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A Thesis for the Master's Degree in Engineering

Physicochemical characteristics of  
*Enteromorpha prolifera* Extract as a Functional Food Additive

기능성 식품첨가물로서 가시파래 추출물의 물리화학적 특성

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# ABSTRACT

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*Enteromorpha prolifera* is a common species among the green algae found all over the globe. Physicochemical characteristics and biological activity of integral, hydrolyzed or fractionated extract of *E.prolifera* has been widely tested *in vitro* and *in vivo*, but not many of the researches had been focused on whether the extract maintains its various functionality in the applied food system.

This study aims to analyze *E.prolifera* extract (EPE) as a supplement in food system carrying retinol. To evaluate the physicochemical characteristics of EPE, chemical composition was investigated prior to any other measurements. The extracts had a yield of 13.47% (g extract/g depigmented raw material), 47% of polysaccharide content, and other minor components consists of 12.4% protein and 19% sulfate attached to the building blocks of polysaccharides. Rheological characteristics depending on temperature and pH of the extract was assessed. EPE did not form gel in low concentrations below 2.5% as well as in strong acidic environment of pH lower than 3.5. The strongest gel was formulated in pH 7 with equilibrated G' value around 800 Pa (pH 5: 79.38 Pa, pH 9: 480 Pa). EPE gels

melted as the temperature rose, and restored the original network as the temperature reached 4 °C again.

A model food system, O/W emulsion (formulated with synthetic emulsifier Tween 20 and protein emulsifier WPI), was fortified with EPE to investigate the effect of the extract as a protective material against instability factors. Addition of over 5% resulted in a gel, which was also thermo-reversible. Emulsions did not set to gel with EPE concentration below 2.5%. In emulsions formulated with neutral buffer, addition of EPE did not affect the emulsion stability nor the retinol retention rate after 6 days of storage ( $p>0.05$ ) up to this concentration except for the synergistic increase in gel strength. On the contrary, reactions in emulsions prepared with pH 3.5 buffer were more diverse. The addition of EPE in acidic Tween 20 emulsion enhanced retinol stability depending on the amount of EPE dissolved (Blank:  $58.16\pm4.7\%$ , EPE 2.5%:  $81.81\pm6.8\%$ ). For WPI emulsions, excessive use of EPE caused undesirable interaction between protein emulsifier and polysaccharide, resulting in retention rate ( $22.91\pm4.3\%$ ) lower than that of the blank ( $40.33\pm6.5\%$ ). The optimum concentration of EPE within the studied conditions was 0.5% ( $77.16\pm6.8\%$ ).

These results suggest a possibility of *E. prolifer*a utilization as a new additive in food system, having desirable rheological properties that leads to a stabilized emulsion in optimum concentration range. It is also a staple species of green algae around the world, and its physiological benefits have already been through in-depth researches. Moreover, the functional fraction of *E. prolifer*a can be extracted without harsh or expensive chemical

treatments, and is generally regarded as safe (GRAS) food material. These functionalities suggests that EPE can be applied to the food system as a universal, multifunctioning stabilizing supplement as well as a drug transport supporter.

Keywords: *Enteromorpha prolifera*, water-soluble sulfated polysaccharide, physicochemical analysis, O/W emulsion, emulsion storage stability

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# 1. Introduction

The concept of welfare is no longer simple, and welfare for the elderly requires extensive insight that can improve the problems of various environments. (Kim 2015) It is important to maintain health and function through proper dietary management for successful aging (Lee, Kim et al. 2013). Elderly people aged over 65 years tend to have multiple diseases including degenerative symptoms. Therefore, eating disorder ratio is higher than that of younger adults, but regular eating habits and balanced nutrient intake are critical to maintain a healthy life (Kim, Lee et al. 2002). However, contrary to its increasing importance, malnutrition is one of the most common causes of poor quality of life in the elderly population. The quality of life declines as malnutrition and lack of meals persist, and as a consequence they do not eat proper meals and the malnutrition state becomes even more severe. Under these circumstances, efforts have been made to develop a new food system with high nutritional density. However, many substances with biological activity are easily broken down by environmental stress; a variety of methods and materials have been presented to protect them from stress causes. In addition, as the preference for natural materials has increased, a variety of studies have also been conducted on naturally occurring substances that are not synthetic stabilizers. In this study, one of them was the choice of a material: water-soluble polysaccharide extract from *Enteromorpha prolifera*.

*Enteromorpha prolifera* is common green algae that belongs to the phylum *Chlorophyta*, class *Chlorophyceae*, order *Ulvaes*, and genus *Enteromorpha*. It has been

consumed among Far East countries through history and is also known for its nutrition profile. *E.prolifera* can enhance immunity and also has anti-bacterial, anti-viral, antioxidant, anti-tumor, anti-Alzheimer's, prebiotic and hypolipidemic activities. To the author's knowledge, no effect on human trials have been reported. Nevertheless, these assays, depending on the specific procedures, were conducted in-depth either on *in vitro* level or on cellular model (Ivanova, Rouseva et al. 1994, Cassolato, Nosedá et al. 2008, Kim, Cho et al. 2011, Ahn, Park et al. 2012, Tang, Gao et al. 2013, Zaporozhets, Besednova et al. 2014, Lin, Wang et al. 2015).

The polysaccharide fraction is known to be the critical physiological functioning component in *E.prolifera* extract as in many other green algae species (Cho, Yang et al. 2010, Kim, Cho et al. 2011, Qi, Mao et al. 2012, Lin, Wang et al. 2015). This polysaccharide is commonly called ulvan; it corresponds up to 50% of the water soluble fraction of green algae showing a structure of great complexity and variability (Cho, Yang et al. 2010). Previous researches verified sulfated rhamnose, xylose and glucuronic acid are the main building blocks of ulvan (Brading, Georg-Plant et al. 1954, Percival and Wold 1963, Quemener, Lahaye et al. 1997, Cho, Yang et al. 2010, Yu, Li et al. 2017). It has also been reported that polysaccharide fraction showed thixotropic characteristics (Qiao, Li et al. 2016), suggesting a possibility for *E.prolifera* extracts to be utilized as stabilizers in food or drug delivery systems.

The basic composition and functionality of crude, hydrolyzed or fractionated extract of *E.prolifera* has been widely tested *in vitro* and *in vivo*. But not many of the

researches had been focused on whether the extract maintains its various functionality in the applied food system. Therefore, this study aims to assess the characteristics of *Enteromorpha prolifera* extract (EPE) in model food system, an O/W emulsion, to improve storage stability and physicochemical characteristics. (Kaplan, Christiaen et al. 1987, Cho, Yang et al. 2010, Costa, Fidelis et al. 2010, Kim, Cho et al. 2011, Tang, Gao et al. 2013, Lin, Wang et al. 2015, Qiao, Li et al. 2016, Chi, Li et al. 2018)

## 2. Objectives

The purposes of this research are;

- To analyze the basic physicochemical characteristics and composition of water soluble extract from *E.prolifera*
- To investigate the gelation mechanism of extract from *E.prolifera*
- To assess the effect on stability *E.prolifera* has on O/W emulsion system during storage



### **3. Literature review & Background**

#### **3.1. Marine resources of bioactive natural compounds and extracts**

##### **3.1.1. *Enteromorpha prolifera***

Green algae are ubiquitous, sharing common physicochemical and biological characteristics across species. However, green algae in general are far less investigated for their functionality and characteristics compared to brown or red algae species, even in areas where the latter two have already proven their value (Cunha and Grenha 2016). *Enteromorpha prolifera* is a common green algae, which belongs to the phylum *Chlorophyta*, class *Chlorophyceae*, order *Ulvales*, and genus *Enteromorpha*. It usually grows in shallow seas or offshore shoals, and is also widely distributed in rock pools in river mouths and tidal zones. *E.prolifera* has been recognized as an edible and medicinal alga since ancient times among Far East countries, but this species has also been identified among marine resources of Europe, America, Africa and Asia.

After the large occurrence of *E.prolifera* in the Qingdao region of China in 2013, recorded as the largest green tide ever seen on Earth (Liu, Keesing et al. 2009), large quantities of the green algal biomass were also investigated for its industrial and environmental usefulness as well as its physiological functionality. Not only the significant ability to bind heavy metal ions, but the additional advantages of biodegradability, safety to human beings and minimal second contamination, natural polymers extracted from this seaweed species have drawn great attention for future applications in water treatment (Zhao, Gao et al. 2014). In particular, a few prevailing metal species were studied; Özer et al.

reported that this species have the ability to adsorb copper (II) ions without any further processing other than inactivation at 105 °C (Özer, Gürbüz et al. 2009). While carbon is already a commonly used material in the purification process of air or water, activation of carbon using *E.prolifera* showed significant enhancement in its ability to remove Pb (II) (Li, Du et al. 2010). The fraction of *E.prolifera* extracted by boiling the raw material in 0.1 N HCl for an hour was able to form a complex by chelating Fe (III) ions up to 9.14%, suggesting a possible natural chelating agent other than EDTA and moreover, as a potential material to help reduce iron deficiency (Chi, Li et al. 2018).

Many research groups over the globe have reported the diverse functions of *E.prolifera*, leading way to possible applications as a functional material in different areas of industry. To maximize the physicochemical and biological functions and to suggest a more concrete evidence on the activity, many researchers had extracted and fractionated the algae; specific chemical and biological reactions are often enhanced when the material is hydrolyzed down to smaller molecules to expose active sites. Immunological tests were done on physiologic markers of RAW 264.7 cell line model using sulfated polysaccharides extracted from *E.prolifera*, validating its immunomodulatory effect by stimulating macrophage cell activity by enhancing mRNA expression that leads to nitric oxide induction and cytokine production (Kim, Cho et al. 2011). Glucose metabolism in type 2 diabetes mellitus improved in rat fed with polysaccharide extracted from *E.prolifera*. The researchers concluded that this result may be due to the antioxidant activity of and its ability to regulate mRNA expression in liver and adipose tissues (Lin, Wang et al. 2015). It was

also notable that most research papers regarding the physiological functions of extracts from *E.prolifera* focuses on its effect to improve lipid metabolism. Teng et al. has tested the effect by feeding the extracts to Sprague-Dawley mice and analyzing blood and fecal samples for triglyceride and cholesterol. Their results indicate that *E.prolifera* polysaccharide can inhibit the increase in weight, decrease liver weight and have a positive effect on serum TG and cholesterol level. They concluded that this result was due to the binding ability of *E.prolifera* polysaccharide to bind with fat and cholesterol (Teng, Qian et al. 2013).

As in many heteropolysaccharides consisting of various sugars with various glycosidic linkage, it was no easy task to accurately assume the structure of the polysaccharide content of *E.prolifera*. For example, methylation assessment for the structural analysis of polysaccharide is also not an option for sulfated polysaccharides due to their insolubility to DMSO. Yet, a number of research groups have went into depth to identify its characteristics. Cho et al. reported that polysaccharide extracted from *E.prolifera* consists mostly of rhamnose which takes up more up to 70% of the total sugar, is highly sulfated (15%), resulting in a negatively charged polysaccharide soluble in cold water (Cho, Yang et al. 2010). Enzymatic hydrolysis enabled the production of controlled oligosaccharides, simplifying the analysis of the wholesome structure. These oligosaccharides were assessed with MS for structural determination and NMR for linkage determination. For monosaccharide analysis liquid or gas chromatography for neutral sugar determination after hydrolyzing polysaccharides were run, and uronic acid determination

was carried out separately. The results of these assays revealed that polysaccharides from *E.prolifera* was composed of sulfated rhamnose, uronic acid and xylose in a ratio of 3.2: 1.1: 1. Sulfate was mainly bonded to rhamnose on the C-3 position, and both  $\alpha$ - and  $\beta$ -(1 $\rightarrow$ 4) bonds were prevalent (Yu, Li et al. 2017). Lin et al. reported the ratio for rhamnose, glucuronic acid, arabinose, fucose, xylose, and glucose are 5.12 : 1.32 : 3.38 : 1.62 : 1 : 1.03, respectively (Lin, Wang et al. 2015). In the paper of Chi et al., ratio of rhamnose, glucuronic acid, glucose, and xylose was 1 : 0.31 : 1.29 : 0.49 in hot-water extracted samples (Chi, Li et al. 2018). Cho et al. reported the rhamnose takes over 70% of the crude polysaccharide, followed by glucose (26.3%) and uronic acid (15.3%) (Cho, Yang et al. 2010). Specific percentage did differ among papers, but the overall trends were similar; all papers included in this review agreed that the most prevalent monosaccharide is rhamnose. Uronic acid (and among them, galacturonic acid), glucose and xylose were also dominant, the four sugars consisting almost up to 80% of the crude polysaccharide. For most papers, sulfate content ranged from 15 to 17%.

### 3.1.2. Other algal species

Polysaccharide extracts from brown and red algae were of more interest throughout the years compared to that of green algae species. Alginate and fucoidan are mainly extracted from brown algae; carrageenan and agar are from red algae.

Alginate is a highly hydrophilic marine polysaccharide that reacts with calcium to form insoluble gel-like beads. This bead is a very promising material that can encapsulate bioactive compound, entrap enzymes and forms artificial seeds for plant tissue culture.

Carrageenan is a very highly sulfated polysaccharide based on a backbone of galactose and 3,6-anhydrogalactose. It is commonly utilized in food and cosmetics industry as a gelling agent and stabilizer. However, a number of researchers has published articles regarding the negative immunological effect of carrageenan. In fact, in many researches, carrageenan is used as a stimulus to induce paw edema in mice as a model for inflammation (Morris 2003). However, this should not be overestimated; in these protocols, carrageenan is directly injected into the mice's paw, which is an unlikely intake process for human, since our intake of carrageenan is mainly through the GI tract.

Agar is one of the materials mostly widely used around the globe. Traditional desserts from Far East Asia contains agar, comparable with jelly or toffees which uses gelatin or high concentration sugar solutions to achieve their unique texture. However, the application of agar is not restricted to culinary arts; the ability to form rigid gel without interacting with other nutrients allows it to provide a growth media for microorganisms. Dried agar powder is insoluble in cold water, but once the gel forms after treatment with autoclave condition of 121 °C for 15 min, the solution will soon set gel and this semi solid network is irreversible. While many other gelling materials share these properties discussed so far, the reason why agar is the staple material is because only very few microorganisms can degrade agar.

Most red and brown algae species mainly consists of polysaccharides and carbohydrates, as in their green counterpart, other bioactive compounds are also present. Balboa et al. had published a well-extended review on antioxidative abilities of brown algae (Balboa, Conde et al. 2013). Aslam et al. reported that the mineral-rich fronds of a red algae species *Lithothamnion calcareum* extracts have the ability to prevent polyp formation and the onset of colon cancer in mice fed with high-fat diet compared to control groups without the supplement of algal extract (Aslam, Paruchuri et al. 2010)

### **3.2. Extraction and analysis of bioactive natural compounds and extracts**

Unlike plant cell walls, which mostly consists of non-soluble and therefore unbreakable cellulose, algae cell walls are rather easier to dissolve, offering scientists to develop new methods for mild-condition extraction of its bioactive components, without the involvement of harsh, organic chemicals. In this method, generally the raw material goes through milling, bleaching process using pure ethanol (optional depending on the colorimetric characteristics of the material) and extraction process using distilled water of 60°C to 80°C. A number of studies has proven such methods are useful to extract water soluble bioactive compounds in various marine resources.

Characterization of natural compounds is no easy task and should be considered in different aspects. Quantification of the composition is important, but this is not possible in all samples especially if the extracts are suspected to be complex. Each extract has to be assessed for their chemical constituents, structure and reactive natures, and if possible, it is recommended to extend the search to their toxicity and functionality in living organisms.

Because extracted materials are not always purified completely down to a singular compound, assessment from various aspects is critical to identify the characteristics of the samples. Chemical bond and molecular structural analysis were done with Fourier transfer infrared spectroscopy (FT-IR), field emission scanning electron microscopy (FE-SEM), nuclear magnetic resonance (NMR), elemental analyzer (EA) to clarify the chemical characteristics and the physical structure responsible for such reactivity (Černá, Barros et al. 2003, Na, Kim et al. 2010, Tabarsa, Han et al. 2012, Tabarsa, Lee et al. 2012, Tabarsa,

Shin et al. 2015, Surayot, Hun Lee et al. 2016). Depending on the expected components of the extract, specific chemical analysis are also done; researchers analyze specific building blocks of macromolecules (ex. monosaccharide verification), antioxidant measures, or other specific interactions within the tested system.

To assess the basic biological function of the desired extract, various *in vitro* test on animal cell lines were previously ran by researchers (Na, Kim et al. 2010, Tabarsa, Han et al. 2012, Tabarsa, Lee et al. 2012, Tabarsa, Shin et al. 2015, Surayot, Hun Lee et al. 2016). Originally isolated from a 72-year-old Caucasian male colon adenocarcinoma, Caco-2 cell line is one of the most widely used in vitro models of physiological analysis in two aspects. Being a colon cancer cell, its immortality enables researchers to apply Caco-2 in various cytotoxicity and apoptosis analysis, especially with medicines and active compounds regarding intestinal health. However, when cultured on a 0.4  $\mu$ m pore filter unit; Caco-2 cells grow in a single layer to form a cellular barrier, which resembles the small intestines. (Hidalgo, Raub et al. 1989) It is not only the morphology of the single layer mucosa that the model resembles; this cell line also possesses the biological function of a normal small intestine cell, including uptake passageways and transporters within the cell membrane. For these reasons, this model is commonly used to analyze the bioavailability (Garrett, Failla et al. 2000) and uptake mechanisms (During and Harrison 2007) for a target bioactive compounds or drugs. To determine whether the filter unit is completely covered with this cell layer, transepithelial electrical resistance value or the permeation of a known chemical (most commonly used is Lucifer Yellow) (Natoli, Leoni et al. 2012). However, Caco-2 cells



only resemble the morphology of monolayer cell barrier and not the mucous layer. Throughout the GI tract, mucin is continuously released from the goblet cells to form a viscoelastic layer that covers the gastrointestinal tract to protect the lumen and to constitute a diffusion barrier to molecules that are to be absorbed during the digestion process. (Kim and Khan 2013) To compensate this lack of function in Caco-2, TC7 cell line, a subclone of Caco-2, is co-cultured due to its ability to differentiate into mucus-secreting cells. (Pontier, Pachot et al. 2001)

3T3-L1 is a fibroblast that originates from mouse embryo, and the cell line does not accumulate lipid droplets until it has been treated with a specific set of chemicals. However, when treated with insulin, IBMX, and DEX at 2 days post-confluence, these fibroblast-like cells will start to accumulate lipid droplets. The samples which are thought to be effective on lipid degradation are then treated to these adipocytes. Remaining lipid droplets are stained with dyes of known absorbance value, most commonly Oil-red O to quantitatively measure the effectiveness of treatment. Garcia-Carrasco et al. had focused on extracts of leaf and root of *Taraxacum officinale*, and reported that the leaves extract has hypolipidemic ability on 3T3-L1 adipocytes (García-Carrasco, Fernandez-Dacosta et al. 2015). Although cells are a simple and reproducible model of physiological analysis, animal tests represent a higher complexity of an intact organism. Ahmed et al. had researched  $\beta$ -glucan extracted from mushrooms in animal feed in depth. It was verified that (Ahmed, Abdullah et al. 2017, Ahmed, Abdullah et al. 2017).

Although 3T3-L1 differentiates into adipocyte using by chemical stimulants, is not the only cell line that indicates hypolipidemic ability.

Hoang and coworkers reported the measurement of intracellular lipid accumulation using Hep G2 cell line (Hoang, Kim et al. 2015). Isolated from human hepatoblastoma, Hep G2 is also a cancer cell line used in various cellular analysis. However, it does not share the same mechanisms as 3T3-L1 cell lines described previously. Unlike 3T3-L1, which differentiated into adipocytes by chemicals and forms lipid droplets within the cell, Hep G2 accumulates lipid in intracellular regions. This is a specific model for the assessment of drugs or compounds for their functionality on improving fat accumulation in liver.

As consumers' preferences for natural materials have increased, search for new functional materials has been an topic of interest for many food, cosmetics and bio industries. In addition, under the pressure of various environmental issues including the controversy over toxic substances in various household goods that have occurred in Korea over the past several years in recent years, methods that can extract target active materials without the use of harsh chemicals are also considered as important research topics. Among the countless research papers regarding the analysis and functionality of natural compounds, polysaccharides extracted using mild extraction procedure with interesting functions were organized in Table 1.

**Table 1** Mild extraction, purification and analysis of polysaccharides

(Abbreviations – MW: molecular weight, DW: distilled water (temperature recorded when higher temperature than normal boiling point was used in the extraction), PS: polysaccharide, AO: antioxidative ability, MIC: metal ion chelating, DM: diabetes mellitus, FT-IR: Fourier transform infrared spectroscopy, SEM: scanning electron microscopy, ASE: accelerated solvent extractor, MeOH: methanol, AFM: atomic force microscope, CD: circular dichroism spectroscopy)

Material	Scientific name	Extraction	Physicochemical assay	Physiological benefit	Reference
Green algae	<i>Capsosiphon fulvescens</i>	0.1N HCl	Composition, MW, FT-IR, monosaccharide analysis	Low cytotoxicity, cytokine secretion	(Na, Kim et al. 2010)
Green algae	<i>Enteromorpha prolifera</i>	DW + Fractionation	Composition, MW, monosaccharide analysis	Anticancer, immunomodulatory	(Cho, Yang et al. 2010)
			-	<i>in vitro</i> and <i>in vivo</i> immunomodulator	(Kim, Cho et al. 2011)
Green algae	<i>Enteromorpha prolifera</i>	DW	IR, monosaccharide analysis	Antioxidant, hypolipidemic -> therapy for DM	(Lin, Wang et al. 2015)
Green algae	<i>Enteromorpha prolifera</i>	DW, enzymatic, acid, alkali	Composition, MW, viscosity, iron(III) complex assay	-	(Chi, Li et al. 2018)
Green algae	<i>Chlorella vulgaris</i>	DW	Composition, monosaccharide analysis, NMR	Immunomodulatory	(Tabarsa, Shin et al. 2015)
Green algae	<i>Ulva fasciata</i>	DW	Composition, viscosity, O/W emulsion stability	-	(Shao, Zhu et al. 2017)

Green algae	<i>Ulva pertusa</i>	DW + Fractionation	Composition, MW, monosaccharide analysis +) FT-IR, glycosidic linkage, NMR	Anticancer, immunomodulatory	(Tabarsa, Han et al. 2012)
Green algae	<i>Cladophora glomerata</i> Kützinger	DW	Composition, MW, monosaccharide analysis, glycosidic linkage, NMR	-	(Tabarsa, Lee et al. 2012)
Green algae	<i>Spirogyra neglecta</i> (Hassall) Kützinger	DW + Fractionation	Composition, MW, monosaccharide analysis, glycosidic linkage	Immunomodulatory	(Surayot, Hun Lee et al. 2016)
Green algae	<i>Monostroma nitidum</i>	DW + Fractionation	Composition, monosaccharide analysis, glycosidic linkage	Immunomodulatory	(Surayot, Wang et al. 2015)
Green algae	<i>Nostoc sphaeroides</i> kütz	DW	Composition, monosaccharide analysis, AO	AO, intracellular lipid, immunomodulatory	(Hoang, Kim et al. 2015)
Brown algae	<i>Laminaria japonica</i>	120 °C DW (Autoclave) + Fractionation	Total sugar, MW, monosaccharide analysis, FT-IR, glycosidic linkage, NMR	Immunomodulatory	(Liu, Su et al. 2018)
Brown algae	<i>Sargassum thunbergii</i>	DW + microwave	Composition, monosaccharide analysis, MIC, AO	AO	(Wang, Zhang et al. 2008)
Plant	<i>Chaenomeles sinensis</i> seed meal	DW + Fractionation	Composition, monosaccharide analysis, IR, AO	AO	(Ren, Chen et al. 2017)
			Monosaccharide analysis, MW, FT-IR, NMR, SEM, glycosidic linkage, AO, MIC	-	(Wang, Liu et al. 2017)
			+) Viscosity, storage stability, AFM, thermal characterization	-	(Wang, Liu et al. 2018)

Plant	<i>Artemisia sphaerocephala</i>	DW + microwave	Monosaccharide analysis, MW, SEM, MIC	AO	(Wang, Zhang et al. 2009)
		+) selenized	Monosaccharide analysis, MW, congo red binding, AFM	Antitumor	(Wang, Li et al. 2016)
Plant	Rice bran	Pre-treatment (acetone, MeOH) + DW	Monosaccharide analysis, MW, MIC, AO	AO	(Zha, Wang et al. 2009)
Plant	<i>Camptotheca acuminata</i> Fruits	DW + ultrasonic, microwave	SEM, MW, UV spectrum, IR, rheology	Antitumor	(Sun, Li et al. 2019)
Plant	<i>Picea abies</i>	140 °C DW (ASE)	Composition, lignin content, MW, glycosidic linkage,	Immunostimulant	(Le Normand, Mélida et al. 2014)
Plant	Corn silk	DW + enzymolysis, ultrasonic	Composition, MW, FT-IR, SEM, CD, AO	Anticancer, AO	(Chen, Chen et al. 2014)
Fungus	<i>Cordyceps gunnii</i>	RT-DW, hot DW, microwave, ultrasound, enzymolysis	Composition, MW, monosaccharide analysis, viscosity, polarimeter, SEM, CD	Antitumor	(Zhu, Dong et al. 2016)
Marine animal	<i>Stichopus japonicas</i>	DW + Fractionation	Composition, MIC, UV spectrum, FT-IR, MW	Immunomodulatory	(Cao, Lee et al. 2014)
Mush- room	<i>Grifola frondosa</i>	DW + Fractionation	Composition, MW, AO, MIC, lipid peroxidation	-	(Chen, Ma et al. 2012)

### **3.3. O/W emulsion system**

#### **3.3.1. Constituents of O/W emulsions**

An emulsion consists of two immiscible liquids, with one of the liquids dispersed as small spherical droplets in the other (Yoshida, Sekine et al. 1999). At this time, the substances are generally water and oil, and the two dispersed states are called the general emulsion system. In most foods, where the system base is water in most cases, the mean droplet size is usually between 0.1 and 100  $\mu\text{m}$ ; generally, if the mean droplet diameter is less than 1  $\mu\text{m}$ , the emulsion may be referred to nano-emulsion. But this standard is not solid and some researchers refer emulsion with larger droplet diameter with the same terminology.

The property of the emulsifier greatly affects the characteristics of the final emulsion. There are a few aspects to be considered for the selection of the emulsifier. Emulsifiers are generally composed of a hydrophilic ‘head’ and a hydrophobic ‘tail’, which allows them to remain stably between water and oil phases. They are classified as anionic, nonionic and cationic emulsifiers depending on the charge of the hydrophilic moiety in the head. However, for some emulsifiers, especially from a protein origin may change its electrostatic characteristics depending on the pH or ion concentration of the phase in which they are solubilized. To select the phase of which the emulsifier should be dissolved in depends on the hydrophile-lipophile balance (HLB) value of the emulsifier. This number indicated the relative affinity of the emulsifier for aqueous or oil phase; emulsifiers with high HLB may dissolve easier in aqueous phase, and vice versa. It is also always important

for an emulsifier to form a protective interfacial membrane that prevents the two immiscible phases from directly contacting each other.

With the adequate amount of emulsifier and oil ratio is incorporated, O/W emulsion is already a stable system itself. However, other materials or methods are integrated into the system to further enhance the stability against harsher environmental stress factors. One of the most common methods for emulsion protection is addition of macromolecules, mainly polysaccharides. These macromolecules are called ‘wall material’ for their protective function by compartmentalizing reactive oxygen species or pro-oxidants that causes oxidative stress to the oil droplets (Shimada, Fujikawa et al. 1992). While there are a number of wall materials available, not all materials are always compatible with all emulsifiers. Protein emulsifiers interact with wall materials and depending on the concentration of the added material; if the concentration is not enough, wall materials may interact with more than one droplet resulting in bridging flocculation. If the concentration is excessive, wall materials may form a polymer gel network, increasing the viscosity and even ‘squeezing out’ the water molecules from the network (Dickinson 1998). Not only wall materials are effective as protection; in fact, metal chelators are more efficient if the researcher is aiming to eliminate the effect of pro-oxidants. Many researchers have discovered that ferrous and ferric ions are major pro-oxidants in O/W emulsions, especially because emulsions droplets are negatively charged since the ions are attracted to the droplet interface (Faraji, McClements et al. 2004, Hu, McClements et al. 2004).

In this study, retinol is encapsulated in the oil droplet of O/W emulsion considering that most food base is water, and one synthetic emulsifier, Tween 20 and a natural emulsifier, whey protein isolate (WPI) was chosen for comparative observation. While Tween 20 relatively maintains its negative charge in different pH conditions, WPI becomes cationic in strong acidic solutions (below pH 4.3). Therefore, two 5mM pH buffers were chosen; phosphate buffer of pH 7 and sodium acetate buffer of pH 3.5, to assess the different interaction mechanisms and the protective ability of the *E.prolifera* extract in different pH conditions.

### 3.3.2. Properties of O/W emulsion filled hydrogel

Although a well-dispersed, homogeneous O/W emulsion is already a stable system solely, the Brownian motion and fluidity may cause the collision of droplets resulting in uneven enlargement of droplets leading to phase instability. Addition of water-soluble macromolecules, mostly polysaccharides or proteins with gelling ability, into the aqueous phase is a widely used method in the industry to overcome such unwanted quality decrease (Geremias-Andrade, Souki et al. 2016).

Emulsion-filled gels are defined as a complex colloidal material formed by the combination of an emulsion dispersion and a gel phase. The combination of these two systems can be emulsion-filled gels or emulsion gels. The most commonly used gelling agents for this complex system was protein, but some recent studies have used polysaccharides to produce gels. The emulsion-filled gels consist of the substitution (partial



or total) of the water of a gel matrix by an emulsion, where the particles of emulsion are incorporated in this matrix and the materials have solid-like rheological properties (Geremias-Andrade, Souki et al. 2016).

The characteristics of filled hydrogel is affected by both gel matrix and emulsion droplets (van Vliet 1988); (1) concentration of gelling macromolecule, (2) oil content and characteristics, (3) emulsion droplet–matrix interactions, (4) morphology of the matrix network, and the (5) gelation process. The replacement of fat while maintaining quality attributes is a need in these foods and is a challenge to the food industry (Geremias-Andrade, Souki et al. 2016). Several bio-macromolecules can be used to replace fat due to their interesting functionality, such as gelling agents that improve structural properties which compensates the unique satiety and mouthfeel of fat. To accomplish this task, studying the rheological, physicochemical, and structural properties of emulsion-filled gels is critical in the development and application of these systems in modern food industry in terms of fat replacers.

## 4. Materials & Methods

### 4.1. Materials

Dried *Enteromorpha prolifera* harvested from the coast of Wando Island in the Jeonam province of Korea was purchased from a harvesting wholesaler. The raw material was washed with deionized water, dried and milled using a blender and stored at  $-65^{\circ}\text{C}$  deep freezer until further extraction process. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferrozine were purchased from Sigma Aldrich. EDTA was purchased from Duksan Chemicals.  $\text{FeCl}_2$ , 95% ethanol, sodium phosphate, sodium acetate, and sodium bicarbonate were purchased from Daejung Chemicals. All-*trans*-retinol and Tween 20 was purchased from Sigma Aldrich Chemical Co. Whey protein isolate (WPI, Product code: 9500) was obtained from Protient, Inc. Soybean oil was purchased from a local retailer and used without further purification. All chemicals were of analytical grade.

### 4.2. Methods

#### 4.2.1. Extraction procedure

Extraction procedure was performed as follows (Cho, Yang et al. 2010); 10 g of milled raw material was depigmented with 500 mL of 95% ethanol in a  $75^{\circ}\text{C}$  water bath with for 5 hrs to remove pigments and other lipophilic components. The sample was centrifuged at 1,800 g,  $10^{\circ}\text{C}$  for 10 min, and then dried at  $40^{\circ}\text{C}$  overnight. The depigmented raw material was then extracted twice with deionized water (5 g sample in 100 mL DW) at  $65^{\circ}\text{C}$  with constant mechanical stirring for 4 hours. The extracts were

centrifuged at 14,392 g, 10 °C for 10 min. 95% ethanol was added to the supernatant to obtain a concentration of 30% (v/v), and then the solution was placed at 4 °C for 4 hrs. After centrifugation at 14,392 g, 10 °C for 30 min, the precipitate was collected and more ethanol was added to the supernatant to obtain a final ethanol concentration of 70% (v/v). The solution was placed at 4 °C overnight and centrifuged again to collect the precipitate. The collected precipitates were filtered while washing with 95% ethanol on a cellulose-nitrate filter membrane (0.45µm pore size, Whatman GmbH., Dassel, Germany) until it became visibly flaky. Crude samples were dried completely until the weight reached an equilibrium, and was kept in a screw cap container at -20 °C until usage.

#### 4.2.2. Composition analysis

##### 4.2.2.1. Carbohydrate analysis

The total carbohydrate content were determined by the phenol-H<sub>2</sub>SO<sub>4</sub> method, using glucose as a standard. 25 µL of 80% phenol solution was added to 1 mL of either glucose or sample solutions of diverse concentrations and vortexed thoroughly. To this mixture, quickly add 2.5 mL of concentrated sulfuric acid and let set until the final mixture reaches room temperature, and absorbance was read at 490 nm. (Nielsen 2010)

The sulfate content of the polysaccharides was determined by the BaCl<sub>2</sub>-gelatin method using K<sub>2</sub>SO<sub>4</sub> as a standard after hydrolyzing the polysaccharide in 0.5M HCl at 95 °C for 5 hr. 0.3% w/v gelatin solution in 100 mL was dissolved in 60 °C, and kept at 4 °C overnight. The following morning, 2g BaCl<sub>2</sub> was dissolved in the gelatin solution. 1.2 mL

of HCl hydrolyzed samples of various concentration was added to 0.3 mL of BaCl<sub>2</sub>-gelatin solution, and incubated in ambient temperature for 10 to 20 min. Absorbance was read at 360 nm, and standard curve was plotted using K<sub>2</sub>SO<sub>4</sub>.

#### 4.2.2.2. Total protein content analysis

Total protein content was measured by Lowry's method. (Gerhardt 1994, Barbarino and Lourenço 2005) Two solutions were formulated prior to the analysis; Solution A: 0.5 g copper sulfate pentahydrate CuSO<sub>4</sub>·5H<sub>2</sub>O and 1 g trisodium citrate dihydrate Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O were dissolved in 100 mL DW; Solution B: 10 g sodium carbonate Na<sub>2</sub>CO<sub>3</sub> and 2 g sodium hydroxide NaOH were dissolved in 500mL DW. For the experiment procedure, 1 mL of solution A and 50 mL of solution B were mixed to make solution C, and 5 mL of Folin-Ciocalteu's phenol reagent were diluted with equal volume of DW to make solution D. 1 mL of solution C was added to 0.2 mL of sample EPE solution and incubated for 15 min away from fluorescent light in room temperature. Then 0.1 mL of solution D was added to initiate color formation and incubated for another 30 min. Absorbance was read at 750 nm. Standard curve ( $R^2 = 0.998$ ) was plotted by measuring the absorbance of known concentration of bovine serum albumin to estimate protein concentration in EPE samples.

#### 4.2.2.3. Organic elemental analysis

To confirm the chemical composition analysis data, elemental analysis was performed for carbon, hydrogen, nitrogen, oxygen and sulfur using FLASH-2000 (Thermo Fischer Scientific. Co.) and thermal conductivity detector. Briefly, a known amount of EPE is combusted at 975°C. The combustion products are passed over a copper reduction tube to convert the oxides of N into molecular N. Carbon dioxide, water vapor and N are homogeneously mixed at a known volume, temperature and pressure. The mixture is released to a series of thermal conductivity detectors/traps, measuring in turn by difference, H (as water vapor), C (as carbon dioxide) and N (as N<sub>2</sub>). The original method was based on sea water analysis of the US Environmental Protection Agency. (Zimmermann, Keefe et al. 1997)

#### 4.2.3. Physicochemical properties of the extract

##### 4.2.3.1. Gel permeation chromatography (GPC) analysis

EPE samples were dissolved in DW to a concentration of 0.5%, and mechanical stirring was applied overnight to ensure full dissolution. The sample was injected into a HPSEC (high performance size exclusion chromatography) system after filtering by a 5.0 µm disposable membrane filter. The HPSEC system was composed of two running columns (OH-Pak 804, OH-806 HQ, Shodex), and a refractive index detector (ProStar 355 RI Detector, Varian Inc., Australia). The columns were run at 50 °C and the flow rate of the

mobile phase (degassed distilled water) was 0.4 mL/min. Pullulans standards (Shodex Standards, Japan) were used as a molecular weight standard.

#### 4.2.3.2. Antioxidant ability analysis

Ferrozine can quantitatively chelate ferrous ions( $\text{Fe}^{2+}$ ) to form a violet colored complex. The presence of other chelators results in a decrease of the formation of the ferrozine-ferrous ion complex. Measurement of the color reduction estimates the chelating activity of the sample to compete with ferrozine for ferrous ions (Dinis, Madeira et al. 1994).

350 $\mu\text{L}$  of extract solution with various concentrations was added to 175 $\mu\text{L}$  of 10mM ferrous chloride. After 10 min incubation at ambient temperature, 252.5 $\mu\text{L}$  aliquots were respectively mixed with 1mL ethanol to precipitate the extract-ferrous ion complex. 525 $\mu\text{L}$  supernatant was then mixed thoroughly with 700 $\mu\text{L}$  of 5mM ferrozine solution. The solution was incubated in dark at room temperature for another 10min. The absorbance was read at 562nm. 1% EDTA solution (pH 9) was taken as positive control.

The % ferrous ion scavenging activity of the extract was calculated using the following formula,

$$\text{Ferrous ion scavenging ability (\%)} = 1 - \frac{(\text{Abs sample} - \text{Abs EDTA})}{(\text{Abs blank} - \text{Abs EDTA})} \times 100$$

The radical scavenging activity of the extract was determined according to the method of Shimada et al. (Shimada, Fujikawa et al. 1992) with some modifications. 0.1 mM DPPH in methanol was prepared 24 hours before the experiment to ensure complete dissolution and was kept in screwcap vial away from fluorescent light. 550  $\mu\text{L}$  DPPH

solution was added to 550  $\mu$ L sample solutions. Blank samples were prepared with respective sample concentrations and pure methanol instead of DPPH to reduce interference with sample hue. All mixtures were incubated in dark at ambient temperature for 20 min. The absorbance of the 1 mL mixture was read at 517 nm. 550  $\mu$ L dw with 550 $\mu$ L DPPH was taken as control.

The % radical scavenging activity of the extract was calculated using the following formula,

$$DPPH \text{ RSA}(\%) = \left[ 1 - \frac{Abs \text{ sample} - Abs \text{ blank}}{Abs \text{ control}} \right] \times 100$$

#### 4.2.3.3. Rheological properties of EPE

Rheological measurements of the emulsion was measured using a rheometer (AR1500ex, TA Instrument Ltd., UK). 20 mm parallel plate geometry was used with a gap of 1mm. To assess the gelation properties of the extract, a concentration of 50 g/L solution was prepared in 5 mM sodium acetate buffer (pH 3 and 5) and 5 mM phosphate buffer (pH 7 and 9). The temperature was equilibrated in a pre-heated 65  $^{\circ}$ C water bath before loading onto the bottom plate of the rheometer. The followings were conducted consecutively to overview the gel-set temperature; (1) time sweep test (duration: 2 hr, frequency: 1 Hz, fixed strain: 0.5%, temperature: 4  $^{\circ}$ C) and (2) frequency sweep test (frequency: 0.1 to 10 Hz, fixed strain: 0.5%. temperature: 4  $^{\circ}$ C). In a preliminary experiment, it was observed that EPE is able to form thermos-reversible gel. Therefor another set of experiment included (1) time sweep test (duration: 2 hr, frequency: 1 Hz, fixed strain: 0.5%, temperature: 4  $^{\circ}$ C) and (2) temperature sweep oscillatory test (temperature range: 4  $^{\circ}$ C to 65  $^{\circ}$ C, ramp rate

2 °C/min, frequency: 1 Hz, fixed strain: 0.5%, equilibration time: 10 min). To observe heat reversibility, the ramp was run backwards down to 4 °C and this cycle was repeated twice.

#### 4.2.4. Physiological properties of the extract

##### 4.2.4.1. Toxic effect of the extract

###### 4.2.4.1.1. Caco-2 cell culture

Caco-2 cells were distributed from Korean Cell Line Bank (KCLB #30037.1) at passage number 26. Cells were cultured in advanced Dulbecco's modified eagle media supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 100 U/mL penicillin-streptomycin solution in a humidified atmosphere (5% CO<sub>2</sub>, 95% air, 37°C). Media was changed every other day. Cells were subcultured at 70-80% confluency. (Natoli, Leoni et al. 2012) Cells from passage number 30~40 were used for all of the experiments.

###### 4.2.4.1.2. WST-1 cytotoxicity assay

Water soluble tetrazolium (WST-1) assay was carried out as follows. To observe the impact the extract has on the viability of cells; Trypsinized Caco-2 cells were diluted with full media to  $8.0 \times 10^4$  cells/cm<sup>2</sup> and 100µL /well was seeded in 96 well plates. After cell growth reached 80~90% confluency, full media change was carried out. Extracts, extract fortified emulsion or the supernatant of the digested emulsion (diluted 1:1 in growth media) was added to each well. The plates were incubated for another 4 hr. 10 µL of WST-1 solution was then applied to all wells, and incubated for 4 hr. (van Meerloo, Kaspers et



al. 2011) Light absorbance at 570 nm was read with microplate reader (Tecan Group Ltd., Männedorf, Zürich, Switzerland). (Yu and Huang 2010) All procedures were done in triplicates on the same plate to minimize the variability of the singular procedure, and a single data was calculated as mean value of respective plates.

#### 4.2.4.2 Hypolipidemic ability of extract fortified emulsion

##### 4.2.4.2.1. 3T3-L1 Cell culture and differentiation

Pre-adipocyte 3T3-L1 cells were distributed from Korean Cell Line Bank (KCLB #10092.1) and were cultured in media previously described (Advanced Dulbecco's modified eagle media supplemented with 10% fetal bovine serum, 4mM L-glutamine and 100U/mL penicillin-streptomycin solution) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Media was changed every other day. Cells were subcultured at 70~80% confluency. Cells from passage number 9 or 10 were used for adipogenesis experiments.

To differentiate cells into adipocytes, two days after reaching confluence, differentiation was initiated by the addition of adipogenic agents (Insulin 5 µg/mL, Dex 0.25 µM, and IBMX 0.5 mM) to the growth media. After the completion of differentiation, full media change was performed with post differentiation media including insulin but not Dex and IBMX. (García-Carrasco, Fernandez-Dacosta et al. 2015)

#### 4.2.4.2.2. Lipid accumulation analysis

Adipogenesis is the process of differentiation of pre-adipocytes into adipocytes (fat cells), the primary fat storage *in vivo*. The accumulation of adipocytes is the basis for obesity, a significant risk factor in many diseases related to metabolic syndrome, including type 2 diabetes mellitus, atherosclerosis, cancer and cardiovascular disease, etc. (Ravussin and Smith 2002) Adipogenesis assay was carried out using Cayman Adipogenesis Assay kit (cat no.10006908) following the manufacturer's instructions.

#### 4.2.5. Extract fortified O/W emulsion system

##### 4.2.5.1. Preparation and storage of fortified O/W emulsion

5 mg of retinol was sonicated with 4 g corn oil to ensure complete dissolution. Tween 20 and WPI were dissolved in 5 mM phosphate buffer (pH 7) or 5 mM sodium acetate buffer (pH 3.5) respectively followed by blending 4% (w/w) corn oil whether with or without retinol using a high speed blender (ULTRA-TURRAX model T25 digital, IKA, Germany) for 1 min at 12,000 rpm. The coarse emulsion was then processed with high-pressure microfluidizer (Picomax MN 250A, Micronox, Seongnam, Korea) three times at 7000 psi to eliminate gravitational effect on droplets. (Lee and Decker 2011) The emulsifier to oil ratio was 1:6 (0.666% w/w emulsifier in total emulsion) and retinol to oil ratio was 1:800 (50 ppm retinol in total emulsion). After the emulsion was prepared, 0.2% sodium azide was added to prevent microbial contamination. 0, 0.5, 1.0, 2.5% of EPE was mixed overnight to ensure complete dissolution in the aqueous phase. All samples were

maintained at either 4, 25, or 40 °C to observe stability over 6 days of storage.

#### 4.2.5.2. Rheological properties of fortified O/W emulsion

The instrument and geometry was as previously mentioned. To measure the effect of EPE on the viscosity of emulsion samples, steady state flow test (shear rate: 0.1 to 100 1/s, temperature: 4 °C, sample period: 10 s) was conducted. To ensure that the cause for the hypothesized difference in emulsion viscosity is EPE, (1) blank pH buffers (pH 7 and 3.5) which consists the aqueous phase of the emulsions, (2) 2.5% EPE solutions prepared with respective buffers, (3) blank emulsions without dissolving EPE and (4) emulsion samples fortified with 2.5% EPE were all taken into account.

#### 4.2.5.3. Stability analysis of fortified O/W emulsion

##### 4.2.5.3.1. Particle size distribution and $\zeta$ - potential analysis

The mean droplet diameters, size distribution and surface  $\zeta$ - potentials were measured every other day using Z-sizer Nano ZS90. (Malvern Instruments, UK) The solutions were analyzed for size distribution and  $\zeta$ - potential tendencies. The mean droplet diameters, size distribution and surface  $\zeta$ - potentials were measured using Zetasizer Nano ZS90 (Malvern Instruments, UK) The mean particle diameters and size distributions were determined by dynamic light scattering using a detection angle of 90°,  $\zeta$  - potentials were determined by photon correlation spectroscopy. All measurements were performed at 25 °C, and the data reported are mean values of triplicate determinations.

#### 4.2.5.3.2. Retinol retention analysis

Retinol concentration in the oil phase was measured every 48 hours. 90µl of each sample was extracted with 1,710 µL of methanol (20-fold dilution) by vortexing. The mixture was then centrifuged at 20,138 xg, 10 °C for 10 min and the supernatant was filtered using 0.45 µm PVDF syringe filter to eliminate large particles or impurities. Absorbance was read at 325 nm and extract fortified emulsion without retinol was taken as blank for corresponding fortified emulsion prepared with retinol. Standard curve ( $R^2 = 0.998$ ) was plotted by measuring the absorbance of known concentration of retinol in methanol to estimate retinol concentration in emulsion samples.

#### 4.2.5.3.3. Creaming analysis

The rate of the emulsion where creaming had occurred during storage at ambient temperature was measured by the volume ratio of the cream layer formed on the surface area of the emulsion. To assess the underlying mechanism for the instability, microscopic photo of the cream layer was taken with an inverted microscope (CKX-53, Olympus, Japan) at 4,000X magnification.

#### 4.2.5.4. Digestion assay of fortified O/W emulsion

##### 4.2.5.4.1. *In vitro* digestion model

The *in vitro* digestibility of fortified emulsion samples were measured using model systems of oral, gastric, and intestinal phases, as reported in previous studies.(Mun,

Kim et al. 2015) In oral phase, 7.5 mL emulsion and equal volume of simulated saliva fluid (SSF) containing mucin and  $\alpha$ -amylase were mixed by continuous stirring in 37 °C water bath for 10min. For the gastric phase, 3.2 g pepsin dissolved in simulated gastric fluid (SGF) was added to the oral digest. (Sarkar, Goh et al. 2009, Sarkar, Goh et al. 2009) Then the pH was adjusted to 2.5 to initiate pepsin activity and incubated at 37 °C for another 2 hr with continuous agitation. In order to mimic the small intestine phase, 1.5 mL of saturated salt stock solution (10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 150 mM NaCl) and 3.5mL of bile extract solution were added with continuous monitoring of the pH. Then 2.5 mL of freshly prepared pancreatin suspension (187.5 mg/2.5 mL 5 mM phosphate buffer, pH 7) was added to the mixture and incubated at 37 °C for 2 h. The final digest was once again adjusted to pH 7.0 for further analysis.

The simulation carried out in this study cannot completely mimic the physiological processes of *in vivo* GI tract. In particular, constant mechanical stirring was applied to samples during the process but it does not match the physical characteristics oral mastication nor gastric/intestinal peristalsis. Gastric lipase, which may play an important role in lipid digestion, was also eliminated. Nevertheless, this model can be used to rapidly analyze and quantify effects of different concentrations of sample formulations within the GI tract. (Lopez-Pena, Zheng et al. 2016)

#### 4.2.5.4.2. Core material bioaccessibility analysis

The bioaccessibility of retinol was determined after the samples had been passed through the simulated GIT tract as previously described.(Qian, Decker et al. 2012, Salvia-Trujillo, Qian et al. 2013) After each step of the digestion, 5 mL of samples were centrifuged at 1,644 g for 40 min at 4 °C. Aliquots of 3 mL of the raw digest and supernatant were respectively mixed with 3 mL of chloroform and vortexed thoroughly until no visible phase separation was observed. The mixture was then centrifuged at 315 g for 10 min, and the bottom chloroform layer was collected. The procedure was repeated twice more for both raw digest and supernatant samples to completely extract the dissolved retinol. The bottom chloroform layer was added to the previous one and absorbance was read at 325 nm. Pure chloroform was used as reference. The concentration of retinol extracted from a sample was determined from a calibration curve of absorbance versus the retinol concentration in chloroform.

### 4.3. Statistical analysis and data display

All data were analyzed using SPSS for WINDOWS (version 25.0, IBM Statistics, Chicago, IL, US). The one-way ANOVA test was performed followed by Duncan's multiple range test, and reported as means  $\pm$  standard deviations. A *p*-value of less than 0.05 was considered statistically significant. Figures were drawn with SigmaPlot, and tables were made using Microsoft Excel. All experiments were done in triplicates unless otherwise mentioned in the method description.

## 5. Results and Discussion

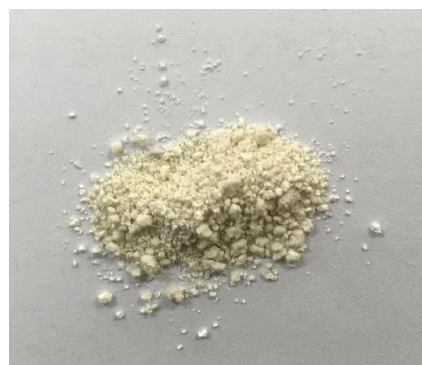
### 5.1. Water-soluble extract of *Enteromorpha prolifera* (EPE)

#### 5.1.1. Composition analysis of EPE

The extracted substance from *E.prolifera* was light, fine powder with little to none yellowish hue. Traces of chlorophylls in the algae cell, which may not have been completely depigmented in the extraction process, are the main cause for this.

**Table 2.** Yield and chemical composition of *Enteromorpha prolifera* extract (EPE).

Criteria	%	
Yield	13.62±1.07	
Total carbohydrate	47.23±4.15	
Total protein	12.43±1.08	
Sulfate	19.09±5.09	
Elemental analysis	C	27.3±1.24
	H	5.14±0.01
	O	36.18±0.19
	N	1.97±0.04
	S	4.22±0.08

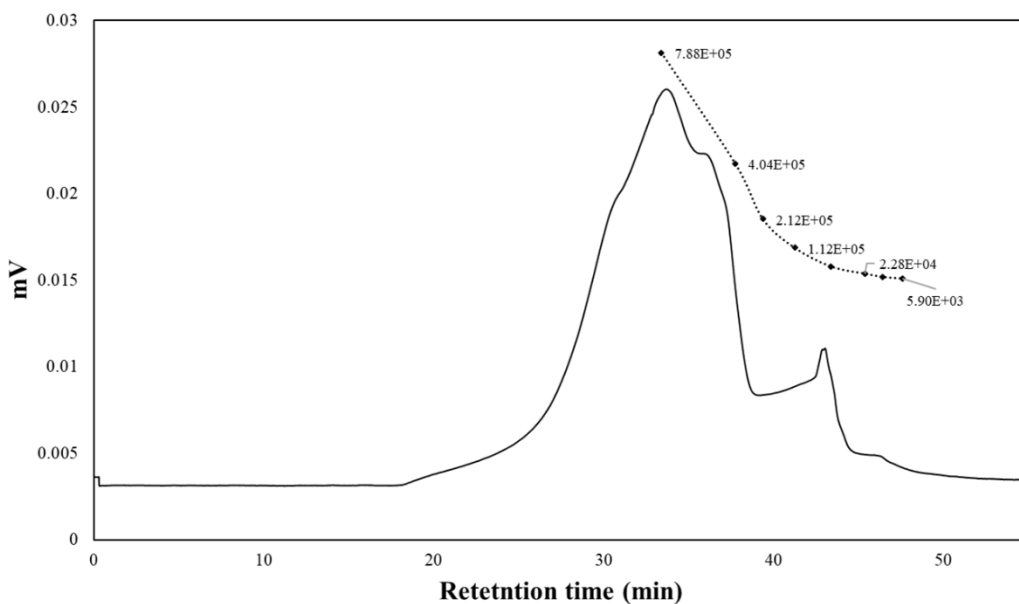


Phenol-sulfate method for total carbohydrate analysis showed reliable OD values within the range 0.2 to 0.9 when the concentration range was between 0.01 ~ 0.1% EPE in DW. However, this calculated value is no solid evidence of the actual total sugar in EPE samples. While the standard curve for the measurement was plotted using glucose, one of the main building blocks of EPE and other algae-derived polysaccharides are acidic sugars including uronic acid, which may cause slight differences in the experimental process and consequently in the colorimetric measurements.

The sulfate content of EPE had a wider range of standard deviation compared to other components. Results from each respective extraction products were distributed within a wide range, and even samples taken from the same extraction product did not show greater uniformity. Algae polysaccharides are usually highly sulfated, but this content may vary depending on the season, environment, and the origin of where the raw material was collected.

### 5.1.2. Physicochemical characteristics of EPE

#### 5.1.2.1. Molecular weight analysis by GPC



**Figure 1.** GPC Analysis data of EPE. Pullulan standard curve was plotted over the sample curve.



Pullulan standard curve was plotted above the sample curve. This indicates that the samples have a molecular weight ranging from 5,000~80,000 Da. The results corresponds well with previous researches regarding the molecular weight of crude polysaccharide extracts from *E.prolifera* (Cho, Yang et al. 2010, Yu, Li et al. 2017).

#### 5.1.2.2. Antioxidative ability of EPE

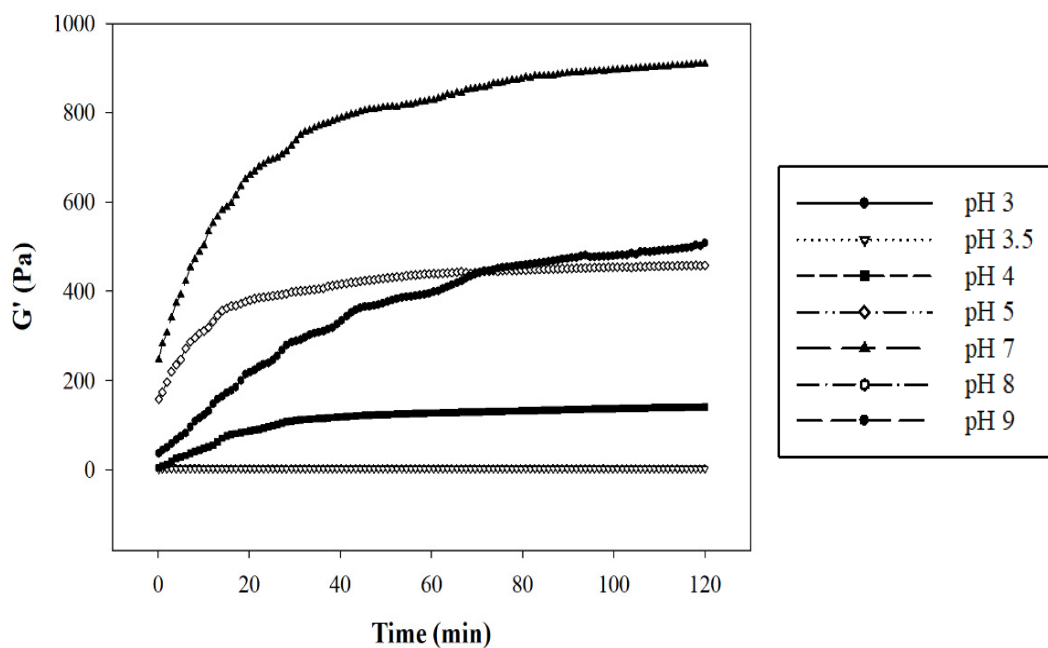
A number of research groups published research and review papers regarding the antioxidant ability of polysaccharide extracted from marine algae (Zhang, Yu et al. 2003, Wijesekara, Pangestuti et al. 2011, Ngo and Kim 2013, Tang, Gao et al. 2013). The term ‘antioxidative activity’ may refer to any mechanism associated with the reduction of oxidative stress by suppressing one of any reasons that induces stress. Oxidation and the mode of action of antioxidants is a highly complicated process where, in most cases, more than one key factors are involved. Thus, different mechanisms must be studied to conclude in which step of oxidation the antioxidant of question takes effect on. Sulfated polysaccharide from algae in particular can scavenge hydroxyl free radical, superoxide anions, and prevent hemolysis in cells induced by  $H_2O_2$ . It is said to be the underlying mechanism of immunomodulatory, anticancer and anticoagulant ability (Wang, Zhang et al. 2008, Zhang, Wang et al. 2010, Ngo and Kim 2013, Tang, Gao et al. 2013, Hoang, Kim et al. 2015).

It was hypothesized that sulfated polysaccharides, consisting of negatively charged monomers, may possess the ability to scavenge, or perhaps even chelate, ferrous

ions. However, the results did not show any significant evidence that EPE binds with metal ions strong enough to reduce oxidative stress derived from it. Nevertheless, it should not be concluded that EPE has no ferrous ion chelating ability. The colorimetric chemical used for the detection of remaining ferrous ion in the solution, ferrozine, is also a chelating agent that binds strongly with metal ion. It was reported in a previous research that EPE extracted with hot water had lower ability to bind with ferric ions compared to the fraction extracted with acid or alkali (Chi, Li et al. 2018). Similar result was observed for DPPH analysis of scavenging free radicals, where EPE did not show significant effect on.

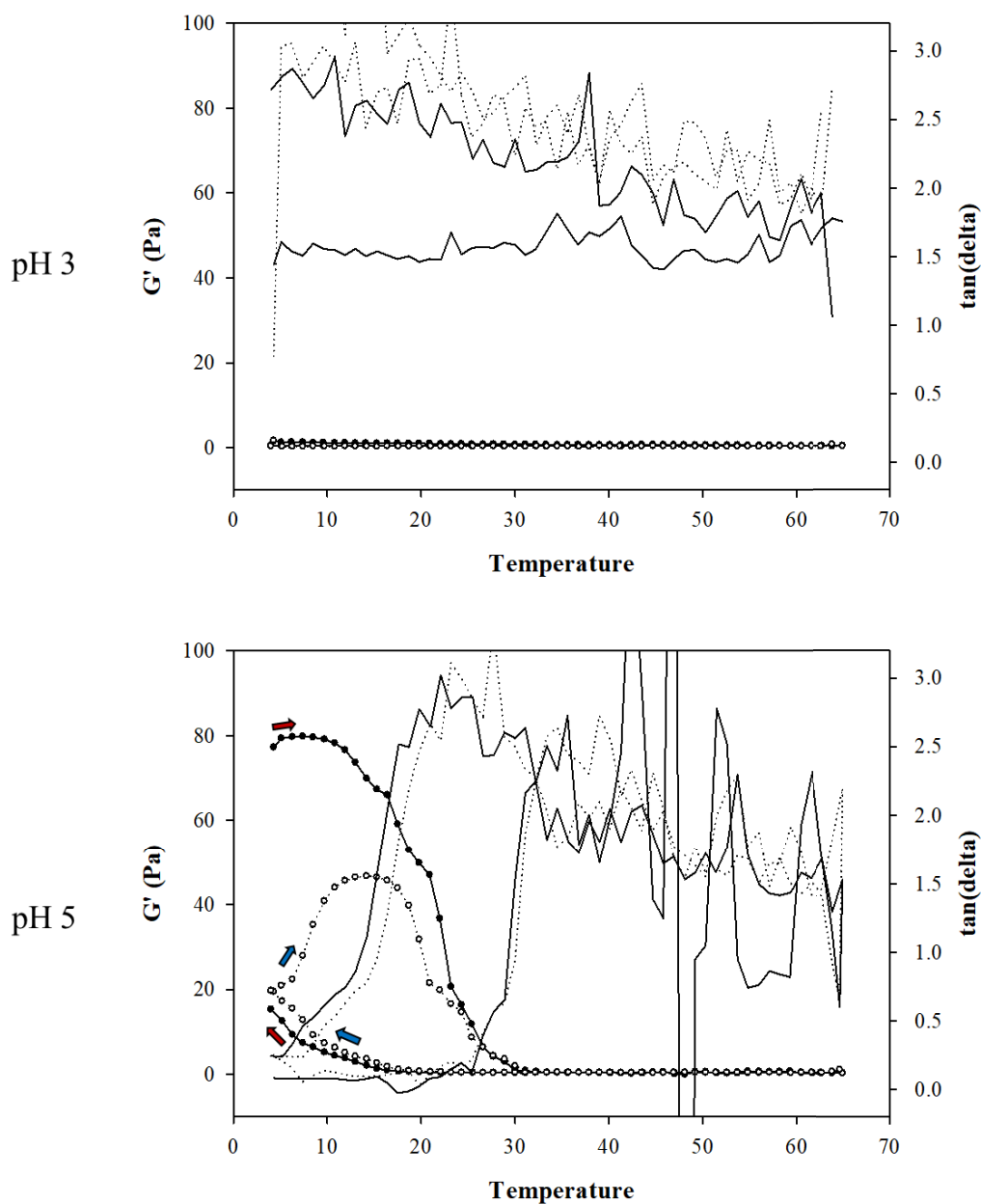
#### 5.1.2.3. Gelling properties of EPE

As discussed shortly before, in many polysaccharides extracted from algae, EPE has a high content of negatively charged monomers and therefore sensitive to environmental pH. In this section, the properties of EPE gel depending on the pH was studied. EPE samples showed a strong tendency to form gel depending on the temperature, concentration and pH. The concentration of EPE to form gel was fixed at 5% in respective pH buffers regarding the data from preliminary experiments in which 2.5% was also taken into consideration. Within the studied pH conditions, 2.5% solutions did not set to gel during 24 hr storage in 4°C. All pH values were given at buffer pH prior to dissolving samples; due to the highly viscous characteristics of 5% EPE solutions, it was not possible to accurately measure the pH of samples after dissolution.

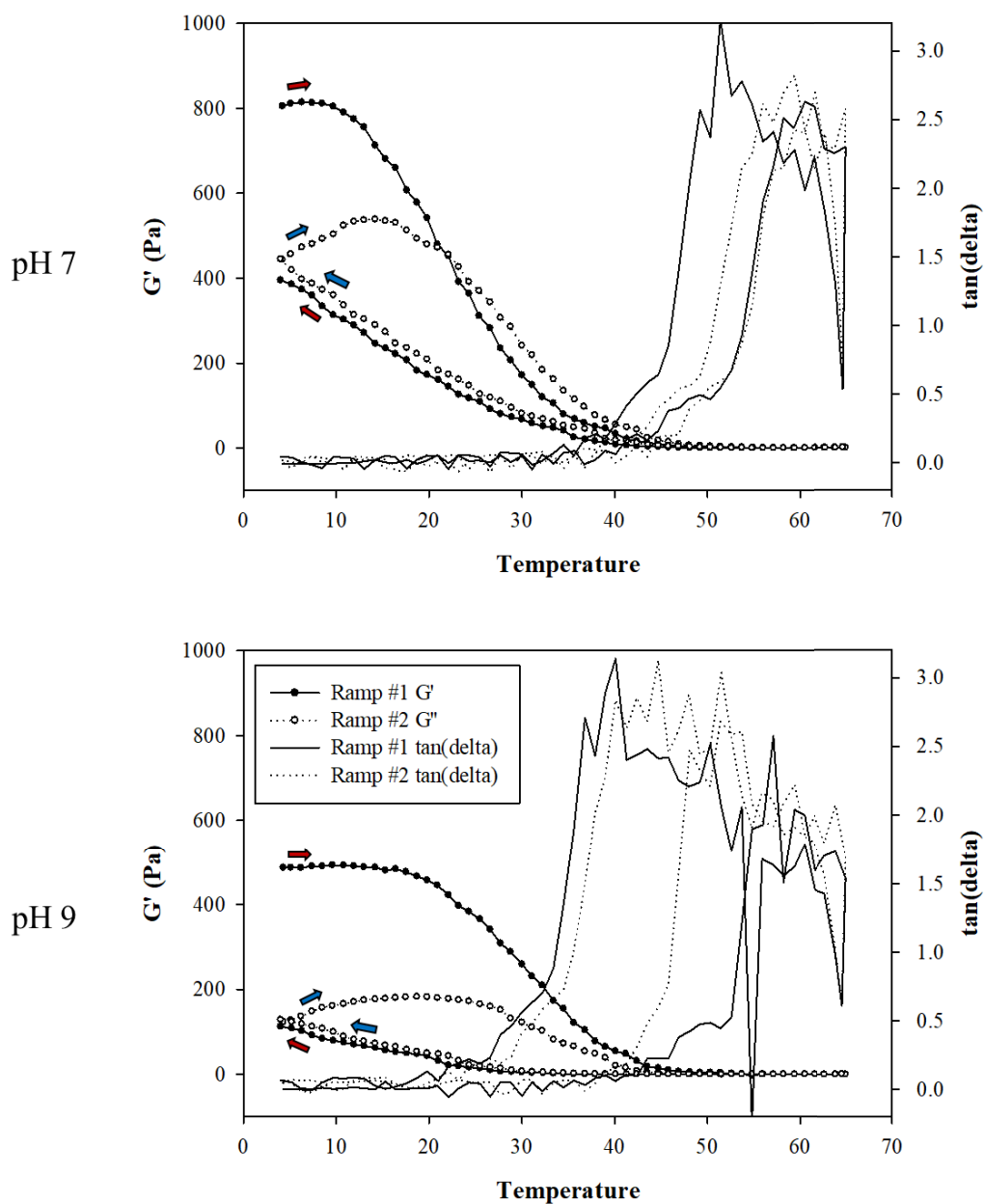


**Figure 2.** G' value during time sweep test of EPE dissolved in pH buffers.

Given that concentrations of EPE is maintained at 5%, gel formulated with neutral buffer showed the greatest G' value during time sweep at 4 °C. Samples dissolved in strong acidic buffers pH 3 and pH 3.5 buffers did not form gel, where G' and G'' plotted a parallel line with G'' higher than G'. Respective G' values reached a plateau after 2hr, and then either temperature ramp or frequency sweep test was run. During the temperature ramp on 5% solutions of EPE dissolved in various pH (3, 5, 7 and 9), modulus and  $\tan \delta$  versus temperature was plotted as in the following figures.



**Figure 3.** Temperature ramp profile of 5% EPE in various pH solutions. Red and blue arrows indicate the ramp direction for ramp cycle #1 and 2, respectively.



**Figure 3(Cont'd).** Temperature ramp profile of 5% EPE in various pH solutions. Red and blue arrows indicate the ramp direction for ramp cycle #1 and 2, respectively.

Uneven, dynamic fluctuation in  $\tan \delta$  was observed after the gel melted to sol. The variables in the calculation of  $\tan \delta$  are the moduli of soft solids, and therefore measurement in sol, with characteristics closer to liquid than solid, cannot be accurate. Aside to that, data presented was clear in point that EPE have the ability to form thermoreversible gels. Thermoresponsive, or also referred to as thermoreversible gels have a significant importance in the field of drug delivery. The formulation of gel matrix leads to changes in gelation properties, including the control of gelling and meltin temperatures of the final product is possible. It is also notable that the critical sol-gel transition temperature differs by pH. For further applications, modifying EPE gels for drug delivery or bioactive molecular protection, this temperature and gel strength may be optimized by simply adjusting the pH of the solution formulated.

As mentioned previously in the literature review section, many polysaccharides from marine organisms including agar and carrageenan are gelling agents. However, not all marine polysaccharides form thermo-reversible gel while being soluble in cold water. This results suggest that EPE is an easily-handled macromolecule that can be manipulated by the handler for the production of a gel with desired characteristics.

## 5.2. Physiological functionality of EPE

### 5.2.1. Cytotoxicity of EPE

Although the species *E.prolifera* is generally regarded as safe (GRAS), to analyze the accurate safety for the extracts, WST-1 test was done on Caco-2 and 3T3-L1 cells treated with different concentrations of EPE. It must be taken into account that macromolecules including polysaccharides are not always suitable for direct cellular assays.

### 5.2.2. Hypolipidemic ability of EPE

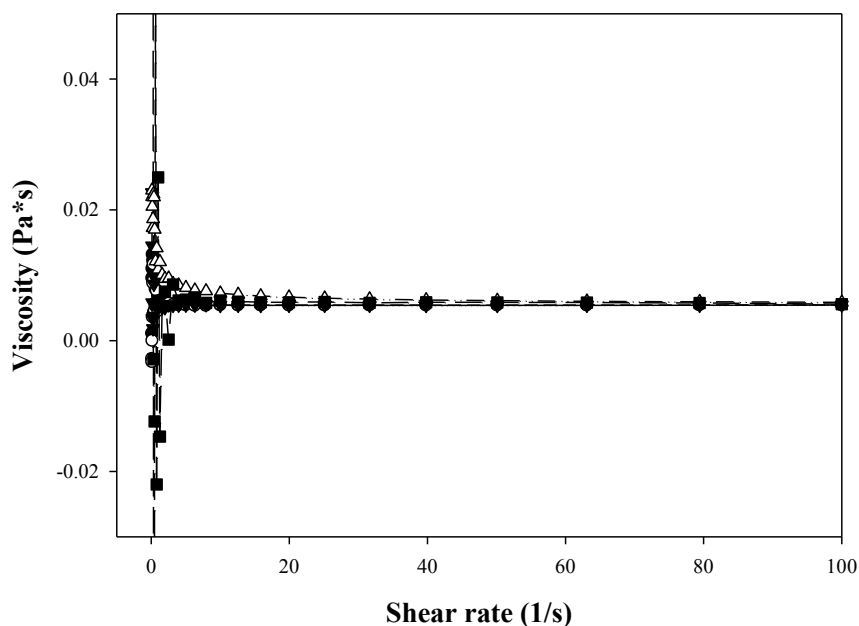
It has been reported in a number of research papers that the polysaccharide fraction from the water-soluble extracts of *E.prolifera* has hypolipidemic activity, reducing lipid accumulation in the circulatory system (Tang, Gao et al. 2013, Teng, Qian et al. 2013). In this study, a test on 3T3-L1 cells was conducted to assess whether the extracts had the same ability in cellular level by directly degrading lipid droplet accumulated in adipocytes. This assay is only able to assess the whether the target material that directly reduces lipid molecules by degrading them, for the assay does not take metabolic pathways and the systematic reaction present in the human body.

When treated with extremely high concentrations of EPE, the data may be deceiving as if the samples exhibited a sudden increase in lipid degradation. However, when 3T3-L1 was treated with more than 1.0% EPE, toxicity was observed on almost 80% of the cells.

### 5.3. Characteristics of O/W emulsion fortified with EPE

#### 5.3.1. Rheological characteristics of emulsion

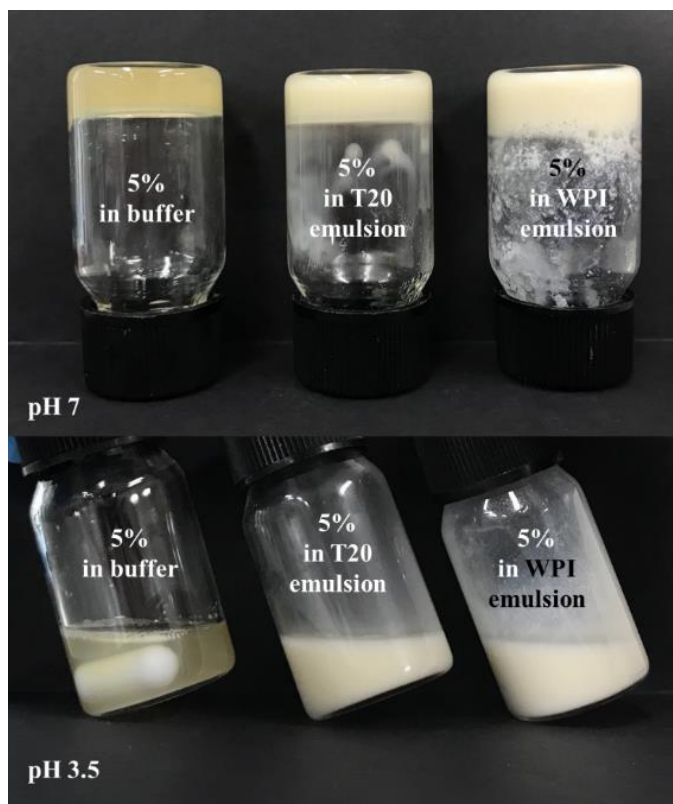
O/W emulsions containing 4% oil formulated with WPI or Tween 20 has a Newtonian flow property.



**Figure 4.** Viscosity measurement of DW and all 4 blank emulsions prepared without the addition of EPE (Tween 20, WPI and pH 7, pH 3.5). Legends were omitted due to the uniformity of plots.

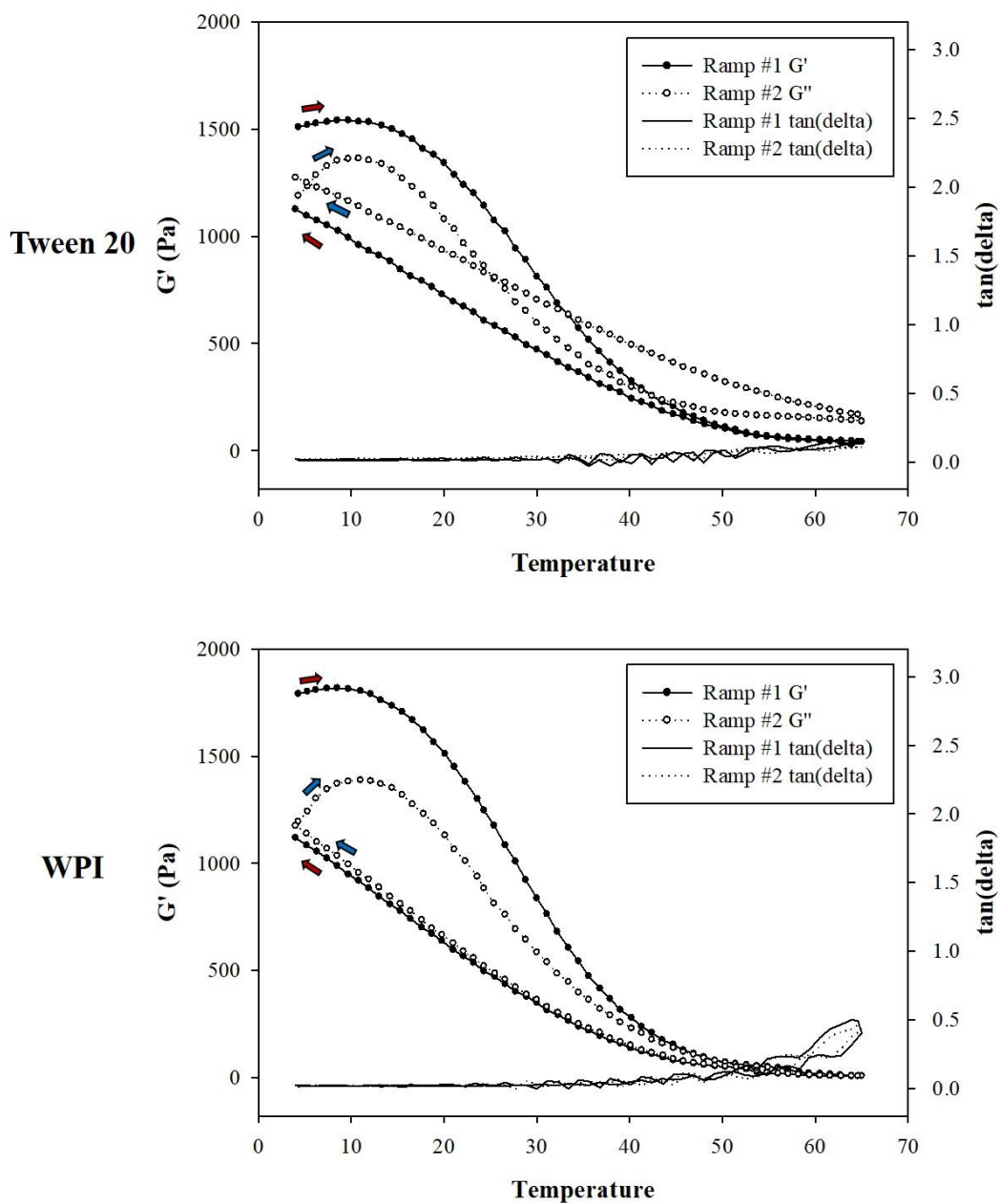
In all blank emulsions prepared without the addition EPE, the flow test results did not differ from DW, resulting in plots almost impossible to tell one from another. However, the addition of EPE in the aqueous phase caused the difference.





**Figure 5.** Images of 5% EPE in respective buffers and emulsion samples of pH 7 and 3.5.

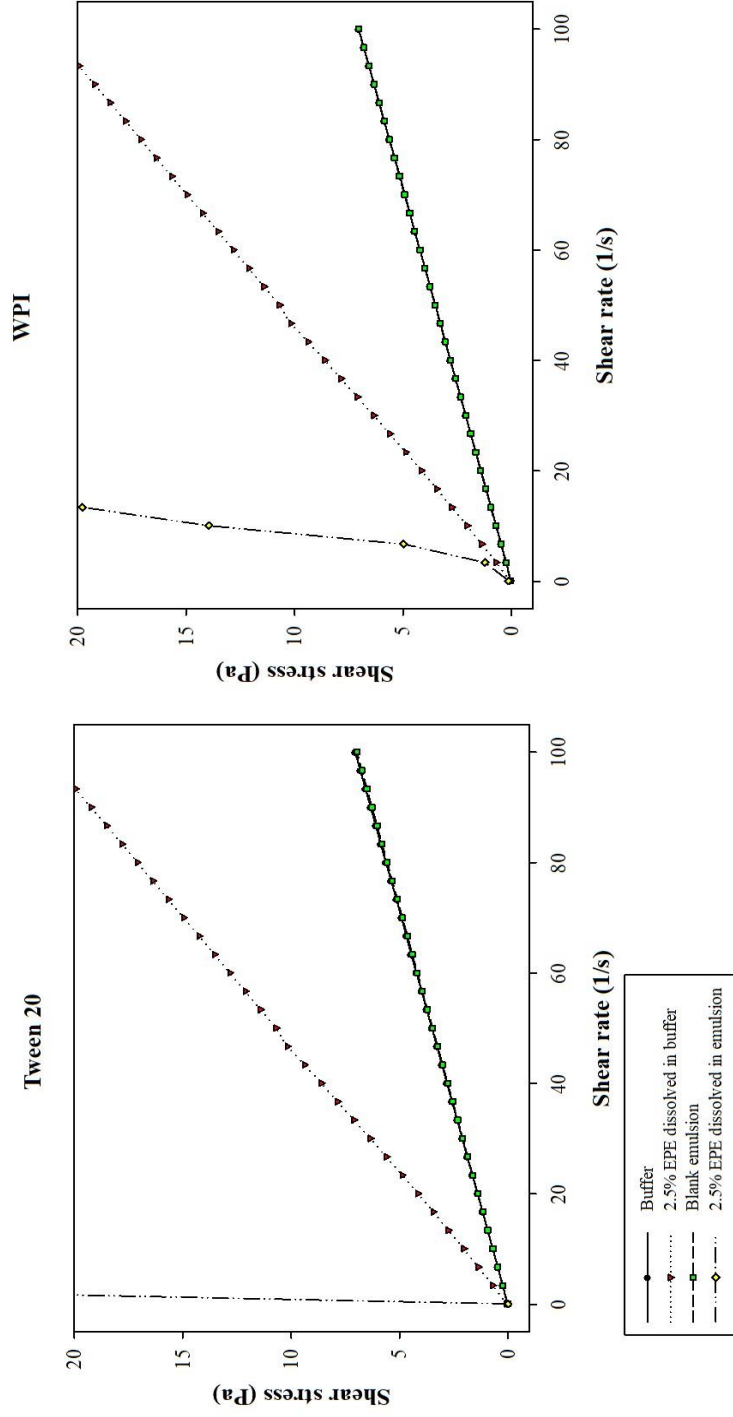
5% (w/v) EPE was dissolved in each emulsions with mechanical stirring. The pH-dependent melting property of EPE did not apparently differ in emulsion samples compared to buffers, in which 5% solution in pH 7 gelled and pH 3.5 solution did not.



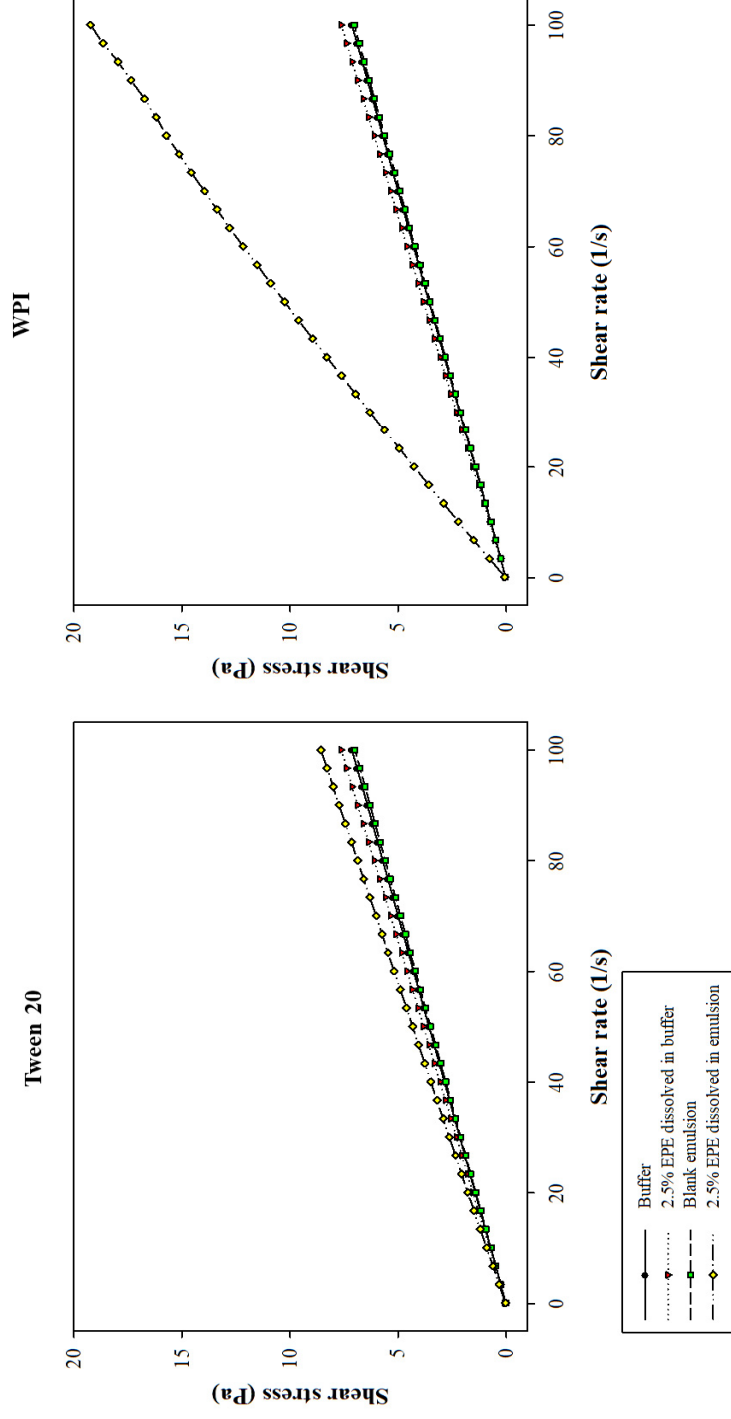
**Figure 6.** Temperature ramp profile of Tween 20 and WPI emulsions (pH 7) fortified with 5% EPE. Red and blue arrows indicate the ramp direction for ramp cycle #1 and 2, respectively.

Temperature ramp test on 5% EPE emulsions also showed thermo-reversibility. Notably, these gels had higher  $G'$  values than that of the corresponding pH buffer gels. It is a commonly observed in filled hydrogel phenomenon when gelation occurs using emulsion as 'filler' material. Oil droplets in emulsion does not interact with the hydrophilic gelling agents, and are much larger than water molecules being sufficient to support the gel network by physically filling up the empty spaces between the junction zone of polymers.

Elastic modulus ( $G'$ ) value of fortified WPI emulsion gel was higher than that of Tween 20 emulsion gels when time sweep of 2 hr ended, but after restoration of gel network  $G'$  recovered to a similar value around 1100 Pa. Also as it was in the temperature ramp test for EPE buffer gels,  $G'$  value of emulsion also seemed to increase until shortly after the second ramp cycle begin.



**Figure 7A.** Synergistic increase of viscosity of Tween 20 and WPI emulsions fortified with 2.5% EPE in pH 7

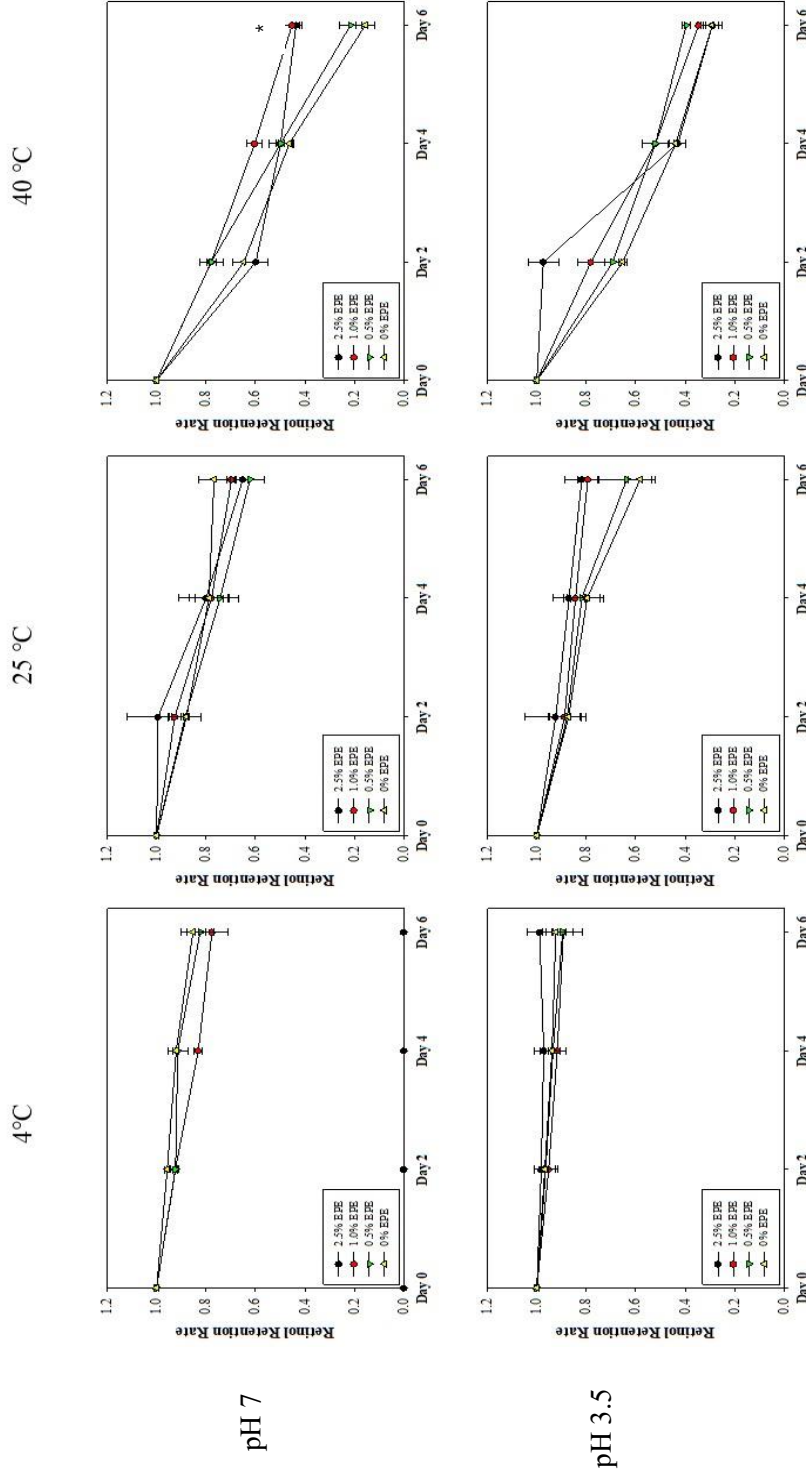


**Figure 7B.** Synergistic increase of viscosity of Tween 20 and WPI emulsions fortified with 2.5% EPE in pH 3.5

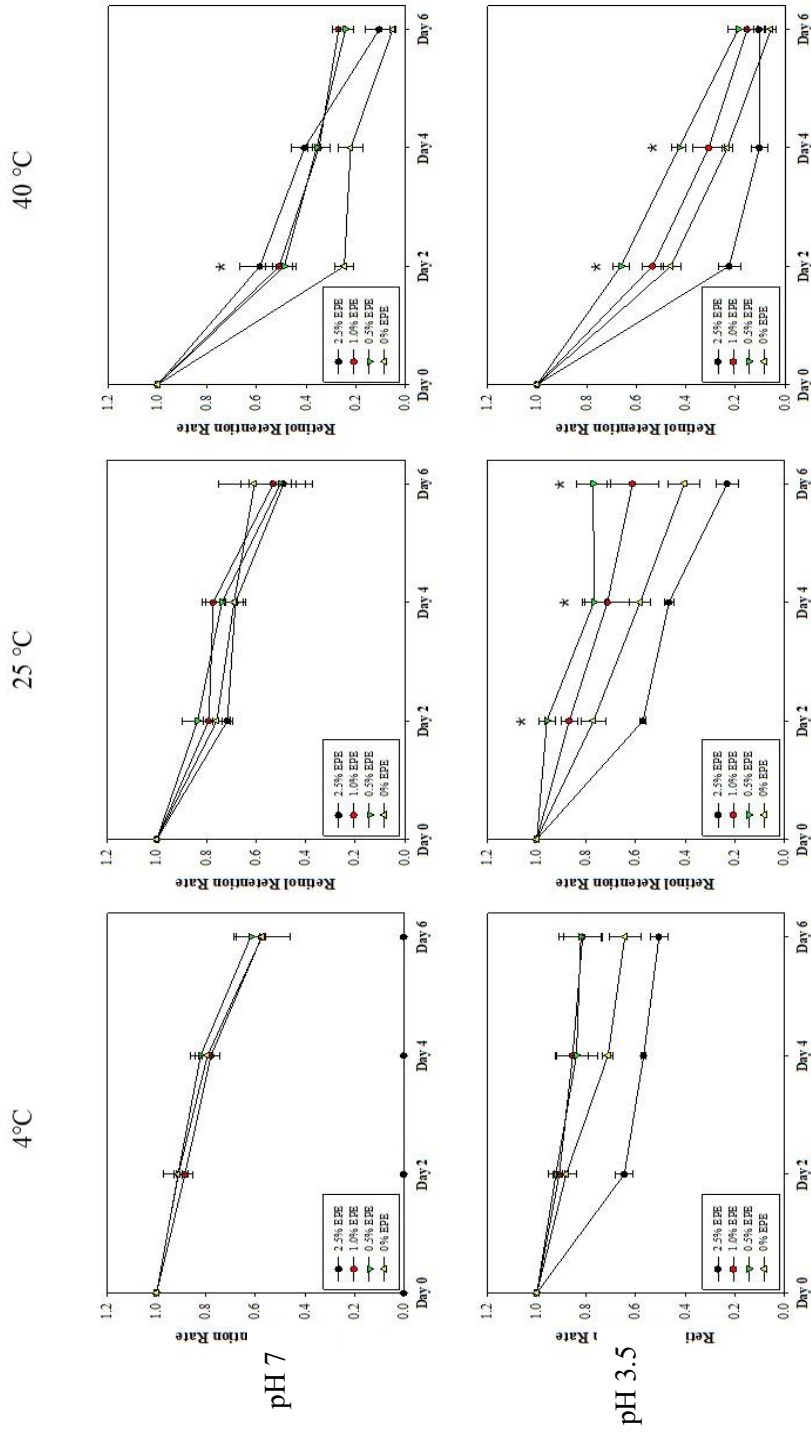
Although EPE did not set gel with 2.5% concentration in all buffers and emulsions, a synergistic increase in the viscosity was observed in emulsion samples. The shear stress of respective pH buffers, 2.5% EPE solution and blank emulsions were plotted for visual comparison on viscosity of the samples. For pH 7 emulsions, both 2.5% Tween 20 and WPI emulsions showed a dynamic increase of shear stress compared to 2.5% EPE solutions. The overall trend was similar for pH 3.5 emulsions, with the viscosity increasing when EPE was dissolved in emulsion rather than in buffers.

### 5.3.2. Storage stability

Emulsions were formulated with emulsifiers Tween 20 or WPI, using 5 mM phosphate (pH 7) or sodium acetate buffer (pH 3.5) as aqueous phase, containing 0, 0.5, 1.0 and 2.5% of the extracts. 4 °C samples were kept refrigerated, 25 °C was in ambient temperature away from air conditioning supplies and 40 °C was met with heat block. The blank data regarding Tween 20 and WPI emulsions with 4% oil prepared in pH 7 buffers corresponds well with previous research published from our lab (Park, Mun et al. 2019). Stability during storage was observed in means of mainly retinol retention within the oil droplets, change in size distribution. The following was plotted for the time-dependent retinol stability during 6 days of storage period.



**Figure 8A.** EPE concentration-dependent retinol retention of emulsion formulated with Tween 20, under different pH (pH 7 and 3.5) and storage temperatures (4, 25, and 40 °C) during 6 days of storage.



**Figure 8B.** EPE concentration-dependent retinol retention of emulsion formulated with WPI, under different pH (pH 7 and 3.5) and storage temperatures (4, 25, and 40 °C) during 6 days of storage.

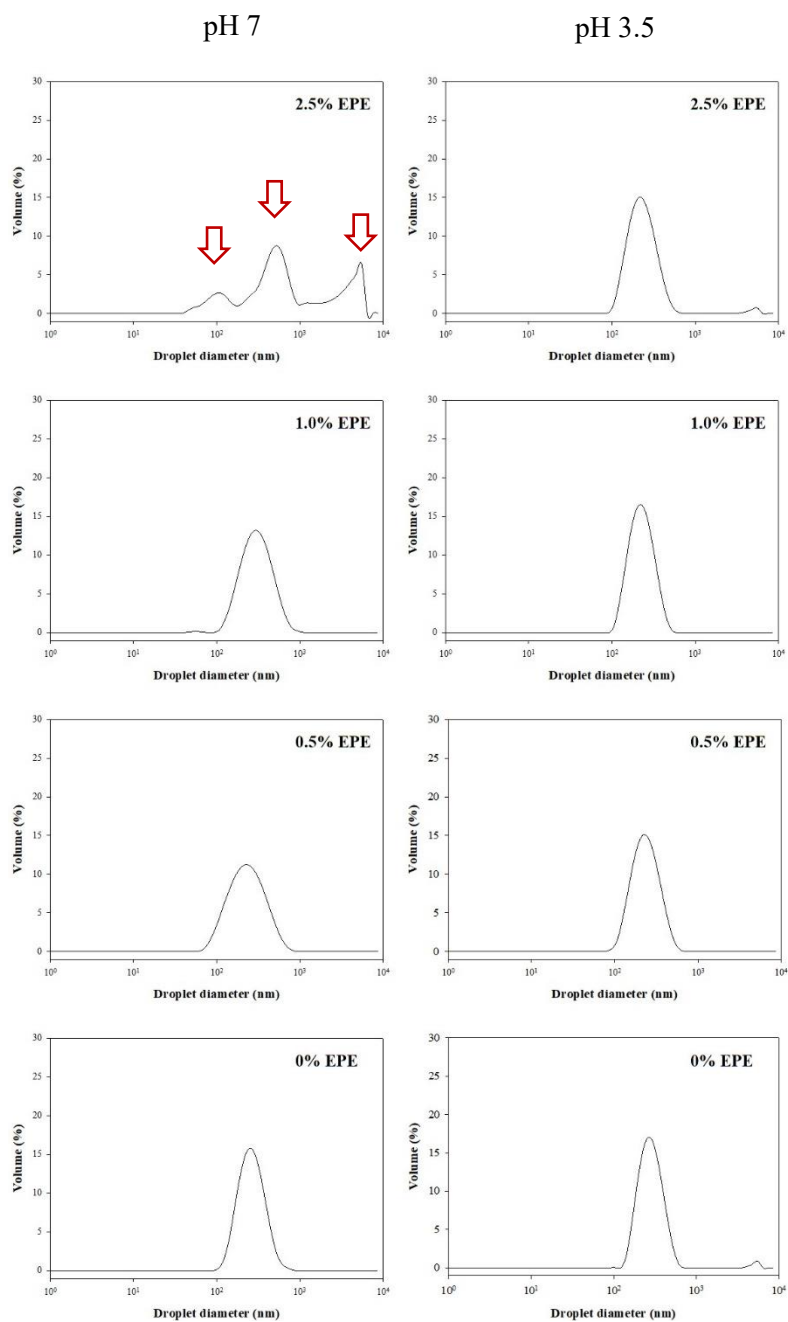




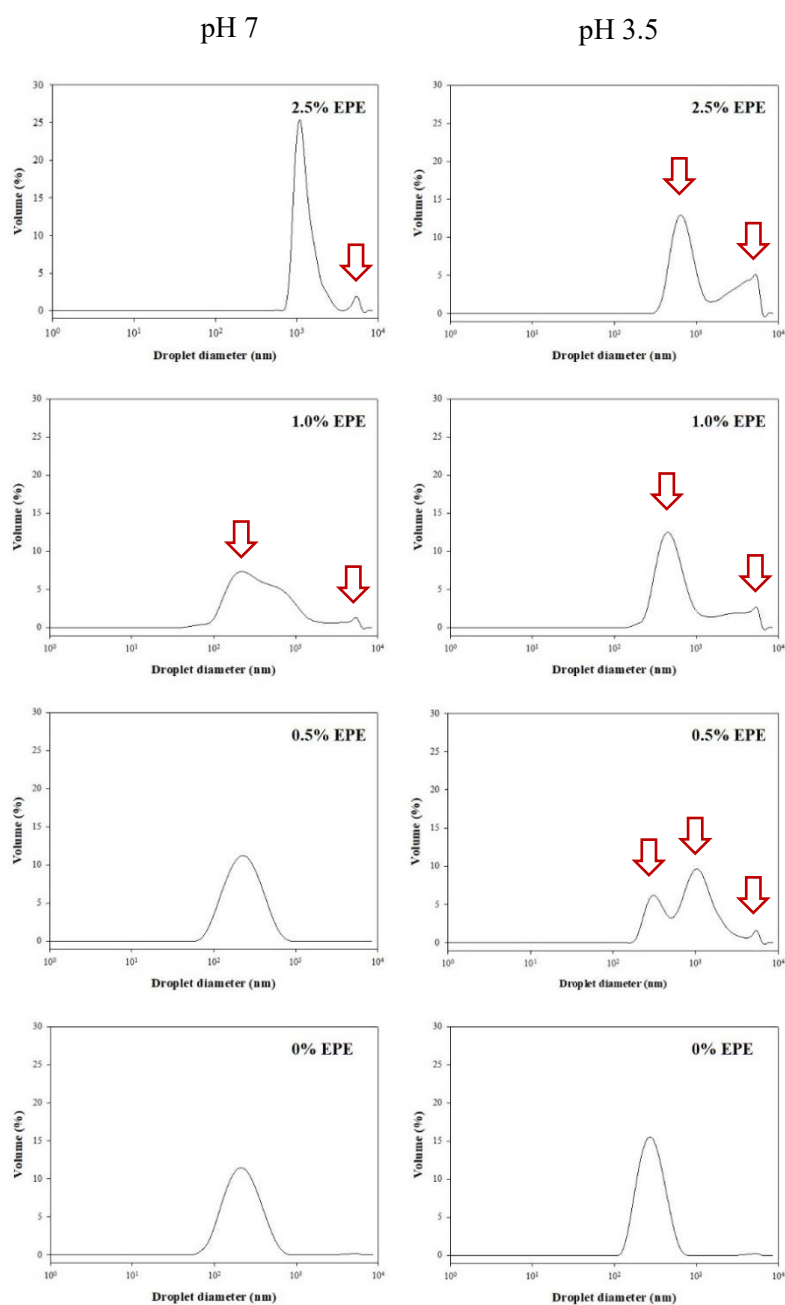
However, fortification by EPE in WPI emulsions has led to meaningful increase in retinol stability especially for pH 3.5 emulsions stored in 25 °C (Retention for blank:  $40.33 \pm 6.46\%$ , 0.5%:  $77.16 \pm 6.81\%$ , 1.0%:  $61.26 \pm 10.49\%$ , 2.5%:  $22.91 \pm 4.34\%$ ). In WPI emulsion in pH 3.5, EPE concentration may had an optimal value around 0.5%, with excessive addition negatively affecting the stability of retinol. In addition to this, it was also notable that in severe conditions where both acidic and temperature stress were present in WPI emulsion, addition of 0.5% and 1.0% EPE did not affect the final retention but decreased the degradation rate of retinol in early storage step. Addition of excessive EPE in pH 3.5 WPI accelerated the degradation of retinol in early stage of storage.

To assess the effect of emulsifier and EPE interaction depending on the pH of the emulsions, Figure 10 was drawn. Each bar represent the final retinol concentration in respective emulsion samples after 6 days of storage. In blank emulsions without any addition of EPE, retinol incorporated in Tween 20, pH 7 emulsions was the most stable as expected. In Tween 20 emulsion, although no dynamic difference was observed under most conditions, in pH 3.5, addition of more than 1.0% EPE increased the retention rate of retinol retention.

All-trans retinol and its relatives readily undergo degradation in the presence of high moisture, low pH, high temperature (Hwang and Ludescher 2002), and oxidative stress. It is also known to be photosensitive, especially under UV light. The degrading reaction, which is common on conjugated double-bond systems, may lead to an overall decrease in total activity of vitamin A incorporated in the system.



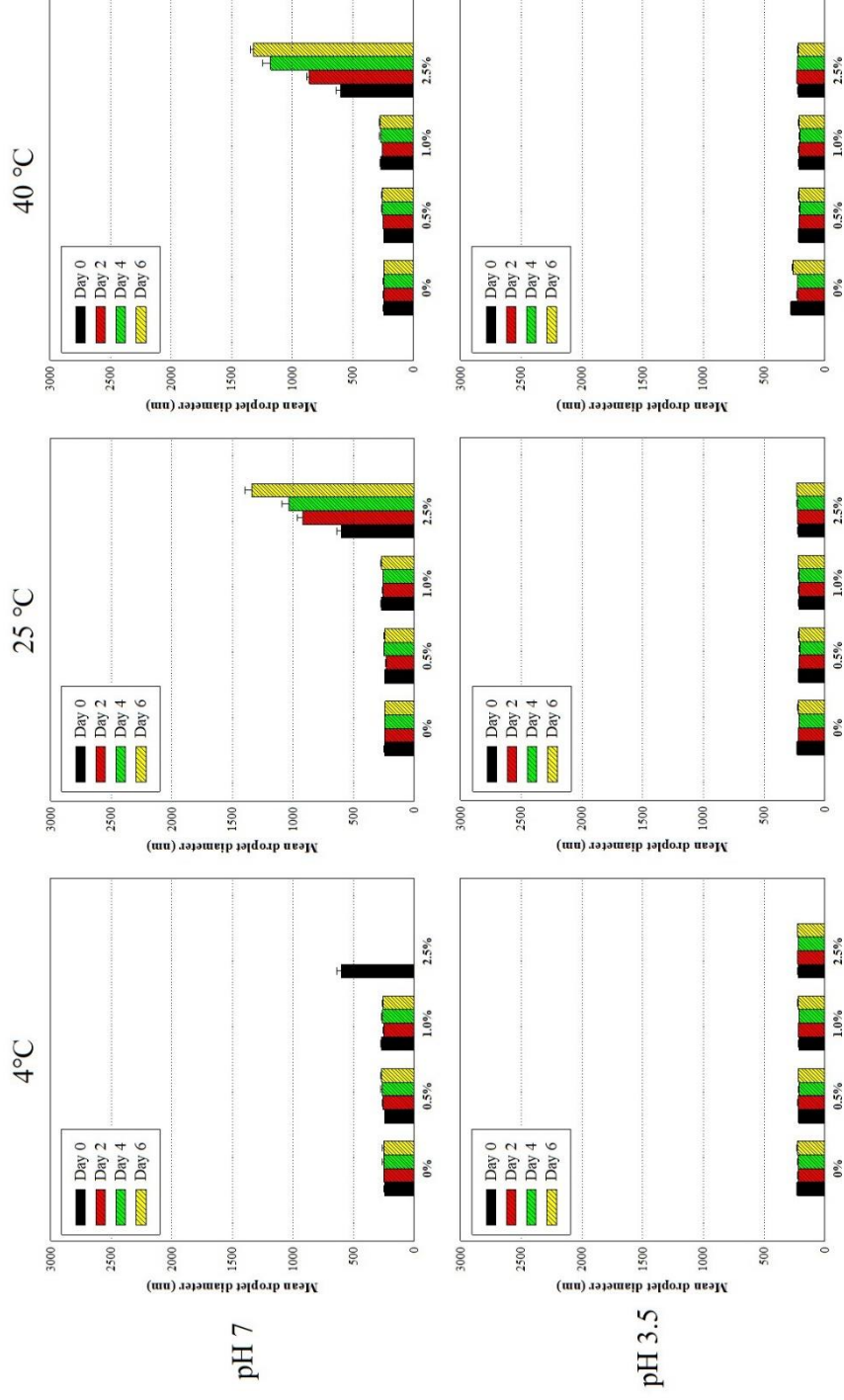
**Figure 10A.** Droplet diameter distribution of Tween 20 emulsions with different pH and EPE concentrations



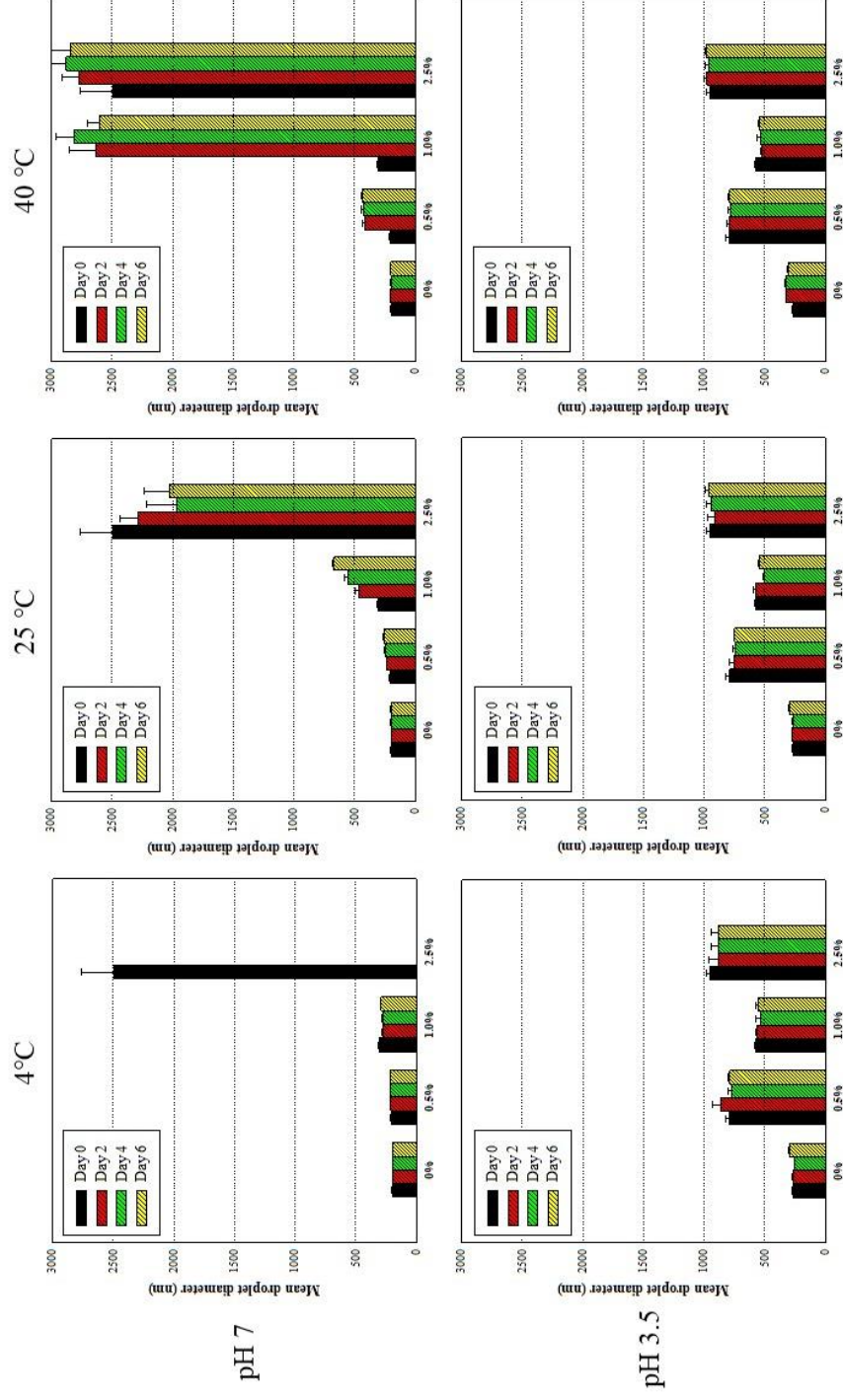
**Figure 10B.** Droplet diameter distribution of WPI emulsions with different pH and EPE concentrations.

Nano-sized emulsions are of great interest as a delivery system of bioactive compounds that are sensitive to environmental stress factors (ie. temperature, pH, UV, reactive oxygen species, prooxidants). Although the definition of nanoemulsion differs from one researcher to another, it can be generally said that the Z-average value of the droplet diameter within the range of 100 nm to 5  $\mu$ m can be referred to as nanoemulsion.

Within this range, core material stability is affected by the homogeneous dispersion of the oil droplets rather than the absolute size of each droplets. This was the reason why the distribution of droplets were plotted in Figure 11. PDI value and distribution of peak is an important indicator that give us predictions on how the droplet size and retinol retention will change. However, this figure alone cannot not give any confirmative information on the actual values.



**Figure 11A.** Change in mean droplet diameter of emulsion formulated with Tween 20, under different pH (pH 7 and 3.5) and storage temperatures (4, 25, and 40 °C) during 6 days of storage.



**Figure 11B.** Change in mean droplet diameter of emulsion formulated with WPI, under different pH (pH 7 and 3.5) and storage temperatures (4, 25, and 40 °C) during 6 days of storage.

**Table 3.** Change in  $\zeta$ -potential value during storage. Data displayed in means  $\pm$  stdev (mV). Lower case alphabets in IpH 7 WPI emulsion indicated groups with no significant difference ( $p>0.05$ ). Only pH 3.5 WPI emulsion without addition of EPE showed positive value in net surface charge.

	Storage period	0%	0.5%	1.0%	2.5%
Tween 20 pH 7	0 Day	-22.3 $\pm$ 1.45	-22.9 $\pm$ 1.32	-19.3 $\pm$ 1.04	-24.2 $\pm$ 0.69
	6 Day	-21.5 $\pm$ 1.63	-24.1 $\pm$ 1.58	-23.9 $\pm$ 2.01	-28.9 $\pm$ 0.72
Tween 20 pH 3.5	0 Day	-6.11 $\pm$ 0.42	-7.28 $\pm$ 0.23	-6.89 $\pm$ 0.44	-8.84 $\pm$ 0.31
	6 Day	-5.52 $\pm$ 0.20	-6.83 $\pm$ 0.31	-7.56 $\pm$ 0.38	-9.94 $\pm$ 0.42
WPI pH 7	0 Day	-51.10 $\pm$ 3.14 <sup>a</sup>	-43.3 $\pm$ 3.27 <sup>b</sup>	-40.2 $\pm$ 3.18 <sup>bc</sup>	-34.8 $\pm$ 3.09 <sup>c</sup>
	6 Day	-52.10 $\pm$ 3.87 <sup>a</sup>	-37.1 $\pm$ 2.86 <sup>c</sup>	-34.5 $\pm$ 1.97 <sup>c</sup>	-34.4 $\pm$ 2.64 <sup>c</sup>
WPI pH 3.5	0 Day	22.80 $\pm$ 1.74 *	-32.10 $\pm$ 2.30	-34.3 $\pm$ 1.73	-33.3 $\pm$ 2.28
	6 Day	23.9 $\pm$ 1.29 *	-28.1 $\pm$ 1.65	-31.4 $\pm$ 1.69	-28.1 $\pm$ 1.65



Changes in size distribution of emulsions are plotted in Figure 12. The data from Day 2 to 6 in both pH 7 emulsions with 2.5% EPE stored at 4 °C was omitted from the graph as in Figure 9 due to gelation and it was unable to accurately analyze the droplet size. In both Tween 20 and WPI emulsion samples, pH 7 emulsions showed greater fluctuation with the droplet size increasing rapidly with the addition of EPE and especially during 40 °C storage. Also addition of 2.5% EPE in pH 7 emulsions caused a sudden increase in mean droplet diameter. This cannot be stated as instability in oil droplets. Instead, the rheological properties of EPE must be taken into account. Flow test profile from Figure 8 confirmed that the addition of 2.5% EPE in emulsion has a synergistic effect on viscosity. Even though EPE does not set to gel in 2.5% concentration, over a certain concentration level in neutral pH might cause self-aggregation within EPE, loosely encapsulating emulsion droplets within its interacting zone and consequently resulting in increased droplet size.

It is also notable that storage temperature had little to no effect on the increase of droplet size in Tween 20 emulsion, while it affected the trend in WPI emulsions. This is explainable with the fact that WPI is a heat-sensitive protein even in natural form.

Contrary to this, pH 3.5 emulsions did not show notable size increase with Tween 20, indicating no self-aggregation occurred in samples. However, size increased in pH 3.5 WPI emulsions, not as dynamically as in pH 7 emulsions, but was statistically significant. This phenomenon is related to opposite charge affinity of EPE and WPI in pH 3.5 rather than self-aggregation. WPI is known to have isoelectric point around pH 4.3, therefore is positively charged dissolved in pH 3.5 buffers.

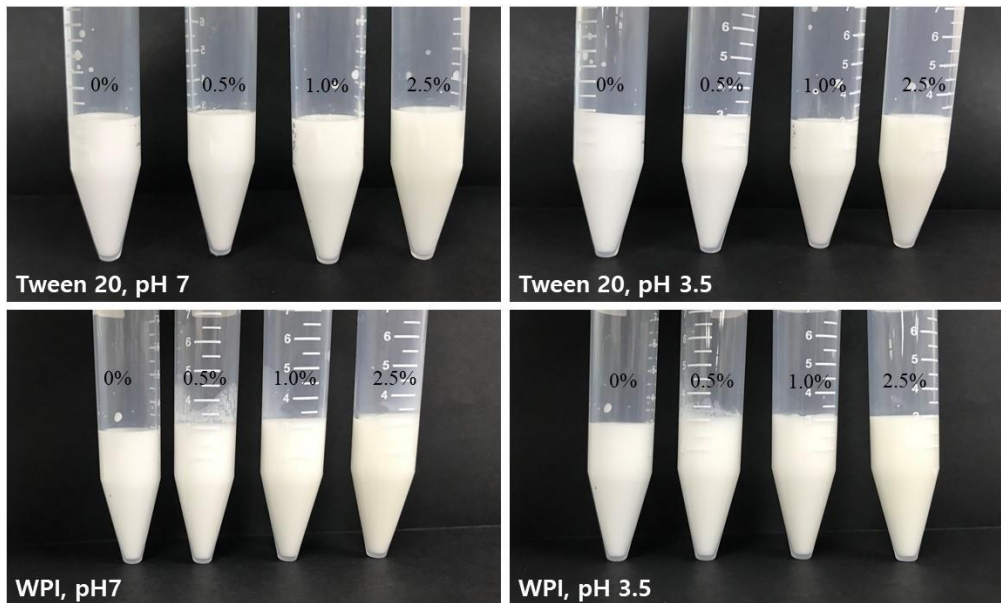
In a preliminary experiment, the  $\zeta$ -potential value of EPE in pH buffers were  $-24.8 \pm 1.47$  mV in pH 7 and  $-19.9 \pm 0.83$  mV in pH 3.5 buffer. It can be seen from Table 3 that the  $\zeta$ -potential value of EPE does not have a significant effect on Tween 20 emulsions, with no significant difference observed among different concentration ranges.

**Table 4A.** Creaming volume of Tween 20 emulsions with EPE in aqueous phase. Values were shown in means  $\pm$  std (mm).  
 NA: not available to measure; ( - ): no creaming observed.

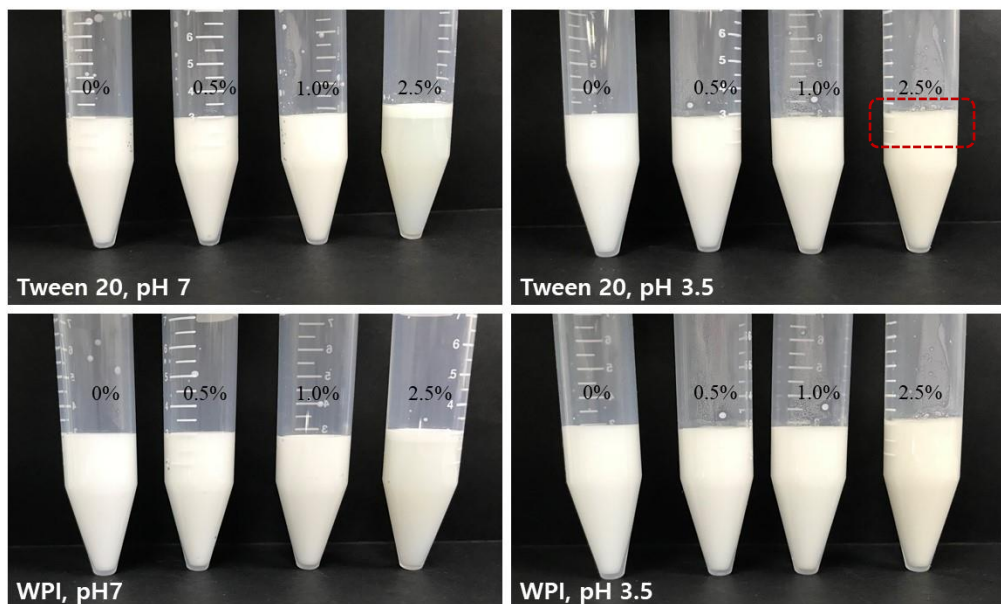
		Time (days)						
		0	1	2	3	4	5	6
pH 7	2.5%	-	3±0.06	3.15±0.05	3±0.06	3.2±0.06	3.2±0.06	3.2±0.05
	1.0%	-	-	-	-	1±0.0	1±0.0	1±0.06
	0.5%	-	-	-	-	-	-	-
	0%	-	-	-	-	-	-	-
Tween 20		-	-	-	-	-	-	-
pH 3.5	2.5%	-	-	124.4±3.8	120.2±1.7	121.7±2.1	120.9±1.8	123.2±2.2
	1.0%	-	-	-	NA	NA	1±0.0	1±0.0
	0.5%	-	-	-	NA	NA	NA	1±0.06
	0%	-	-	-	-	-	-	-

**Table 4B.** Creaming volume of WPI emulsions with EPE in aqueous phase. Values were shown in means (mm). NA: not available to measure; (-): no creaming observed..

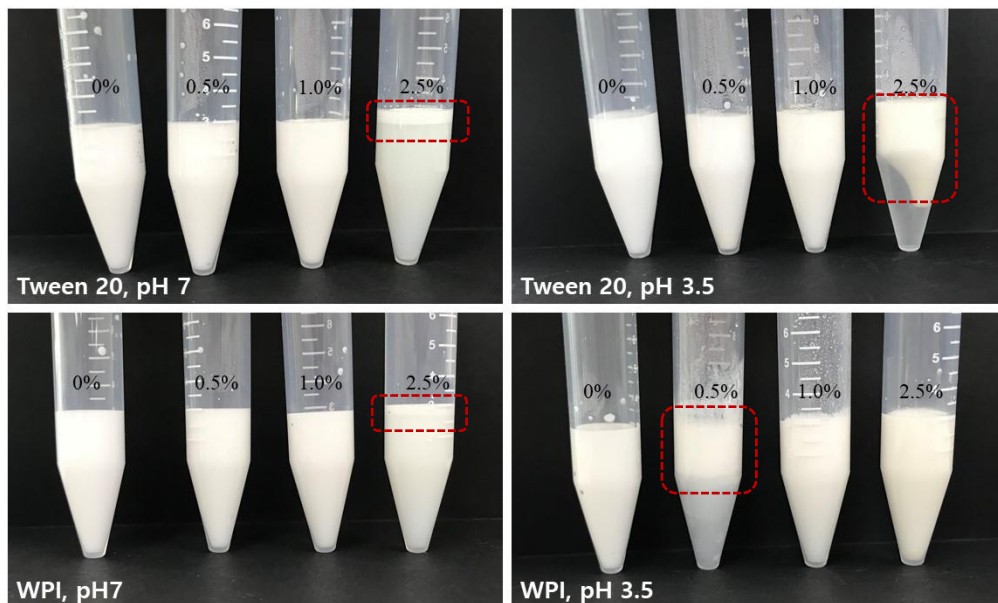
pH 7	2.5%	-	-	2±0.0	2.03±0.06	2.1±0.0	2.1±0.1	2.1±0.1
	1.0%	-	-	-	1±0.0	1±0.0	1±0.06	1±0.08
	0.5%	-	-	-	-	NA	NA	NA
	0%	-	-	-	-	-	-	-
pH 3.5	2.5%	-	-	-	-	NA	1±0.0	1±0.06
	1.0%	-	-	-	-	NA	NA	NA
	0.5%	-	-	120.6±3.7	140.9±5.9	142.2±7.1	141.4±11.4	143.2±7.2
	0%	-	-	-	-	-	-	-



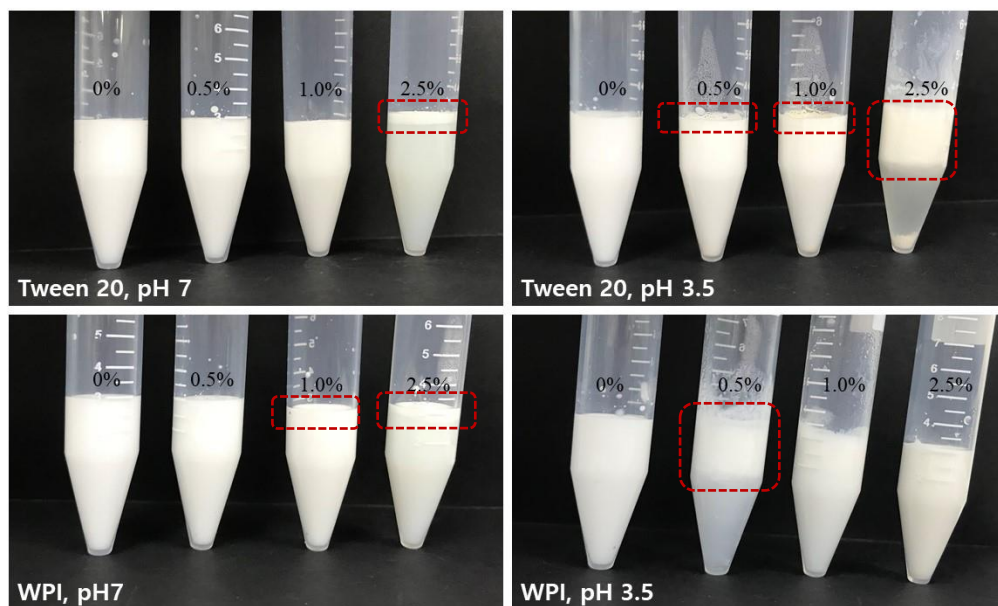
**Figure 12A.** Creaming volume analysis - Day 0



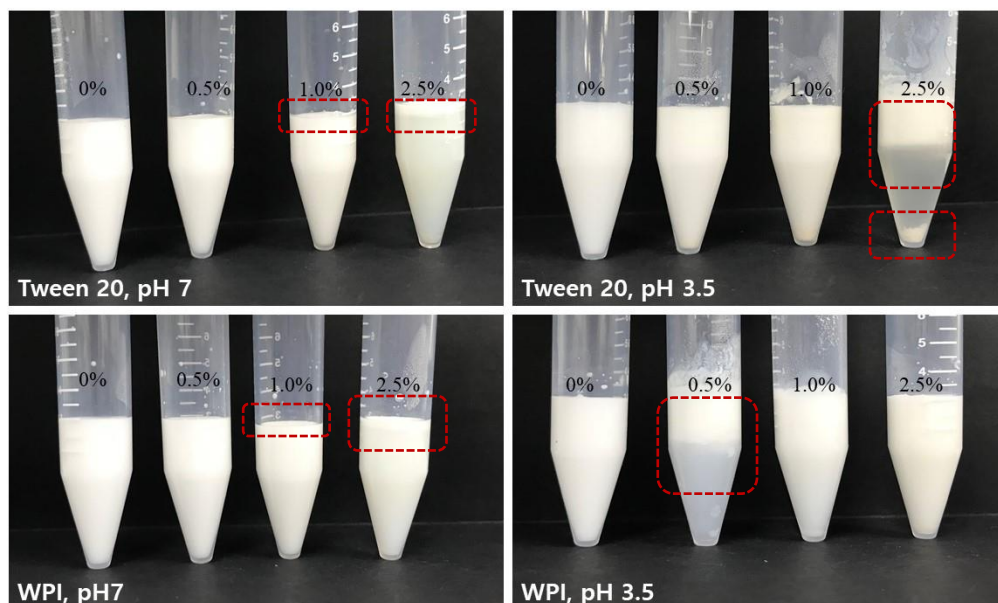
**Figure 12B.** Creaming volume analysis – Day 1



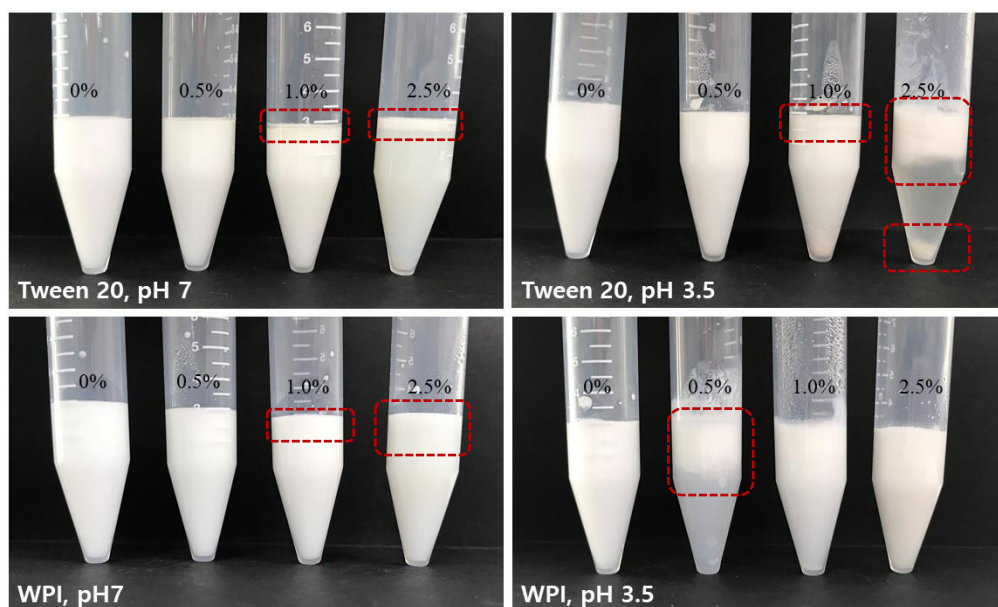
**Figure 12C.** Creaming volume analysis - Day 2



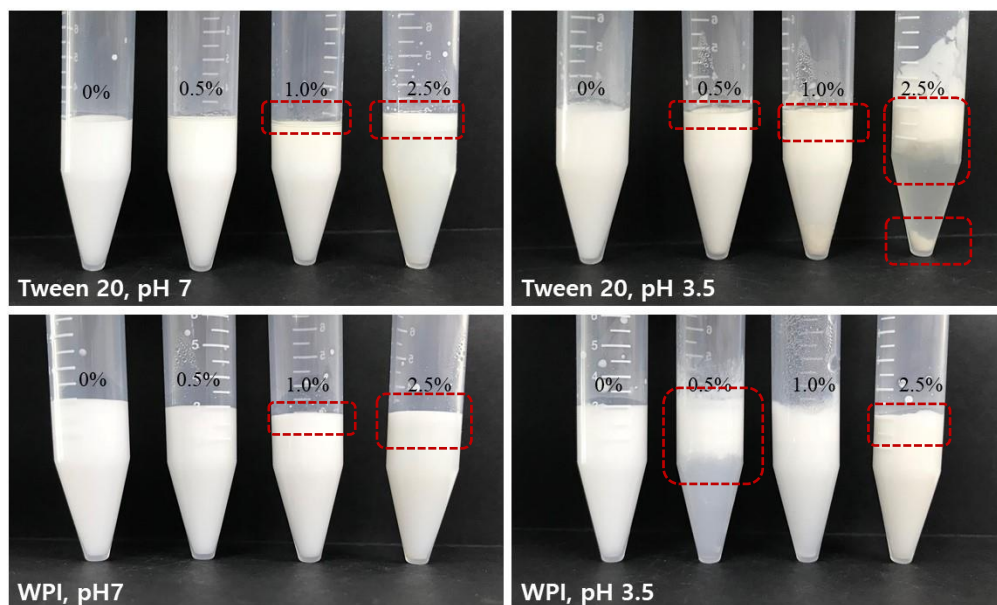
**Figure 12D.** Creaming volume analysis – Day 3



**Figure 12E.** Creaming volume analysis – Day 4



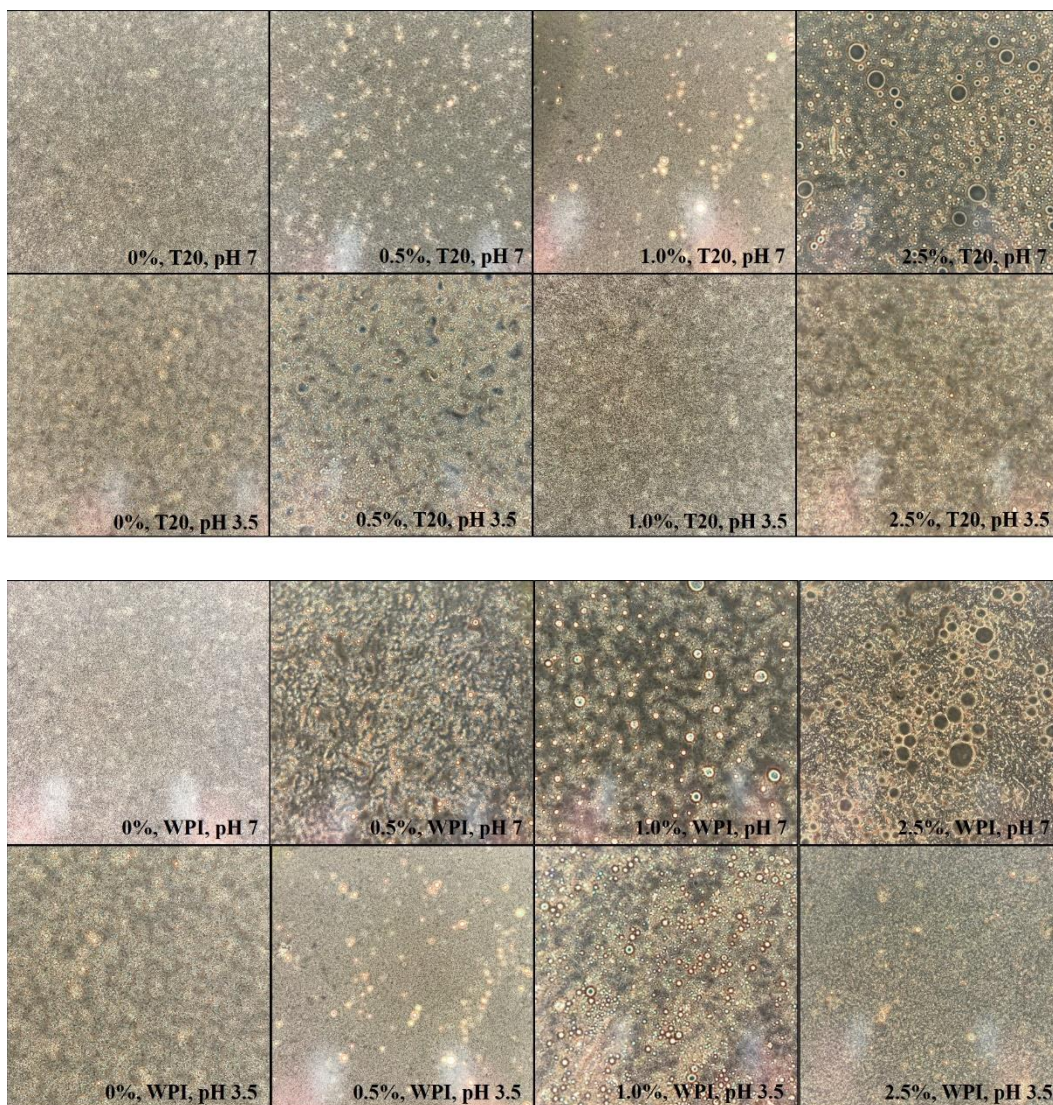
**Figure 12F.** Creaming volume analysis – Day 5



**Figure 12G.** Creaming volume analysis – Day 6

Storage of emulsion samples in ambient temperature resulted in phase separation in certain conditions emulsion was formulated. Figures 13A to 13G shows the changes of creamed layers that formed on the surface of the emulsions during 6 days of storage. The thickness of the layer during storage was as in the Table 4. The most prevalent creaming was observed in 2.5% pH 7 Tween 20 emulsion and 0.5% pH 3.5 WPI emulsion.

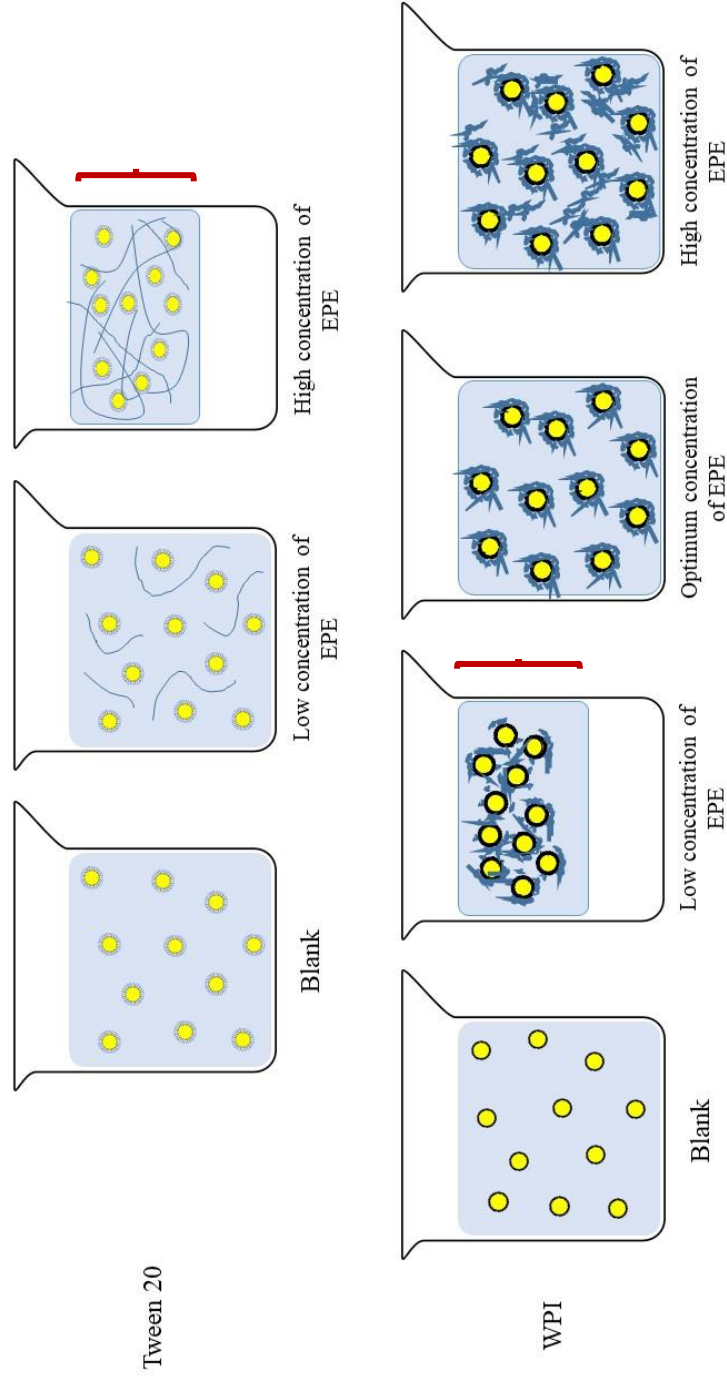




**Figure 13.** Microscopic images (400 x) of emulsions after 6 days of storage in 25 °C.

The size distribution change and creaming process indicates that the mechanism for acidic and neutral emulsions reacting with EPE is different. Therefore microscopic images were taken to visually verify the mechanism of the change in size. After 6 days of

storage oil droplet did not visually vary largely in size except in pH 7 emulsion with 2.5% EPE. In other conditions, increase in the mean droplet diameter indicates that EPE caused by the flocculation of fine oil droplets to flocculate, which was reversible with simple agitation. Yet, the overall average of the absolute value of  $\zeta$ -potential is the lowest among all samples, which may be the cause of instability when foreign molecules with uneven particle size were introduced to the system in pH 3.5 Tween 20 emulsion with 2.5% EPE.

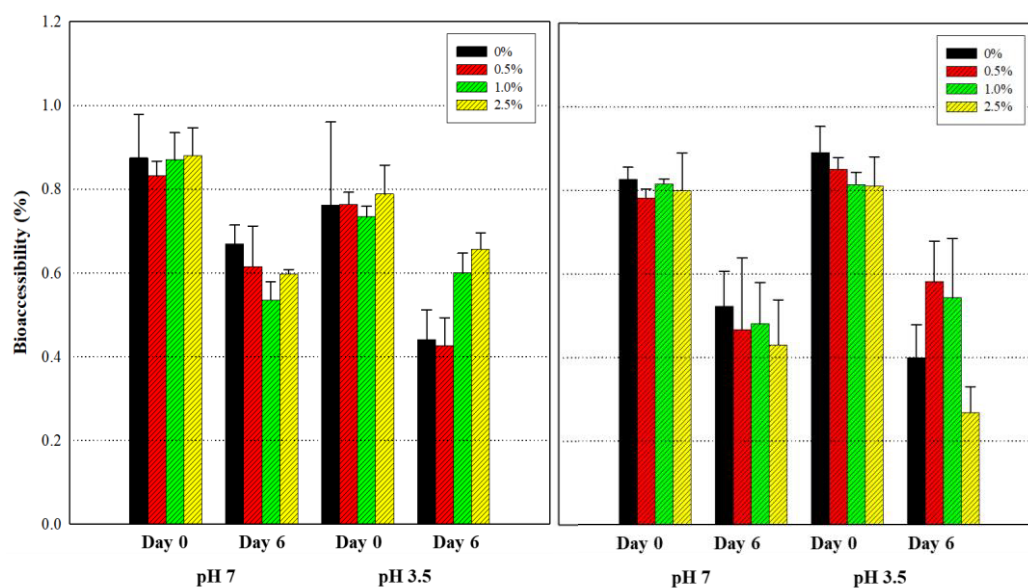


**Figure 14.** Theoretical schematic of the creaming mechanism in pH 3.5 emulsion fortified with EPE. Red indicated creaming layer formed atop the surface of emulsion.

Schematic drawing of Figure 15 depicts the mechanism of creaming occurred in pH 3.5 emulsion samples prepared with respective emulsifiers. As shown in the drawing, due to the non-reactivity of Tween 20 and EPE, no significant creaming occurs up to a certain concentration. However, when the concentration reached 2.5%, severe rate of creaming was observed. Although EPE did not form gel in neither pH 3.5 nor 2.5% conditions, it can only be explained that during the 6 day storage period, EPE molecules started to self-interact to form a network not stable enough to gel but enough to form interaction zones entrapping emulsion droplets, and consequently resulting in phase separation. This phenomenon, “depletion flocculation”, is most prevalent in circumstances where polymer concentration is excessive in the emulsion aqueous phase (Dickinson 1998). On the contrary, the reaction in WPI emulsion was different from Tween 20. As mentioned previously, WPI emulsion droplets are positively charged in acidic condition of pH 3.5. Contrasting net charge of EPE and emulsion droplet molecules result in larger mean droplet size which is stable throughout storage (Figure 12B). However, the overall phenomenon is not the same in terms of retinol retention; the overall scheme indirectly presents that an optimum concentration exists for the protection of core material (around 0.5%).

### 5.3.3. Bioaccessibility of retinol in fortified emulsion

The bioaccessibility of retinol in O/W emulsions fortified with 0, 0.5, 1.0, 2.5% EPE is shown in Figure 16. The trend was most closely related to retinol retention rate after 6 days of storage rather than EPE concentration or other factors.



**Figure 15.** Bioaccessibility of Tween 20 (LEFT) and WPI (RIGHT) emulsions fortified with 0, 0.5, 1.0, 2.5% EPE at Day 0 and 6.

## 6. Conclusion

### 1) Physicochemical characteristic of EPE

- Water-soluble *Enteromorpha prolifera* extract (EPE) was extracted using only DW as solvent. The extracted material consisted of sulfated and negatively charged polysaccharide with molecular weight range of 5 to 80 kDa.

- EPE is a gelling material with the ability to form thermoreversible gel over 5% concentration dissolved in buffers in the pH range of 4 to 9. The strongest gel was observed around neutral pH, and the sol-gel transition temperature range was affected by pH seeming relevant with gel strength in each pH conditions.

- Similar results was observed when the samples were dissolved in emulsions formulated in pH 7 using Tween 20 and WPI as emulsifiers, but the gel strength in emulsion was significantly higher compared to buffer gels. Oil droplets in emulsion act as supportive filler materials between the gel matrixes resulting in the increased gel strength.

### 2) Effect on O/W emulsion phase stability

- Addition of EPE in pH 7 emulsion does not greatly affect, if not negative, the stability of retinol in both Tween 20 and WPI conditions. However, in concentration of 2.5%

EPE in either of the emulsions seems to cause self-aggregation of EPE. This uneven distribution of particle size causes the mean diameter of oil droplets to increase.

- EPE dissolved in pH 3.5 emulsions do not self-aggregate as readily as in pH 7; the increase in viscosity relatively small to that of pH 7. Yet creaming was more prevalent compared to pH 7 emulsions. Although the observed result was similar, the underlying mechanism seem to differ between two emulsifiers. For Tween 20 emulsions, having the lowest absolute value of  $\zeta$ -potential, lack of electric repulsion force may have been the reason. In WPI emulsions, which is the only case in this study to have positively charged droplets, interacts with negatively charged EPE causing increase in droplet diameter. When the concentration of EPE is not enough, it will only partially cover the surface of emulsion and causes bridging flocculation, resulting in phase separation.

- In terms of retinol stability, optimum condition was different among emulsion formulation conditions. In both of the pH 7 emulsions, the concentration of EPE did not seem to greatly affect the retention rate. However, in an acidic condition, Tween 20 emulsion showed better protection as the concentration of EPE increases, while in WPI emulsions an optimum concentration was set between 0.5% to 1.0%.

To the authors' knowledge, this study was the first to report the analysis of water-soluble extracts from *Enteromorpha prolifera* in an applied system. The physicochemical characteristics of EPE was investigated to apply the material into a model food system,

O/W emulsion. The health function of EPE has already been researched in many aspects, and is a material generally accepted as safe. Although further optimization is required within a more complex system, the results of this study suggests that EPE has the possibility to become a universal protective material with multifunctioning abilities.



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## 국 문 초 록

가시파래는 갈파래목 파래과에 속하는 녹조류의 일종으로, 흔히 녹조류는 종간에 물리화학적, 생물학적 특성이 비슷하고, 흔하며 독성이 없으며 주로 얕은 바다나 하구 근처에 서식하는 것으로 알려져 있다. 특히 다당류를 포함한 수용성 추출물들은 항산화능, 금속 이온 배위 결합능이 입증되었으며 지질 대사에 도움이 된다고 한다. 그러나 이러한 효능은 소재 자체의 기능성으로서 연구되어 온 것에 비해 실제 식품 모델 시스템 내에서 거동과 효과를 검증한 연구는 상대적으로 그 수가 적었다.

이 연구의 목적은 기능성 식품 첨가물로서 가시파래 추출물의 특성을 분석하고 식품 모델 시스템 내에서의 기능성을 확인하는데 있다. 구체적으로는 가시파래 추출물의 성분을 확인하고, 추출물 농도 및 pH 에 따른 젤화 거동을 분석하여 추출물의 물리화학적 특성을 파악하였다. 가시파래 추출물은 다당류 함량이 평균적으로 47%로 가장 높았으며, 그 외에 12.4%의 단백질과 다당류에 연결된 황산염 또한 약 19% 정도를 가지고 있다. 추출물은 2.5% 이하의 낮은 농도 또는 pH 3.5 이하의 강한 산성 환경에서는 젤을 형성하지 않았으며, 중성에서 가장 강도 높은 젤을 형성하였다. 형성된 젤은 온도를 높임에 따라 흐름성을 가지는 졸 상태가 되었으며, 다시 온도를 낮추면 초기의 젤 구조를 복원하는 열 가역성을 가지고 있는 것이 확인되었다.

이러한 특성을 활용해 식품 모델로 선정된 수중유적형 에멀션 시스템에의 적용 가능성을 실험하였다. 추출물 5%를 함유한 에멀션 젤은 동일한 pH 의 완충 용액에서 형성된 젤보다 훨씬 높은 강도를 가지고 있었으며, 2.5%에서는 젤이 형성되지는 않았지만 점도에 있어 상승 효과를 나타냈다. 이는 에멀션 방울이 젤 네트워크 사이를 지지하는 이른바 ‘필러’ 역할을 한 것으로 보인다. 마지막으로 에멀션의 저장 중 상과 기름층 내 존재하는 레티놀의 안정성 평가를 통해 실질적으로 에멀션 보호능에 있어서의 효과를 검증하였다. 에멀전만으로도 레티놀에 대한 보호능이 충분한 경우에는 추출물의 첨가가 도리어 안정성의 저해로 이어진다는 결과가 있었지만, 산성 환경 등 외부적 스트레스가 가해지는 상황에서는 적정량 (0.5 ~ 1.0%)의 추출물의 첨가가 상 안정성과 레티놀 안정성에 긍정적인 영향을 미치는 것을 확인하였다.

해양 자원이 앞으로의 시대에 지속적인 연구가 필요한 친환경 소재가 될 것이라는 전망은 이미 오래 전부터 제시되어 왔다. 그 중에서도 가시파래 수용성 추출물은 에멀션에 첨가했을 때 다양한 메커니즘의 물리, 화학적 보호 기능을 가지고 있으며 독한 약품을 사용하지 않으며 비교적 단순한 공정으로 추출할 수 있기 때문에, 산업적인 장점도 가지고 있다. 특히 젤화 특성을 가지고 있으므로 수용성 다당류로서 에멀전과의 네트워크 형성을 통해 지방 대체제로 사용할 수 있는 증점제로서 활용 가능성이 있으며, 젤 자체에도 기능성을 가지고 있으므로 천연 소재에 대한 소비자의 관심이 증가하는 최근의 추세를 볼 때 식품 산업에 폭넓게 활용될 수 있는 우수한 소재로서의 가능성이 충분하다고 볼 수 있다.