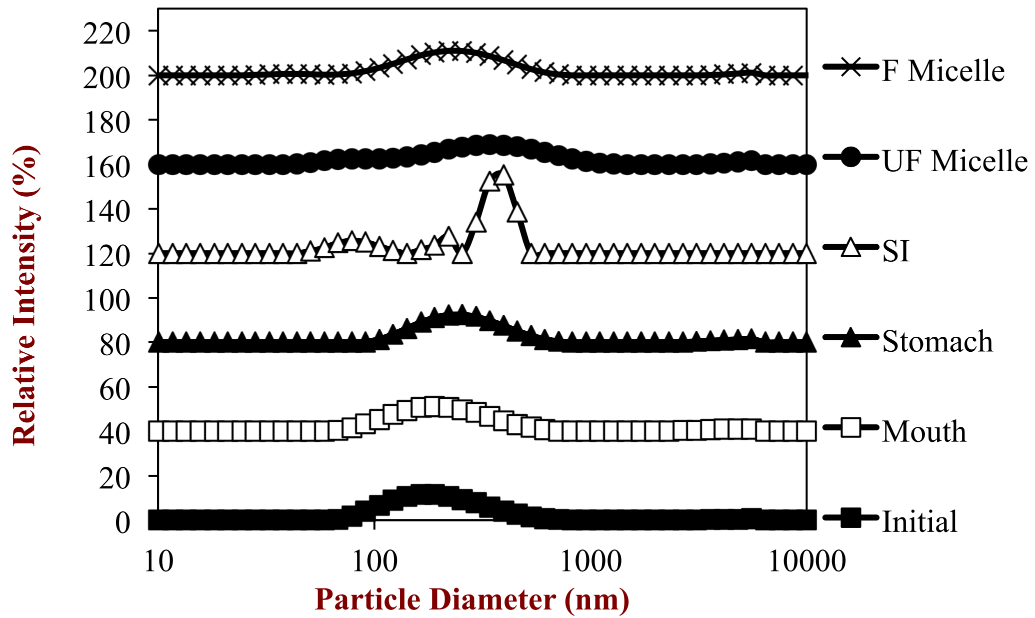

Figure 1c. Particle size distribution of β-carotene crystals group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. The physical state of β-carotene is crystal

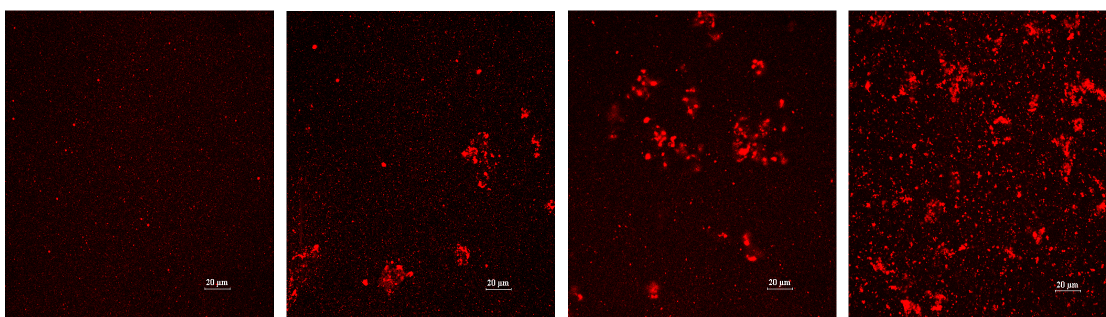

Figure 2. The changes of morphologies of lipid. The changes of morphologies of lipid in emulsion system during *in vitro* digestion. Images were taken by confocal microscopy. The scale bars are 20um long. Pictures from left to right: original phase, mouth phase, stomach phase, small intestine phase.

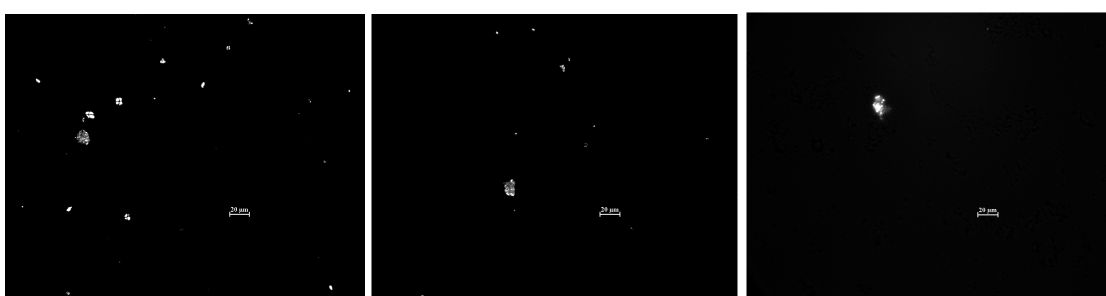

Figure 3. The change of microstructure of β-carotene crystals. The change of microstructure of β-carotene crystals measured by crossed polarizer microscopy at various *in vitro* digestion stages. The scale bars are 20um long. Pictures from left to right are: after mouth digestion/after stomach digestion/after small intestine digestion.

All the initial nanoemulsions in solubilized β-carotene emulsion group (group 1) and crystalized emulsion group (group 2) had relatively small mean particle diameters (**Figure 1a**), monomodal particle size distributions (**Figure 1b, 1c**), which suggested that under the initial solution condition (pH 7), they were stable enough to prevent droplet aggregation. In the microscopy images, only small fine distributed particles can be observed (**Figure 2**). After the mouth and stomach digestion, the particles still remained in small size (d<270nm), which is also demonstrated in microscopy images. The non-ionic surfactant (Tween 20) is a highly surface active molecule with a relatively large hydrophilic head group. In mouth and stomach conditions, there is no highly surface-active substance, and therefore the non-ionic surfactant molecules

could still remain on the surface of the droplet. From the confocal microscopy images, we can also tell most of the droplets still stayed in small sizes (**Figure 2**). The good physical stability of the nanoemulsions under neutral (oral) and acidic (gastric) environments observed in this study may therefore be attributed to the relatively strong steric repulsion generated by the neutral hydrophilic head groups. As previous studies have reported that the strong steric repulsion generated by the neutral hydrophilic head groups is the contributor of the physical stability under neutral (oral) and acidic (gastric) environments (Golding, Wooster et al. 2011, Nik, Langmaid et al. 2012, Qian, Decker et al. 2012).

After simulated small intestine digestion, the mean particle size of nanoemulsions showed a large increase (**Figure 1a**), which was caused by droplet aggregation. It can be proved by microscopy image (**Figure 2**) that until small intestine stage, small droplets started to coalescent and aggregate. This suggested that when the nanoemulsions exposed to small intestinal condition, their physical stability decreased significantly. According to previous studies, several physicochemical mechanisms may have been responsible for this change (Qian, Decker et al. 2012). The replacement of the surface active species (i.e., phospholipids, bile salts, lipase, free fatty acids, monoacyglycerols) with original non-ionic surfactant (Tween 20) may lead to decrease of steric repulsion between droplets. On the other hand, after lipase bound to the surface of the oil droplet, it digested triacylglycerols to free fatty acids and monoacylglycerols and then changed the internal composition, structure and properties of the lipid droplets. After the samples were centrifuged and the micelles

phase was separated, the droplet sizes decreased dramatically (228nm, 279nm of two groups respectively), which means the large aggregations were removed. After micelle phase was filtered (pore size: 0.45um), the size went down further (107nm, 205nm of two groups respectively). The images of the β-carotene crystals were captured by crossed polarizer lens (**Figure 3**). The amount of crystals in the images decreased obviously through *in vitro* digestion, we would assume that the β-carotene crystals were solubilized. However, the decrease is because of the dilution of the system instead of solubilization, which will be discussed in the bioaccessibility session.

We also measured the electrical charge of the sample of each step. The initial nanoemulsions of both groups had little negative charges (**Figure 2**). Tween20 is known as a non-ionic surfactant, however the anionic species in the lipid phase (i.e., phospholipids, free fatty acids) or the anionic impurities in surfactant, even the hydroxide ions in water could contribute to the negative charge of the system (Qian, Decker et al. 2012).

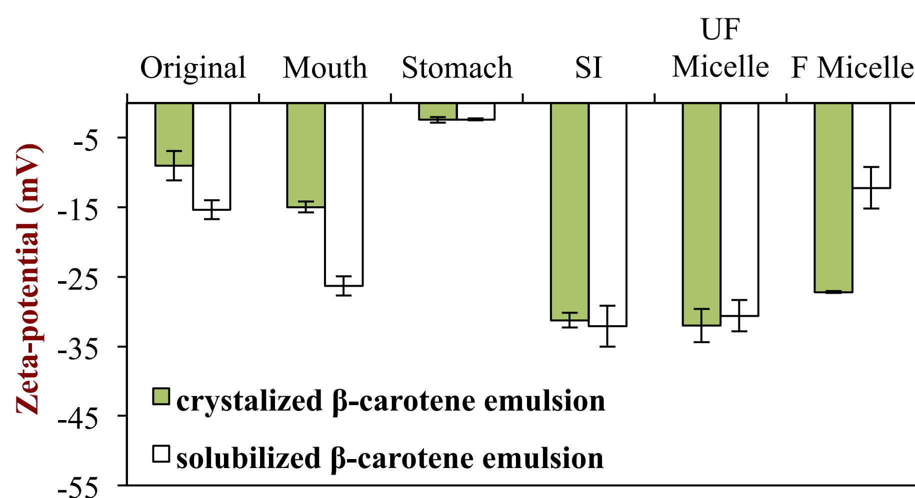

Figure 4. Charge of the particles. Influence of physical state of β-carotene and *in vitro* digestion on the charge (ζ-potential) of the particles in oil-in-water emulsions subjected to a simulated gastrointestinal model.

After simulated small intestine digestion, the digesta were collected and centrifuged. The middle “micelle” phase was collected and its particle size and electrical charges were measured (**Figure 1a, 1b, 1c, 4**). The data of both the filtered (0.45um pore size) and unfiltered were showed. The sizes in the unfiltered groups were larger than filtered groups indicated there was large particles or vesicles that scattered light weakly in micelle phase. After filtration the size decreaed as expected, suggesting that some large vesicles were filtered out (**Figure1**). The size in the solubilized group is larger than the crystalized group mat be due to the β-carotene molecule presenting more in the solubilized group and contributing to the formation of micelles; in the crystalized group, most of the unsolubilized β-carotene crystals sedimentated to the bottom after centrifugation and less amount participating in forming micelles, therefore the micelles sizes are smaller. The electrical charges of the unfiltered and filtered micelles are also negative (**Figure 4**) because of the anionic species, such as bile salts, phospholipids, free fatty acids. In filtered micelle phase of the solubilized β-carotene group, the number of electrical charge is less negative than the crystalized β-carotene emulsion group, which is due to the average size of micelles in the former group was larger than that of the latter group. The larger size lead to less surface area compared with the smaller micelles, resulting in less negative charge exposed.

2.3.2 Lipid Digestion via *In Vitro* Digestion

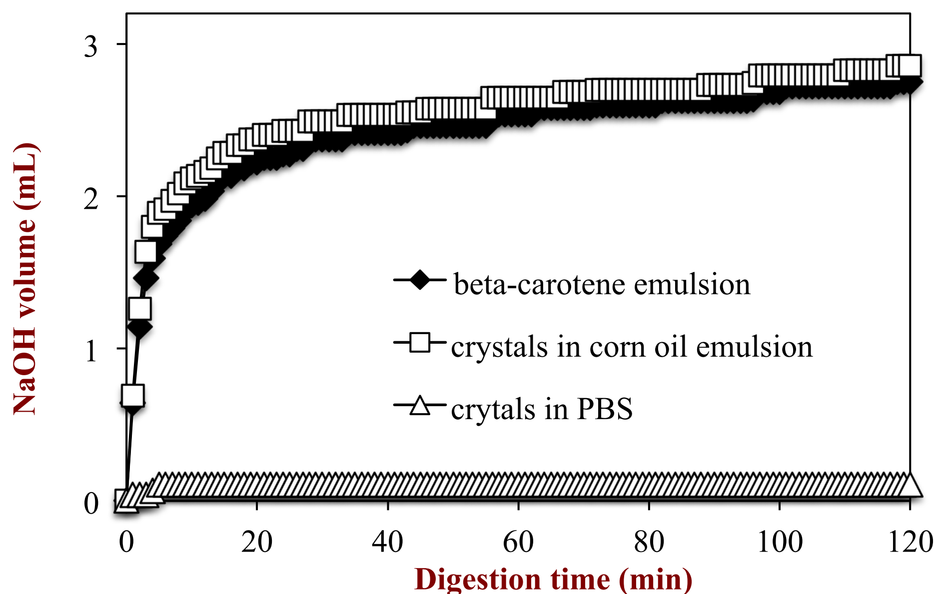

Figure 5. Volume of NaOH. Influence of physical state of β-carotene on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the volume of 0.25 M NaOH solution that had to be added to the samples to maintain the pH at a constant value of 7.

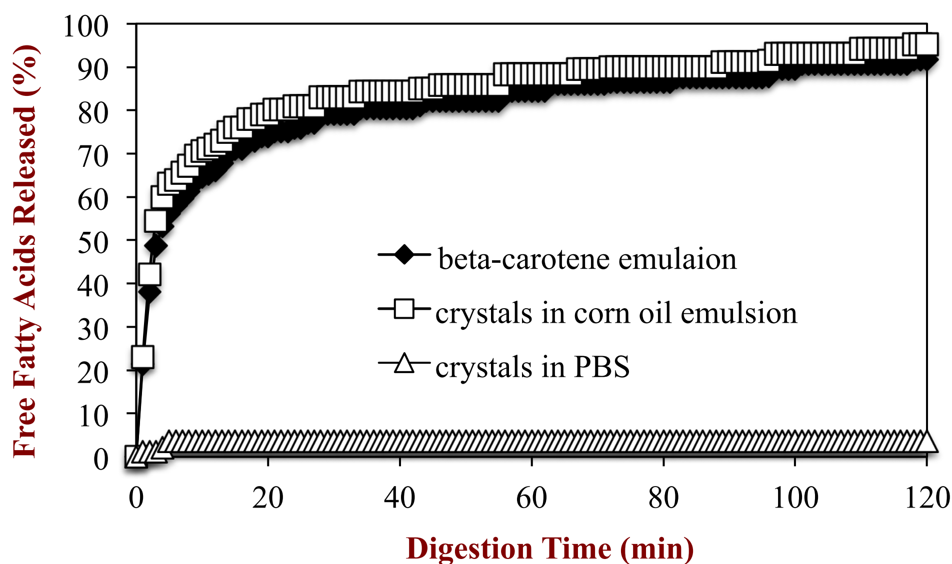

Figure 6. Free fatty acids released percentage. Influence of physical state of β-carotene on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the calculated percentage of free fatty acids (FFA) released from the nanoemulsions after digestion of the triacylglycerol phase by lipase.

In this section, the rate and extent of lipid digestion in the small intestinal digestion was monitored via pH-stat. This method measured the amount of free fatty acids (FFAs) released from triglycerides under the digestion of lipase(McClements and Li 2010). As expected, there was little change in the pH of the sample only contained β-carotene crystals and PBS, because there is no fat in the system and no FFAs released during digestion (**Figure 5,6**). The slight consumption of NaOH may due to the impurities in non- ionic surfactant. A recent study showed that several non-ionic surfactants have ester bonds that are susceptible to lipase digestion leading to FFAs release (Li and McClements 2011). In the other two systems, there was a dramatic increase in the first 20 minutes of digestion and then it started to level off (**Figure 5**). The FFAs release profiles followed a similar trend as the NaOH consumption (**Figure 6**). The trend suggested that lipase attached to the surface of the lipid droplets rapidly and promoted triacylglycerol digestion.

2.3.3 In Vitro Digestion Bioaccessibility

In this section, we examined the β-carotene bioaccessibility under solubilized status and crystalized status after it passed through the simulated gastrointestinal tract model. Theoretically, the portion of β-carotene in “micelle” phase over the portion of original β-carotene can represent the β-carotene bioaccessibility. The bioaccessibilty was measure before and after filtration. It was assumed that the filtered sample could be more practically represent the actual bioaccessibility of β-carotene because it could remove any particles larger than 450nm that could not be absorbed by small intestine epithelium cells. Practically, using filter may also lead to absorb β-carotene on its surface, consequently decreased the concentration of β-carotene in “micelle” phase.

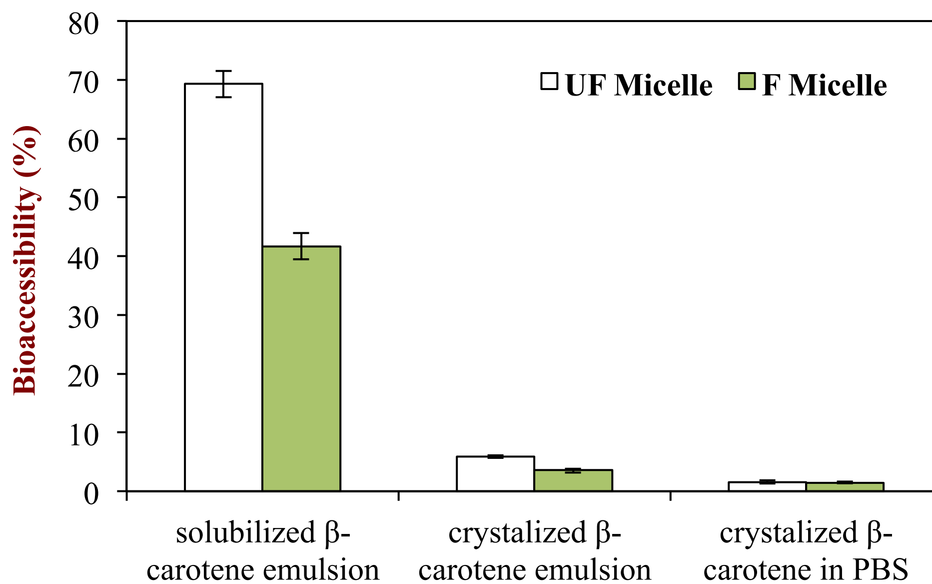

Figure 7. Beta-carotene bioaccessibility. Effect of physical states of β-carotene on bioaccessibility of β-carotene measured after centrifugation of emulsions passed through an *in vitro* digestion model.

The lowest bioaccessibility (1.39%) was measured in filtered β-carotene crystals with PBS (**Figure 7**). There are also comparatively low bioaccessibilities of β-carotene crystals in corn oil emulsion (5.74%, 3.64%, unfiltered, filtered respectively). The solubilized β-carotene emulsion enjoyed the highest bioaccessibility. This is because when added β-carotene crystals in either corn oil emulsion or PBS, most of the β-carotene still stayed unsolubilized. The β-carotene crystals are too large for small micelles to solubilize and only solubilized β-carotene molecules are small enough to be enraptured in micelles. Although β-carotene better in corn oil emulsion than only in PBS, however compared with the solubilized β-carotene emulsion, the solubilized part was still too few to have a high bioaccessbility. These results suggested people to take solubilized β-carotene supplements to get the best absorption in gastrointestinal tract.

2.4 Conclusions

This study showed the bioaccessibility of different physical states of β-carotene are significantly different. In the three compare groups: (1) solubilized β-carotene emulsion, (2) β-carotene crystals in corn oil emulsion, (3) β-carotene crystals in PBS, the rate and extent of free fatty acid production in the small intestine decreased in the order (2)>(1)>(3); whereas the β-carotene bioaccessibility decreased in the order (1)>>(2)>(3). In system (3), even without any fat, there was still noticeable consumption of NaOH, which was attributed to hydrolysis of the ester bonds in the non-ionic surfactant (Tween 20). The low bioaccessibility (1.3%) of system (3) was attributed to the fact that there were few free fatty acids produced to form mixed micelles to solubilize β-carotene. The bioaccessibility of system (2) was also relatively low (3.6%) compared to system (1) (42%), which suggests that crystalline β-carotene is much less bioaccessible than soluble β-carotene .

The bioaccessibility of solubilized β-carotene entrapped in nanoemulsions was the highest is because the free fatty acids produced through small intestine digestion formed mixed micelles capable of solubilizing and transporting lipophilic compounds. Crystalline β-carotene was not incorporated into mixed micelles easily, leading to a reduced bioaccessibility. In the system with crystals in water, there was no free fatty acids produced, so the bioaccessibility of β-carotene was low.

This study provides useful suggestions of how to take lipophilic nutraceutical supplements: the best way is taking the bioactive components encapsulated fat-contained emulsion products (*i.e.*, functional food or functional drink) ; the second choice is taking nutraceutical tablets with fat-contained emulsion (*i.e.*, milk or salad

dressing) or after eating meals (oil in meal forms natural emulsion through digestion). The least effective way is taking the tablets with only water and an empty stomach. The results are also important for designing effective delivery systems to encapsulate and stabilize b-carotene for application within food, beverage, and pharmaceutical products.

CHAPTER 3

THE INFLUENCE OF OIL CONCENTRATION ON BETA-CAROTENE BIOACCESSIBILITY

3.1 Introduction

It is suggested by epidemiological studies that there is a positive link between higher dietary intake and tissue concentrations of carotenoids and lower risk of chronic diseases including cardiovascular diseases, cancer and other chronic diseases (Agarwal and Rao 2000, Johnson 2002, Elliott 2005). However, due to its high hydrophobicity, β-carotene is reported to cause low solubility in aqueous systems and lead to poor uptake in the body, which limits its use in food formulations. The bioavailability of β-carotene in human body is also affected by many factors, i.e. the state of the food matrix, food processing and the conditions in the gastrointestinal (GI) tract (Castenmiller and West 1998). We assumed that the higher amount of oil we put in emulsion system, the higher amount of β-carotene we can encapsulate in emulsion system, consequently, higher bioaccessibility we can get.

The objective of this study is to compare the influence of high oil concentration with low oil concentration on β-carotene nanoemulsion bioaccessibilty.

3.2 Materials and Methods

3.2.1 Materials

Corn oil was used as an example of a long chain triglyceride (LCT) and was purchased from a local supermarket. Beta-carotene (Type I, C9750) was purchased from Sigma Chemical Company (St. Louis, MO) and Tween 20 was purchased from Fisher Scientific Company (Fair Lawn, NJ). Sucrose was purchased from Sigma

Chemical Company (St. Louis, MO). Their purities were > 98% based on HPLC analysis. Lipase (from porcine pancreas pancreatin) and bile extract (porcine) and Nile Red used for confocal microscopy work were also purchased from Sigma. All other chemicals used were of analytical grade. Double distilled water was used to prepare all solutions and emulsions.

3.2.2 Preparation of Beta-carotene Enriched Emulsion

For the high oil concentration group, the oil phase was prepared by dispersing 0.07% (w/w) of crystalline β-carotene into corn oil 20% (w/w) followed by mild heating at 50 ºC for 5 min and stirring at ambient temperature for about 1 hour to make sure they were fully dissolved. The aqueous phase was formed by mixing 5% (w/w) Tween 20 with aqueous buffer solution (10.0 mM phosphate buffer saline, pH 7.0) contained 60% (w/w) sucrose, which was heated for 10 min with stirring to make sure completed dissolution. Coarse emulsion was formed by mixing oil phase with aqueous phase in 1:16 ratio, then use a high-speed blender (Tissue-Tearor, Biospec Products, Bartlesville, OK) blending for 2 minutes at ambient temperature to make the well homogenized emulsion, the coarse emulsion was then passed through a high pressure microfluidizer (Model 101, Microfluidics, Newton, MA) three times at 9,000psi.The freshly made nanoemulsion was t stored at 20 ºC. The low fat group was made by diluting the high fat group, which was made by above procedures, five folds to make the fat concentration to be 4% (w/w) in the system.

3.2.3 Particle Characterization

The mean particle diameters (d_{43}) and particle size distribution (PSD) of the

nanoemulsions were measured after mouth, stomach and small intestine digestion using a static light scattering instrument (Mastersizer 2000 Malvern Instruments). A few drops of sample were dispersed in phosphate buffer saline (PBS) in the sample chamber with agitation. The size of the particles in the filtered and unfiltered micelle phase was measured using dynamic light scattering (ZetaSizer Nano, Malvern Instruments). The original size of the nanoemulsion was measured through both static and dynamic light scattering.

The electrical charge (ζ-potential) of the emulsion after each step digestion was monitored using a micro-electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). The same diluted samples used above were used for measuring electrical charge.

3.2.4 *In vitro* Digestion

Two compare groups were created (20% oil, 4% oil). All the following steps were applied as same to the two groups. Each sample was passed through a three-step simulated GI model that consisted of a mouth, gastric, and small intestine phase. This model was established and described in previuos study (Mun and McClements 2006).

Mouth phase: Simulated saliva fluid (SSF), containing 3% mucin and various salts, was prepared as described in previous studies(Sarkar, Goh et al. 2009, Mao and McClements 2012). Ten ml of sample was placed in a beaker and mixed with 10 ml of SSF to make the oil concentration to be 2%. The mixture was then adjusted to pH 6.8 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic oral conditions (Innova Incubator Shaker, 132 Model 4080, New Brunswick Scientific, New Jersey,

USA).

Gastric phase: Simulated gastric fluid (SGF) was prepared according to a protocol described before (Sarkar, Goh et al. 2009). The "bolus" sample from the mouth phase was mixed with SGF at a 1:1 mass ratio to make the final oil concentration to be 1%. The mixture was then adjusted to pH 2.5 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic gastric conditions.

Small Intestine phase: Gastric digesta (30 ml) was incubated in a water bath (37 ºC) for 10 min and then used NaOH solution to adjust the pH to 7.0. The 1.0 ml of $CaCl_2$ solution, containing 110 mg of $CaCl_2$ (pH 7.0, PBS) was added. Then 4 ml bile extract, containing 187.5 mg bile extract (pH 7.0, PBS), was first added into the 30 ml digesta with stirring and the resulting system was adjusted to pH 7.0. Finally, 2.5 ml of pancreatic suspension, containing 60 mg of lipase (pH 7.0, PBS) was added to the solution. At this point, titration was started automatically. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7.0) of the digestion solution. As the triacylglycerol of fat was digested into free fatty acids by lipolysis, the NaOH solution (0.25 M) was added in to neutralize the pH (7.0). With the volume of NaOH, the amount of free fatty acids released in the system can be calculated. The whole digestion lasted for 2 hours. The calculation of free fatty acids released was described in the previous study (Li and McClements 2010).

3.2.5 Bioaccessibility Determination

After *in vitro* digestion, digesta of each sample group was collected and centrifuged (14000 rpm, Thermo Scientific, CL10 centrifuge) at 25 ºC for 40 min. Centrifuged

samples separated into three phases: sediment phase at the bottom, a relatively clear water phase contained micelles in the middle, and sometimes an undigested oil phase at top. The mixed micelles in the water phase were considered that entrapped the bioactive components. The β-carotene was extracted using organic solvent isopropanol-isooctane solution (1:1 volume ratio). Micelle phase or total digesta (0.2 ml) from the small intestine was added into 4.8 ml isopropanol-isooctane solution and centrifuged at 4000 rpm at 25ºC for 10 min. Then the middle micelle phase was passed through a syringe filter with the pore size of 0.45 um. Both the filtered and unfiltered β-carotene concentration was analyzed using a UV-visible spectrophotometer (Ultrospec 3000 pro, GE Health Sciences, USA) at the spectrum of 450 nm. The concentration of β-carotene was converted from the absorbance by the standard curve. The bioaccessibility was calculated using the following equation:

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{Digesta}} \right)$$

In this equation, $C_{Micelle}$ and $C_{Digesta}$ are the concentrations of β-carotene in the filtered and unfiltered micelle fraction and in the overall sample (total digesta) after the pH-stat experiment, respectively.

3.2.6 Statistical analysis

All measurements were performed at least twice on freshly prepared samples and were reported as calculated means and standard deviations.

3.3 Results and discussion

3.3.1 Nanoemulsion stability in digestion model

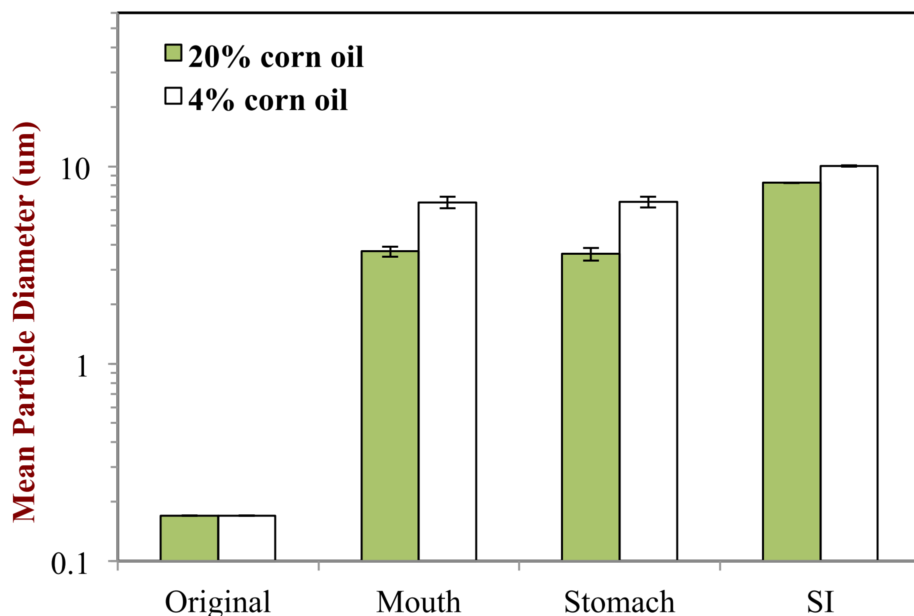

Figure 8a. Influence of oil concentration on mean particle size. Influence of oil concentration and *in vitro* digestion on the mean diameter of the particles in oil-in-water nanoemulsions subjected to a simulated gastrointestinal model.

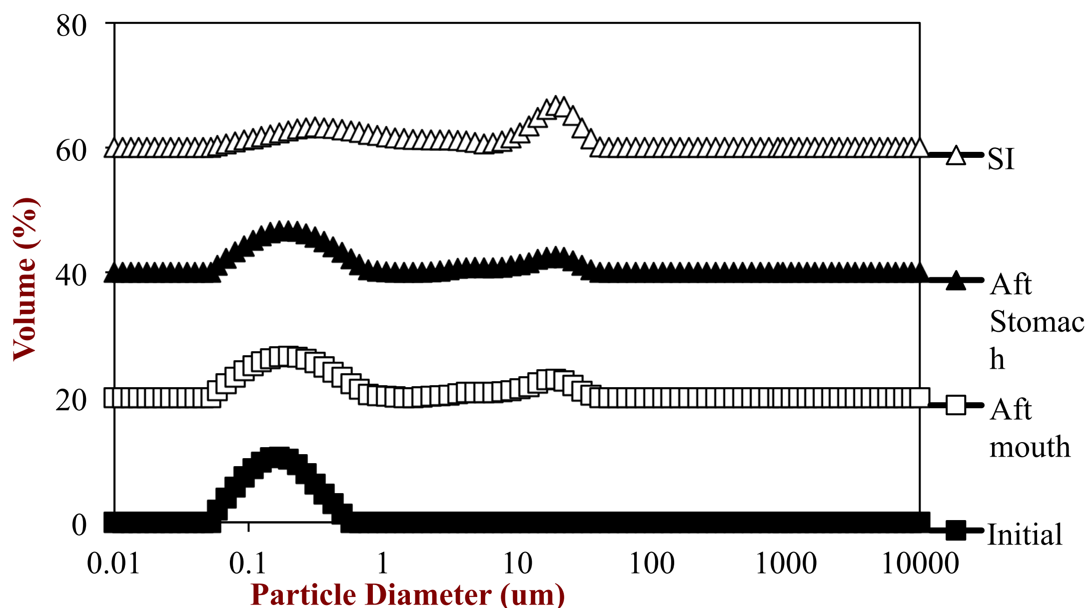

Figure 8b. Particle size distribution of 20% fat group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. The oil concentration is 20%.There are four stages: initial, after (Aft) mouth, after (Aft) stomach, small intestine (SI).

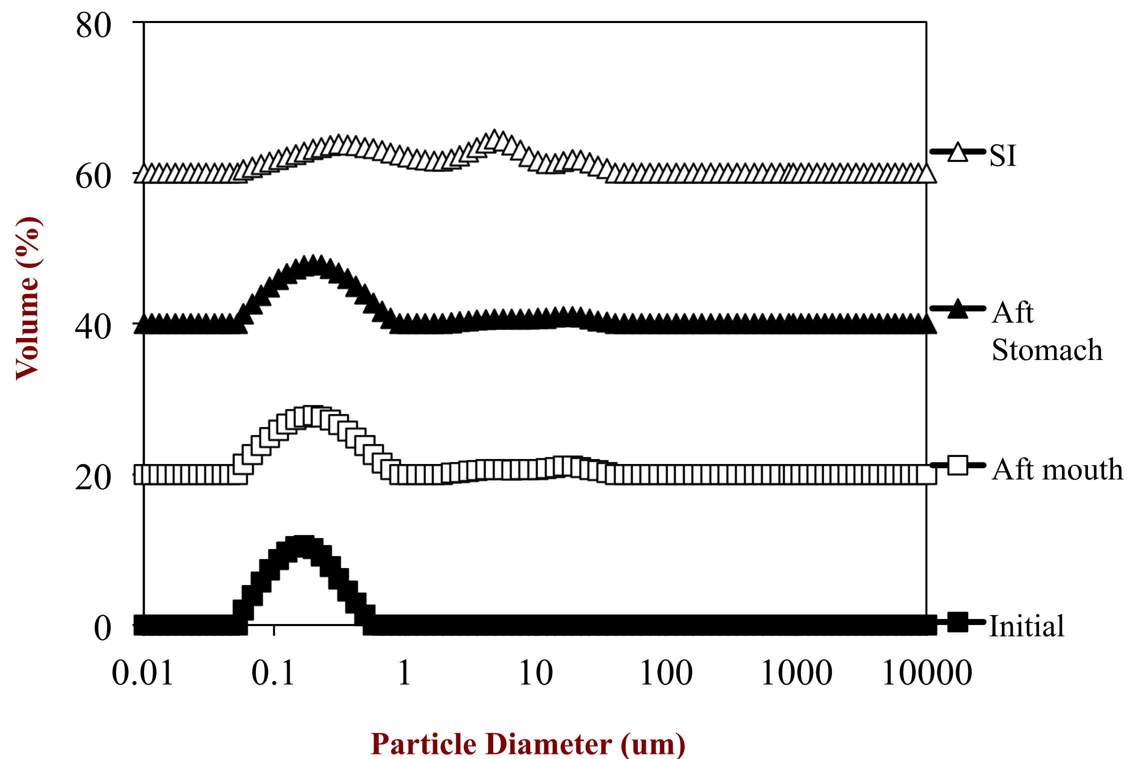

Figure 8c. Particle size distribution of 4% fat group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. The oil concentration is 4%.

All the initial nanoemulsions had relatively small mean particle diameters (d_{43}=0.17, **Figure 8a**), monomodal particle size distributions (**Figure 8b, 8c**), which suggested that they were stable to droplet aggregation under the initial solution conditions (pH 7). After the mouth and stomach digestion, the main particle diameter increased appreciably (d_{43}>3). After incubated in simulated small intestine fluid, the mean particle size of nanoemulsions showed a large increase, which can be explained by droplet aggregation (**Figure 8a**). This suggested that when the nanoemulsions exposed to small intestinal condition, their physical stability decreased a lot.

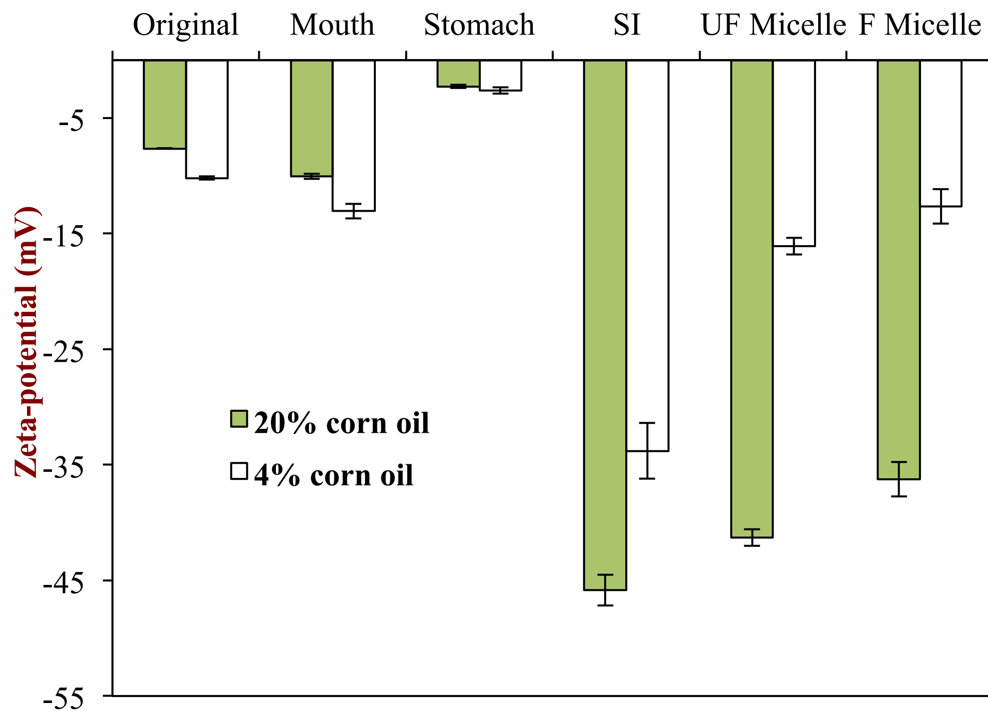

Figure 9. Influence of oil concentration on particle charge. Influence of oil concentration and *in vitro* digestion on the charge (ζ-potential) of the particles in oil-in-water emulsions subjected to a simulated gastrointestinal model.

The initial nanoemulsions both had negative charges (-7.64mV/ -10.02mV, **Figure 9**). Although Tween20 is a non-ionic surfactant, however, there may be anionic impurities (*e.g.* free fatty acids and phospholipids) entrapping within the surfactant or lipid phase that could adsorb to the oil-water interface and therefore giving a negative charge to the droplets. After the small intestinal stage, the particles charges appeared to be the most negative, which is due to adsorption of anionic species (*e.g.*, bile salts, phospholipids, lipases) to the droplets surfaces.

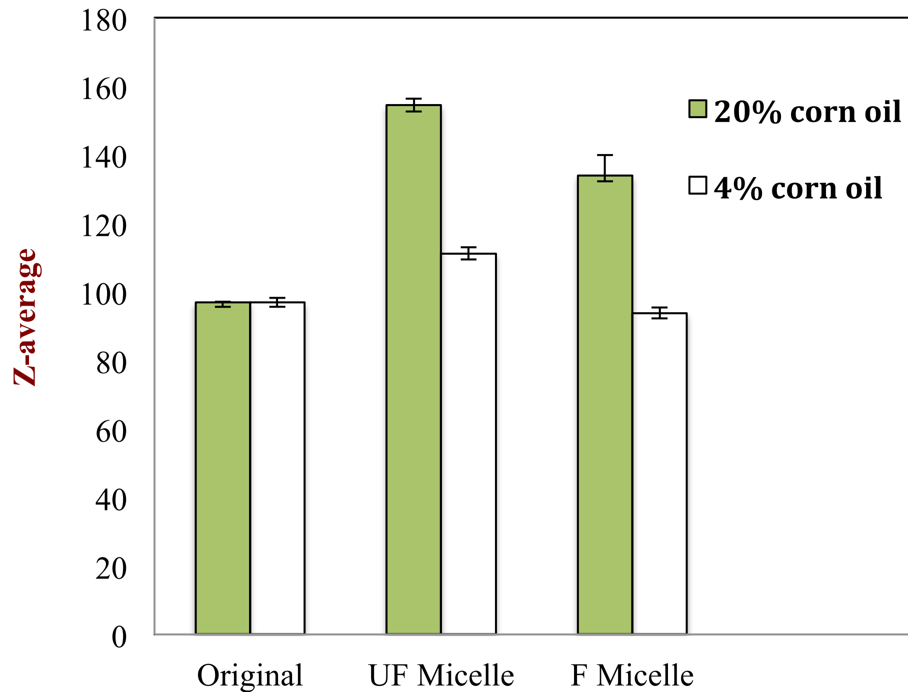

Figure 10. Influence of oil concentration on micelles size. Influence of oil concentration on the original particle size and micelle phase (represent by Z-average) collected after digestion and centrifugation. Measurements were made before and after filtration.

After simulated small intestine digestion, the digesta were collected and centrifuged. Three layers formed afterwards: bottom sediment phase, middle micelle phase and sometimes the undigested oil floated on the top. The "micelle" phase was collected and filtered through a 0.45 um pore size filter. The particle size and electrical charges of filtered and unfiltered micelles were measured (**Figure 9, 10**). And the results were showed as unfiltered and filtered. The sizes in the unfiltered groups are larger indicated that the middle phase are mainly mixed micelles and thin-walled vesicles. After filtration the size decreased as expected, suggesting that some large vesicles were filtered out. The electrical charges of the unfiltered and filtered micelles are also negative (**Figure 9**) because of the anionic species, such as bile salts, phospholipids, free fatty acids.

3.3.2 Lipid Digestion via *In Vitro* Digestion

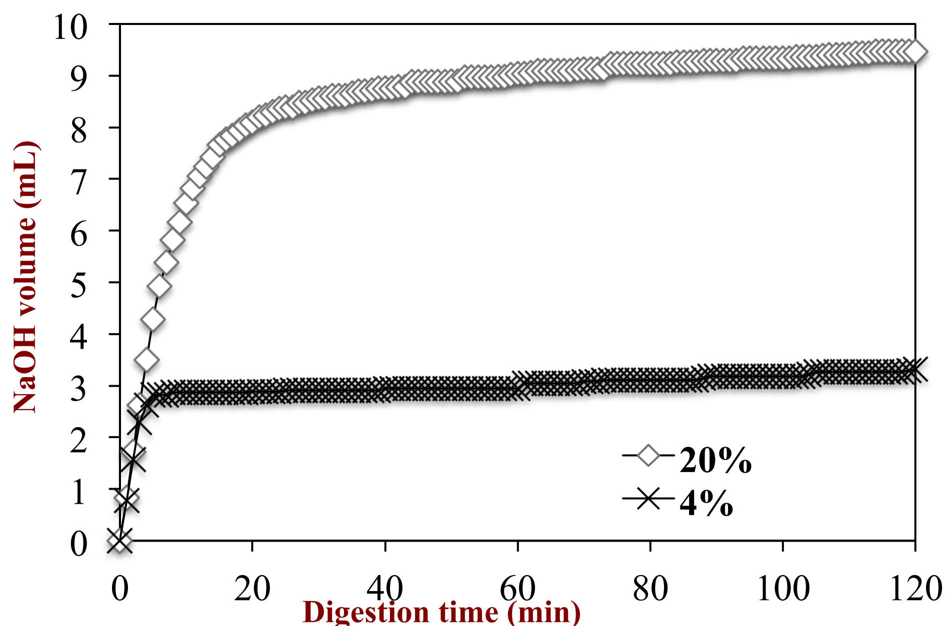

Figure 11. Volume of NaOH. Influence of oil concentration on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the volume of 0.25 M NaOH solution that had to be added to the samples to maintain the pa constant value of 7.

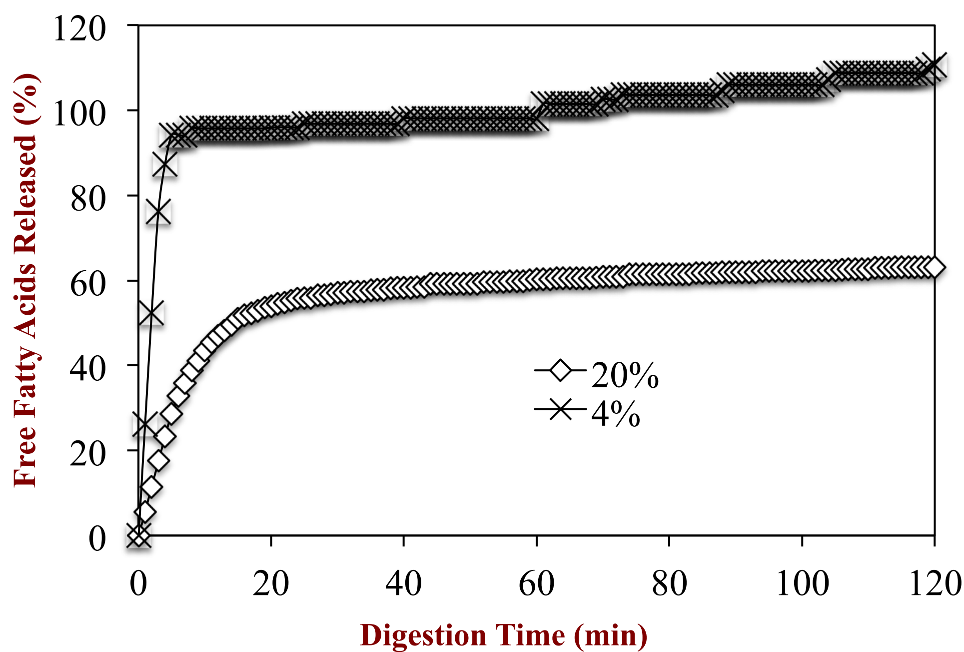

Figure 12. Free fatty acids released percentage. Influence of oil concentration on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the calculated percentage of free fatty acids (FFA) released from the nanoemulsions after digestion of the triacylglycerol phase by lipase.

In this section, the rate and extent of lipid digestion in the small intestinal digestion was measured via pH-stat. This method measured the amount of free fatty acids (FFAs) released from triglycerides under the digestion of lipase(McClements and Li 2010). As expected, the high oil content group (20%) consumed more NaOH than the low oil content group (4%) (**Figure 11**). There was a dramatic increase in the first 20 minutes of digestion and then it gradually leveled off (**Figure 11**). The FFAs release profiles were a reverse of the NaOH consumption profiles. The high oil content group was with a high NaOH consumption but a low FFAs percentage; the low oil content was with a low NaOH consumption but a high FFAs percentage. This is due to the bile salts concentration (46.88 mg/ml) used in the simulated digestion system, which limited the concentration of fat (~4%) in the system. The FFAs release profiles followed a similar trend as the NaOH consumption (**Figure 12**). The trend suggested that lipase attached to the surface of the lipid droplets rapidly and promoted triglycerol digestion.

3.3.3 In Vitro Digestion Bioaccessibility

In this section, we examined the β-carotene bioaccessibility under high fat condition and low fat condition, after it passed through the simulated gastrointestinal tract model. Theoretical, the portion of β-carotene in "micelle" phase over the portion of original β-carotene can represent the β-carotene bioaccessibility. The bioaccessibilty was measure before and after filtration. It was assumed that the filtered sample could be more practically represent the actual bioaccessibility of β-carotene because it could remove any particles larger than 450nm that could not be absorbed by

small intestine epithelium cells. It simulated the functions of mucus layer on the small intestine epithelial cells.

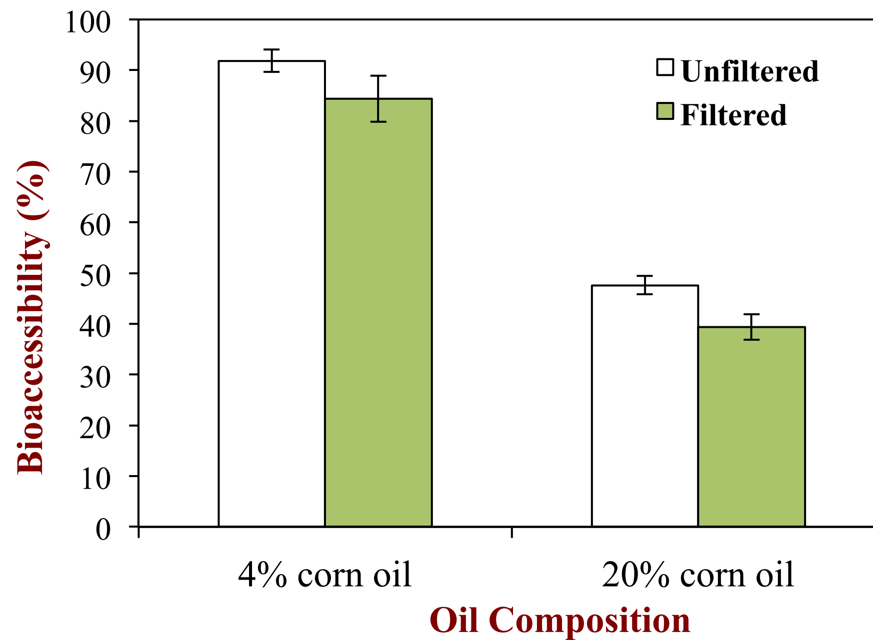

Figure 13. Influence of oil concentration on β-carotene bioaccessibility. Effect of oil concentration on bioaccessibility of b-carotene measured after centrifugation of emulsions passed through an *in vitro* digestion model.

The bioaccessibility of the filtered micelles of the 20% oil content sample is 84.4%, the same group of the 4% oil content sample is 39.4% (**Figure 13**). This is because some of the β-carotene molecules were still entrapped in the undigested oil. The bioaccessbility is proportional to the FFAs released. In the FFAs profile (**Figure 12**), the FFAs percentage of the 20% oil group is only half of 4% oil group; correspondingly, the bioaccessibility of the high fat group is also only half of low fat group (**Figure 13**). These results suggest us the high fat content oil with bioactive compounds encapsulated, even can solubilize more bioactive components, after digestion the bioavailable parts are smaller than the low fat content part.

3.4 Conclusions

This study showed the bioaccessibility of β-carotene in the delivery systems with different oil concentration. In the two compare groups: (1) 20% oil concentration group (2) 4% oil concentration group, the rate and extent of free fatty acid production in the small intestine decreased in the order (1)>(2); whereas the β-carotene bioaccessibility decreased in the order (2)>(1). In the high fat group even the total concentration of β-carotene is higher than the low fat concentration due to the higher solubilization of β-carotene in the former group. However, the bioaccessibility of the high fat group is only half of the low fat group due to the insufficient digestion of fat in the former group.

The bioaccessibility of high fat concentration group (40%) is almost half of it is in high fat group. This is because without enough bile salts and lipase, fat is not able to be digested into free fatty acids and monoglycerids. Free fatty acids and monoglycerids are the main components of micelles, not enough micelles means not enough carriers can transfer β-carotene molecules. This end up with a lower bioaccessibility of β-carotene in high fat group.

CHAPTER 4

INFLUENCE OF PHYSICAL STATES AND SIZE OF EMULSION ON NOBILETIN BIOACCESSIBILITY

4.1 Introduction

Nobiletin is an O-methylated flavone, which is a flavonoid isolated from citrus peels like in tangerine. Like other polymethoxylated flavones found in citrus peel, nobiletin was found to have anti-inflammatory and anti-tumor invasion, proliferation, and metastasis in vitro and in vivo. Nobiletin was also found to have the potential ability to inhibit cartilage degradation. (Henrotin, Lambert et al. 2011) Despite it's health-beneficial biofunctionalities, the lipophilic nature of nobiletin limits its water solubility and oral bioavailability.

Emulsion-based delivery systems are being developed to encapsulate, protect, and release these non-polar compounds. *In vitro* digestion models are needed to test the efficacy of different systems and control lipid digestion under conditions that simulate the human gastrointestinal tract. The pH stat method used in this study is particularly useful for the rapid screening of food emulsions and emulsion-based delivery systems with different compositions and structures. The candidates successfully tested from this digestion model can then be tested with more rigorous *in vitro* digestion models, or using animal or human feeding studies(McClements and Li 2010).

The objective of this study is to compare the bioaccessibility differences between crystalized nobiletin and solubilized nobiletin and between coarse nobiletin emulsion and well-homogenized emulsion.

4.2 Materials and Methods

Standard nobiletin was bought from Quality Phytochemicals, LLC (New Jersey, USA).

4.2.1 Preparation of Nobiletin Enriched Emulsion and Corn Oil Emulsion

Bioactive Compounds Enriched Nanoemulsion Preparation: An oil phase was prepared by dispersing 0.08% (w/w) nobiletin crystal into corn oil. An aqueous phase was formed by mixing 0.5% (w/w) Tween 20 with aqueous buffer solution (10.0 mM phosphate buffer saline, pH 7.0). Coarse emulsion was formed by mixing oil phase (4% w/w) with aqueous phase (96% w/w) in a container, then use a high-speed blender (Tissue-Tearor, Biospec Products, Bartlesville, OK) blending for 2 minutes at ambient temperature. To make the well-homogenized nanoemulsion, the coarse emulsion was then passed through a high-pressure microfluidizer (Model 101, Microfluidics, Newton, MA) three times at 9,000psi.

Corn Oil emulsion Preparation: Oil-in-water nanoemulsions were prepared by homogenizing 4%(w/w) corn oil with 96%(w/w) aqueous phase that was mixed by dispersing 0.5% (w/w) Tween 20 with aqueous buffer solution (10.0 mM phosphate buffer saline, pH 7.0). The following steps are as same as making nobiletin enriched coarse emulsion and nobiletin enriched nanoemulsion.

4.2.2 Particle Characterization

The mean particle diameters (d_{43}) and particle size distribution (PSD) of the nanoemulsions were measured after mouth, stomach and small intestine digestion using a static light scattering instrument (Mastersizer 2000 Malvern Instruments). The

sizes of the particles in the unfiltered and filtered micelle phase were measured using dynamic light scattering (ZetaSizer Nano, Malvern Instruments).

The electrical charge (z-potential) of the emulsion and the digesta of each step were measured using a micro-electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK).

4.2.3 *In vitro* Digestion

Five comparative groups were prepared, which were nobiletin-enriched nanoemulsion; nobiletin-enriched coarse emulsion; nobiletin crystals in corn oil nanoemulsion and nobiletin crystals in coarse emulsion; nobiletin crystals in phosphate buffer saline (PBS). The concentrations of nobiletin in the three groups were the same. The methods of making the coarse and nanoemulsion were the same as described in chapter two. Each sample was passed through a three-step simulated gastrointestinal (GI) model that consisted of a mouth, gastric, and small intestine phase. This model was established and described previously (Mun and McClements 2006).

Mouth phase: Simulated saliva fluid (SSF), containing 3% mucin and various salts, was prepared as described in previous studies(Sarkar, Goh et al. 2009, Mao and McClements 2012). Ten ml of sample was placed in a beaker and mixed with 10 ml of SSF to make the oil concentration to be 2%. The mixture was then adjusted to pH 6.8 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic oral conditions (Innova Incubator Shaker, 132 Model 4080, New Brunswick Scientific, New Jersey, USA).

Gastric phase: Simulated gastric fluid (SGF) was prepared according to a protocol described before (Sarkar, Goh et al. 2009). The "bolus" sample from the mouth phase was mixed with SGF at a 1:1 mass ratio to make the final oil concentration to be 1%. The mixture was then adjusted to pH 2.5 and placed in a shaker at 100 rpm and 37 ºC for 10 min to mimic gastric conditions.

Small Intestine phase: Gastric digesta (30 ml) was incubated in a water bath (37 ºC) for 10 min and then used NaOH solution to adjust the pH to 7.0. The 1.0 ml of $CaCl_2$ solution, containing 110 mg of $CaCl_2$ (pH 7.0, PBS) was added. Then 4 ml bile extract, containing 187.5 mg bile extract (pH 7.0, PBS), was first added into the 30 ml digesta with stirring and the resulting system was adjusted to pH 7.0. Finally, 2.5 ml of pancreatic suspension, containing 60 mg of lipase (pH 7.0, PBS) was added to the solution. At this point, titration was started automatically. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7.0) of the digestion solution. As the triacylglycerol of fat was digested into free fatty acids by lipolysis, the NaOH solution (0.25 M) was added in to neutralize the pH (7.0). With the volume of NaOH, the amount of free fatty acids released in the system can be calculated. The whole digestion lasted for 2 hours. The calculation of free fatty acids released was described in the previous study (Li and McClements 2010).

4.2.4 Bioaccessibility Determination

After *in vitro* digestion, digesta of each sample group was collected and centrifuged (14000 rpm, Thermo Scientific, CL10 centrifuge) at 25 ºC for 40 min. Centrifuged samples separated into three phases: sediment phase at the bottom, a relatively clear

water phase contained micelles in the middle, and sometimes an undigested oil phase at top. The mixed micelles in the water phase were considered that entrapped the bioactive components. The β-carotene was extracted using organic solvent isopropanol-isooctane solution (1:1 volume ratio). Micelle phase or total digesta (0.2 ml) from the small intestine was added into 4.8 ml isopropanol-isooctane solution and centrifuged at 4000 rpm at 25ºC for 10 min. Then the middle micelle phase was passed through a syringe filter with the pore size of 0.45 um. Both the filtered and unfiltered β-carotene concentration was analyzed using a UV-visible spectrophotometer (Ultrospec 3000 pro, GE Health Sciences, USA) at the spectrum of 450 nm. The concentration of β-carotene was converted from the absorbance by the standard curve. The bioaccessibility was calculated using the following equation:

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{Digesta}} \right)$$

In this equation, $C_{Micelle}$ and $C_{Digesta}$ are the concentrations of β-carotene in the filtered and unfiltered micelle fraction and in the overall sample (total digesta) after the pH-stat experiment, respectively.

4.2.5 Statistical analysis

All measurements were performed at least twice on freshly prepared samples and were reported as calculated means and standard deviations.

4.3 Results and Discussion

4.3.1 Nanoemulsion stability in digestion model

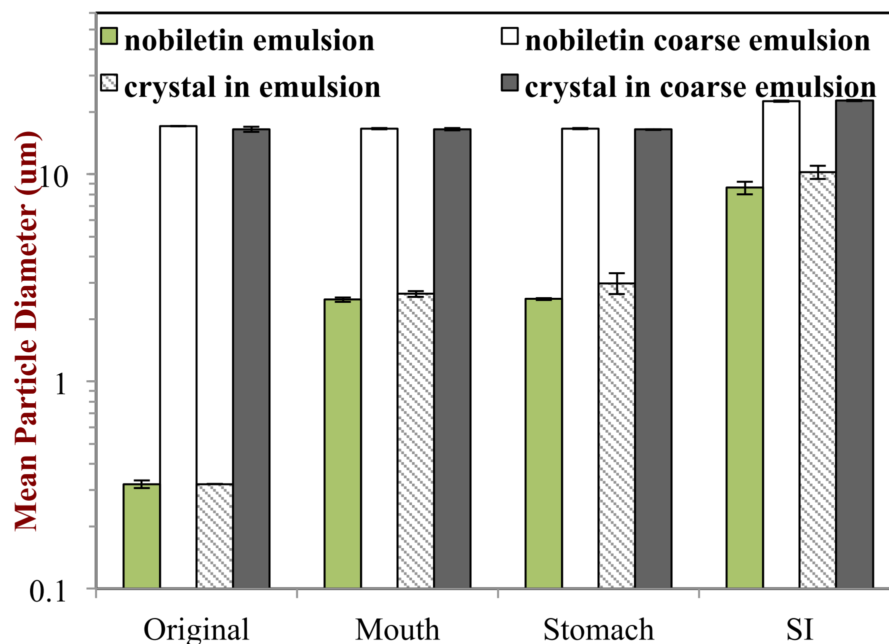

Figure 14a. Influence of nobiletin on mean particle size. Influence of physical state of nobiletin and size of emulsion and *in vitro* digestion on the mean diameter of the particles in oil-in-water nanoemulsions subjected to a simulated gastrointestinal model.

Both the initial particle size of the nanoemulsion groups, no matter with soulubilized nobiletin or crystalized nobiletin, are comparatively small (d_{43}=0.32 μm). The initial particle sizes of the coarse emulsion groups are large (d_{43}>16). After mouth and stomach digestion, the sizes of nanoemulsion groups increased relatively (d_{43}>2.0). Meanwhile, after mouth and stomach digestion, the sizes of the coarse emulsion groups didn't increase a lot (d_{43}>16). After small intestine digestion, sizes in nanoemulsion groups increased dramatically (d_{43}>8.0). There was also an obvious increase of size in coarse emulsion groups (d_{43}>22) (**Figure 14a**). The size distributions of the nanoemulsion groups are monomodal. The size distributions after mouth, stomach and small intestine are bimodal. The size distributions of all the

digestion stages of coarse emulsion are monomodal. Particles all distributed at large sizes (**Figure 14b, 14c, 14d, 14e**).

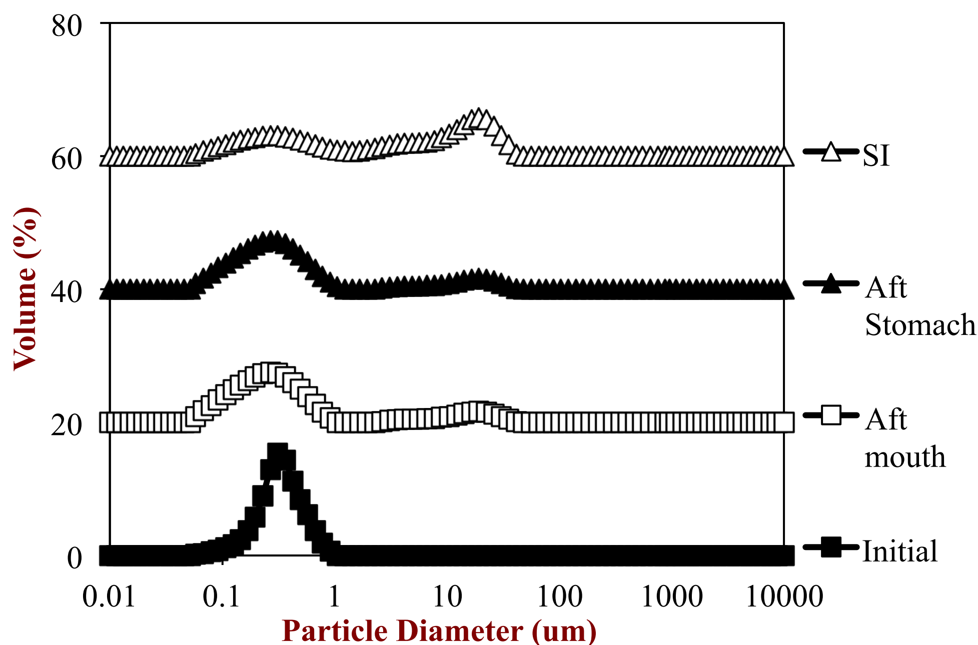

Figure 14b. Particle size distribution of nobiletin nanoemulsion group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. Solubilized nobiletin nanoemulsion.

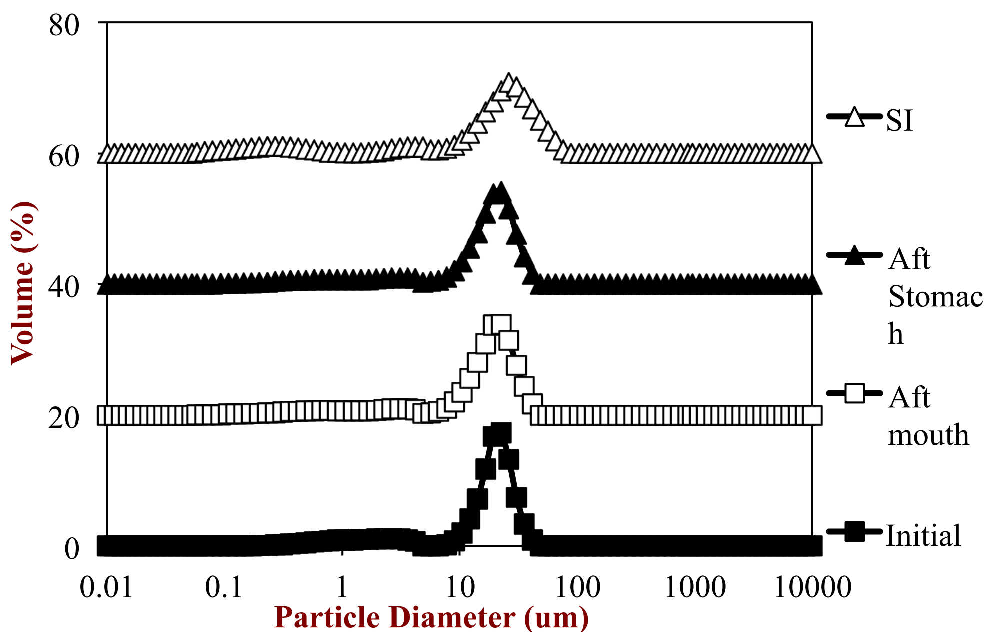

Figure 14c. Particle size distribution of nobiletin coarse emulsion group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. Solubilized nobiletin coarse emulsion.

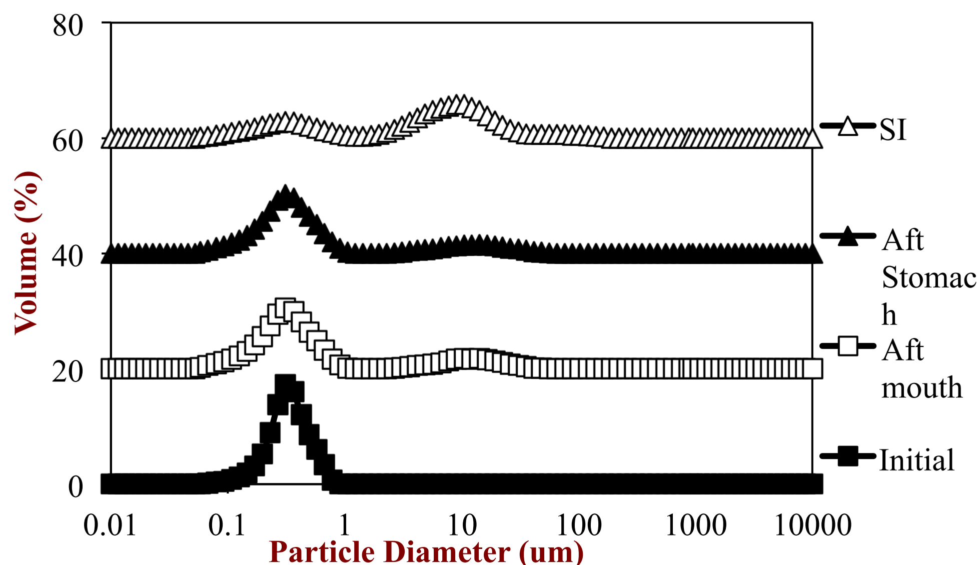

Figure 14d. Particle size distribution of nobiletin crystal nanoemulsion group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. Crystalized nobiletin in nanoemulsion.

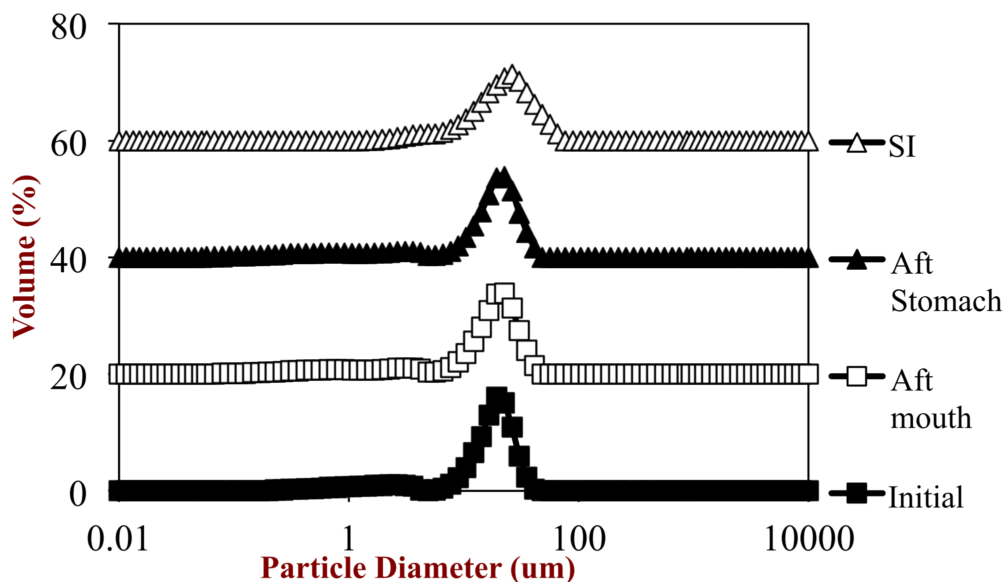

Figure 14e. Particle size distribution of nobiletin cryastal coarse emulsion group. Influence of *in vitro* digestion on the particle size distribution of oil-in-water emulsions subjected to a simulated gastrointestinal model. Crystalized nobiletin in coarse emulsion.

The initial nanoemulsions of all the groups are negatively charged, but the magnitude is small. Although Tween20 is a non-ionic surfactant, however, there may be anionic impurities (*e.g.* free fatty acids and phospholipids) presenting within the surfactant or lipid phase that could adsorb to the oil-water interface and gave a negative charge on the droplets. After the small intestinal stage, the particles charges appeared to be the most negative, which is due to adsorption of anionic species (*e.g.*, bile salts, phospholipids, lipases) to the droplets surfaces (**Figure 15**).

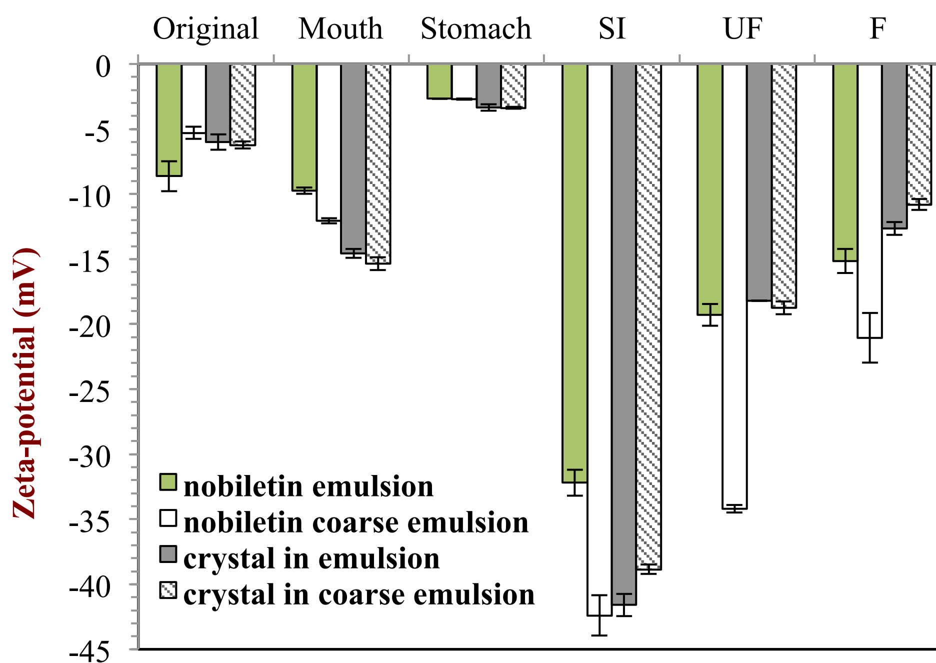

Figure 15. Influence of nobiletin on particle charge. Influence of physical state of nobiletin and size of emulsion and *in vitro* digestion on the charge (ζ-potential) of the particles in oil-in-water emulsions subjected to a simulated gastrointestinal model.

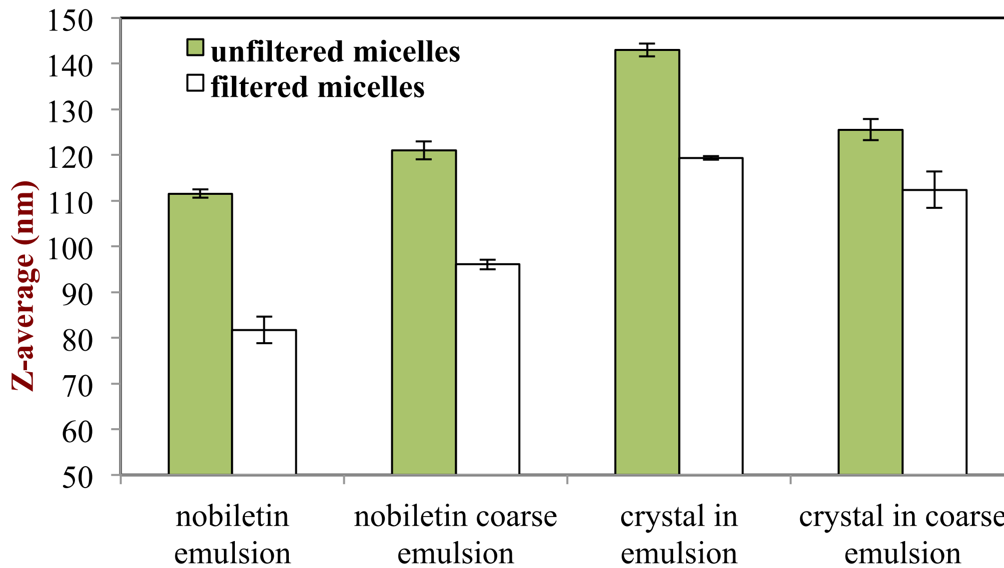

Figure 16. Influence of nobiletin on micelles size. Influence of physical states of nobiletin on the micelle phase sizes (represent by Z-average) collected after digestion and centrifugation. Measurements were made before and after filtration.

After simulated small intestine digestion, the digesta were collected and centrifuged. Three layers formed: the bottom sediment phase, the middle micelle phase and the top undigested oil phase. The "micelle" phase was collected and filtered through 0.45 pre size filters. The particle size and electrical charges of both the filtered and unfiltered micelles were measured (**Figure 15,16**). The sizes in the unfiltered groups are larger indicated that the middle phase are mainly mixed micelles and thin-walled vesicles. After filtration the size decreased as expected, suggesting that some large vesicles were filtered out. The sizes of the crystalized groups are larger than the solubilized groups. The sizes of the solubilized nobiletin groups are smaller, regardless of the original sizes of emulsion. The electrical charges of the unfiltered and filtered micelles are also negative (**Figure 15**) because of the anionic species, such as bile salts, phospholipids and free fatty acids.

4.3.2 Lipid Digestion via *In Vitro* Digestion

In this section, we monitored the rate and extent of lipid digestion in the small intestinal digestion via pH-stat. This method measured the amount of free fatty acids (FFAs) released from triglycerides under the digestion of lipase(McClements and Li 2010). As expected, there was little change in the pH of the sample only contained nobiletin crystals and PBS, because there is no fat in the system and no FFAs released during digestion (**Figure 17**). In this sample, the slightly increase in the amount of NaOH due to the digestion of Tween 20. In the nanoemulsion systems, there was a dramatic increase in the first 20 minutes of digestion and then it gradually reached a plateau; the increases in the coarse groups were relatively slow (**Figure 17**). The FFAs release profiles followed a similar trend as the NaOH consumption (**Figure 18**). The trend suggested that lipase attached to the surface of the lipid droplets rapidly and promoted triglycerol digestion.

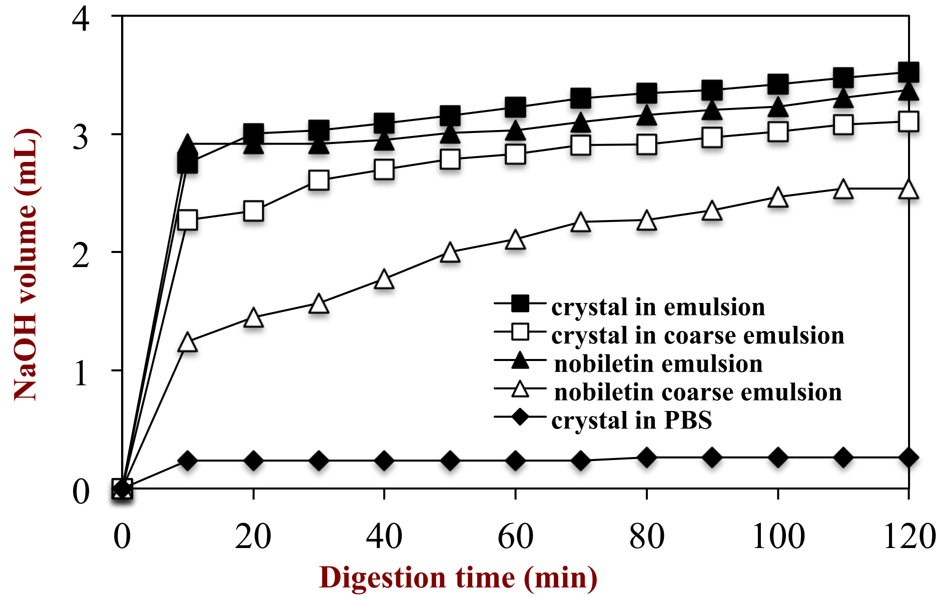

Figure 17. Volume of NaOH. Influence of physical state of nobiletin and emulsion size on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the volume of 0.25 M NaOH solution that had to be added to the samples to maintain the pH at a constant value of 7.

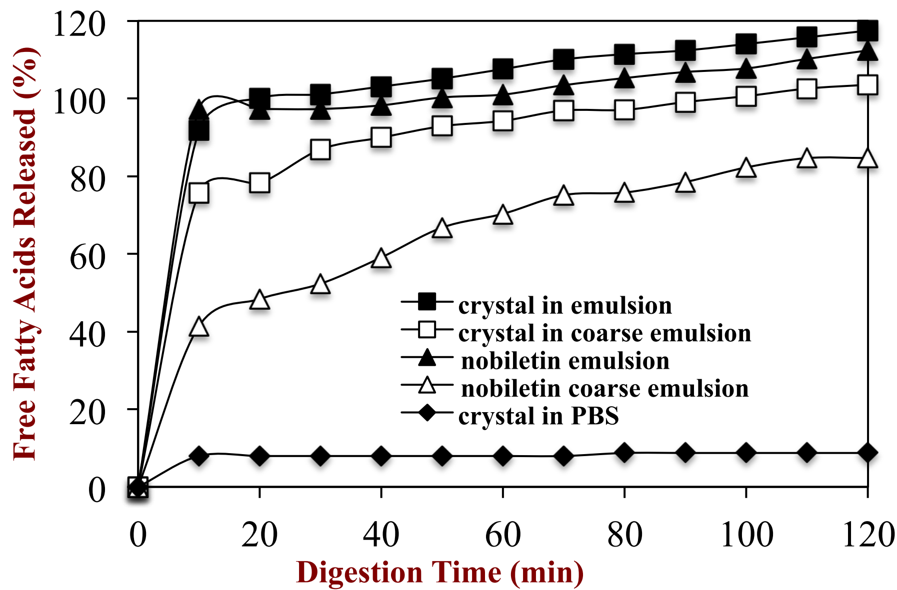

Figure 18. Free fatty acids released percentage. Influence of physical state of nobiletin and emulsion size on the pH-stat titration curves of oil-in-water nanoemulsions. The curves show the calculated percentage of free fatty acids (FFA) released from the nanoemulsions after digestion of the triacylglycerol phase by lipase.

4.3.3 In Vitro Digestion Bioaccessibility

In this section, we examined the nobiletin bioaccessibility under solubilized state and crystalized state and with nanoemulsion and conventional emulsion after it passed through the simulated gastrointestinal tract model. Theoretical, the portion of nobiletin in “micelle” phase over the portion of original nobiletin can represent the nobiletin bioaccessibility. The bioaccessibilty was measured before and after filtration. It was assumed that the filtered sample could be more practically represent the actual bioaccessibility of nobiletin because it could remove any particles larger than 450nm that could not be absorbed by small intestine epithelium cells. The filter mimicked the function of the mucus layer above the small intestine.

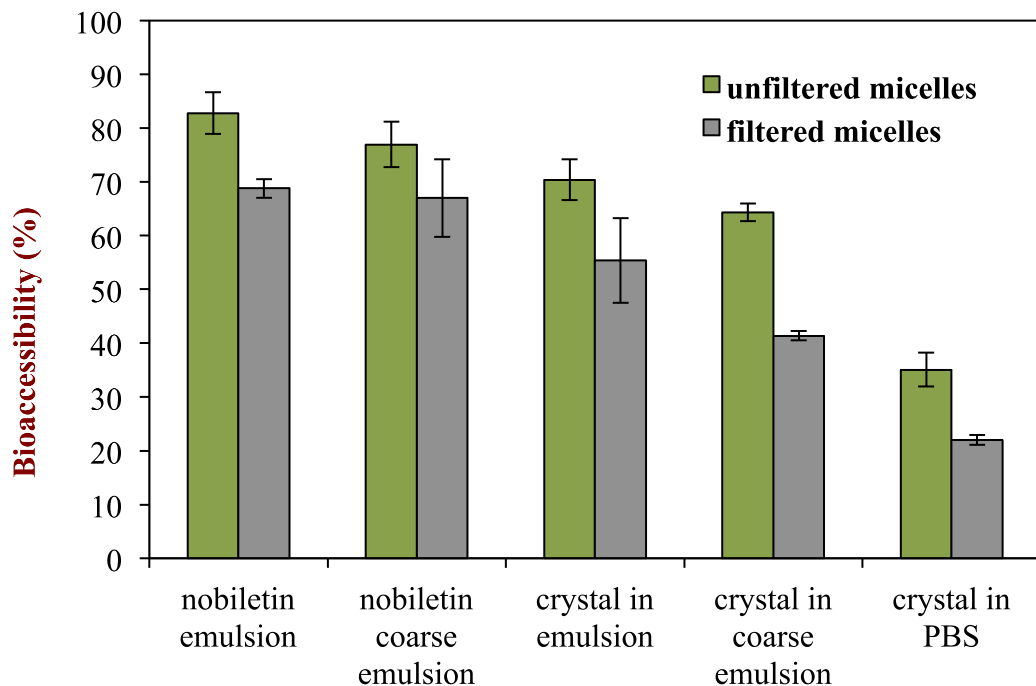

Figure 19. Nobiletin bioaccessibility. Effect of physical states of nobiletin and emulsion size on bioaccessibility of nobiletin measured after centrifugation of emulsions passed through an *in vitro* digestion model.

The lowest bioaccessibility (22.0%) was measured in filtered nobiletin crystals with PBS (**Figure 19**). The bioaccessiblity of solubilized nobiletin in nanoemulsion is higher than coarse emulsion. The bioaccessibility of nobiletin crystals in coarse emulsion is lower than nanoemulsion. Comparing the solublized ones with the crystalized ones, the bioaccessibilies of the solubilized groups are larger than the crystalized groups. The solubilized nobiletin nanoemulsion enjoyed the highest bioaccessibility. This is because when added nobiletin crystals in either corn oil emulsion or PBS, most of the nobiletin still stayed unsolubilized. Although nobiletin better in corn oil emulsion than only in PBS, however compared with the solubilized nobiletin emulsion, the solubilized part was still too few to have a high bioaccessibilty. These results suggested people to take solubilized nobiletin supplements to get the best absorption in gastrointestinal tract.

4.4 Conclusions

This study showed the bioaccessibility of different physical states of nobiletin and with different delivery systems is different. In the five compare groups: (1) solubilized nobiletin nanoemulsion, (2) solubilized nobiletin conventional emulsion, (3) nobiletin crystals in nanoemulsion, (4) nobiletin crystals in conventional emulsion, (5) nobiletin crystals in PBS, the rate and extent of free fatty acid production in the small intestine decreased in the order (3)>(1)>(4)>(2)>(5); whereas the nobiletin bioaccessibility decreased in the order (1)>>(2)>(3)>(4)>(5). In system (5), even without any fat, there was still noticeable consumption of NaOH, which was attributed to hydrolysis of the ester bonds in the non-ionic surfactant (Tween 20). The low bioaccessibility (22%) of system

(5) was attributed to the fact that there were few free fatty acids produced to form mixed micelles to solubilize β-carotene. The bioaccessibility of nobiletin crystals groups are generally lower than soulubilized groups, which suggests that crystalline nobiletin is less bioaccessible than soluble nobiletin.

The bioaccessibility of solubilized nobiletin entrapped in nanoemulsions was the highest is because the free fatty acids produced through small intestine digestion formed mixed micelles capable of solubilizing and transporting lipophilic compounds. Crystalline nobiletin was not incorporated into mixed micelles easily, leading to a reduced bioaccessibility. In the system with crystals in water, there was no free fatty acid produced, so the bioaccessibility of nobiletin was low.

BIBLIOGRAPHY

Agarwal, S. and A. Rao (2000). "Carotenoids and chronic diseases." Drug metabolism and drug interactions **17**(1-4): 189-210.

Alam, B. S., et al. (1990). "Influence of dietary fats and vitamin E on plasma and hepatic vitamin A and β-carotene levels in rats fed excess β-carotene." Nutrition and Cancer **14**(2): 111-116.

Bevernage, J., et al. (2010). "Drug Supersaturation in Simulated and Human Intestinal Fluids Representing Different Nutritional States." Journal of Pharmaceutical Sciences **99**(11): 4525-4534.

Boon, C. S., et al. (2010). "Factors Influencing the Chemical Stability of Carotenoids in Foods." Critical Reviews in Food Science and Nutrition **50**(6): 515-532.

Borel, P. (2003). "Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols)." Clin Chem Lab Med **41**(8): 979-994.

Brewster, M. E. and T. Loftsson (2007). "Cyclodextrins as pharmaccutical solubilizers." Advanced Drug Delivery Reviews **59**(7): 645-666.

Brouwers, J., et al. (2009). "Supersaturating drug delivery systems: The answer to solubility-limited oral bioavailability?" Journal of Pharmaceutical Sciences **98**(8): 2549-2572.

Brubacher, G. B. and H. Weiser (1985). "The vitamin A activity of β-carotene." International Journal for Vitamin and Nutrition Research **55**(1): 5-15.

Castenmiller, J. J. and C. E. West (1998). "Bioavailability and bioconversion of carotenoids." Annual review of nutrition **18**(1): 19-38.

Castenmiller, J. J. M. and C. E. West (1998). "Bioavailability and bioconversion of carotenoids." Annual Review of Nutrition **18**: 19-38.

Castenmiller, J. J. M. and C. E. West (1998). "Bioavailability and bioconversion of carotenoids." Annual review of nutrition **18**(1): 19-38.

Chen, L. Y., Remondetto, G. E., and Subirade, M (2006). "Food protein-based materials as nutraceutical delivery systems." Trends in Food Science & Tech- nology **17**(5): 272-283.

Elliott, J. G. (1999). "Application of antioxidant vitamins in foods and beverages." Food Technology **53**: 46-49.

Elliott, R. (2005). "Mechanisms of genomic and non-genomic actions of carotenoids." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease **1740**(2): 147-154.

Golding, M., et al. (2011). "Impact of gastric structuring on the lipolysis of emulsified lipids." Soft Matter **7**(7): 3513-3523.

Goltz, S. R., et al. (2012). "Meal triacylglycerol profile modulates postprandial absorption of carotenoids in humans." Molecular nutrition & food research **56**(6): 866-877.

Gonnet, M., et al. (2010). "New trends in encapsulation of liposoluble vitamins." Journal of Controlled Release **146**(3): 276-290.

Hauss, D. J. (2007). "Oral lipid-based formulations." Advanced Drug Delivery Reviews **59**(7): 667-676.

Henrotin, Y., et al. (2011). "Nutraceuticals: do they represent a new era in the management of osteoarthritis? – a narrative review from the lessons taken with five products." Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society **19**(1): 1-21.

Hörter, D. and J. Dressman (2001). "Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract." Advanced drug delivery reviews **46**(1): 75-87.

Huo, T., et al. (2007). "Impact of fatty acyl composition and quantity of triglycerides on bioaccessibility of dietary carotenoids." Journal of agricultural and food chemistry **55**(22): 8950-8957.

Johnson, E. J. (2002). "The role of carotenoids in human health." Nutrition in Clinical Care **5**(2): 56-65.

Karppi, J., et al. (2012). "Low beta-carotene concentrations increase the risk of cardiovascular disease mortality among Finnish men with risk factors." Nutrition Metabolism and Cardiovascular Diseases **22**(10): 921-928.

Kawabata, Y., et al. (2011). "Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: Basic approaches and practical applications." International Journal of Pharmaceutics **420**(1): 1-10.

Kleberg, K., et al. (2010). "Characterising the behaviour of poorly water soluble drugs in the intestine: application of biorelevant media for solubility, dissolution and transport studies." Journal of Pharmacy and Pharmacology **62**(11): 1656-1668.

Li, S., et al. (2006). "Hydroxylated Polymethoxyflavones and Methylated Flavonoids in Sweet Orange (Citrus sinensis) Peel." Journal of Agricultural and Food Chemistry **54**(12): 4176-4185.

Li, S., et al. (2009). "Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones." Journal of Functional Foods **1**(1): 2-12.

Li, Y., et al. (2011). "Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised pH-stat method." Food Chemistry **126**(2): 498-505.

Li, Y. and D. J. McClements (2010). "New Mathematical Model for Interpreting pH-Stat Digestion Profiles: Impact of Lipid Droplet Characteristics on in Vitro Digestibility." Journal of Agricultural and Food Chemistry **58**(13): 8085-8092.

Li, Y. and D. J. McClements (2011). "Inhibition of lipase-catalyzed hydrolysis of emulsified triglyceride oils by low-molecular weight surfactants under simulated gastrointestinal conditions." European Journal of Pharmaceutics and Biopharmaceutics **79**(2): 423-431.

Mao, Y. and D. J. McClements (2012). "Influence of electrostatic heteroaggregation of lipid droplets on their stability and digestibility under simulated gastrointestinal conditions." Food & Function.

McClements, D. J., et al. (2009). "Structural Design Principles for Delivery of Bioactive Components in Nutraceuticals and Functional Foods." Critical Reviews in Food Science and Nutrition **49**(6): 577-606.

McClements, D. J. and Y. Li (2010). "Review of in vitro digestion models for rapid screening of emulsion-based systems." Food & Function **1**(1): 32-59.

McClements, D. J. and H. Xiao (2012). "Potential biological fate of ingested nanoemulsions: influence of particle characteristics." Food & Function **3**(3): 202-220.

Miller, J. M. and A. Dahan (2012). "Predicting the solubility-permeability interplay when using cyclodextrins in solubility-enabling formulations: Model validation." International Journal of Pharmaceutics **430**(1-2): 388-391.

Miller, P. E. and D. C. Snyder (2012). "Phytochemicals and Cancer Risk: A Review of the Epidemiological Evidence." Nutrition in Clinical Practice **27**(5): 599-612.

Mittal, P. C. (1983). "β-Carotene utilization in rats fed either vitamin A or carotene in early life." Nutrition Reports International **28**(1): 181-188.

Muller, R. H. and C. M. Keck (2004). "Challenges and solutions for the delivery of biotech drugs - a review of drug nanocrystal technology and lipid nanoparticles." Journal of Biotechnology **113**(1-3): 151-170.

Mun, S. and D. J. McClements (2006). "Influence of Interfacial Characteristics on Ostwald Ripening in Hydrocarbon Oil-in-Water Emulsions." Langmuir **22**(4): 1551-1554.

Nik, A. M., et al. (2012). "Digestibility and beta-carotene release from lipid nanodispersions depend on dispersed phase crystallinity and interfacial properties." Food & Function **3**(3): 234-245.

Omenn, G. S., et al. (1996). "Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease." New England journal of medicine **334**(18): 1150-1155.

Poor, C. L., et al. (1993). "The accumulation of α- and β-carotene in serum and tissues of preruminant calves fed raw and steamed carrot slurries." Journal of Nutrition **123**(7): 1296-1304.

Qian, C., et al. (2012). "Nanoemulsion delivery systems: Influence of carrier oil on β-carotene bioaccessibility." Food Chemistry **135**(3): 1440-1447.

Rao, A. V. and L. G. Rao (2007). "Carotenoids and human health." Pharmacological Research **55**(3): 207-216.

Rao, J., et al. (2013). "Nutraceutical nanoemulsions: influence of carrier oil composition (digestible versus indigestible oil) on beta - carotene bioavailability." Journal of the Science of Food and Agriculture **93**(13): 3175-3183.

Sarkar, A., et al. (2009). "Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model." Food Hydrocolloids **23**(6): 1563-1569.

Sarkar, A., et al. (2009). "Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva." Food Hydrocolloids **23**(5): 1270-1278.

Sato, T., et al. (2002). "Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and-9 in human fibrosarcoma HT-1080 cells." Cancer research **62**(4): 1025-1029.

Shefer, A. a. S., S (2003). "Novel encapsulation system provides controlled release of ingredients." Food Technology **57:40–43**.

Thakkar, S. K., et al. (2007). "beta-Carotene micellarization during in vitro digestion and uptake by Caco-2 cells is directly proportional to beta-Carotene content in different genotypes of cassava." Journal of Nutrition **137**(10): 2229-2233.

Tyssandier, V., et al. (2001). "Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles." Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids **1533**(3): 285-292.

Ubbink, J. (2002). "Flavor delivery systems: Trends, technologies and applica- tions. ." Abstracts of Papers of the American Chemical Society **223**: U34-U34.

Ubbink, J. a. K., J (2006). "Physical approaches for the delivery of active ingredients in foods." Trends in Food Science & Technology **17**(5): 244-254.

van Het Hof, K. H., et al. (2000). "Dietary factors that affect the bioavailability of carotenoids." The Journal of nutrition **130**(3): 503-506.

Wang, J. M. and T. J. Hou (2011). "Recent Advances on Aqueous Solubility Prediction." Combinatorial Chemistry & High Throughput Screening **14**(5): 328-338.

Warren, D. B., et al. (2010). "Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility." Journal of Drug Targeting **18**(10): 704-731.

Wiseman, H. (1999). "Bioactive components of foods." Journal of Chemical Technology & Biotechnology **74**(4): 371-372.

Yonekura, L. and A. Nagao (2007). "Intestinal absorption of dietary carotenoids." Molecular nutrition & food research **51**(1): 107-115.

Ziani, K., et al. (2012). "Encapsulation of functional lipophilic components in surfactant-based colloidal delivery systems: Vitamin E, vitamin D, and lemon oil." Food Chemistry **134**(2): 1106-1112.