FATE AND EFFECT OF FOOD-GRADE TITANIUM DIOXIDE PARTICLES/POLYSTYRENE NANOPLASTICS ON NUTRIENTS BIOACCESSIBILITY OF FOOD IN THE SIMULATED HUMAN GASTROINTESTINAL TRACT

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https://doi.org/10.7275/27751199 https://scholarworks.umass.edu/dissertations_2/2448

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FATE AND EFFECT OF FOOD-GRADE TITANIUM DIOXIDE PARTICLES/POLYSTYRENE NANOPLASTICS ON NUTRIENTS BIOACCESSIBILITY OF FOOD IN THE SIMULATED HUMAN GASTROINTESTINAL TRACT

A Dissertation Presented

By

CHUNYANG LI

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

DOCTOR OF PHILOSOPHY

February 2022

Environmental Conservation
FATE AND EFFECT OF FOOD-GRADE TIO$_2$ PARTICLES/POLYSTYRENE NANOPLASTICS ON NUTRIENTS BIOACCESSIBILITY OF FOOD IN THE SIMULATED HUMAN GASTROINTESTINAL TRACT

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ACKNOWLEDGMENTS

Throughout pursuing a Ph.D. degree in the University of Massachusetts, Amherst, I received a great deal of support and assistance. I would like to express my thanks and appreciation to all wonderful individuals for being part of this journey and making this thesis possible.

First and foremost, I owe my deepest gratitude to my esteemed advisor Dr. Baoshan Xing for his invaluable supervision, marvelous advice, patience, continuous support during my Ph.D. study and research. His expertise, immense knowledge, and insightful methodology to research have pushed me to sharpen my thinking, brought my research at a higher level, and carried me through all the stages of my projects. Many thanks to my committee members, Dr. David Julian McClements (Department of Food Science, University of Massachusetts Amherst) and Dr. Jason C. White (The Connecticut Agricultural Experiment Station), for their constructive suggestions to my research and they helpfully authorize me on the usage of instruments in their labs. It is my honor to collaborate with their labs in my research.

I am deeply thankful to all the colleagues and fellow graduate students in Dr. Xing’s group for their generous support and friendship. I gratefully acknowledge the constant support and valuable suggestion of my seniors Dr. Chuanxin Ma, Dr. Huiyuan Guo, and Dr. Heping Shang towards to my dissertation research project. Thanks to Dr. Hamid Mashayekhi and Mr. Ian Eggleston for helpfully training me on the instruments in the lab. Also, it is a pleasure to thank my lab mates including Sicheng Xiong, Aoze Li, Shang Gao, Anahita Khosravi, Xiupei Zhou, and many more for their help and accompany.

I would like to express my gratitude to my department and program, Stockbridge School of Agriculture and Environmental Conservation, for providing me the best doctoral training. I also
acknowledge the financial support by the China Scholarship Council for part of my study at the University of Massachusetts, Amherst.

Last but not least, my deep and sincere gratitude to my parents for their continuous and unparalleled love, help and support. Without their encouragements, it would be impossible for me to complete my Ph.D. study in the United States, and I dedicate this milestone to them.
ABSTRACT

FATE AND EFFECT OF FOOD-GRADE TITANIUM DIOXIDE PARTICLES/POLYSTYRENE NANOPLASTICS ON NUTRIENTS BIOACCESSIBILITY OF FOOD IN THE SIMULATED HUMAN GASTROINTESTINAL TRACT

FEBRUARY 2022

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Food grade titanium dioxide particles (E171), as whitening agent, are commonly used in chewing gums, candies, sauces, salad dressings, and powdered milks. Recently, nanoplastics (NPs) (defined as < 1 µm), which are degraded from plastic debris undergoing environmental process, have received a global attention. Because nanoplastics are ubiquitous in aquatic and terrestrial systems, and have been detected in marine animals, table salts, drinking water, and air. Thus, the presence of E171 as additives in the food, and the nanoplastics in food chains, both pose potential risks to human health through retarded processes in the gastrointestinal tract (GIT).

However, the knowledge about the fate of E171 or nanoplastics and their impact on digestive enzymes activity or nutrient bioaccessibility are currently limited. Therefore, it is necessary to investigate potential impacts of E171 or nanoplastics on digestion processes and nutrient bioaccessibility in the simulated human gastrointestinal tract, which includes mouth, stomach and intestinal phases, to mimic the digestion processes of food incubating with E171 or
polystyrene (PS) nanoplastics. The objectives of my dissertation and major findings are presented below:

1) The impacts of E171 on lipid digestion and vitamin D$_3$ (VD$_3$) bioaccessibility encapsulated within oil-in-water emulsions in a simulated human gastrointestinal tract model were explored. VD$_3$ bioaccessibility significantly decreased from 80 to 74% when raising E171 from 0 to 0.5 wt%. The extent of lipid digestion was reduced by E171 addition in a dose-dependent manner. VD$_3$ bioaccessibility was positively correlated to the final amount of free fatty acids (FFAs) produced by lipid digestion ($R^2 = 0.95$), suggesting that the reduction in VD$_3$ bioaccessibility was due to the inhibition of lipid digestion by E171. Further experiments showed that E171 interacted with lipase and calcium ions, thereby interfering with lipid digestion. These findings improved our understanding towards the potential impact of E171 on nutritional attributes of foods for human digestion health.

2) The impacts of E171 on the bioaccessibility of minerals (Ca, K, Mg, Fe, Mn, Zn, P and S) released from spinach leaves using a simulated human digestion tract were investigated. The digestive enzymes used in the GIT model, including α-amylase, pepsin, and pancreatin, prompted mineral release from spinach leaves (except for Ca). E171 particles did not affect the bioaccessibility of most minerals, except for Fe. The final bioaccessibility of Fe decreased from 59% in the absence of E171 to 53% in the treatment with 0.2 wt% E171. Interestingly, the decrease in Fe bioaccessibility mainly occurred within the oral phase, rather than the gastric or intestinal phases. Mechanistic studies indicated that the reduction in Fe bioaccessibility was due to the following two processes: (i) inhibition of α-amylase activity by E171, thereby interfering with Fe release from the spinach leaves and (ii) adsorption of Fe onto E171 particles. The results of this
study are useful for assessing the potential impact of E171 on the human digestive processes and the nutritional value of foods.

3) The impacts of various concentrations or different functional group (-COOH, -NH₂) of PS NPs on the starch hydrolysis in the simulated INFOGEST model were examined. The extent of starch hydrolysis was significantly decreased from 95% to 74% upon exposure to 0-0.3wt% of PS NPs, as indicated by the glucose released. Additionally, PS, PS-COOH, and PS-NH₂ exhibited inhibitory effects on the digestion of starch. The mechanism of PS NPs in reducing the corn starch hydrolysis was illustrated: 1) PS NPs or PS NPs agglomerates were adsorbed onto starch granules, thereby inhibiting the digestive enzymes to access to the surface of starch granules; 2) PS NPs decreased the digestive enzymatic activities (i.e., amylase, pepsin, and pancreatin) via adsorption and changed the secondary structures of enzymes (i.e., π-π interaction or hydrophobic interaction), subsequently reducing the capacity of enzymes in starch hydrolysis.
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CHAPTER

CHAPTER 1

1. BACKGROUND

1.1 The production and application of TiO$_2$ in food industry

In recent years, potential applications of nanotechnology in food industry involve food package to enhance stability of food during storage, and food additives to improve food appearance.\(^1\) For example, nano-engineered food additives are intentionally incorporated into food to optimize qualities such as taste, color, texture or consistency.\(^2\) Among the worldwide nanomaterials in food products, titanium dioxide nanoparticles (TiO$_2$ NPs) are particularly popular engineered nanomaterials. TiO$_2$ used as a food additive for the first time can date back to 1969, where Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that TiO$_2$ was an extremely insoluble compound.\(^3\) TiO$_2$ particles naturally have three crystal forms, including anatase, rutile as well as brookite, and their chemical and physical properties are very stable. From 1916 to 2011, the global production of titanium dioxide pigment was totally 165 million metric tons.\(^3\) The production rate was estimated to continuously increase until at least 2025.\(^4\)

TiO$_2$ is commonly incorporated into various products such as sauces, cheeses, skimmed milk, ice-cream, and confectionary products.\(^5\) Particularly, the highest level of TiO$_2$ in confectionary products i.e., sweets, chewing gum, and other white-coated products, has been reported to reach up to 2.5 mg Ti/g of food.\(^6\) A food industry compared the dosage of TiO$_2$ in various food and found that highest content of TiO$_2$ in chewing gum, food supplements, nuts, and ready-to-use salads was up to 16 mg/g, 12 mg/g, 7 mg/g, and 3 mg/g, respectively.\(^7\) Chen et al investigated that the size of TiO$_2$ particles in commercial gums was 40% in nanoscale and easily swallow them as chewing.\(^8\) Particle size of five E171 products from worldwide food suppliers is
Weir et al. characterized E171 in a broad size distribution (30 - 400 nm), with up to approximately 36% of E171 less than 100 nm in at least one dimension. In addition, they used a Monte Carlo human exposure analysis to estimate that U.S. adult daily consumption of E171 might be 1 mg Ti/kg body weight per day. The daily consumption of TiO₂ varies depending upon age, body weight, and residential area. It is estimated that children could consume up to 2–4 times higher TiO₂ NPs per body weight than an adult. Currently, safety regulations on TiO₂ as a whitening colorant used in food according to legislation from 1969 are controversial and out of date. The European Food Safety Authority (EFSA) has re-assessed the risk of E171 used as a food additive.

1.2 The production and exposure of microplastics/nanoplastics to human and environments

Nowadays, the plastic pollution has become a globally severe environmental issue. Because of massive production and usage of plastics, as well as their inappropriate disposal, the plastic wastes have attracted attention among scientists, politicians, and the public. Since the 1950s, the production of plastics has been begun with a steady growth annually. Recently, the worldwide production of plastics increased from 16.5 million tons to 368 million tons, and the output was predicted to be tripled by 2050. However, the recycling of these plastics is only 6–26%, implying that at most 94% of the plastics may enter ecosystems via multi-routines. Subsequently, these plastics wastes mostly exacerbate the environmental pollution. For instance, 5-13 million tons of plastics were estimated to leaks into the oceans per year, and > 5 trillion plastic pieces (weight > 250,000 tons) were estimated to float on the oceans. The most common plastics in the environment are carbon-based polymers, i.e., polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), polyethylene (PE), and polyethylene terephthalate (PET).
Plastics undergo environmental weathering, e.g., mechanical abrasion, biodegradation, hydrolysis, thermal, and light irradiation, to break into smaller plastic fragments, termed as microplastics (MPs) and nanoplastics (NPs).\textsuperscript{18, 19} Thompson et al. (2004) firstly introduced the definition of microplastics to document small plastic pieces in the oceans.\textsuperscript{20} Arthur et al. (2009) proposed to set up an upper size limit (5 mm) for microplastics (MPs).\textsuperscript{21} To the present, researchers recommended that microplastics are defined as the size of plastic debris in the range of 1 µm to 1 mm and nanoplastics (NPs) are termed as the size of plastic particles less than 100 nm.\textsuperscript{22, 23} Nowadays, MPs and NPs are considered as a potential threat to human health and the environment, attracting a huge scientific and public interest.\textsuperscript{24} Because these tiny particles are ubiquitous in the environment as well as in drinking water and food.\textsuperscript{24} Additionally, da Costa Araujo & Malafaia have verified the accumulation of plastic particles the organisms at upper tropic levels, thus, it is possible for plastics ultimately accumulate in food chain exposure to human.\textsuperscript{25} Shi et al. summarized that inhalation, ingestion and skin were three major pathways for human to expose to MPs and NPs due to their ubiquitous existence in the environment or personal care products.\textsuperscript{26} In 2019, the World Health Organization (WHO) has proposed questions and concerns about MPs exposure to human via drinking water.\textsuperscript{17} In comparison with MPs, nanoparticles particularly display different physical and chemical properties.\textsuperscript{27, 28} Because of tiny size, higher specific surface area, and strong biological penetration, NPs may penetrate organs of organisms, and cause adverse effects on biological functions.\textsuperscript{29, 30} Therefore, it is necessary and urgent to explore the potential impacts of MPs and NPs on human health.

1.3 Toxicities studies of TiO$_2$ particles and MPs/NPs

1.3.1 Toxicities of TiO$_2$ particles
Potential risks of chronic intestinal inflammation and carcinogenesis induced by TiO$_2$ have become a growing concern.$^{31}$ Heringa et al. found that ingestion of TiO$_2$ NPs through food, supplements and toothpaste potentially damaged liver and reproductive organs.$^{32}$ It has been reported that human oral consumption of 23 or 46 mg of TiO$_2$ in capsules resulted in an increase of TiO$_2$ concentration (5-10 times) in blood.$^{33}$ In addition, smaller particles were easily absorbed by the gastrointestinal tract as compared to larger particles.$^3$ Healthy human volunteers orally consumed 100 mg pharmaceutical/food grade TiO$_2$ and the presence of Ti in blood were confirmed by ICP-MS, suggesting uptake of particles by human ingestion.$^{34}$ Exposure to 200 µg/mL of nano-TiO$_2$ extracted from gum had mild impacts on gastrointestinal cells within 24 hours. Another study investigated cytotoxicity and translocation of TiO$_2$ NPs pretreated with digestion fluid or bovine albumin in Caco-2 cells, and showed that TiO$_2$ NPs below 200 µg/mL had no toxicity to Caco-2 cells but the concentration of TiO$_2$ NPs beyond 200 µg/mL inhibited the growth of undifferentiated Caco-2 cells.$^{35}$ Bettini et al. found that food-grade TiO$_2$ deteriorate intestinal and systemic immune homeostasis, induced preneoplastic lesions and developed aberrant crypt in the rat colon upon exposure to E171 at human related levels (10 mg/kg of BW/day).$^{31}$ E171 has been reported to elevate ROS levels and cause DNA damages in an in vitro model using human Caco-2 cell and HCT116 cells.$^{36}$

1.3.2 Toxicities of MPs / NPs

Microplastics as an emerging contaminant have caused worldwide attention in biological toxicity.$^{37, 38}$ Both in vitro or in vivo toxicity studies of micro- and nanoplastics showed that they may cause physical stress and damage, oxidative stress and apoptosis, inflammation, and impair cellular metabolism.$^{39}$ In general, microplastic pollution has occurred in the oceans, rivers, and soils.$^{40, 41}$ MPs and NPs may initially attack the digestive system of organisms, since
gastrointestinal tract is the primary exposure pathway of MPs and NPs, then causing toxic effects.\textsuperscript{42} The existence of microplastics has been detected in the stomachs and guts of marine animals.\textsuperscript{43} Yin et al. summarized the toxicity of MPs and NPs in the digestive system, including inflammation, oxidative stress, and serious intestinal flora disorders.\textsuperscript{44} PE MPs caused a notable histological change and a strong inflammatory response in blue mussel \textit{Mytilus edulis L}.\textsuperscript{45} Jin et al. also found that exposure to 1000 mg/L of PS MPs increased the intestinal mucus volume and remarkably declined the abundance of bacteroidetes and proteobacteria in gut microbiota of zebrafish.\textsuperscript{37} NPs have been reported to show high proneness to pass through the GIT barrier using a model of intestinal epithelium.\textsuperscript{46} The length of human GIT is 6–8 meters and its surface area is 200 square meters, which is likely to be the primary exposure way for plastic particles to human.\textsuperscript{26} PS nanoplastics impaired cell viability and upregulated inflammatory gene expressions associated with gastric pathology cytokines in gastric adenocarcinoma cells.\textsuperscript{47} Most toxicity studies focus on the toxic effect of MPs/NPs in gastrointestinal tract at cellular level; however, the knowledge for the impacts of these macro-/nano-scale plastics on nutrient assimilation in the digestive system is limited. Therefore, it is necessary to explore the interaction between MPs or NPs with the food components during nutrient assimilation.

\textbf{1.4 Simulated gastrointestinal tract}

Over last few decades, to establish the possible impacts of food on human health attracts interests in understanding food's digestive fate.\textsuperscript{48} In theory, investigation of the food digestion should be carried out on human \textit{in vivo} to obtain reliable and accurate results. But clinical studies performed on human are not always ethically, technically, financially possible, and low repeatability because of individual variations.\textsuperscript{49-51} Additionally, fancy and pricey instruments such as nuclear magnetic resonance and ultrasonic scanner need to be used for clinical human tests.\textsuperscript{52}
Therefore, simple and fast in vitro digestion models to simulate the gastrointestinal tract have been introduced as substitutes to in vivo experiments. In vitro digestion analysis under the similar conditions of in vivo digestion is valuable to investigate and understand modification, interactions, as well as the bioaccessibility of nutraceuticals. The method has been commonly used in various fields, i.e., nutrition, pharmacology and food chemistry since it is rapid, cheap, less labor intensive without ethical restrictions. More than 2500 published articles used in vitro digestion assays in the past four decades (85% of which published in the last two decades) to illustrate scope such as lipid digestion, protein digestibility, nutrient. Typically, simulated digestion tract includes the mouth, stomach and small intestine phase. And in vitro method mimicking in vivo physiological conditions involves the presence and concentration of digestive enzymes, pH, digestion time, and salt concentrations. Static models of simulated human gastrointestinal tract have been applied in diverse researches such as the digestibility of macronutrients such as proteins, carbohydrates and lipids. Also, it has been widely used to study the bioaccessibility of various nutrient compounds, e.g., tocopherols, carotenoids and vitamin.

1.5 Objectives

Specific Aim 1: To investigate the potential fate of E171 particles within a simulated human gut, as well as to assess their impact on lipid digestion and the bioaccessibility of VD3 in model food emulsions.

Specific Aim 2: To study impact of E171 particles on mineral bioaccessibility (Ca, K, Fe, Mg, Mn, Zn, P and S) released from spinach leaves in simulated human gastrointestinal tract as well as reveal the mechanism of E171 on the minerals released from food matrix.

Specific Aim 3: To investigate the gastrointestinal fate of PS NPs, examine their impact on the digestion of corn starch and their interaction with digestive enzymes (i.e., amylase, pepsin,
pancreatin), and further reveal the mechanisms for PS NPs-reduced corn starch hydrolysis in the INFOGEST model.
CHAPTER 2

2. FOOD-GRADE TITANIUM DIOXIDE PARTICLES DECREASED THE BIOACCESSIBILITY OF VITAMIN D₃ IN SIMULATED HUMAN GASTROINTESTINAL TRACT

2.1 Introduction

Titanium dioxide (TiO₂) is widely used as a whitening agent in dairy, bakery, and confectionery products because of its strong light scattering properties and resistance to discoloration.⁶⁶ The global production of TiO₂ was around 165 million metric tons from 1916 to 2011, and this production rate is predicted to rise until at least 2025.³ ⁴ As a food additive, TiO₂, designated “E171” by the European Union, is authorized to be used in *quantum satis* levels (as much as sufficient, but not more) in Europe.⁹ In the U.S., addition of TiO₂ is permitted up to 1 % by overall food weight.³ A human exposure analysis of TiO₂ use in food showed a U.S. adult’s daily consumption at around 1 mg Ti kg⁻¹ body weight per day, and that children typically have higher exposure levels than adults due to the greater amounts of TiO₂ found in the foods for children, such as confectionary.⁶ Humans are therefore exposed to significant levels of E171 through their diet.⁶⁷

Oil-in-water emulsions consist of oil phase dispersed within an aqueous phase.⁶⁸ In the food industry, this kind of colloidal dispersion is an integral part of many kinds of food products, including beverages, milks, creams, dressings, sauces, soups, and dips.⁶⁹ Moreover, they can be used to encapsulate lipophilic bioactive substances, protect them from degradation, and increase their bioaccessibility, *e.g.*, curcumin,⁷⁰ carotenoid,⁷¹ and oil-soluble vitamins.⁶³ Vitamin D₃ (VD₃) is an oil-soluble micronutrient that has essential roles in maintaining bone, teeth, and cartilage health, as well as for the proper function of the immune system and protection against cancer.⁷² It
has been estimated that one-third of the U.S. population suffers from the risk of vitamin D inadequacy (30 - 49 nmol L\(^{-1}\)) or deficiency (< 30 nmol L\(^{-1}\)) based on blood levels.\(^{73, 74}\) The relatively poor chemical stability and low bioaccessibility of this oil-soluble vitamin contribute to this problem. It has been reported that oil-in-water emulsions may be used as vehicle for VD\(_3\) delivery to promote its stability and bioaccessibility in food products.\(^{75}\)

The bioaccessibility of encapsulated oil-soluble vitamins is highly dependent on the digestion of the lipid droplets encapsulated within emulsions, which is regulated by various factors, including droplet characteristics (oil phase composition, emulsifier type, and particle size) and gastrointestinal conditions (lipase, bile salts, and calcium levels).\(^{76}\) Lipase is a key factor for lipid digestion because it converts the triglycerides inside the lipid droplets into monoglycerides and free fatty acids (FFAs).\(^{77}\) Digestion of the lipid is important because it is required to release the vitamins from the oil droplets, as well as to solubilize them within the mixed micelles that convey them to the epithelium cells. Consequently, any factor that interferes with lipid digestion would impact vitamin bioaccessibility. When there is insufficient lipase present, the extent of lipid digestion is shown to be relatively low.\(^{77}\) Certain types of emulsifier may inhibit lipid digestion by interfering with the ability of lipase to access the lipid droplet surfaces, by denaturing lipase, or by prompting lipid droplet aggregation.\(^{76}\) Bile salts also have an essential role in lipid digestion due to their capacity to bind to the lipid droplet surfaces and displace emulsifiers, as well as their role in forming mixed micelles.\(^{78}\) In addition, calcium plays a crucial role in lipid digestion due to its ability to remove lipid digestion products (long-chain FFAs) from the lipid droplet surfaces.\(^{77}\)

E171 particles can be co-ingested with fatty food products, which may contain lipid droplets or form new ones within the human gastrointestinal tract. For instance, E171 particles can be added to powdered milks or sprinkled on bakery products to improve their appearance. Then, these E171
particles could interfere with the digestion of ingested lipids, thereby reducing the bioaccessibility of any oil-soluble vitamins encapsulated within them. The importance of this kind of food matrix effect has been demonstrated in numerous previous studies. For instance, casein, EDTA, and alginate have all been shown to strongly bind calcium ions, thus decreasing the concentration of free calcium within the gastrointestinal fluids, and reducing in the rate and extent of lipid digestion.\textsuperscript{79} Moreover, TiO\textsubscript{2} particles have been shown to bind calcium ions to their surfaces under simulated GIT conditions.\textsuperscript{80, 81} Calcium ions could increase the positively charged surface of rutile TiO\textsubscript{2}, thereby attracting glutamate but repelling lysine.\textsuperscript{82} Other food and gastrointestinal components that influence lipid digestion and bioaccessibility may also be impacted by the presence of E171 particles. For instance, calcium ions of small intestine fluid could modify the $\zeta$ potential of E171 particles; thus, anionic bile salts and lipase might adsorb onto positively charged E171 particles through static electrostatic interaction. Furthermore, surface active agents containing -OH and -COOH functional group, such as Tween 80 (an emulsifier in this study) or bile salts might also adsorb to titanium dioxide surfaces via chemical bonding and hydrophilicity.\textsuperscript{83} The knowledge from this study is useful for evaluating the potential impact of TiO\textsubscript{2} particles on the nutritional value of foods and human health.

We hypothesized that the binding ability of surfactants, lipase, bile salts, and calcium to the surface of E171 particles may interfere with lipid digestion, thereby decreasing VD\textsubscript{3} bioaccessibility. In this study, we used an \textit{in vitro} gastrointestinal tract (GIT) model to investigate the potential fate of E171 particles within a simulated human gut, as well as to assess their impact on lipid digestion and the bioaccessibility of VD\textsubscript{3} in model food emulsions. The \textit{in vitro} simulated GIT included mouth, gastric and small intestinal phases.\textsuperscript{77} Initially, the impact of E171 (0-0.5 wt\%) on lipid digestion as well as VD\textsubscript{3} bioaccessibility were examined.\textsuperscript{84} Then, additional experiments
were implemented to detect the physicochemical origin of the observed effects. In particular, the interactions of the E171 particles with the surfactant used to coat the vitamin-loaded lipid droplets were studied, as well as with the bile salts, lipase, and calcium ions in the gastrointestinal fluids.

2.2 Materials and methods

2.2.1 Materials

Food-grade TiO$_2$ particles (E171, purity 99%) were purchased from Precheza (Přerov, Czech Republic). Vitamin D$_3$ (1.0 Mill. I.U. /g) was provided by D-BASF (Ludwigshafen, Germany). Tween 80 (Acros Organics) were purchased from Fisher Scientific (Hampton, NH). Corn oil were purchased from a commercial food supplier. Porcine mucin, porcine pepsin from gastric mucosa ($\geq 250$ units mg$^{-1}$), porcine pancreas lipase (100 - 500 units mg$^{-1}$), and porcine bile extract were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals used were of analytical grade and ultrapure deionized water were used to prepare all solutions.

2.2.2 Emulsions preparation

Emulsion preparation were based on the previous studies with slight mortification.$^{85, 86}$ Initially, coarse emulsions were prepared by mixing 10 % (w/w) oil phase and 90 % (w/w) aqueous phase together using a high-shear mixer (M133/1281-0, Biospec Products, Inc., ESGC, Basel, Switzerland) for 2 min at ambient temperature. The oil phase consists of 10 % (w/w) vitamin D$_3$ dissolved in 90 % (w/w) corn oil. The aqueous phase consists of 1 % (w/w) Tween 80 mixed with 99 % (w/w) buffer solution (Na$_2$HPO$_4$-NaH$_2$PO$_4$, 5 mM phosphate, pH 7.0). Oil-in-water nanoemulsions were prepared by passing the coarse emulsions through a microfluidizer (M110L, Microfluidics, Newton, MA, USA) with a 75 μm interaction chamber (F20Y) for 3 cycles at 12,000 psi. Emulsions containing 4 wt % oil phase (0.4 wt % Vitamin D$_3$) were prepared by diluting stock nanoemulsions with phosphate buffer solution. Stock oil-in-water nanoemulsions consisted of 10
wt% oil phase (1 wt% vitamin D₃ + 9 wt% corn oil) and 90 wt% aqueous phase (0.9 wt% Tween 80 + 89.1 wt% 5 mM phosphate buffer solution, pH 7). Emulsions containing 4 wt % oil phase (0.4 wt % vitamin D₃) were obtained by diluting stock emulsion in phosphate buffer solution.

2.2.3 Simulated GIT model

Food-grade TiO₂ particle suspensions were prepared by adding 1 wt% of powdered E171 ingredient into ultrapure deionized water, and then sonicating for 15 min (Fisherbrand™ 120 Sonic Dismembrator) to reduce particle agglomeration. Model food emulsions containing a range of different E171 concentrations were prepared by mixing (1:1 w/w) aqueous E171 suspensions (0, 0.01, 0.5, 1, 5, 10 wt%) with stock emulsions (0.4 wt% VD₃, 3.6 wt% oil) and then stirred for another 20 min. The final samples used in the simulated GIT experiments therefore contained 0.2 wt% VD₃, 1.8 wt% oil and 0, 0.005, 0.25, 0.5, 2.5, or 5 wt% E171 particles, respectively.

The test samples were then gone through the simulated GIT to simulate the passage upon mouth (pH 6.8, 2 min), gastric (pH 2.5, 2 hours) and intestinal phases (pH 7.0, 2 hours). The composition of this simulated GIT model were based on the previous study and described in Table S1 with some slight modifications. The details and the procedure of this simulated GIT model were based on the previous studies with some slight modifications. Mouth phase: An aliquot (20 g) of simulated saliva fluid containing 0.06 g mucin were preheated to 37 °C, and then mixed with the same amount (20 g) of the test sample. The pH were adjusted to 6.8 and the mixture were shaken for 2 min at 100 rpm to mimic oral conditions. Stomach phase: An aliquot (20 g) of samples obtained from the mouth phase were mixed with 20 g of simulated gastric fluids (2 g L⁻¹ NaCl, 7 ml L⁻¹ of HCl, and 3.2 g L⁻¹ of pepsin). The pH of the resulting mixture were then adjusted to 2.5 and the system were shaken for 2 hours at 37 °C at 100 rpm to mimic the stomach phase. Small intestine phase: An aliquot (30 g) of the sample collected from the stomach phase were adjusted
to pH 7.0 and mixed with intestinal fluid containing 1.5 mL salt solution, 3.5 mL of bile salts. The mixture were then adjusted back to pH 7.0, and 2.5 mL of freshly prepared lipase solution were added to the samples. An automatic titration unit (Metrohm, USA Inc., Riverview, FL, USA) were used to monitor and maintain the pH at 7.0 by titrating 0.25 N NaOH solution into the reaction vessel at 37 °C for 2 hours to mimic the small intestine phase. The percentage of free fatty acids (FFAs) produced were calculated using the following equation:

\[ FFA(\%) = 100 \times \frac{V_{NaOH} \times C_{NaOH} \times M_{oil}}{2 \times m_{oil}} \]  

(1)

where, \( V_{NaOH} \) (L) is the volume of NaOH solution required to neutralize the released FFAs during small intestine digestion, \( C_{NaOH} \) (mol / L) is the molarity of the consumed NaOH solution, \( M_{oil} \) (g / mol) is the molecular weight of the oil, and \( m_{oil} \) (g) is the total weight of the oil initially presented in the incubation cell.

### 2.2.4 Particle size and zeta potential

Average hydrodynamic diameters and \( \zeta \)-potential values were measured by dynamic light scattering and laser doppler velocimetry by a 90Plus Particle Size Analyzer (Brookhaven, USA). The samples were diluted with buffer solutions or distilled water to provide an optimum light scattering signal. Diluted mouth and intestinal samples were prepared in phosphate buffer (pH 7.0), and diluted gastric samples were prepared in distilled water (pH adjusted to 2.5).

### 2.2.5 Vitamin D\(_3\) bioaccessibility

The bioaccessibility of lipophilic substances is regularly described as the fraction that is dissolved within the mixed micelle phase after small intestine digestion. Raw digesta samples (15g) were centrifuged at 12,000 rpm, 25 °C for 30 min (Thermo Scientific, CL10 centrifuge). The clear supernatant were obtained as the micelle fraction. According to the previous studies, the
bioaccessibility were calculated as the vitamin D$_3$ concentration in the micelle fraction relative to that in the overall digesta.$^{76}$

### 2.2.6 Vitamin D$_3$ extraction and High-performance liquid chromatography analysis

The VD$_3$ concentration in micelle and digesta were determined following the method by Abbasi et al.$^{88}$ The VD$_3$ calibration curve were established over the range of 2 to 10 μg g$^{-1}$ with acceptable linearity ($R^2 = 0.9989$). One milliliter of sample (micelle and digesta) were mixed with 2 mL of absolute ethanol and 2 mL of hexane and then vortexed for 10 s. The mixture were centrifuged at 1,750 rpm for 10 min. One mL of supernatant (organic phase) were transferred into a HPLC glass vial and concentrated under nitrogen atmosphere. One mL of methanol were added to the vial to re-suspend the vitamin D$_3$. All the samples were then filtered through a 0.20 um filter prior to High-performance liquid chromatography (HPLC, Shimadzu Prominence, Japan) containing a reverse-phase C18 column (ZORBAX Eclipse Plus, 4.6 × 150 mm, 5.0 μm, Agilent) and coupled to a UV detector set at 265 nm. The mobile phase were acetonitrile–water (95/5; v/v) at a flow rate 1 mL min$^{-1}$. The bioaccessibility were then calculated using the following equation$^{76, 85}$:

$$\text{Bioaccessibility} (\%) = 100 \times \frac{C_{\text{micelle}}}{C_{\text{digesta}}} \quad (2)$$

where $C_{\text{micelle}}$ (μg g$^{-1}$) and $C_{\text{digesta}}$ (μg g$^{-1}$) are the concentrations of vitamin D$_3$ in the micelle phase and in the total digesta after the simulated intestinal digestion, respectively.

### 2.2.7 Ultraviolet–visible spectroscopy measurements

*Tween 80*: Tween 80 were measured based on the method by Brown et al.$^{89}$ Tween 80 were detected at 318.5 nm using Ultraviolet–visible spectroscopy (UV-VIS, Agilent 8453, USA). Samples containing 0.36 wt % Tween 80 (a food-grade nonionic surfactant) and various E171 concentrations (0, 0.005, 0.5 wt %) were carried out through the simulated mouth, stomach, and
small intestine phases. The standard curve were prepared by eluting tween 80 in phosphate buffer to various concentrations (0 - 0.27 mM) \((R^2 = 0.997)\).

**Bile salts**: Bile salts were determined according to Wang et al.\(^9\) The bile salts were detected at a wavelength of 309 nm using a UV-VIS. Samples containing 12 mM bile salts were mixed with aqueous E171 suspensions (0, 0.005, or 0.5 wt\%) and then passed through the small intestine phase. A standard curve were prepared by diluting a series of bile salt in phosphate buffer solutions (0-7 mM) \((R^2 = 0.9994)\).

**Lipase**: The amount of lipase were detected by measuring the absorbance at 260 nm using a UV-Vis. The samples containing 3125 mg L\(^{-1}\) lipase were mixed with aqueous E171 suspensions (0-1526.5 mg L\(^{-1}\)) and then passed through the small intestine phase. A standard curve were prepared by diluting a series of lipase in phosphate buffer solutions (0 – 600 mg L\(^{-1}\)) \((R^2 = 0.9998)\).

2.2.8 **Inductively coupled plasma optical emission spectrometry measurements**

The solution composed of saliva, gastric, and small intestinal fluid with 0, 0.005 or 0.5 wt\% E171 were passed through the small intestine phase (only mineral ions). After incubation in small intestine phase (0, 0.5 or 2 hours), the mixtures were centrifuged at 12,000 rpm for 30 min and the supernatants were collected to measure the calcium (Ca) and phosphorus (P) concentration. Briefly, 0.5 g supernatant were digested with 0.50 mL of HNO\(_3\) at 115 °C for 40 mins using a digestion unit (DigiPREP MS digestor, SCP Science, Quebec, CND). Prior to analysis, 0.5 g sample were diluted to 14 mL using distilled water. Inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 3300 DV, Norwalk, CT, USA) were used to determine the Ca and P concentration in the supernatant.

2.2.9 **Fourier transform infrared spectroscopy and x-ray diffraction**
Fourier transform infrared spectroscopy (FT-IR spectrometer, PerkinElmer Spectrum, USA) were used to obtain the spectra of E171 and Tween 80 in each simulated GIT phase, as well as the presence of calcium-phosphate precipitates in the small intestine phase. Transmission spectra were scanned 200 times from 4000 to 500 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). The composition and crystallinity of raw powder E171, and of calcium phosphates sedimentation in the presence and absence of E171 in the small intestine phase were characterized by X-ray diffraction (XRD) and data were recorded with constant scanning 2\(\theta\) from 10\(^{\circ}\) to 80\(^{\circ}\) at 0.0263\(^{\circ}\) per step.

### 2.2.10 Statistical analysis

Each measurement were carried out on three samples (triplicates). The results were calculated as averages and standard deviations on Microsoft Excel 2019 (Microsoft Corp., USA). Analysis of variances (ANOVA) were carried out on OriginPro 2018 statistical software (OriginLab Corp., USA). A Tukey test at a \(p\)-value of < 0.05 were regarded as significantly different across all treatments.

### 2.3 Results and Discussion

#### 2.3.1 Characterization of E171 particles and emulsion droplets

*Particle size and zeta potential of E171 particles*

In the distilled water, the \(\zeta\)-potential of E171 steadily decreased from highly positive (+39 ± 7 mV) at pH 2 to highly negative (-54 ± 2 mV) at pH 9 (Figure 2-1A). The isoelectric point (IEP) of the E171 particles was around pH 3.5. At pH close to the IEP, the E171 particles were unstable as indicated by the large increase in hydrodynamic diameter (794 ± 6 nm). In simulated digestion fluids (only mineral ions), the hydrodynamic diameters of E171 particles were relatively large (> 1000 nm) at pH < 7, indicating that extensive particle aggregation occurred (Figure 2-1B). The \(\zeta\)-potential of E171 particles changed from + 10 to -10 mV with increasing the pH in the digestion
fluids. The lower surface potential and higher aggregation of the E171 particles in the digestion fluids can be ascribed to the relatively high levels of NaCl and CaCl$_2$ (Table 2-S1). These mineral ions would have decreased the magnitude and range of the electrostatic interactions through electrostatic screening, thereby promoting particle aggregation.$^{91}$ At pH $\geq$ 7, calcium and phosphate formed precipitates on the surfaces of the E171 particles, as confirmed by FTIR and XRD analysis (as presented later), as a result, the large rise in hydrodynamic diameters (> 2000 nm) was observed when the pH was increased above 7.

**Particle size and zeta potential of E171 particles and lipid droplet mixtures in simulated GIT fluids**

The emulsions were stable in each phase because Tween 80 (non-ionic surfactant) coated the lipid droplets was able to prevent droplet aggregation by generating strong steric repulsive forces (Figures 2-1C-D). Even though Tween 80 is supposed to be nonionic, the $\zeta$-potentials of the emulsions in the mouth and intestinal phases were -13 ± 1 mV and -13 ± 2 mV, respectively. It has been reported that lipid droplets emulsified by nonionic surfactants are negatively charged at neutral pH likely due to impurities presented in the surfactants and/or oil, or the preferential adsorption of hydroxyl groups onto the droplet surfaces.$^{92}$ The E171 particles rapidly formed large agglomerates after exposure to stomach and small intestine phases. Under these conditions, the $\zeta$-potentials of E171 particles became close to zero, which was likely due to the high ionic strength (Figure 2-S1).

**2.3.2 Impact of E171 particles on Vitamin D$_3$ bioaccessibility**

The impact of E171 particles on the bioaccessibility of VD$_3$ in the emulsions was measured upon passage through the simulated GIT (Figure 2-1E). VD$_3$ bioaccessibility was around 80% in the control, consistent with a previous study.$^{85}$ In comparison with oil type, lipid-bioactive bioaccessibility was higher in long chain triglycerides (LCT) than medium chain triglycerides
In this study, corn oil, as LCT, was used to solubilize VD₃ for emulsion preparation as a result of high bioaccessibility. Additionally, tween 80 as emulsifier used in this study prompted bioaccessibility since small lipid droplets were stable entering small intestine phase, which is consistent with the stable particle size of emulsions in the three phases (Figure 2-1C). The bioaccessibility of VD₃ was 81% and 74% in the presence of 0.005 and 0.5 wt% of E171, respectively, and the latter treatment was significantly lower than the control. These results indicate that comparatively high amounts of E171 particles can cause a significant reduction in VD₃ bioaccessibility. Previous studies have demonstrated that the bioaccessibility of nutraceuticals is strongly linked to the extent of lipid phase digestion they are dissolved in. In our study, the bioaccessibility of VD₃ was positively correlated ($R^2 = 0.95$) to the final extent of FFAs released from the lipid droplets (Figure 2-1F). It is observed that at higher level of final FFAs releases, the VD₃ bioaccessibility was above 80%, while VD₃ bioaccessibility was less than 72% at the lower level of final FFAs released. The VD₃ bioaccessibility is likely to depend on lipid digestion: since lipid phase must be digested before the vitamin is released from the droplets, then VD₃ could be solubilized in mixed micelles to become accessible. We therefore hypothesized that the reduction in VD₃ bioaccessibility was a result of inhibition of lipid digestion by E171. In addition to droplet characteristics (oil phase composition, emulsifier type, and particle size), the digestion of the lipid droplets is also regulated by gastrointestinal conditions, i.e., lipase, bile salts, and calcium level. Thus, we further explored impact of the E171 particles on lipid digestion, as well as interaction between E171 and some critical constituents known to impact lipid digestion (such as emulsifier, bile salts, calcium ions, and lipase).

**2.3.3 Impact of E171 particles on lipid digestion**
The effect of E171 particles on lipid digestion was determined by measuring the generation of FFAs produced from the lipid droplets during the small intestine phase. The lipid digestion profiles of VD₃ emulsions exhibited a similar pattern in the absence or presence of E171 (Figure 2-2A). More than 70% of the FFAs were released during the first 10 min, with the remainder being released more slowly at longer times, until a comparatively steady level was reached after 2 hours.

The maximum amount of FFAs released ranged from around 79% to 95% depending on the E171 concentration (Figure 2-2A). In the absence of E171 particles, the maximum extent of the FFAs released was around 95%. The addition of 0.005 wt% of E171 had little influence on lipid digestion. However, the addition of 0.5 wt% of E171 led to an appreciable decrease to 88% by the end of digestion. Figure 2-2B shows that the final extent of FFAs released reduced with increasing E171 concentration (R² = 0.91). These results indicate that high amounts of E171 particles inhibited lipid digestion during intestinal phase. Li et al. reported that emulsions with TiO₂ particles showed a significant decrease in final extents of lipid digestion compared to the one in the absence of TiO₂ particles.⁸⁷ In addition, pectin, as a dietary fiber, significantly decreased the rate and extent of lipid digestion, which was attributed to its binding with gastrointestinal components, i.e., bile salts, calcium, and lipase.⁹⁹ Given this reason, we implemented further experiments to identify the origin of these effects.

It is known that surfactants, bile salts, lipase, and calcium ions impact the rate and extent of lipid digestion in oil-in-water emulsions, as well as the bioaccessibility of nutraceuticals.⁷⁶⁻⁷⁸ We hypothesized that increasing the amount of E171 might inhibit lipid digestion, thereby decreasing vitamin bioaccessibility, through various mechanisms: (1) E171 particles might adsorb Tween 80 molecules to their surfaces, altering the aggregation stability of lipid droplets, which can change the surface area of lipids exposed to lipase; (2) E171 particles might interact with bile salts,
reducing bile salt adsorption onto the lipid droplet surfaces and vitamin solubilization within the intestinal fluids; (3) E171 might interact with lipase and decrease the amount available to digest the lipids; (4) E171 particles might interact with calcium ions to reduce their ability on removal of long-chain FFAs from the lipid droplet surfaces. A sequence of experiments was therefore executed to establish the relative importance of these different mechanisms.

**Tween 80:** Tween 80 is a nonionic surfactant commonly used as an emulsifier in the food industry. In this study, the vitamin-loaded lipid droplets were coated with this surfactant. The concentrations of Tween 80 in simulated GIT fluids containing different E171 levels were measured (Figure 2-2C). For each GIT phase, no significant difference in Tween 80 concentration was seen in samples containing different E171 levels, suggesting that there was no appreciable surfactant adsorption onto the E171 particles. The Tween 80 concentration used in these experiments was above the critical micelle concentration (around 13 μg mL⁻¹), suggesting that the surfactant should form micelles in the mouth, gastric, and intestinal phases. The significant differences in different GIT phases were due to the dilution factor: 0.5 for the stomach phase and 0.8 for the small intestine phase.

**FTIR** analysis provided additional evidence that no Tween 80 was adsorbed onto the E171 particles in any of the simulated GIT phases. The FTIR spectrum of pure Tween 80 is shown in Figure 2-2D. The peak at 1737 cm⁻¹ is consistent with a C=O stretching vibration associated with the ester structure. Interestingly, no characteristic band associated with Tween 80 was found in the mixed E171 particles/Tween 80 system after incubation in the mouth (2 min), gastric (2 hours), and intestinal (2 hours) phases. The peak around 3500 - 3000 cm⁻¹ and the peak around 1650 - 1600 cm⁻¹ are associated with hydroxyl groups from water molecules adsorbed onto the titanium dioxide surfaces. After passing through the small intestine phase, the spectrum of the E171
particles/Tween 80 system showed distinct peaks around 1200 – 900 cm\(^{-1}\), which can be attributed to the P-O bands arising from calcium-phosphate precipitates formed in the intestinal phase.\(^{103}\) Hence, there was no strong interaction between the Tween 80 and E171 in any of the simulated GIT phases.

**Bile Salts:** Bile salts are natural anionic surfactants which have hydrophilic and hydrophobic regions on their surfaces. During the lipid digestion, bile salts can bind to lipid droplet surfaces and remove the original emulsifiers from the oil-water interface (such as proteins, phospholipids, or surfactants), thereby facilitating the capability of lipase to bind to the droplet surfaces and digest the oil phase.\(^ {104}\) In addition, bile salts promote lipase activity by removing lipid digestion products, such as monoglycerides and FFAs, from the lipid droplet surfaces.\(^ {105}\) This is because accumulated lipolysis products can impede the access of lipase to the droplet surfaces and slow down digestion.\(^ {106}\) The lipid digestion products can also form mixed micelles that increase the solubility of VD\(_3\) in the intestinal liquids.\(^ {105}\) No significant difference in the concentrations of bile salts was observed when different levels of E171 were used or across the different time points (Figure 2-2E). Thus, there was no interaction between E171 particles and bile salts. Interestingly, the measured bile salts concentrations (< 8 mM) were appreciably lower than the initial concentrations (12 mM) in the small intestine phase, indicating that some of the bile salts settled down to the bottom of the tubes after centrifugation. This effect can be attributed to the formation of electrostatic complexes (“calcium soaps”) between cationic calcium ions and anionic bile salts, which was supported by the change in the ζ-potential and concentration of the bile salt solutions with or without calcium (Figure 2-S2). Without calcium ions, the concentration of bile salts was around 11 mM, close to the initial concentration, suggesting that the anionic E171 particles did not
strongly bind the anionic bile salts. In the presence of 10 mM calcium, bile salt concentrations decreased to around 8 mM, suggesting that calcium ions could bind to bile salts and precipitate.

\textit{Lipase:} Lipase plays a critical role in lipid digestion by converting triglycerides to monoglycerides and FFAs.\textsuperscript{77} A recent study reported that microplastics (MPs) reduce lipid digestion via two mechanisms: one is the formation of lipid-MPs aggregates decreasing the bioavailability of lipid droplets, and another is the adsorption of lipase onto microplastics reducing enzyme activity.\textsuperscript{107} In our study, tween 80, as surfactant to coat on lipid surface, showed no interaction with E171 across all the phases. But the free lipase concentration in the small intestine phase decreased as the concentration of E171 particles increased ($R^2 = 0.98$). In particular, the presence of 0.5 wt% E171 particles decreased the lipase concentration by 11% (Figure 2-3A-B). Thus, the ability of E171 to bind lipase could be responsible for the observed decrease in lipid digestion observed in our study.

To better understand the origin of the interaction between lipase and E171 particles, we examined the role of calcium ions. Without calcium ions, lipase concentration decreased significantly with increasing the concentration ratio (E171 over lipase) larger than 0.02, and free lipase decreased by 2% with increasing the ratio at 0.5; However, in the presence of calcium ions, the lipase concentration decreased significantly with increasing the ratio beyond 0.005, and free lipase decreased by 11% at the ratio of 0.5 (Figure 2-S3a). The $\zeta$-potential determination exhibited that the magnitude of the negative charge on E171 particles decreased appreciably with increasing CaCl$_2$ concentration, from around -20 to nearly 0 mV (Figure 2-S2a). In comparison with E171, the $\zeta$-potential of lipase was significantly decreased from -21.5 ± 2.2 mV to -14.4 ± 0.8 mV by adding 10 mM calcium (Figure 2-S3b). These results suggest that the cationic calcium ions can reduce the surface potential of E171 particles by charge screening and ion binding.\textsuperscript{82} In addition,
the calcium ions may act as bridges between the surfaces of the anionic lipase and anionic E171, thereby holding them together. After incubating with 10 mM CaCl₂, E171 immediately formed large agglomerates. Thus, lipase binding to the flocs could impede the capability of lipase to access the lipid droplet surfaces, further decreasing lipid digestion.⁹⁹

_Calcium Ions_: Characteristic peaks appeared around 1200 - 900 cm⁻¹ were assigned to P-O bonds across all three levels of E171 in the small intestine phase (Figure 2-3C).¹⁰³ Significantly, the spectra of these specific peaks were similar to the FTIR spectrum of hydroxyapatite¹⁰⁸, suggesting calcium-phosphate deposited as hydroxyapatite in the small intestine phase. The strongest peak for all the treatments was observed in the range from 1030 - 1010 cm⁻¹, which corresponds to the ν₃ PO₄³⁻ band of apatite. A shoulder peak corresponding to the ν₁ mode of PO₄³⁻ was observed at approximately 960 cm⁻¹ in the samples containing 0 and 0.005 wt% E171. In addition, an obvious peak was present between 1120 and 1110 cm⁻¹ in the samples containing 0.5 wt% E171 but only a broad shoulder was seen in the other treatments. This peak can be found in the poorly crystalline hydroxyapatite and may correspond to the ν₆’ and ν₆’’ PO₄³⁻ degenerate stretching of the HPO₄²⁻.¹⁰⁸

Importantly, XRD analysis showed peaks at 2Θ = 32° and 45°, which are characteristic of hydroxyapatite (Figure 2-3D).¹⁰⁹ Also, precipitates formed in the presence of 0.005 and 0.5 wt% E171 particles had a strong peak at 2Θ = 24.5°, which is consistent with the anatase form of titanium dioxide. Thus, both FTIR and XRD analysis demonstrated the presence of calcium-phosphate precipitates (hydroxyapatite) in the small intestine phase. In addition, XRD analysis of the powdered E171 particles indicated that the major composition of the E171 ingredient used in our experiments was anatase rather than rutile (Figure 2-S4). It has been reported that apatite
preferably deposited on the surfaces of anatase gels rather than rutile gels, implying that TiO$_2$ with anatase crystal structure could be more effective for apatite deposition.$^{80}$

The amount of phosphorus deposited at the initial stage of the small intestine phase increased with E171 concentration, being 37%, 37%, and 57% for 0, 0.005, and 0.5 wt% E171, respectively (Figure 2-3F). This suggests that some of the phosphate anions were able to adsorb onto the E171 surface. The amount of calcium present in the precipitates also increased with increasing E171 concentration, being 23%, 24%, and 30% for 0, 0.005, and 0.5 wt% E171, respectively (Figure 2-3E). Thus, our results suggest that calcium-phosphate precipitated onto the surface of E171 particles at the beginning of small intestine phase. Previous researchers have reported that titanium dioxide can serve as a reactive substrate to accelerate calcium-phosphate precipitation.$^{110}$ Calcium and phosphate deposition reached maximum levels (around 41% and 95%, respectively) after 30 mins in the small intestine phase for each treatment. Nevertheless, the percentage of deposited calcium and phosphate by the end of the small intestine phase were not significantly different, indicating no extra adsorption of calcium in the presence of E171 particles. Thus, the mechanism by which E171 decreased the VD$_3$ bioaccessibility was that lipase could adsorb onto the surface of E171 via electrostatic attraction in the presence of calcium ions, then inhibiting lipid digestion and reducing the bioaccessibility of VD$_3$. Our findings improved our understanding of the interaction mechanism of E171 particles with gastrointestinal constituents during lipid digestion, as well as the impact of E171 on lipid digestion profile and nutraceutical bioaccessibility. Further experiments should be conducted using animals (i.e., in vivo studies) to provide a better understanding of impacts of E171 on the nutritional attributes of foods for human health and wellness.

2.4 Conclusions
Taken together, the addition of 0.5 wt% E171 significantly decreased the bioaccessibility of VD₃ from 80 to 74% as compared to 0 and 0.005 wt% E171 in the simulated GIT. The bioaccessibility of VD₃ was positively correlated with the amount of FFAs produced during lipid digestion (R² = 0.95). Furthermore, with increasing amount of E171 in the system, the final amount of FFAs released from the emulsions gradually decreased. E171 had no significant impact on the concentrations of Tween 80, bile salts, and calcium ions under simulated digestion conditions. However, we found that lipase could adsorb onto the surface of E171 via electrostatic attraction in the presence of calcium ions, with the free lipase concentration decreasing as E171 concentration increased (R² = 0.98). Additionally, E171 particles rapidly agglomerated in the presence of calcium ions, attributed to ion binding and electrostatic screening. Consequently, the binding of lipase molecules to E171 could inhibit lipid digestion, thereby reducing the bioaccessibility of VD₃.

Our findings enhanced our understanding of the interaction mechanism of E171 particles with gastrointestinal constituents during lipid digestion, as well as the impact of E171 on lipid digestion profile and nutraceutical bioaccessibility. Further experiments should be implemented in animals in vivo studies to provide a better understanding of impact of E171 on the nutritional attributes of foods for human health and wellness.
Figure 2-1. Food grade TiO$_2$ (E171) hydrodynamic particle size and ζ potential values in distilled water (A) and simulated gastrointestinal fluid (B) with only mineral ions as a function of pH. The isoelectric point of E171 is at pH 3.5; (C) particle size (nm) of emulsions, 0.005 wt% and 0.5 wt% E171 in the mouth phase, stomach phase and small intestine phase (only mineral ions); (D) ζ potential values (mV) of emulsions, 0.005 and 0.5 wt% E171 in the mouth phase, stomach phase and small intestine phase (only mineral ions); (E) vitamin D$_3$ bioaccessibility in emulsions with 0, 0.005 and 0.5 wt% E171; (F) the correlation ($R^2 = 0.95$) between final free fatty acid (%) released from lipid droplets and vitamin D$_3$ bioaccessibility (%). Bars with different capital letters (A-C) are significantly different ($p < 0.05$) when comparing between different GIT phases for same treatment. Bars with different lower-case letters (a-c) are significantly different ($p < 0.05$) when compared to emulsions, 0.005 and 0.5 wt% E171 in the same region.
Figure 2-2. (A) Free fatty acid release from lipid droplets in the small intestine phase over 2 hours under 0, 0.005, 0.25, 0.5, 2.5 and 5 wt% E171; (B) the correlation ($R^2 = 0.91$) between E171 addition (wt%) released from lipid droplets and final released FAAs (%); (C) Tween 80 concentration (mM) in the mouth phase (2 mins), stomach phase (2 hours), and small intestine phase (2 hours) under 0, 0.005 and 0.5 wt% E171 (only mineral ions); (D) FTIR spectra of Tween 80, E171, E171-Tween 80 samples in the mouth, stomach, small intestine phases; (E) bile salts concentration (mM) in the small intestine phase (0 and 2 hours) under 0, 0.005 and 0.5 wt% E171. Bars with different capital letters (A-C) are significantly different ($p < 0.05$) when compared between different GIT phases for the same treatment. Bars with the same capital letters are not significantly different ($p < 0.05$) when compared between 0 hour and 2 hours (same treatment); Bars with same lower-case letters are not significantly different when compared 0, 0.005 and 0.5 wt% E171 in the same phase or same time period.
Figure 2-3. (A) UV-vis spectra of lipase (initial concentration 3125 mg L\(^{-1}\)) under various initial concentrations of E171 (0, 15.625, 62.5, 156.25, 625, 937.5, 1562.5 mg L\(^{-1}\)) in the small intestine phase; (B) the correlation \((R^2 = 0.98)\) between lipase concentration (mg L\(^{-1}\)) in the supernatant and initial concentration ratio of E171 to lipase (0, 0.005, 0.02, 0.05, 0.2, 0.3, 0.5); (C) FTIR spectra and (D) XRD patterns of Ca-P precipitates at 0, 0.005, and 0.5 wt% E171; (E) deposited calcium (%) and (F) phosphorus (%) in the small intestine phase under 0, 0.005 and 0.5 wt% E171 after 0, 0.5 and 2 hours. Bars with different capital letters (A-C) are significantly different \((p < 0.05)\) when compared between different GIT phases for the same treatment. Bars with lower-case letters (a-b) are significantly different \((p < 0.05)\) when compared 0, 0.005 and 0.5 wt% E171 at the same time period.
Table 2-S1. Chemical composition of simulated gastrointestinal fluids (stock solution) used to prepare the mouth, stomach and small intestine phases of the simulated gastrointestinal tract (GIT) model.

<table>
<thead>
<tr>
<th>Gastrointestinal stock solution</th>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva</td>
<td>Sodium chloride</td>
<td>1.594 g/L</td>
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<tr>
<td></td>
<td>Ammonium nitrate</td>
<td>0.328 g/L</td>
</tr>
<tr>
<td></td>
<td>Monopotassium phosphate</td>
<td>0.636 g/L</td>
</tr>
<tr>
<td></td>
<td>Potassium chloride</td>
<td>0.202 g/L</td>
</tr>
<tr>
<td></td>
<td>Potassium citrate</td>
<td>0.308 g/L</td>
</tr>
<tr>
<td></td>
<td>Uric acid sodium salt</td>
<td>0.021 g/L</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0.198 g/L</td>
</tr>
<tr>
<td></td>
<td>Lactic acid sodium salt</td>
<td>0.146 g/L</td>
</tr>
<tr>
<td>Gastric fluid</td>
<td>Sodium chloride</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td></td>
<td>Hydrochloric acid</td>
<td>7 mL/L</td>
</tr>
<tr>
<td>Small intestinal fluid</td>
<td>Calcium Chloride Dihydrate</td>
<td>36.667 g/L</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>219.133 g/L</td>
</tr>
</tbody>
</table>
Figure 2-S1. The ζ potentials of E171 in DI water at pH 7.0 as a function of CaCl$_2$ concentration (0-10 mM). Bars with lower case letters (a-c) are significantly different ($p < 0.05$) between different CaCl$_2$ concentrations.
Figure 2-S2. (a) ζ potentials of bile salts and E171 under 0, 10 mM CaCl2 in the small intestine phase. Bars with different capital letters (A-B) are significantly different ($p < 0.05$) when compared to different CaCl2 concentrations. Bars with different lowercase letters (a-b) are significantly different ($p < 0.05$) when compared to bile salts and E171 under a same CaCl2 concentration; (b) bile salts concentration in the small intestine phase (0 hour and 2 hours) under 0.5 wt% E171, 10 mM CaCl2, and 0.5 wt% E171 + 10mM CaCl2. Bars with capital letter A were not significantly different ($p < 0.05$) when compared between 0 hour and 2 hours phases (same treatment). Bars with different lowercase letters (a-b) were significantly different ($p < 0.05$) when compared to different treatments at the same period.
Figure 2-S3. (a) Lipase concentration (mg L⁻¹) under various ratios of initial concentrations of E171 to lipase (0, 0.005, 0.02, 0.50, 0.2, 0.3, 0.5) in the absence or presence of calcium (0, 10 mM). Bars with different capital letters (A-E) are significantly different ($p < 0.05$) when compared to different concentration ratio of E171 to lipase. Bars with different lowercase letters (a-b) are significantly different ($p < 0.05$) when compared to different CaCl₂ concentration; (b) $\zeta$ potentials of lipase under 0, 10 mM CaCl₂ in the small intestine phase. Bars with different lowercase letters (a-b) are significantly different ($p < 0.05$) when compared to different CaCl₂ concentration.
Figure 2-S4. XRD pattern of the E171 particles.
CHAPTER 3

3. FOOD-GRADE TITANIUM DIOXIDE PARTICLES DECREASE THE BIOACCESSIBILITY OF IRON RELEASED FROM SPINACH LEAVES IN SIMULATED HUMAN GASTROINTESTINAL TRACT

3.1 Introduction

Titanium dioxide (TiO\(_2\)) particles have been used as an additive in paints, personal care products, and foods.\(^{102}\) As a food additive, titanium dioxide (E171) has been reported to be present in more than 900 commonly consumed food products, which is mainly because of its ability to improve their aesthetic properties, especially their appearance.\(^{111}\) In the USA, the Food and Drug Administration (FDA) limits the use of E171 as a food additive to 1% of the overall food weight.\(^{112}\) In Europe, E171 can be used in foods at a quantum satis level, \textit{i.e.}, the quantity required to achieve the intended purpose.\(^{113}\) TiO\(_2\) particles are typically used as whitening or brightening agents because they have a relatively high refractive index and scatter light strongly. They are used for this purpose in numerous foods, including confectionery, dairy products, sauces, salad dressings, and pastries.\(^6\) Their level is particularly high in many candies, with TiO\(_2\) content reaching 2.5 mg Ti g\(^{-1}\) of food. It is estimated that the average adult can consume 0.7-5.9 mg of TiO\(_2\) kg\(^{-1}\) of body weight (BW) per day, and children can consume up to 5 times that of an adult person using this same metric.\(^7\) These studies have highlighted that there may be appreciable levels of E171 in the human diet depending on the nature of the foods consumed.\(^{67}\)

Spinach, a leafy green vegetable, is one of the most common ingredients of salads consumed worldwide.\(^{114}\) Its consumption is claimed to have a number of positive biological impacts, including antioxidant, anti-inflammatory, antiproliferative, and anti-obesity effects, which can
protect humans against chronic disease. Spinach is also an important source of vitamins, minerals, phenolic compounds, carotenoids, and dietary fibers. Minerals are necessary for the maintenance of certain physiological processes that are essential to human life. Ca, as a cofactor for many enzymes, is also a messenger in intracellular cascade signaling reactions. Fe, Mn, and Zn are cofactors participating in the activity of numerous physiological processes, such as cellular homeostasis and survival, as well as organ and tissue development. Mineral deficiencies are a worldwide health problem affecting people in developing and some developed countries. It is estimated that Ca, Zn and Fe deficiencies lead to health issues in billions of people globally. Low Zn blood levels may lead to clinical symptoms, including growth retardation, impaired brain development and cognitive performance, infertility, and increased risk of infections. Fe deficiency is the major cause of anemia associated with respiratory weakness, as well as abnormal mental and motor development. Previous studies have focused on the total amount of minerals present in the food matrix, but this may not provide an inaccurate evaluation of the nutritional potential of the minerals. The term “bioaccessibility” is defined as nutrients that are released from the food matrix into the gastrointestinal tract and are available for absorption within the small intestine. The low bioaccessibility of minerals in some food products can contribute to inadequate mineral intake.

As mentioned earlier, E171 particles are commonly used as additives in salad dressings to improve their visual appearance. Salad dressings are designed to be consumed with salads, which contain leafy greens that are rich in essential minerals. Titanium dioxide is known to interact with digestive enzymes and minerals (see below). We therefore hypothesized that E171 particles may impact the bioaccessibility of essential minerals in the leafy greens found in salads.
A simulated human gastrointestinal tract (GIT) model, which included mouth, stomach and small intestine phases, were used to digest spinach leaves mixed with E171 and to evaluate the effects of E171 particles on the bioaccessibility of minerals in spinach. In the GIT model, salivary \( \alpha \)-amylase were included in the mouth phase to initialize the digestion of any starch.\(^{127}\) Pepsin were added in the gastric fluid to degrade proteins by cleaving peptide bonds under acidic conditions.\(^{128}\) Pancreatin, a gastrointestinal extract containing \( \alpha \)-amylase, lipase and protease, were added into small intestinal fluid to digest fats, proteins, and carbohydrates.\(^{129}\) Spinach leaves are mechanically, chemically, and enzymatically degraded as they pass through each GIT phase, thereby leading to the release of the minerals into the gastrointestinal fluids. Studies have reported that the activity of numerous kinds of enzymes are decreased after they adsorb onto the surfaces of TiO\(_2\) particles, including \( \beta \)-galactosidase, alkaline phosphatase, \( \beta \)-glucosidase, L-leucin andaminopeptidase.\(^{130-132}\) Researchers have also reported that TiO\(_2\) particles significantly decreased pepsin activity due to particle-enzyme interactions.\(^{133}\) In addition, several studies have demonstrated that TiO\(_2\) nanoparticles can interact with heavy metals, including As,\(^{134}\) Cd,\(^{135}\) Zn,\(^{135}\) and Cu\(^{136}\) via electrostatic interactions or chemical bonding.\(^{138}\) Our results provide useful information about the potential impact of E171 additives on the human digestive system and nutritional value of foods. We hypothesized that E171 could decrease mineral bioaccessibility in the GIT model via two mechanisms: (i) E171 particles may inhibit enzyme activities (\textit{e.g.}, \( \alpha \)-amylase, pepsin, and pancreatin), thereby reducing the digestion of the spinach leaves and the release of the minerals; and (ii) E171 particles may bind with the minerals released from the spinach leaves, thereby decreasing their bioaccessibility.

To test these hypotheses, a mixture of spinach leaves and E171 were passed through the GIT model and the bioaccessibility of the minerals released from the spinach were determined. The
concentrations of macronutrients (Ca, K, Mg, P and S) and micronutrients (Fe, Zn, Mn) in the digesta from each stage of the GIT model were measured and their bioaccessibility were then calculated. Mechanistic studies were then carried out using a range of methods to characterize the nature of any interactions between E171 particles, enzymes, and minerals.

3.2 Materials and Methods

3.2.1 Materials

Food-grade TiO$_2$ particles (E171, purity 99%) were purchased from Precheza (Přerov, Czech Republic). Baby spinach (Dole fresh, Dole Food Company, Inc, Boston) were purchased from a local supermarket. Mucin from porcine stomach, bile salts, pepsin from porcine gastric mucosa ($\geq$ 250 units mg$^{-1}$), $\alpha$-amylase from porcine, and pancreatin from porcine pancreas (100 - 500 units mg$^{-1}$) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals or reagents used in this study were of analytical grade and were purchased from either Sigma-Aldrich or Fisher Scientific.

3.2.2 Simulated GIT model

The simulated GIT model (Table S1 of the Supporting Information, SI) has been widely used in a number of previous studies with some minor modifications. A 1% (w/w) of food grade TiO$_2$ (E171) particle stock suspension were prepared by adding E171 powder into distilled water, and then sonicating for 15 min using a sonicator (FisherbrandTM 120 Sonic Dismembrator) to reduce particle agglomeration.

Different concentrations of E171 (0, 0.002, 0.05 and 0.2 wt%) were prepared by diluting the stock E171 suspension in nano-pure water. The concentrations of E171 in the gastrointestinal system were selected and used based on around 1:2 ratio of a salad dressings (TiO$_2$ 113 mg per serving)$^6$ and spinach leaves (85 g per serving). Fresh spinach leaves were selected and then cut
into 10 mm × 10 mm pieces. Two g of spinach leaves and 13 g of E171 suspensions with different concentrations were placed in a glass vial containing 15 g of simulated saliva fluid (SSF), in which the concentrations of mucin and α-amylase were 3.0 and 0.5 g L\(^{-1}\), respectively. The mixture were adjusted to pH 6.8 and then shaken for 5 min in a shaker maintained at 37 °C to simulate mouth agitation. Then 15 g of oral digesta solution were collected and stored for measuring the amount of minerals released in the mouth phase. An aliquot of simulated gastric fluid (15 g) containing pepsin (3.2 g L\(^{-1}\)) were then added into the vials. The mixture were then adjusted to pH 2.5 and shaken at 37 °C and 100 rpm to mimic the stomach phase. After a 2-hour incubation, 5 g of gastric digesta solution were collected and stored in the refrigerator for element concentration determination in the gastric phase. The remaining sample were then adjusted to pH 7.0 and 5 mL of simulated intestinal fluid (SIF), which contained pancreatin (14.4 g L\(^{-1}\)) and bile salts (30 g L\(^{-1}\)), were added. The pH were then adjusted to 7.0 and the sample were shaken at 37 °C and 100 rpm to simulate small intestine phase. After a 2-hour incubation, the intestinal digesta were collected and stored in the refrigerator for element concentration determination in the small intestine phase.

To test the adsorption of released minerals onto E171 particles, an identical experiment were conducted but without the addition of E171. The collected samples at each phase were centrifuged and then separated into two portions: one portion were used to measure the elemental content in the absence of E171; the other portion were mixed with suspensions containing different concentrations of E171 (0.002, 0.05, 0.2 wt%). Briefly, the mixture was shaken for either 5 min or 2 hours to simulate each phase. After incubation, a sample of digesta solution (1 or 2 g) were collected from mouth, stomach, and small intestine phases for ICP-OES measurement. The amount
of minerals adsorbed onto the E171 particles were determined by the differences between the two corresponding portions.

3.2.3 Element measurement by ICP-OES

The samples collected from each phase were passed through the simulated GIT tract and then centrifuged at 4000 rpm for 30 min. The supernatant were used to measure the concentration of macronutrients (Ca, K, Mg, P, S) and micronutrients (Fe, Mn, Zn) released from the spinach leaves in the mouth, stomach, and small intestine phase. The supernatants and leaf debris collected from each phase were digested with HNO₃ at 115 °C for 40 min. The digesta were diluted to 15, 20 and 30 mL using deionized water for mouth, stomach, and small intestine phases, respectively. Inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 6500, Thermo Fisher Scientific, Waltham, MA) were used to measure the element concentrations. The bioaccessibility of the minerals in each phase were calculated as follows:

\[ \text{Bioaccessibility (\%)} = \frac{\text{mineral in collected supernatant (\(\mu\)g)}}{\text{total mineral (\(\mu\)g)}} \times 100 \]

3.2.4 UV-visible spectroscopy measurements

The amounts of digestive enzymes that had adsorbed to the surfaces of E171 particles were determined using UV-visible spectroscopy.

\(\alpha\)-Amylase: Initially, a standard curve \((r^2 = 0.9999)\) of \(\alpha\)-amylase were prepared by dissolving different concentrations of \(\alpha\)-amylase (0 to 3 mg mL\(^{-1}\)) in simulated saliva solution. The absorbance of these solutions were then measured at 260 nm using a UV-Vis spectrophotometer. The interactions between E171 and \(\alpha\)-amylase were assessed by mixing 1.5 mL of E171 suspensions (0, 0.2, 1, 2, 5, 20 mg mL\(^{-1}\)) with 1.5 mL of \(\alpha\)-amylase solution (5 mg mL\(^{-1}\)). The resulting mixtures (pH 6.8) were then shaken at 37 °C for 5 min prior to measurement. After
centrifugation, the concentration of non-adsorbed α-amylase remaining in the solution were then determined from the calibration curve.

**Pepsin:** A calibration curve were established by dissolving a range of concentrations (0-1.6 mg mL\(^{-1}\)) of pepsin in gastric solution (NaCl-HCl) and measuring the absorbance at 296 nm (r\(^2\) = 0.9996). The enzyme-E171 interactions were assessed by mixing 1.5 mL of E171 suspension (0, 0.02, 0.1, 0.2, 0.5, 1, 2 mg mL\(^{-1}\)), 1.5 mL of saliva solution (without α-amylase) and 3.0 mL of pepsin solution (3.2 mg mL\(^{-1}\)) at pH 2.5 and then shaking at 37 °C for 2 hours. The resulting mixture were centrifuged at 4000 rpm for 15 min. The amount of pepsin in the supernatant were then determined by measuring the absorbance at 296 nm.\(^{143}\)

**Pancreatin:** A calibration curve were prepared by dissolving various concentrations of pancreatin (0-0.6 mg mL\(^{-1}\)) in SIF solution (pH 7.0) and then measuring the absorbance at 260 nm using a UV-visible spectrometer (r\(^2\) = 0.9995). The pancreatin-E171 interactions were then assessed by mixing 1.5 mL of E171 suspension (0, 0.0025, 0.0125, 0.025, 0.0625, 0.125, or 0.25 mg mL\(^{-1}\)), 1.5 mL of salivary solution (without α-amylase), and 3.0 mL of gastric solution (without pepsin) and then adjusting the system to pH 7.0. Then, 1.2 mL of small intestine solution containing pancreatin (1.8 mg mL\(^{-1}\)) were added to this mixture and the pH were adjusted to 7.0 again. The total system were then incubated in shaker at 37 °C for 2 hours. After centrifugation, the amount of pancreatin in the supernatant were determined from the calibration curve by measuring the absorbance at 260 nm.

### 2.3.5 Enzymatic Activity Assay (α-amylase, pepsin, and pancreatin)

*α-Amylase:* The α-amylase activity were measured as described in Jiang et al.\(^{35}\) and Terra et al.\(^{144}\) The method used were based on the quantification of the reducing sugars released from the starch hydrolysis reaction, which is catalyzed by α-amylase. The calculation details for α-amylase
activity were described in Experiment S2. A 1% (w/v) soluble starch solution were prepared by dissolving 1.0 g soluble starch into 100 mL boiling buffer solution (20 mM sodium phosphate, 6.7 mM sodium chloride, pH 6.9). A series of concentrations of E171 (0, 0.006, 0.031, 0.062, 0.154, 0.308, or 0.615 mg mL$^{-1}$) were separately mixed with an equal volume (0.5 mL) of $\alpha$-amylase solution (2 unit) at pH 6.8 (by adding 12 µl 0.1N NaOH and around 20 µl 0.01N NaOH) and shaken at 37 ºC for 5 mins. Afterwards, 1 mL of 1.0 % (w/v) soluble starch solution were added into a tube containing the above mixture. After incubation in a shaker at 37 ºC for exact 3 min, the reaction were terminated by adding 1 mL of color reagent solution (20 mL of 96 mM 3,5-Dinitrosalicylic acid solution, 8 mL of 5.3 M potassium sodium tartrate, tetrahydrate solution in 2 M NaOH solution, 12 mL of DI water) into the final product and then all tubes were placed into hot water (90 ºC) for 15 min. After cooling to room temperature, 9 mL of deionized water were added into each tube and mixed thoroughly. After centrifugation (4000 rpm, 15 min), the concentration of maltose in the supernatant were then measured at 540 nm.

Pepsin: The method used to determine pepsin activity were based on that of Zhu et al. with some minor modifications.$^{133}$ Pepsin can convert hemoglobin to tryptophan and tyrosine, both of which are easily detected at 280 nm by UV-vis spectrophotometry. The calculation details for pepsin activity were described in Experiment S3. Samples were prepared by mixing 187.5 µL of E171 suspension (0, 0.002, 0.01, 0.02, 0.05, 0.1, or 0.2 mg mL$^{-1}$), 187.5 µL of saliva solution (without $\alpha$-amylase) and 375 µL of pepsin solution (0.32 mg mL$^{-1}$) and then adjusting to pH 2.5 (by adding 2 µl 6 N NaOH and 16 µl 1 N NaOH) and shaking at 37 ºC for 30 min. Then, 750 µL of pepsin-E171 mixture were incubated with 3.75 mL of bovine hemoglobin solution (5 mg mL$^{-1}$) at 37 ºC. After 30 min, 7.5 mL of 5% (w / v) trichloroacetic acid were added into the mixture to stop the reaction and the sample were shaken for another 10 min at 37 ºC. After centrifugation, the
supernatant were measured at 280 nm. The inhibition rate of α-amylase and pepsin as affected by E171 were calculated as the percentage of the control:

\[
\% \text{ Inhibition rate} = [1 - (\text{OD of test} / \text{OD of control})] \times 100
\]

**Trypsin:** Trypsin, rather than pancreatin, were used in this experiment to determine the impact of E171 on enzyme activity in the small intestine phase. This were done to simplify interpretation of the results since pancreatin contains a complex mixture of different enzymes. Trypsin activity were measured using a vendor-supplied (Sigma) method with some modifications. \(\alpha\)-Benzoyl-L-arginine ethyl ester (BAEE), the final product of the reaction, were used to determine trypsin activity. A mixture containing 0.5 mL of E171 suspension (0 or 0.278 mg mL\(^{-1}\)), 0.5 mL of saliva solution, 1.0 mL of gastric solution and 0.4 mL of trypsin (2 mg mL\(^{-1}\)) were incubated at pH 7.0, 37 ℃ for 10 min. After centrifugation, 1.2 mL of supernatant were immediately mixed with 2.0 mL of BAEE (0.13 mg mL\(^{-1}\)). The increase in absorbance at 253 nm were recorded for 5 min at 1 min intervals. The rate in the increase of the absorbance (\(\Delta A_{253} \text{ min}^{-1}\)) were then used to calculate the amount of BAEE produced (unit mL\(^{-1}\)).

### 3.2.6 FTIR spectroscopy

The FTIR spectra of E171, \(\alpha\)-amylase, pepsin, E171+\(\alpha\)-amylase, E171+pepsin, and E171+pancreatin were recorded using a PerkinElmer Spectrum instrument (USA) that were operated in the attenuated total refeciton (ATR) mode at a resolution of 4 cm\(^{-1}\). Samples of E171+\(\alpha\)-amylase, E171+pepsin, and E171+pancreatin were collected from the mouth phase (pH 6.8, 37 ℃, 5 mins), stomach phase (pH 2.5, 37 ℃, 2 hours), and small intestine phase (pH 7.0, 37 ℃, 2 hours), respectively. The supernatants of these samples were collected after centrifugation. The target compounds, including E171+\(\alpha\)-amylase, E171+pepsin, and E171+pancreatin, were rinsed three times with the corresponding buffer solution (saliva fluid, gastric solution, intestinal
fluid, respectively). The collected samples were re-suspended in the corresponding solutions for FTIR spectra analysis. Each spectrum were collected over the range from 4000 to 500 cm\(^{-1}\) with 200 scans. The final spectra of each sample were an average of three replicates.

### 3.2.7 Fluorescence spectroscopy

Fluorescence spectroscopy were used to investigate the impact of E171 concentration (0, 1, 2, 4, 6, 8, 10 mg L\(^{-1}\)) on the spectra/availability of digestive enzymes (\(\alpha\)-amylase and pepsin) to provide some insights into binding interactions. Mixtures of 1.5 mL of \(\alpha\)-amylase (150 mg L\(^{-1}\)) and 1.5 mL of E171 suspension (pH 6.8) were shaken and incubated at 37 °C for 5 min. For pepsin, 3 mL of pepsin solution (75 mg L\(^{-1}\)) were mixed with 1.5 mL of saliva fluid (only ions) and 1.5 mL of E171 suspension (pH 2.5), and then the mixture was shaken and incubated at 37 °C for 2 hours. The fluorescence emission spectra of all samples were excited at 280 nm and recorded from 300 to 500 nm using a fluorescence spectrophotometer (Hitachi, Ltd., Japan). Since blank E171 also exhibited detectable signals, which can interfere with fluorescence intensity of enzymes, the intensity of fluorescence used were corrected by subtracting emission spectra of the corresponding E171 solution from that of E171-enzymes. Fluorescence quenching were described by the Stern-Volmer’s equation as follows\(^{142}\):\

\[
\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0[Q]
\]

Where, \(F_0\) and \(F\) represent the fluorescence intensities in the absence and presence of E171, respectively; \([Q]\) is the concentration of the quencher (E171); \(K_{sv}\) is the Stern-Volmer quenching constant; \(K_q\) is the bimolecular quenching constant; and \(\tau_0\) is the lifetime of the fluorophore (for tryptophan, \(\tau_0\) is 5.7 ns). In addition, the interaction between the quencher and enzyme were described using the Scatchard equation\(^{145}\):

\[
\log((F_0-F)/F) = \log K_a + n \log[Q]
\]
where, $K_a$ is the binding constant associated with the E171-enzyme interactions and $n$ is the number of binding sites.

### 3.2.8 Statistical analysis

All experiments were conducted at least in triplicate. Significant differences between treatment groups were determined by an analysis of ANOVA with Turkey’s test on OriginPro 2018 statistical software (OriginLab Corp., USA). We used $p < 0.05$ for being statistically significant.

### 3.3 Results and Discussion

#### 3.3.1 Impact of digestive enzymes on mineral bioaccessibility

Digestive enzymes (amylase, pepsin and pancreatin) significantly increased mineral bioaccessibility in spinach leaves, except for Ca (Figure 3-1 and 3-S1). For most of the minerals, more than 60% was released after passing through the simulated GIT model. In the absence of the digestive enzymes, only about 40% of K, Mg, Zn and Mn was released and about 20% of P, S and Fe was released. In the oral phase, $\alpha$-amylase can catalyze the hydrolysis of internal $\alpha$-1,4-glycosidic linkages in polysaccharides into glucose, maltose, and maltotriose units. $\alpha$-Amylases act on starch, glycogen, and related polysaccharides or oligosaccharides in a random manner. It is noted that spinach does not contain much starch. In addition to constituents of cellulose in the cell wall, other sources of polysaccharides and oligosaccharides are the vacuoles and plastids of plant cells in leafy vegetables. Thus, $\alpha$-amylase may still hydrolyze polysaccharides or oligosaccharides within vacuoles and plastid of the plant-tissue matrix to facilitate mineral release. This could explain the observation that the addition of amylase significantly increased the bioaccessibility of K, Mg, Mn, Zn, Fe, P and S from 1.52- to 7.08-fold compared to the corresponding blank. In the gastric phase, pepsin, which degrades proteins, had no impact on the
bioaccessibility of Ca, K, Mg, Mn, and Zn, but significantly increased the bioaccessibility of Fe, P and S by 1.25- to 2.47-fold. According to their biochemical function, K, Mg, and Mn ions are known to participate in establishing cell turgor and maintaining cell electroneutrality, while Fe, P and S are important constituents of many proteins in tissues. Consequently, the presence of pepsin may have hydrolyzed the proteins and facilitated the release of these minerals under simulated gastric conditions. In the small intestinal phase, the presence of pancreatin that can digest fats, proteins, and carbohydrates significantly elevated the bioaccessibility of K, Mg, Mn, Zn, Fe, P and S by 5.17- to 19.43-fold. Thus, these enzymes may have facilitated spinach leaf digestion and mineral release.

It is noted that the final bioaccessibility of Ca in the spinach leaves was only around 10% after passing through the full simulated GIT model, even in the presence of digestive enzymes (Figure 3-1A and 3-S1). This value was significantly less than any of the other minerals, which had bioaccessibility of at least 20%. A possible explanation is that Ca is a constituent of the middle lamella of cell walls, and the upper GIT digestive enzymes used could not digest cellulose, hemicellulose, or pectin, which are important structural components in primary cell walls. The measured bioaccessibility (0.3-5%) of Ca in spinach was quite low compared to reported values for its bioaccessibility in kale and wheat. This may have occurred because spinach contains relatively high levels of oxalate, which is known to be an antinutrient that can bind calcium and reduce its bioaccessibility and absorption. All these results suggest that digestive enzymes can facilitate the breakdown of spinach leaves, which enhances the release of some minerals. However, the extent of enhancement depends on the characteristics of the minerals and their location within the plant tissue.

3.3.2 Impact of titanium dioxide on mineral bioaccessibility
The final Fe bioaccessibility decreased significantly from 59% without E171 to 53% in the presence of 0.2 wt% E171 after passing through simulated GIT model (Figure 3-1D). Interestingly, in the presence of E171, an appreciable decrease in Fe bioaccessibility was evident in the mouth phase rather than gastric and intestinal phase. Specifically, in the mouth phase samples containing 0.2 wt% E171 significantly decreased Fe and Zn bioaccessibility by 11% and 6%, respectively; samples containing 0.05 wt% E171 significantly decreased Fe bioaccessibility by 5% compared to controls. E171 could decrease mineral bioaccessibility via two pathways: one is that E171 could interact with enzymes and reduce the enzymatic activities, thereby inhibited mineral released from spinach leaves. However, exposure to different concentrations of E171 (0-0.2 wt%) had no impact on the bioaccessibility of other minerals. In addition, the elements released from spinach leaves was independent or minor dependent on enzyme additions, thus, even though E171 reduced the enzymatic activity in the specific phase, it was unlikely to decrease their bioaccessibility through inhibiting mineral releases from spinach. The amount of minerals released from spinach was varied, ranging from 10-79% in the presence of digestive enzymes, with the lowest Ca and the highest P (Figure 3-1). The release extent of minerals was distinct because of their characteristics and location within plant tissues. As shown in Table S2, the released minerals from spinach as affected by digestive enzymes can be separated into three levels by their bioaccessibility as compared to the blank. Level one is no change in the mineral bioaccessibility as affected by enzymes. For instance, the total bioaccessibility of Ca was only 10% in all the three phases across all the treatments, and the most of Ca was remained in the spinach residues. The bioaccessibility of K, Mg, Mn, and Zn was not affected by pepsin in the stomach phase as compared to the blank. Level two is minor increase (3-5%) in the mineral bioaccessibility as affected by enzymes. In the mouth phase, K, Mg, and Mn bioaccessibility was 7-10% in the absence of amylase, but the presence of
amylase only increased their bioaccessibility by 3-5%. In the stomach phase, the bioaccessibility of Fe and P was 18 and 14%, respectively, in the absence of pepsin, and increased by 4-5% by adding pepsin. Level three is large increase (12-22%) in the mineral bioaccessibility as affected by enzymes. For example, in the mouth phase, P, S and Fe bioaccessibilities as affected by amylase was increased by 18-22% in comparison with the one without the amylase addition. In the stomach phase, S bioaccessibility as affected by pepsin was increased by 22%. Digestive enzymes can break the structure of food, and subsequently release the elements that are involved in structural integrity and composition of carbon compounds, i.e., P and S, can be initially released from spinach leaves than Fe and Zn that are constituent of proteins or enzyme participated in redox reactions. In the simulated GIT model, the concentration of E171 was gradually decreased, resulting in weaker interaction between E171 and enzymes overtime. In particular, in the small intestine phase, although the addition of pancreatin exhibited notable effect on increasing the bioaccessibility of all the minerals (except Ca) by over 12-34% as compared to the one without pancreatin, E171 addition showed insignificant change in their bioaccessibility, which could be ascribed as no inhibitory effects of E171 on the pancreatin activity. Thus, it is necessary to investigate the interaction between E171 and amylase, pepsin, and pancreatin, as well as inhibition effect of E171 on enzymatic activities in the specific phase as discussed below. In addition, E171 could decrease Fe bioaccessibility via another pathway: the free Fe released from spinach leaves could adsorb on E171 particles. We therefore further investigated the adsorption of free elements released from spinach leaves onto E171 particles in the simulated GIT model.

**3.3.3 Adsorption of minerals onto titanium dioxide particles**

To examine the potential cause of the observed decrease in Fe bioaccessibility in the presence of E171, mineral adsorption onto E171 particles was measured (Table 3-1). At 0.002 wt%
E171, no mineral adsorption was observed in any of the three GIT phases, suggesting that this low concentration of E171 particles did not lead to significant mineral adsorption. Additionally, a common finding in the stomach phase is that no mineral sorption across all the treatments, with the exception being S in the 0.2 wt% E171 treatment, which may because the charge of E171 was close to zero, and likely aggregated in the stomach phase. Among all minerals, a minor amount (less than 5%) of elements was adsorbed onto E171 particles in the mouth, stomach or small intestine phase, but no K adsorption was observed in all the three phases regardless of doses of E171. Previous studies also reported that divalent cations might be more efficient in destabilizing nanoparticles than monovalent cations\textsuperscript{91,151}, and implied the specific adsorption of divalent cations on the particle surfaces in addition to charge screening.\textsuperscript{84} Thus, in the presence of other elements, K ions was less prone to adsorb onto E171 particles, suggesting that E171 was not expected to reduce K bioaccessibility. The amount of Mg, Ca, and P adsorbed onto E171 was less than 1% in the mouth, or small intestine phase, respectively. While the amount of Fe, Mn, Zn, and S adsorbed onto E171 was ranging from 1-5%: in the mouth phase, 0.2 wt% E171 adsorbed 5.30% Fe, 4.47% Mn, 3.66% Zn and 1.26% S, as well as 0.05 wt% E171 adsorbed 3.02% Fe, 1.41% Mn and 1.60% Zn; in the small intestine phase, 4.01% Mn and 4.27% Zn was adsorbed in the presence of 0.2 wt% E171, respectively. These results suggest that the addition of E171 above 0.05 wt% resulted in approximately 1-5% decrease in the bioaccessibility of Fe, Mn, Zn, and S via the E171 adsorption in the specific phase. In fact, no significant difference of the mineral bioaccessibility was evident, except for Fe. In the experiment for the bioaccessibility of minerals as affected by E171, when the minerals were gradually released from spinach by digestive enzymes, part of the enzymes was already adsorbed onto E171 (see discussion below), then free minerals were adsorbed onto E171. In the adsorption experiment of minerals by E171 was used to demonstrate whether E171 additions
could decrease the amount of free minerals released from spinach. The spinach samples were firstly digested in the simulated GIT model, and then in each phase, a fraction of the digest was used to interact with different concentrations of E171. In this case, free minerals and enzymes could simultaneously interact with E171. Hence, the decreased amount of released minerals from adsorption by E171 could be overestimated. Part of the decrease can be attributed to the binding of Fe to the surface of E171, while this cannot entirely explain the observed reduction. Thus, it is necessary to further explore whether E171 could exhibit inhibitory effects on enzymatic activities, leading to inhibition in mineral releases from spinach leaves.

3.3.4 Adsorption of enzymes onto titanium dioxide particles

_α-Amylase_: As the E171-to-amylase ratio increased, the absorbance of amylase decreased (Figure 3-2A), suggesting that amylase bound to the surface of E171 particles in the mouth phase, thereby leading to a reduction in the amount of free α-amylase in the supernatant. The concentration of amylase in the supernatant was closely related to the initial concentration ratio (E171-to-amylase) with $R^2 = 0.88$ (Figure 3-2B). The ζ-potential of E171 and α-amylase in the mouth phase were -37 and -20 mV, respectively (Figure 3-S2); consequently, one would not expect an electrostatic attraction between them. However, proteins are known to bind to the surface of inorganic nanoparticles due to other interactions, such as van der Waals, hydrophobic, or hydrogen bonding interactions. Thus, we used FTIR to examine the potential binding of the digestive enzymes to E171, since this technique has previously been shown to be useful for this type of analysis. The FTIR spectra of E171, α-amylase, and E171+α-amylase in the mouth phase are shown in Figure 3-3A. A broad peak at 3400 cm$^{-1}$ and a clear peak at 1640 cm$^{-1}$ were observed in the E171 spectra, which was assigned to O-H stretching and bending modes. Some characteristic peaks were observed in the spectra of α-amylase that was in agreement with those reported in
previous studies (Table 3-S3).

The strong and broad peak at 3400 cm\(^{-1}\) was due to the N-H and O-H stretching vibrations. A slight and clear peak at 2910 cm\(^{-1}\) was attributed to C-H stretching because of the presence of methylene. The band at 1637 cm\(^{-1}\) (amide I) corresponds to C=O stretching vibrations of the peptide bonds. The broad absorption bands in the range of 1413 and 1258 cm\(^{-1}\) refer to amide II and III, respectively. The other peaks at 1042 and 1038 cm\(^{-1}\) may be ascribed to the stretching and bending vibrations of aliphatic amines. Thus, the appearance of characteristic bands of α-amylase at approximately 2910, 1404, 1067 and 1032 cm\(^{-1}\) in the spectra of E171+ amylase indicate the adsorption of α-amylase to the E171 surface.

**Pepsin:** A characteristic absorption peak in the UV-visible spectrum at 269 nm arose from the \(\pi-\pi^*\) transition in the benzene rings of Trp, Tyr, Phe residues in pepsin. The intensity of the peak decreased with increasing E171 concentrations (Figure 3-2C), which can be attributed to enzyme binding to E171. These results are supported by a previous study the adsorption of pepsin onto E171 particles. The pepsin concentration in the supernatant was negatively correlated with the E171-to-pepsin ratio used (\(R^2 = 0.99\), Figure 3-2D), indicating that increasing additions of E171 reduced the amount of pepsin in the supernatant. The FTIR spectra of E171, pepsin, and E171+pepsin in the small intestine phase were shown in Figure 3-3B. The broad peak observed at 3277 cm\(^{-1}\) in the FTIR spectra of pepsin corresponds to amide A, which is associated with N-H stretching and hydrogen bonding; the peak at 2935 cm\(^{-1}\), referred to as amide B, is from an asymmetrical stretch of CH\(_2\) (Table 3-S3). The peaks at 1661 cm\(^{-1}\) and 1537 cm\(^{-1}\), corresponding to amide I and amide II groups, respectively, can be attributed to N-H in-plane bending coupled with C=N stretch bending. The peaks at 1413 and 1233 cm\(^{-1}\) can be ascribed to the amide III groups. The absorption band of the amide group at 1558 cm\(^{-1}\) in the spectrum of E171+pepsin as compared to the spectrum of E171 indicates the adsorption of pepsin onto the E171 surfaces.
Pancreatin: No significant differences in pancreatin concentration was evident across all the E171-to-pancreatin ratios used (0–0.139), suggesting that no adsorption of this enzyme onto the surfaces of the E171 particles in the small intestine phase (Figure 3-2E). The FTIR spectrum of pancreatin+E171 showed no characteristic peaks of pancreatin, which further confirms the lack of interaction. Given that pancreatin is a mixture of α-amylase, lipase and protease, this result is surprising since both α-amylase and pepsin was shown to bind to E171 in the mouth and gastric phases. It is possibly because the E171 concentration was considerably reduced in the small intestine due to dilution with simulated oral, stomach, and intestinal fluids.

3.3.5 Effect of titanium dioxide particles on enzymatic activity

Measurements of enzyme activity showed that E171 exhibited potential inhibition of α-amylase and pepsin in the mouth and stomach phases, respectively (Figure 3-3D and E). The degree of inhibition for both α-amylase and pepsin increased in a dose-dependent manner. For example, the activity of α-amylase decreased from 5.72 to 3.99 U mL\(^{-1}\) when the E171 concentration was increased from 0 to 0.615 mg mL\(^{-1}\). Jiang et al. reported that both spherical and polygonal starch nanoparticles (SNPs) inhibited α-amylase activity in phosphate buffer solution (pH 5.8); mechanistically, the SNPs interacted with the active site of the α-amylase, thereby inhibiting its ability to interact with soluble starch molecules.\(^{142}\) However, some minerals as ionic forms in plant tissues, i.e. the bioaccessibility of Ca, K, Mg and Mn, showed no change or minor increase by adding amylase. In addition, minerals such as P and S are essential in structural integrity or part of carbon compounds, which could be easily accessible to α-amylase than Fe and Zn, which are constituents of protein interior of plant tissues.\(^{148}\) Although E171 inhibited 35% of amylase activity, the remaining amylase could still breakdown spinach, and prompt the releases of these minerals except Fe and Zn. As mentioned earlier, the bioaccessibility of Fe and Zn in the
mouth phase decreased significantly in the presence of E171 particles, *i.e.*, by 11% and 6%, respectively, for 0.2 wt% E171 (Figure 3-1D). Part of this decrease can be attributed to binding of Fe and Zn to the surface of E171, *i.e.*, by 5% and 4% for 0.2 wt% E171 (Table 3-1) but this does not fully account for the observed reduction. Hence, it is possible that part of the observed decrease in Fe and Zn bioaccessibility was due to the ability of the E171 to decrease the activity of important digestive enzymes, thereby reducing the amount of minerals released.

The enzyme activity of pepsin decreased from 62.37 to 34.74 U mL\(^{-1}\) as the E171 concentration increased from 0 to 0.2 mg mL\(^{-1}\). Others have also reported that E171 particles can significantly inhibit pepsin activity.\(^{158}\) Interestingly, although the addition of 0.2 wt% E171 inhibited pepsin activity, no significant difference in mineral bioaccessibility was observed for any of the samples in the stomach phase. Among these minerals, the bioaccessibility of Ca, K, Mg, Mn, Zn was not affected by the pepsin addition; the bioaccessibility of Fe and P was 14% and 18%, respectively, in the absence of pepsin, while only increased by 3-5% in the presence of pepsin; S bioaccessibility exhibited a larger increase (8% to 20%) after adding enzyme, suggesting that there was still sufficient pepsin remaining to digest protein and release the three elements in the stomach phase. In addition, the stomach phase is a strong acidic environment where spinach leaves could easily release these minerals as determined by the bioaccessibility of all minerals that was notably higher than other two phases without the addition of enzymes. No significant difference was found in BAEE concentration between the two E171-to-trypsin ratios used (Figure 3-3F), suggesting that E171 had no impact on trypsin activity. This result is consistent with the findings of the bioaccessibility study, which showed that E171 concentration did not impact mineral bioaccessibility in the small intestine phase.
These results suggest that addition of E171 may have an inhibitory effect on \( \alpha \)-amylase activity in the mouth phase, thereby impeding the release of minerals from the spinach leaves. It is known that both the conformation and microenvironment of enzymes play a crucial role in determining their catalytic efficiency.\(^{159}\) The adsorption of enzymes onto the surface of E171 particles may have promoted conformational changes due to alterations in the new microenvironment.\(^{160}\) For this reason, fluorescence spectroscopy was used to further explore the inhibitory effect of E171 on digestive enzyme activity.

### 3.3.6 Fluorescence analysis

Fluorescence spectroscopy is a powerful tool to obtain information about the microenvironment of the tryptophan groups in proteins by monitoring changes in their intrinsic fluorescence intensity.\(^{161}\) We used this method to evaluate the ability of E171 particles to promote conformational changes in amylase and pepsin. Fluorescence emission spectra of \( \alpha \)-amylase was measured in the absence and presence of E171 (Figures 3-4A and C). The emission peak observed around 350 nm was assigned to the tryptophan residues located in the protein interior.\(^{162}\) The fluorescence intensity of \( \alpha \)-amylase and pepsin was quenched substantially by addition of E171. This quenching effect is consistent with a strong interaction between E171 and the tryptophan groups of the enzymes.\(^{145}\) This interaction may have been from hydrogen bonding or hydrophobic attraction between the E171 and enzymes, leading to a modification in the microenvironment of the tryptophan groups.

The quenching effect of E171 on the fluorescence intensity of \( \alpha \)-amylase and pepsin was concentration dependent as described by the Stern-Volmer relation. A linear relationship between \( F_0/F \) of \( \alpha \)-amylase or pepsin and E171(\( Q \)) was evident (Figure 3-4B and D), which is consistent with previous studies.\(^{143, 145}\) The mechanisms of fluorescence quenching include dynamic
quenching and static quenching, where the former involves the excited fluorophore quenched upon collision with a quencher molecule in solution, and the latter is due to non-covalent complex formation between the fluorophore and quencher.\textsuperscript{163} The $K_q$ values, serving as an important parameter for the efficiency of quenching or the availability of the fluorophore to the quencher, derived from the plots of $F_0/F$ versus $[Q]$, are listed in Table 3-S4. The $K_q$ values can be used to establish whether the quenching results from the formation of protein-quencher complexes.\textsuperscript{164} The $K_q$ values of E171 with amylase and pepsin was approximately 50- and 30- fold, respectively, higher than the maximum constant ($2 \times 10^{10}$ L mol$^{-1}$ s$^{-1}$) for dynamic quenching.\textsuperscript{165} Hence, the high $K_q$ values obtained in our study suggest that both amylase and pepsin were statically quenched due to complex formation with E171. Assuming E171 could bind to a set of equivalent sites on an active enzyme, the binding constant ($K_a$) and number of binding sites ($n$) between the quencher and enzymes were calculated using the Scatchard equation (Table 3-S4). The $K_a$ value implies affinity of E171 to enzymes.\textsuperscript{166} Additionally, the value of $n$ was approximately equal to 1, indicating a single binding site mode for the interaction between enzymes and E171.

**3.4 Conclusions**

This study showed that these digestive enzymes promoted the release of minerals from spinach leaves under simulated gastrointestinal conditions (except for Ca). This promotion was mainly attributed to the ability of the enzymes to break down the plant tissue structure. The addition of E171 (above 0.05 wt%) significantly decreased Fe bioaccessibility by about 5%-11% in the oral phase. This effect was attributed to two processes: (1) inhibition of $\alpha$-amylase activity by E171 particles and (2) binding of Fe to the surface of E171 particles. In this study, $\alpha$-amylase used was from porcine pancreas. $\alpha$-Amylases from different sources have considerable similarity in amino acid sequence.\textsuperscript{167} In addition, Pajic et al. found that animals (such as mice, rats, pigs and dogs) that
have lived closely with people for centuries, consume a large amount of starch, similar to human food.\textsuperscript{168} X-ray crystallography has been used to visualize 3D structures of $\alpha$-amylase enzymes from human pancreas and saliva, and from porcine pancreas, both of which are closely related.\textsuperscript{169} Previous studies also suggested that both human salivary and pancreatic $\alpha$-amylase are similar to rat pancreatic $\alpha$-amylases, especially similar to porcine pancreas.\textsuperscript{170, 171} Thus, the finding of inhibitory effect of E171 on amylase activity could be extrapolated to human, or is informative for future \textit{in vivo} studies. Fe is an essential element that participates in hundred enzymatic reactions, and Fe deficiency is a global health problem that can cause anemia and immune system disorders. Vegetables are the major sources of vitamins, minerals, and dietary fibers for the human diet. Thus, the significant decrease of Fe bioaccessibility may cause health problems. It has been reported that metal oxide nanoparticles, i.e., CuO, Fe$_2$O$_3$, and TiO$_2$, could alter peanut crop yield as well as nutritional quality such as amino acid, fatty acid profile\textsuperscript{172}; exposure to TiO$_2$ nanoparticles disturbed antioxidant defense system in rice, and also inhibited carbohydrate synthesis, thereby decreased crop yield and quality\textsuperscript{173}; cucumber fruit treated with TiO$_2$ nanoparticles exhibited macromolecule modification, including amide, lignin, and carbohydrates.\textsuperscript{174} Most previous studies have been focused on the effect of TiO$_2$ nanoparticles on the physiological response and nutritional quality of crop and implied that TiO$_2$ could be introduced into the food chain via accumulation in the plant tissue. However, the information about TiO$_2$ exposure to the human gastrointestinal tract through food additives and its impact on nutrient absorption for humans is limited when co-ingested with vegetables. The results of this study enhance our understanding of the impact of E171 on the performance of digestive enzymes within the GIT and the nutritional profile of foods. Also, the findings provide valuable information to guide the development and use of mineral-fortified food and supplements when E171 is involved
Figure 3-1. The amount (\%) of mineral nutrients (Ca, K, Mg, Fe, Mn, Zn, P and S) released in the mouth, stomach, and small intestine phase, as well as that remaining in spinach leaves, under 0 (in the absence (blank) or presence (control) of digestive enzymes), 0.002, 0.05, or 0.2 wt\% E171. Ca released in the small intestine is shown in Figure 3-S1. Bars with different capital letters (A-C) are significantly different ($p < 0.05$) when comparing between different regions for a same treatment. Bars with different lower-case letters (a-c) are significantly different ($p < 0.05$) when compared among 0, 0.002, 0.05 and 0.2 wt\% E171 in the same region.
Figure 3-2. (A) UV-vis spectra of α-amylase (initial concentration 5 mg mL⁻¹) under various initial concentrations of E171 (0, 0.2, 1, 2, 5, 10, 20 mg mL⁻¹) in the mouth phase; (B) The correlation ($R^2 = 0.88$) between α-amylase concentration (mg mL⁻¹) in the supernatant and initial concentration ratio of E171 to α-amylase (0, 0.04, 0.2, 0.4, 1, 2, 4); (C) UV-vis spectra of pepsin (initial concentration at 3.2 mg mL⁻¹) under various initial concentrations of E171 (0, 0.02, 0.1, 0.2, 0.5, 1, 2 mg mL⁻¹) in the stomach phase; (D) The correlation ($R^2 = 0.99$) between pepsin concentration (mg mL⁻¹) in the supernatant and initial concentration ratio of E171 to pepsin (0, 0.00625, 0.03125, 0.0625, 0.15625, 0.3125, 0.625); (E) Pancreatin concentration (mg mL⁻¹) in the supernatant under various initial concentration ratios of E171 to pancreatin (0, 0.001, 0.007, 0.014, 0.035, 0.069, 0.139).
Figure 3-3. (A) FTIR spectra of α-amylase, E171, α-amylase + E171 in the mouth phase; (B) FTIR spectra of pepsin, E171, pepsin + E171 in the stomach phase; (C) FTIR spectra of pancreatin, E171, pancreatin + E171 in the small intestine phase; Inhibitory effects of E171 on (D) α-amylase activity as affected by E171 in the mouth phase; (E) pepsin activity as affected by E171 in the stomach phase; (F) pancreatin activity in the small intestine phase.
Figure 3-4. Changes in the intrinsic (A) α-amylase and (C) pepsin fluorescence at different concentrations of E171 (0-10 mg L⁻¹); the Stern-Volmer curves of (B) α-amylase and (D) pepsin quenched by E171. The initial concentration of α-amylase and pepsin was 150 and 75 mg L⁻¹, respectively.
Table 3-1. Mineral Adsorption (%) onto 0.2, 0.005, and 0.002 wt% E171 in the mouth, stomach, and small intestine phase. Bars with different capital letters (A-C) are significantly different ($p < 0.05$) when comparing between different regions for a same treatment. Bars with different lower-case letters (a-c) are significantly different ($p < 0.05$) when compared various E171 additions (0.002, 0.05 and 0.2 wt%) in the same region.

<table>
<thead>
<tr>
<th>Region</th>
<th>Ca (%)</th>
<th>K (%)</th>
<th>Mg (%)</th>
<th>Fe (%)</th>
<th>Mn (%)</th>
<th>Zn (%)</th>
<th>P (%)</th>
<th>S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouth Phase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% E171</td>
<td>0.48 ± 0.31</td>
<td></td>
<td>5.30a ± 1.09</td>
<td>4.47Aa ± 0.63</td>
<td>3.66Aa ± 1.20</td>
<td>–</td>
<td>1.26B ± 0.84</td>
<td></td>
</tr>
<tr>
<td>0.05% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.02b ± 1.08</td>
<td>1.41b ± 0.31</td>
<td>1.60a ± 0.75</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.002% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.10A ± 1.42</td>
</tr>
<tr>
<td>0.05% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.002% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td><strong>Small Intestine Phase</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% E171</td>
<td>0.70a ± 0.35</td>
<td>–</td>
<td>–</td>
<td>4.01A ± 2.74</td>
<td>4.27A ± 3.35</td>
<td>0.90a ± 0.49</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.05% E171</td>
<td>0.19b ± 0.16</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.07a ± 0.60</td>
<td>–</td>
</tr>
<tr>
<td>0.002% E171</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

“–” = No measurable mineral adsorbed on E171 particles
Table 3-S1. Chemical composition of simulated gastrointestinal fluids (stock solution) used to prepare the oral, gastric and small intestinal phases of the simulated gastrointestinal tract (GIT) model.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>mucin</td>
<td>3.0 g/L</td>
</tr>
<tr>
<td>α-amylase</td>
<td>0.5 g /L</td>
</tr>
<tr>
<td><strong>Gastric fluid</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>HCl</td>
<td>7 mL/L</td>
</tr>
<tr>
<td>pepsin</td>
<td>3.2 g/L</td>
</tr>
<tr>
<td><strong>Small intestinal fluid</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>52.6 g/L</td>
</tr>
<tr>
<td>bile salts</td>
<td>30 g/L</td>
</tr>
<tr>
<td>pancreatin</td>
<td>14.4 g/L</td>
</tr>
</tbody>
</table>
Table 3-S2. Mineral Amount (%) in the oral, gastric, and small intestinal phases. Bars with different capital letters (A-C) are significantly different ($p < 0.05$) when comparing between different regions for a same treatment. Bars with different lower-case letters (a-c) are significantly different ($p < 0.05$) when comparing each treatment (blank, control, 0.002, 0.05 and 0.2 wt% E171) in the same region.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ca (%)</th>
<th>K (%)</th>
<th>Mg (%)</th>
<th>Fe (%)</th>
<th>Mn (%)</th>
<th>Zn (%)</th>
<th>P (%)</th>
<th>S (%)</th>
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<tr>
<td>Oral Phase</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>2.23Ca ± 0.97</td>
<td>6.91Cb ± 1.26</td>
<td>9.81Cb ± 1.23</td>
<td>3.87Cc ± 1.6</td>
<td>8.5Cb ± 0.98</td>
<td>11.15Cb ± 1.2</td>
<td>2.89Db ± 0.97</td>
<td>3.99Cb ± 1.46</td>
</tr>
<tr>
<td>Control</td>
<td>3.05Ca ± 0.33</td>
<td>10.05Da ± 1.87</td>
<td>14.9Ba ± 3.29</td>
<td>23.45Ba ± 3.61</td>
<td>13.27Ca ± 3.19</td>
<td>18.19Ca ± 1.69</td>
<td>20.49Ba ± 1.37</td>
<td>26.45Aa ± 1.14</td>
</tr>
<tr>
<td>0.2 wt% E171</td>
<td>3.23Ca ± 0.31</td>
<td>9.94Ca ± 1.63</td>
<td>13.47Da ± 1.06</td>
<td>12.87Cb ± 0.87</td>
<td>11.53Da ± 0.9</td>
<td>12.03Cb ± 1.9</td>
<td>20.4Ba ± 1.2</td>
<td>26.71Aa ± 1.4</td>
</tr>
<tr>
<td>0.05 wt% E171</td>
<td>3.02Ca ± 0.54</td>
<td>11.65Da ± 2.16</td>
<td>14.48Ca ± 2.01</td>
<td>18.11Cb ± 1.52</td>
<td>13.44Ca ± 1.97</td>
<td>15.46Da ± 1.3</td>
<td>20.64Ba ± 1.44</td>
<td>27.21Aa ± 1.64</td>
</tr>
<tr>
<td>0.002 wt% E171</td>
<td>3.26Ca ± 0.64</td>
<td>11Da ± 0.85</td>
<td>14.14Ca±1.4</td>
<td>25.14Ba ± 2.1</td>
<td>14.8Ca ± 0.77</td>
<td>18.05Ca±0.71</td>
<td>21.19Ba ± 0.69</td>
<td>27.37Aa ± 0.81</td>
</tr>
<tr>
<td>Gastric Phase</td>
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<tr>
<td>Blank</td>
<td>5.64Ba ± 1.37</td>
<td>26.81Ba ± 4.36</td>
<td>29.14Ba ± 4.91</td>
<td>18.34Bb ± 1.41</td>
<td>26.78Ba ± 3.61</td>
<td>27.48Bb ± 1.07</td>
<td>14.45Bb ± 0.84</td>
<td>8.22Bb ± 0.28</td>
</tr>
<tr>
<td>Control</td>
<td>6.87Ba ± 0.82</td>
<td>28.04Ba ± 2.18</td>
<td>31.12Aa ± 2.22</td>
<td>23.13Ba ± 2.06</td>
<td>29.92Aa ± 2.61</td>
<td>28.2Aab ± 0.94</td>
<td>18.12Ba ± 1.69</td>
<td>20.33Ba ± 1.52</td>
</tr>
<tr>
<td>0.2 wt% E171</td>
<td>6.74Ba ± 0.5</td>
<td>25.16Ba ± 1.01</td>
<td>28.9Ba ± 0.65</td>
<td>26.68Ba ± 2.18</td>
<td>31.11Ba ± 0.88</td>
<td>31.35Aa ± 1.26</td>
<td>17.13Ca ± 0.76</td>
<td>19.31Ba ± 1.59</td>
</tr>
<tr>
<td>0.05 wt% E171</td>
<td>6.47Ba ± 1.25</td>
<td>29.02Ba ± 3.28</td>
<td>32.42Aa ± 2.54</td>
<td>27.27Bb ± 2.92</td>
<td>31.22Aa ± 1.81</td>
<td>32.55Aa ± 1.26</td>
<td>18.25Ba ± 1.37</td>
<td>21.02Ba ± 0.94</td>
</tr>
<tr>
<td>0.002 wt% E171</td>
<td>6.37Ba ± 0.54</td>
<td>27.25Ba ± 3.32</td>
<td>29.81Aa ± 3.39</td>
<td>24.01Ba ± 2.51</td>
<td>29.01Aa ± 3.79</td>
<td>28.59Aab ± 0.42</td>
<td>17.97Ca ± 0.29</td>
<td>20.64Ba ± 0.2</td>
</tr>
<tr>
<td>Small Intestinal Phase</td>
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<tr>
<td>Blank</td>
<td>1.13Ca ± 0.69</td>
<td>6.26Cb ± 0.59</td>
<td>4.42Db ± 0.6</td>
<td>1.01Cb ± 2.46</td>
<td>3.61Db ± 1.04</td>
<td>1.27Db ± 2.53</td>
<td>5.74Cb ± 0.49</td>
<td>4.89Cb ± 0.49</td>
</tr>
<tr>
<td>Control</td>
<td>0.55Da ± 0.39</td>
<td>38.09Aa ± 2.39</td>
<td>27.79Aa ± 4.48</td>
<td>12.76Ca ± 1.35</td>
<td>23.52Ba ± 3.9</td>
<td>24.59Ba ± 1.52</td>
<td>40.72Aa ± 2.1</td>
<td>25.28Aa ± 2.07</td>
</tr>
<tr>
<td>0.2 wt% E171</td>
<td>1.11Da ± 0.6</td>
<td>41.68Aa ± 1.83</td>
<td>32.54Aa ± 1.89</td>
<td>13.49Ca ± 2.36</td>
<td>22.43Ca ± 1.48</td>
<td>26.3Ba ± 1.3</td>
<td>42.11Aa ± 0.62</td>
<td>26.7Aa ± 1.52</td>
</tr>
<tr>
<td>0.05 wt% E171</td>
<td>0.45Da ± 0.32</td>
<td>40.09Aa ± 4.05</td>
<td>31.08Aa ± 4.72</td>
<td>11.25Da ± 3.08</td>
<td>25.21Ba ± 3.42</td>
<td>24.23Ca ± 1.79</td>
<td>43.02Aa ± 2.67</td>
<td>26.94Aa ± 3.05</td>
</tr>
<tr>
<td>0.002 wt% E171</td>
<td>0.31Da ± 0.60</td>
<td>40.86Aa ± 2.88</td>
<td>32.08Aa ± 3.22</td>
<td>11.02Ca ± 0.29</td>
<td>25.8Ba ± 3.35</td>
<td>24.8Ba ± 0.51</td>
<td>41.69Aa ± 1.64</td>
<td>25.49Aa ± 0.67</td>
</tr>
<tr>
<td>Residue in Leaf</td>
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</tr>
<tr>
<td>Blank</td>
<td>90.99Aa ± 0.84</td>
<td>60.03Aa ± 5.38</td>
<td>56.63Aa ± 6.12</td>
<td>76.76Aa ± 3.49</td>
<td>61.11Aa ± 4.31</td>
<td>60.11Aa ± 1.55</td>
<td>76.92Aa ± 2.2</td>
<td>82.89Aa ± 1.71</td>
</tr>
<tr>
<td>Control</td>
<td>89.53Aab ± 1.43</td>
<td>23.37Cb ± 2.07</td>
<td>26.19Ab ± 1.56</td>
<td>40.66Ac ± 2.79</td>
<td>33.3Ab ± 2.51</td>
<td>29.03Ab ± 1.26</td>
<td>20.67Bb ± 1.66</td>
<td>27.95Ab ± 1.78</td>
</tr>
<tr>
<td>0.2 wt% E171</td>
<td>88.92Ab ± 0.79</td>
<td>23.22Bb ± 2.63</td>
<td>25.09Cb ± 2.7</td>
<td>46.95Ab ± 0.36</td>
<td>34.94Ab ± 1.82</td>
<td>30.32Ab ± 2.1</td>
<td>20.35Bb ± 1.72</td>
<td>27.27Ab ± 1.84</td>
</tr>
<tr>
<td>0.05 wt% E171</td>
<td>90.05Aab ± 1.73</td>
<td>19.23Cb ± 2.81</td>
<td>22.02Bb ± 2.93</td>
<td>43.38Aabc ± 1.76</td>
<td>30.13Ab ± 3.28</td>
<td>27.76Bb ± 1.59</td>
<td>18.09Bb ± 2.71</td>
<td>24.83Bb ± 2.77</td>
</tr>
<tr>
<td>0.002 wt% E171</td>
<td>90.06Aab ± 0.52</td>
<td>20.88Cb ± 1.59</td>
<td>23.96Bb ± 1.75</td>
<td>39.82Ac ± 0.48</td>
<td>30.39Ab ± 1.36</td>
<td>28.56Ab ± 0.73</td>
<td>19.15BCb ± 1.19</td>
<td>26.5Ab ± 0.95</td>
</tr>
</tbody>
</table>
Table 3-S3. FITR major peak assignments of α-amylase and pepsin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Characteristic Peaks Wavenumber</th>
<th>Functional Group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>α-amylase</td>
<td>Pepsin</td>
</tr>
<tr>
<td>Amide A</td>
<td>3400</td>
<td>3277</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-H stretch and hydrogen bond</td>
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<tr>
<td>Amide B</td>
<td>2910</td>
<td>2935</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-H stretch</td>
</tr>
<tr>
<td>Amide I</td>
<td>1637</td>
<td>1661</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=O stretch</td>
</tr>
<tr>
<td>Amide II</td>
<td>1413</td>
<td>1537</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-H bend coupled with C=N stretch</td>
</tr>
<tr>
<td>Amide III</td>
<td>1258</td>
<td>1233</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-H bend coupled with C=N stretch</td>
</tr>
</tbody>
</table>
Table 3-S4. Binding constants $K_a$ and the number of binding sites ($n$) for interaction of E171 with $\alpha$-amylase, pepsin and $R^2$ of Stern–Volmer quenching at 310 K.

<table>
<thead>
<tr>
<th></th>
<th>$K_a$ ($\times 10^{11}$ L mol$^{-1}$ s$^{-1}$)</th>
<th>$R^2$</th>
<th>$K_a$ ($\times 10^3$ L mol$^{-1}$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-amylase</td>
<td>10.16</td>
<td>0.97</td>
<td>15.79</td>
<td>1.1</td>
</tr>
<tr>
<td>pepsin</td>
<td>5.79</td>
<td>0.98</td>
<td>1.23</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Figure 3-S1. The amount (%) of Ca released in the small intestinal phase under 0 (in the absence (blank) or presence (control) of digestive enzymes), 0.002, 0.05, or 0.2 wt% E171. Bars with capital letters (A-C) are significantly different \((p < 0.05)\) when comparing between different regions for a same treatment. Bars with different lower-case letters (a-c) are significantly different \((p < 0.05)\) when compared among 0, 0.002, 0.05 and 0.2 wt% E171 in the same region.
Figure 3-S2. The ζ potentials of E171, α-amylase, E171+α-amylase in the oral phase.
4. POLYSTYRENE NANOPLASTICS DECREASED STARCH HYDROLYSIS IN SIMULATED INFOGEST MODEL

4.1 Introduction

Worldwide plastic production has exceeded 359 million tons in 2018, and plastic wastes would triple to 270 million tons from 2015 to 2060.\textsuperscript{175, 176} Plastics undergo UV radiation, mechanical abrasion, and biodegradation that cause plastic debris degradation and fragmentation into smaller plastics, i.e., microplastics (MPs) and nanoplastics (NPs).\textsuperscript{177} Plastics debris have raised a global attention due to their ubiquity in the environment and potential risks to human.\textsuperscript{178} Both MPs and NPs are present in seawater, freshwater, sediment, terrestrial systems, soil, and even air.\textsuperscript{178} After being fragmented under environmental processes, MPs/NPs ultimately could expose to human through various pathways, such as inhalation, ingestion, and dermal contact.\textsuperscript{179} A recent study reported billions of MPs in hot water that were released from a plastic tea bag.\textsuperscript{180} Additionally, Zhou et al. extracted polystyrene (PS) NPs and poly(methyl methacrylate) (PMMA) in tissues of aquatic animals and detected the concentration of PS NPs in a range of 0.093–0.785 μg/g.\textsuperscript{176} MPs have also been found in the gastrointestinal tract and in human stools.\textsuperscript{181} These studies highlighted the potential high exposure of MPs/NPs to human digestive system. Stock et al. underlined that the primary phase of artificial gastrointestinal tract did not decompose plastic particles into smaller fragments or altering their shape and texture, and implied the adsorption of organic compounds (lipids, mucin, and proteins) onto plastic particles.\textsuperscript{182}

Recently, a standardized \textit{in vitro} gastrointestinal model, defined as INFOGEST model, has been introduced to simulate food digestion inside the human gut.\textsuperscript{140} \textit{In vitro} digestion models are
inexpensive, simple, rapid, and repeatable and can provide insights into the physicochemical mechanisms of food digestion.\textsuperscript{183} Starch is a dominant energy source of human diet (i.e., carbohydrate) and can provide 17 kJ/g energy.\textsuperscript{184} Starch contains two types of molecules, amylose and amylopectin, and loose helical amylose chains have a hydrophobic interior that can bind to hydrophobic molecules such as lipids and aromatic compounds.\textsuperscript{185} In order to simulate starch digestion in the INFOGEST model, three major enzymes including amylase, pepsin, and pancreatin, were applied to the model. PS MPs reduced lipase activities by changing the secondary structure and disturbing the essential open conformation; pepsin activities were not changed after incubation with PS MPs.\textsuperscript{107} It is known that aromatic residues (Trp58, Trp59, Tyr151 and Phe256) prominently locate at the active site of amylase and plays an important role in substrate binding, enzyme activity and catalysis.\textsuperscript{186} In addition, hydroxyl and carbonyl groups were developed in weathered plastic pellets by photo-oxidative degradation upon exposure to UV irradiation in air and water environments.\textsuperscript{187} Plastics with functional groups may be more efficient to adsorb digestive enzymes than pristine plastics. However, the knowledge for impact and fate of NPs in the simulated gastrointestinal model is limited. This investigation provides a new understanding of potential risks of NPs towards to human gastrointestinal system.

We hypothesized that PS NPs could bind to both starch and digestive enzymes via noncovalent interaction (e.g., π-π or hydrophobic interaction), thus inhibiting the starch hydrolysis in the simulated INFOGEST model, and PS NPs with functional groups (-COOH or NH\textsubscript{2}) might show higher inhibition in starch hydrolysis due to hydrogen bonding with starch or enzymes. In this study, different concentrations or different functional groups of PS NPs were used to explore the PS NP impacts on corn starch digestion in the simulated gastrointestinal model. The aims were to investigate the gastrointestinal fate of PS NPs, to examine their interactions with corn starch,
digestive enzymes (e.g., amylase, pepsin, pancreatin) and to further illustrate the mechanisms by which PS NPs reduced the corn starch digestion in the INFOGEST model.

4.2 Materials and Methods

4.2.1 Materials

PS nanoparticles, and PS nanoparticles with functional groups, -COOH, or -NH$_2$ (0.08 µm) were ordered from BaseLine Chromtech Research Centre (Tianjin, China). Corn starch (Argo) were purchased from a local supermarket. Mucin from porcine gastric, α-amylase from porcine pancreas (≥ 10 units/mg solid), pepsin from porcine gastric mucosa (≥ 250 units mg$^{-1}$), pancreatin from porcine pancreas (100 - 500 units mg$^{-1}$), bile salts, and amyloglucosidase were all purchased from Sigma Aldrich Ltd. (St Louis, USA). Other chemicals for preparing simulated fluid used in this study were of analytical grade.

4.2.2 Corn starch digestion

Starch digestibility was analyzed by the INFOGEST in vitro digestion model, which includes oral, gastric, and small intestinal phases with some minor modifications. The composition of simulated saliva fluid (SSF), simulated gastric fluid (SGF), and small intestinal fluid (SIF) were described in Tan et al. The corn starch (150 mg/mL) was mixed with different concentrations (0, 0.1, 0.2, 0.6%, w/v) of PS NP suspensions or 0.6% (w/v) PS NPs suspensions with different functional groups (-COOH, -NH$_2$,). The mixtures were shaken at 37 °C for 15 min. The designated concentrations of PS NPs suspension or PS NPs suspension with different functional group at the same level were prepared by diluting PS, PS-NH$_2$, PS-COOH stock solution with DI water. The final mixtures, which contain corn starch (75 mg/mL) with 0, 0.05, 0.15, 0.3% (w/v) PS NPs, or 0.3% (w/v) of PS, PS-COOH, PS-NH$_2$ respectively, were passed through the in vitro INFOGEST digestion model.
Mouth phase  The simulated saliva fluids containing α-amylase (5.6 mg/mL), mucin (3 mg/mL), 1.5 mM CaCl\(_2\) (H\(_2\)O) were added into the tested samples on a 1:1 (v/v) ratio. The mixture was adjusted to pH 6.8 and shaken for 5 min at a speed of 100 rpm in an incubator shaker setting at 37 °C. After 5 min, the aliquots (0.5mL) of sample were withdrawn for testing the glucose released in the mouth phase.

Gastric phase  The resulting samples from the mouth phase were transferred into the gastric fluid, a mixture of 3.2 mg/mL pepsin and 0.15 mM CaCl\(_2\), on a 1:1 (v/v) ratio. The mixture pH was adjusted to 3.0. The mixtures were shaken for 2 h at 100 rpm and 37 °C. A volume of 0.5 mL of mixtures was collected at 30 and 120 min in order to monitor the hydrolysis rate of the starch.

Small intestine phase  Gastric samples were then mixed on a 1:1 (v/v) ratio with the simulated intestinal fluid containing pancreatin (32 mg/mL), amylloglucosidase (7.5 U/mL), 10 mM bile salts and 0.6 mM CaCl\(_2\). The mixtures were adjusted to pH 7.0 and then incubated for 150 min at 100 rpm, 37 °C.

To test glucose content released in the small intestine phase, aliquots (0.5 mL) of sample were collected at different time points (10, 20, 30, 60, 90, 100, 120, and 150 min). Each aliquot collected from the INFOGEST model was then mixed with absolute ethanol (4.0 mL) to inhibit the activity of enzymes and centrifuged at 4000 ×g for 3 min. Afterwards, the glucose content in the supernatant was measured using the 3,5-dinitrosalicylic acid (DNS) method.\(^{188}\) Starch hydrolysis (%) were calculated using the following equation (1):

\[
\text{Starch hydrolysis (\%)} = \frac{S_h}{S_i} = \frac{(0.9 \times G_h)}{S_i} \times 100\% \quad (1)
\]
where \( S_h \) is the amount of hydrolyzed starch, \( S_i \) is the initial amount of starch, and \( G_h \) is the amount of the produced glucose. A factor of 0.9 is calculated from conversion of the molecular weight of starch over monomer glucose.

4.2.3 Scanning electron microscopy (SEM) imaging

Scanning electron microscopy (SEM Magellan 400, USA) was used to characterize the microstructure change of starch granules during hydrolysis.\(^{189}\) The starch samples with or without PS NPs before or after passing through the INFOGEST model was prepared for the SEM observation. A half mL of samples was collected in the initial, mouth, stomach, and intestine phase, respectively. Then 50 \( \mu \)L of samples were placed and dried overnight on a mica with double-sided conductive adhesive carbon tapes. All the samples were coated with a 4 nm gold layer on the surface under vacuum by a 108 Manual Sputter Coater prior to the SEM observation at an accelerating voltage of 1 kV.

4.2.4 Laser scanning confocal microscopical imaging

Confocal laser scanning microscope was used to visualize the process of starch hydrolysis as affected by PS.\(^{190}\) The normal maize starch samples with non-PS NPs or PS NPs were passed through the INFOGEST digestion model, and samples were collected and measured at mouth, stomach, and intestine phase, respectively. Rhodamine B was used as a dye for the noncovalent staining of starch. The dye was mixed with the pastes or digesta. A drop of the stained paste was placed on a glass slide; a coverslip was applied and then the prepared samples were examined under a fluorescence confocal laser scanning microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA).

4.2.5 Enzymatic activity assay (\( \alpha \)-amylase, pepsin, and pancreatin)
**α-Amylase**  The α-amylase activity was described in previous studies,\textsuperscript{142,144} which quantifies the amount of reducing sugars released from the starch hydrolysis catalyzed by α-amylase. A soluble starch solution (1%, w/v) was prepared by dissolving 1.0 g soluble starch into 100 mL boiling buffer solution (20 mM sodium phosphate, 6.7 mM sodium chloride, pH 6.9). Aliquots of 0.5 mL of different concentrations PS NPs, PS-NH\textsubscript{2}, PS-COOH (0, 14, 42, 84 mg/L) were separately diluted with 0.5 mL of α-amylase solution (2 unit) into a tube, and then the mixtures were shaken at 37 ℃ for 5 mins. Afterwards, 1 mL of 1.0 % (w/v) soluble starch solution was added into the mixture. After incubation in a shaker at 37 ℃ for 3 min, the reaction of soluble starch and amylase was stopped by adding 1 mL of DNS color reagent into the final product. All tubes were placed into hot water (90 ℃) for 15 min. After cooling to room temperature, 9 mL of deionized water were added into each tube and mixed thoroughly. The resulting samples were centrifuged at 4000 rpm for 15 min. The amount of maltose in the supernatant was calculated from the calibration curve (r) by measuring the absorbance at 540 nm using a UV-vis spectrometer. The α-amylase activity is calculated using the equation below:

\[
\text{The enzyme activity (Units/mL)} = \frac{(\text{mg of maltose released}) \times (\text{df})}{(\text{mL of enzyme})}
\]

Where df is the dilution factor.

**Pepsin**  The method for the pepsin activity measurement was described in Zhu et al. with some minor modifications.\textsuperscript{133} Pepsin can convert hemoglobin to tryptophan and tyrosine, both of which are easily detected at 280 nm by UV-vis spectrophotometry. A volume of 375 μL of PS, PS-NH\textsubscript{2}, PS-COOH suspensions (0, 31.25, 93.75, 187.5 mg/L) was mixed with 375 μL of pepsin solutions (0.32 mg/mL), respectively. Afterwards, the samples were adjusted to pH 2.5 and placed in an incubator for 30 min at 37 ℃. Then, 750 μL of pepsin-PS mixture were incubated with 3.75 mL of bovine hemoglobin solution (5 mg/mL) at 37 ℃. After 30 min, 7.5 mL of 5% (w/v)
trichloroacetic acid were added into the mixture to cease the reaction and the samples were shaken for another 10 min at 37 °C. After centrifugation, the supernatant was measured at 280 nm. The pepsin activity was calculated according to a vendor-supplied (Sigma) method as follows:

\[
\text{The enzyme activity (Units/mL)} = \frac{(A_{280 \text{ Test}} - A_{280 \text{ Blank}}) \times (df)}{T \times V \times U}
\]

Where \( T \) is the time (min) for assay incubation, \( V \) is the volume (mL) of enzyme solution, \( U \) is \( \Delta A_{280} \) per unit of pepsin.

**Pancreatin** The pancreatin activity measurement was referred to the trypsin activity measurement according to a vendor-supplied (Sigma) method with some modifications. The \( N_\alpha \)-Benzoyl-L-arginine ethyl ester (BAEE) as substrate was used to determine the trypsin activity. Two mL of PS NP, PS-NH\(_2\), PS-COOH (0, 0.5, 1.5, 3 mg/L) suspensions were mixed with 0.4 mL of pancreatin (800 mg/L), and the mixtures were then incubated at pH 7.0, 37 °C for 30 min. After centrifugation, 1.2 mL of supernatant were immediately mixed with 2.0 mL of BAEE (0.13 mg/mL). Increases in absorbance at 253 nm was recorded for 5 min at 1 min intervals. The rate in the increase of the absorbance (\( \Delta A_{253} \text{ min}^{-1} \)) was used to calculate pancreatin activity described below:

\[
\text{The enzyme (Units/mL)} = \frac{\Delta A_{253/\text{minute Test}} - \Delta A_{253/\text{minute Blank}}}{\text{df}} \times (0.001 \times V)
\]

Where \( \text{df} \) is dilution factor, 0.001 is the change in \( A_{253/\text{minute}} \) based on unit definition, and \( V \) is the volume of enzyme solution used in assay.

**4.2.6 Circular dichroism (CD) spectroscopy**

Circular dichroism spectroscopy was applied to analyze the changes occurred in the secondary structure of \( \alpha \)-amylase, pepsin pancreatin.\(^{142}\) For amylase, \( \alpha \)-Amylase (0.4 mg/mL) was individually mixed with 0.214 mg/mL of PS, PS-COOH, PS-NH\(_2\) NPs on a 1:1 (v/v) ratio for 5 min. Pepsin (0.4 mg/mL) was individually incubated with 0.234 mg/mL of PS, PS-COOH, PS-
NH₂ NPs on a 1:1 (v/v) ratio for 2 h. Pancreatin (0.4 mg/mL) were respectively incubated with 0.09 mg/mL of PS, PS-COOH, PS-NH₂ NPs on a 1:1 (v/v) ratio for 2 h. The mixture was centrifuged at 12000 rpm for 30 min and the clear supernatant was collected for CD measurement. The CD spectra were recorded using a Jasco J1500 Spectropolarimeter (Tokyo, Japan) in the far-UV (190–260 nm) region under constant nitrogen flush. The path length was 0.1 mm for the far-UV region. Ellipticity was recorded at a 50 nm min⁻¹ speed, 3 accumulations, and 1.0 nm bandwidth.

4.2.7 FTIR spectroscopy

A PerkinElmer Spectrum instrument (USA) was used to record the FTIR spectra of PS NPs, PS-NH₂, PS-COOH, α-amylase, pepsin, pancreatin, as well as mixture of PS NPs with enzymes using the attenuated total reflection (ATR) model. Samples of PS, PS-NH₂, PS-COOH with α-amylase, pepsin, pancreatin, were collected from the oral phase (pH 6.8, 37 ℃, 5 mins), gastric phase (pH 2.5, 37 ℃, 2 hours), and small intestinal phase (pH 7.0, 37 ℃, 2 hours), respectively. The supernatants of these samples were removed after centrifugation. The target compounds, including PS NPs with or without coatings mixed with different enzymes, were rinsed three times with the corresponding buffer solution and then re-suspended for the FTIR spectra analysis. Each spectrum was collected over the range from 4000 to 500 cm⁻¹ with 50 scans at a resolution of 4 cm⁻¹.

4.2.8 Fluorescence spectroscopy

Fluorescence spectroscopy was used to provide some insights into binding interactions between enzymes and PS NPs. PS NPs (90 mg/L) were mixed with α-amylase (150 mg/L) at 37 ℃ for 5 min. PS NPs (1 mg/L) were incubated with pancreatin (50 mg/L) at 37 ℃ for 2 h. The fluorescence emission spectra of all the samples were excited at 280 nm and recorded from 300 to
500 nm using a fluorescence spectrophotometer (Hitachi, Ltd., Japan). Since blank PS NPs also exhibit detectable signals, which can interfere with the fluorescence intensity of enzymes, the intensity of fluorescence used was calibrated by subtracting emission spectra of PS solution from that of PS-enzymes.

4.2.9 Statistical analysis

All experiments were performed in triplicates. An analysis of ANOVA with Sidak’s test \((p < 0.05)\) was used to determine significant differences across all the treatments using OriginPro 2018 statistical software (OriginLab Corp., USA).

4.3 Results and Discussion

4.3.1 Corn starch hydrolysis in the INFOGEST model affected by PS NPs

Corn starch hydrolysis in the INFOGEST model in the absence or presence of PS NPs (0.05, 0.15, 0.3% PS, w/v or 0.3% PS, PS-COOH, PS-NH₂) is shown in Figure 4-1. Time-dependent starch hydrolysis across all the treatments exhibited a similar pattern that a small amount of glucose released in the mouth and gastric phase; a rapid increase of glucose released in the small intestine phase. A total small extent of starch (<12%) hydrolysis occurred after passing through mouth and stomach phases, and no significant difference for the starch hydrolysis was found between the starch alone and the starch amended with PS NPs. In the small intestinal phase, approximately 60% of corn starch (without NPs) was hydrolyzed in the first 10 min, and then a gradual increase was shown at later times (20-150 min). This result was expected as corn starch contains 73% percent amyllopectin and 27% amylose, both of which can be highly and rapidly digested.\(^{191}\) However, in the first 10 min, the extent of starch hydrolysis in the presence of 0.15% and 0.3% (w/v) PS NPs was significantly reduced. The high dose of PS NPs significantly reduced the starch hydrolysis after 150 min digestion. The final degree of the starch digestion in the 0.15
and 0.3% PS treatments was 85% and 73%, respectively, both of which were significantly lower than the control (95%).

Additionally, the effects of PS NPs with different function groups (-COOH or -NH₂) were further investigated. In comparison with non-PS NPs, the addition of PS, PS-COOH, and PS-NH₂ significantly reduced the starch hydrolysis by approximately 20% within 150 min. However, different functional groups did not result in significant differences in reducing starch hydrolysis, which indicated that functional groups (-COOH and -NH₂) of PS NPs showed little impact in starch hydrolysis. Green tea extract (epicatechin, epigallocatechin gallate, epicatechin gallate) was used to reduce starch digestion because of its inhibitory effect on human pancreatic a-amylase activity through binding with amino acid residues via hydrogen bonds and Van der Waals forces. We expected that PS NPs interacted with starch, inhibiting the enzymes access to the surface of starch granules, or PS NPs reduced the enzymatic activities, i.e., amylase, pepsin, pancreatin. Further experiments were therefore carried out to determine interaction between starch and PS as well as the impacts of PS on the enzymatic activities.

4.3.2 SEM and confocal images

Scanning electron microscopy (SEM) and confocal laser scanning microscopy were used to visualize surface morphology and microstructural characteristics of hydrolysis of corn starch granules. SEM and confocal images of corn starch granules without or with PS NPs in the initial, mouth, stomach, and intestine phase are presented in Figure 4-2 and Figure 4-3, respectively. It is observed that corn starch granules were spherical and in irregular polygonal shape with a smooth surface before the digestion, aligning with previous studies. However, in the presence of PS NPs, the surface of starch granules became rough and grainy, and SEM images confirmed that the small round particles were PS NPs (Figure 4-S1A-D). These micrographs demonstrated that the
adsorption of PS NPs or PS NPs agglomerates onto the surface of starch granules, which may inhibit digestive enzymes to have access to surface of the corn starch.

The irregular shape of corn starch granule still existed after applying digestive enzymes (amylase and pepsin) into the system, which was confirmed by both SEM and confocal images. Rhodamine B as a fluorescent probe was used to stain starch granules due to its affinity to starch. Red areas in the confocal image correspond to the fluorescence of rhodamine B, implying the presence of starch granules. But some subtle microstructure of change can be observed using SEM images: the surface of starch granule became loose and less smooth; and some filamentous as well as small pits can be observed on its surface (Figure 4-2I-L, 4-S1B-D). Microstructure changes implied that starch has been partially hydrolyzed by digestive enzymes, which is consistent with the small extent of starch hydrolysis (12%) after mouth and stomach phases. Govindaraju et al reported that the degradation of corn starch is visible in the form of small pinholes on granule surface, and as increasing digestion time, the pores on the surface became larger and the granule surfaces became rougher. Glucoamylase could firstly access to the susceptible surface regions of granule and ex-corrode external part of granule; as the pores or pits became larger, canals of endo-corrosion extended into the granules were formed, and then enzymes penetrated and endo-corroded the internal part of the granules via small pores. The hydrolysis of corn starch granules exhibited loose packing with voids and cracks. PS NPs were observed to adsorb onto the surface of starch granule in the mouth phase and stomach phase (Figure 4-S1A-D), leading to a decrease in enzymes accessing the surface of starch granule. However, amylase and pepsin still can access to the partial surfaces of granules; consistently, some pinholes were found on the surface of starch in the presence of NPs (Figure 4-2J-K). This result demonstrated no significant differences in the glucose released among non-PS NPs and PS NPs during the mouth
phase and stomach phase. This is also consistent with the confocal observation (Figure 4-3C-D, and F-G). Moreover, due to short digestion time (5 min in mouth phase), large amounts of starch, and pepsin were incapable of degrading starch, the total extent of starch hydrolysis was only approximately 12% after passing through the mouth and stomach phase.

Lv et al. reported that corn starch granules upon enzymatic hydrolysis for 120 min were broken into gel pieces. As granules were treated with pancreatin for 150 min, most of starch granules were hydrolyzed. Confocal image confirmed the disappearance of granules after 150 min of digestion as displayed by the homogenous background (Figure 4-3E). Granules were hardly observed, or very few granules were found (Figure 4-2D). These micrography was in line with the results that approximately 95% starch was hydrolyzed into glucose. Interestingly, a few threadlike substances formed outline similar with irregularly polygonal granules with empty interior can be found by SEM (Figure 4-S1E). The similar threadlike structure was also observed in the confocal images (Figure 4-S1G). This residue is compatible with corn starch digestion by endo-corrode pattern, where the presence of surface pores and internal channels provide pathway for enzyme access to interior granule. Therefore, it implies that the threadlike substances may be the residues of undigested starch granules. Corn starch consists of 27% amylose, which is relatively hard to be degraded and intertwines with amylopectin increasing the integrity of granules. In the presence of PS NPs, an amount of unhydrolyzed starch granules residues with irregular polygonal shape were still existed after150 min (Figure 4-2H). Besides, threadlike substances were also observed in the treatments with NPs in SEM and confocal images (Figure 4-S1F, H). As compared to the ones treated with non-NPs, this substance was mostly undigested with a hole. Therefore, these results demonstrated that the presence of PS NPs could significantly inhibit the hydrolysis of corn starch likely via the adsorption of PS onto surface of granule, leading to limitation of enzyme
access to granule surface or directly inhibit enzymatic activity, thereby reducing the starch hydrolysis.

4.3.3 Effect of PS NPs on enzymatic activity

The enzymatic activities as a function of PS NP concentrations are shown in Figure 4-3A. Both the activities of amylase and pancreatin were significantly decreased by PS NPs across all the concentrations; the pepsin activity was significantly decreased by 40% at the level of 0.3% PS NPs. The amylase activity was significantly reduced by 25%-54% in the treatments with PS NP ranging from 0.05% to 0.3%. The pancreatin activity was remarkably decreased by 28-33% upon exposure to 0.15% and 0.3% PS NPs. Tan et al. reported that PS MPs adsorbed both lipase and pepsin but only exhibited the inhibitory effects on the lipase activity. In this study, the pepsin activity was not changed by PS NPs at the concentration below 0.15%, but its activity was significantly reduced in the 0.3% PS NP treatment. Polymer typed plastics such as polystyrene have been reported to significantly reduce the activities of carbohydrase enzymes (i.e., amylase and xylanase) in the marine bivalves. Additionally, the enzymes activity was also measured upon exposure to PS NPs with different functional groups (-COOH and -NH₂) (Figure 4-3B). All PS NPs with various functional group showed a significantly inhibitory effect on activities of amylase, pepsin, and pancreatin in the corresponding phases. After incubating with these PS NPs, the activities of amylase, pepsin, and pancreatin significantly decreased by at least 55%, 19%, and 50%, respectively. Interestingly, amylase activity exhibited 39% after incubating with PS-NH₂ which was significantly lower than ones (45%) treated with PS and PS-COOH; pepsin activity was 68% with the addition of PS-COOH, which was significantly less than ones (around 80%) treated PS and PS-NH₂. Skoluda et al. found that salivary amylase activity decreased in polystyrene containers compared to glass ones, and suggested that PS adsorbs and inhibits amylase due to its
hydrophobic surface.\textsuperscript{200} PS NPs with aromatic benzene ring could bind to aromatic residues located at the active site of enzyme, thus, inhibiting the enzyme activity. Further experiments were conducted to explore effect of PS NPs on the secondary structure or microenvironments of enzymes.

4.3.4 FTIR analysis

FTIR spectra of PS, PS-COOH, PS-NH\textsubscript{2}, enzyme, PS/PS-COOH/PS-NH\textsubscript{2} + enzyme are shown in Figure 4-4A-C. FTIR spectra of PS, PS-COOH, PS-NH\textsubscript{2} showed a characteristic band at 3100 - 3000 cm\textsuperscript{-1}, attributing to the C-H stretching from the aromatic ring; peaks at 2924 and 2846 cm\textsuperscript{-1} correspond to the symmetrical and asymmetrical stretching vibration of CH\textsubscript{2}; bands at 1602, 1490 and 1447 cm\textsuperscript{-1} correspond to stretching vibration of the C=C bond on the benzene ring; bands at 1023 cm\textsuperscript{-1}, 902 cm\textsuperscript{-1} are assigned to C-H out-of-plane bending vibration of the benzene ring.\textsuperscript{201} The FTIR spectra of enzymes (i.e. amylase, pepsin, and pancreatin) were consistent with our previous study.\textsuperscript{202} The FTIR spectrum of amylase shows characteristic peaks: a strong and broad peak at 3400 cm\textsuperscript{-1} corresponds to the N-H and O-H stretching vibrations; a slight and clear peak at 2910 cm\textsuperscript{-1} attributes to C-H stretching because of methylene; the band at 1637 cm\textsuperscript{-1} (amide I) corresponds to C=O stretching vibrations of the peptide bonds; the broad absorption band at 1413 and 1258 cm\textsuperscript{-1} refers to amide II and III, respectively; the other peaks at 1042 and 1038 cm\textsuperscript{-1} may be ascribed to the stretching and bending vibrations of aliphatic amines.\textsuperscript{154-156} Individual FTIR spectra of PS, PS-COOH, PS-NH\textsubscript{2} after mixed with amylase show the characteristic bands of \(\alpha\)-amylase at approximately 3297 and 1655 cm\textsuperscript{-1}.

In addition, the broad peak observed at 3277 cm\textsuperscript{-1} in the FTIR spectra of pepsin corresponds to amide A associated with N-H stretching and hydrogen bonding; the peak at 2935 cm\textsuperscript{-1} referred to as amide B is from an asymmetrical stretch of CH\textsubscript{2}.\textsuperscript{157} The peaks at 1661 cm\textsuperscript{-1} and 1537 cm\textsuperscript{-1},
corresponding to amide I and amide II group, respectively, can be attributed to N-H in-plane bending coupled with C=\text{N} stretch bending. The peaks at 1413 and 1233 cm\(^{-1}\) can be ascribed to the amide III group. The appearance of peak at 3270 cm\(^{-1}\) and 1642 cm\(^{-1}\) in the PS, PS-COOH, PS-NH\(_2\) after incubated with pepsin individual indicates the adsorption of pepsin onto PS NPs. The FTIR spectrum of pancreatin shows characteristic peak at 3349 cm\(^{-1}\) assigned to the N-H and O-H stretching vibrations, the band at 1636 cm\(^{-1}\) and 1357 cm\(^{-1}\) corresponds to amide I and amide II, respectively. PS, PS-COOH, PS-NH\(_2\) after incubated with pancreatin, respectively, showed the appearance of typical bands at 3276 and 1638 cm\(^{-1}\). These results suggested the adsorption of enzymes to the PS, PS-COOH, and PS-NH\(_2\). Taken together, the adsorption of enzymes onto PS NPs can be confirmed by appearance of characteristic peaks of enzymes at approximately 3270 cm\(^{-1}\) and 1640 cm\(^{-1}\) in the spectra of PS NPs after incubating with enzymes.

4.3.5 Circular dichroism analysis

Circular dichroism spectroscopy is a powerful and sensitive technique to measure the secondary structure and conformational change of proteins. The secondary structural change enzymes (i.e., amylase, pepsin, and pancreatin) as affected by PS, PS-COOH, and PS-NH\(_2\) were assessed by CD spectra (Figure 4-4D-F). The CD spectra of amylase and pepsin were similar to previous studies. Two peaks of amylase at 208 and 220 nm are typical features of \(\alpha\)-helices, which were attributed to \(\pi-\pi^*\) and \(n-\pi^*\) transitions of amide groups, respectively. With the addition of PS, PS-COOH, PS-NH\(_2\), respectively, the intensity of the negative ellipticities of amylase was significantly reduced, suggesting a loss in \(\alpha\)-helix structure. In addition, the band at 208 nm was red shifted. The \(\alpha\)-helical content of amylase upon interaction with PS NPs significantly decreased. The CD spectra of amylase without or with PS NPs suggest that PS could potentially induce a change in the secondary structure of amylase. Chlorogenic acid has been
reported to bind to amylase and alter the secondary structure of amylase via hydrogen bonding, inhibiting the substrate contacting active sites, thereby leading to inactivation of amylase.\textsuperscript{145} A strong negative band at 200 nm in the CD spectra of pepsin was characteristic peak of β-sheet configuration arisen from n-π * transition, indicating that the pepsin is mainly composed of β-sheet.\textsuperscript{205} Although the shape of CD spectra in the range of 190-260 nm had no change, a significant decline in negative ellipticity was observed in the spectra of pepsin incubating with all PS NPs types, implying a loss in β-sheet structure. Pepsin's β-sheet content significantly decreased in the presence of all the PS types. Wang et al. showed that binding of imidacloprid with pepsin altered the conformation of pepsin due to a decrease in β-sheet using CD spectra.\textsuperscript{203} The CD spectra of pancreatin showed strong negative ellipticities at around 190 nm, and for individual interaction with PS NPs, PS-COOH, PS-NH\textsubscript{2} NPs, the intensities were decreased. These results indicated that the addition of PS, PS-COOH, PS-NH\textsubscript{2} could alter the secondary structure of enzymes, break down the α-helices or β-sheet. Further experiments were performed on amylase, pancreatin incubation with PS NPs using fluorescence spectra. Fluorescence spectra displayed fluorescence quenching of tryptophan (Trp) residues with the addition of PS NPs (Figure S4-2). The aromatic residues, Trp58, Trp59, Tyr151 and Phe256, are prominently located at the active sites.\textsuperscript{206} Trp has benzene rings in its molecular structure. Aromatic residues in the vicinity of the active site of human salivary α-amylase are closely related to amylase activity. The quenching of Trp residues indicated that PS NPs interacted with enzymes may via noncovalent interaction (i.e., π−π and hydrophobic interactions), and PS NPs also may change the microenvironment of the Trp residue. Therefore, PS NPs could decrease the activity of pancreatin by changing the secondary structures of enzymes and Trp residues via adsorption, consequently leading to a decrease in starch digestion.

4.4 Conclusions
In this study, PS or PS with functional groups (i.e., -COOH or -NH$_2$) significantly inhibited the hydrolysis of corn starch, leading to a significant decrease in glucose release (74%) relative to the control (95%). SEM images suggested that as enzyme catalyze corn starch, pinholes and cracks were firstly formed on the surface of starch granule, and a canal could be formed with increasing digestion time, enzyme could access to internal granules, and continuously breakdown the starch. Our investigation revealed that PS NPs significantly reduced the corn starch hydrolysis through two possible pathways: 1) PS NPs or PS NPs agglomerates were adsorbed onto starch granules, thereby inhibiting the digestive enzymes access to the surface of starch granules; 2) PS NPs decreased the digestive enzymatic activities, subsequently reducing the capacity of enzymes in starch hydrolysis. Additionally, PS, PS-COOH, and PS-NH$_2$ NPs showed inhibitory effects on enzymatic activities (i.e., amylase, pepsin, and pancreatin) via the disruption of the secondary structure and microenvironments of enzymes. These results indicate that adverse effects of PS NPs on nutrient assimilation and human gastrointestinal tract. Also, our study provided an instructional information for future investigation on the effects of NPs on the human gastrointestinal tract in-vivo studies.
Figure 4-1. (A) Changes in starch hydrolysis of corn starch with different PS NPs concentration (0.05, 0.15, 0.3%) in the simulated *in vitro* INFOGEST model. (B) Final glucose released (%) from starch with 0, 0.5, 0.15, 0.3% PS NPs. Bars with different lower-case letters (a-c) are significantly different (*p* < 0.05) when compared to various concentrations of PS or PS with different functional groups.
Initial phase

(Corn starch)

(A)

(Corn starch + PS NPs)

(E)

(I)

Mouth phase

(B)

(F)

(J)
Figure 4-2 SEM images of corn starch digestion (A-D) and PS+corn starch (E-H) and enlargement of the PS+corn starch (I-L) in the initial stage, mouth phase, stomach phase, small intestine phase, respectively.
(C) Corn starch

Mouth phase

(D)

Stomach phase

(E)

Intestine phase

(F) Corn starch + PS NPs

2 µm

2 µm

2 µm

2 µm
Figure 4-3 Changes in the activities of amylase, pepsin, and pancreatin during interaction with (A) different concentrations (0.05 wt%, 0.15 wt%, 0.3 wt%) or (B) different functional group (-COOH, -NH₂) of PS NPs; confocal images of corn starch digestion in the (C-E) absence or (F-G) presence of PS NPs in the mouth, stomach, and intestinal phase. Bars with different lower-case letters (a-c) are significantly different (p < 0.05) when compared to various concentrations and different functional group.
Figure 4-4. FTIR spectrum (A-C) of amylase, pepsin, and pancreatin in the absence or presence of PS, PS-COOH, PS-NH$_2$ in the mouth phase, stomach phase, and intestine phase, respectively; The CD spectra (D-F) of amylase, pepsin, and lipase in the absence or presence of PS, PS-COOH, PS-NH$_2$. 
Figure 4-S1 The SEM image of the PS NPs on the surface of starch in the initial stage (A), mouth phase (B), stomach phase (C), intestine phase (D); starch residues in the absence (E) and presence (F) of PS NPs in the small intestine phase; confocal image of corn starch residue in the absence (G) and presence (H) of PS NPs in the intestine phase.
Figure 4-S2. Fluorescence spectra of amylase (A) and pancreatin (B) incubating with PS NPs.
CHAPTER 5

5. CONCLUSIONS AND PERSPECTIVES

5.1 Conclusions

In this dissertation, we investigated the impact of E171/PS NPs on macronutrients digestion (i.e., lipid, starch), and bioaccessibilities of nutraceutical and minerals (i.e., Vitamin D₃, Ca, K, Mg, Mn, Fe, Zn, S, P) from diverse food matrix in the simulated human gastrointestinal tract. Both E171 and PS NPs showed inhibitory effects on the digestion process of food. For instance, the extent of lipid digestion was reduced by E171 addition in a dose-dependent manner; PS NPs significantly inhibited the hydrolysis of corn starch, as indicated by a decrease of 21% of glucose content compared to the control. In addition, E171 particles reduced bioaccessibilities of nutraceutical and minerals released from specific foodstuffs: bioaccessibility of VD₃ encapsulated in oil-water emulsion was significantly decreased from 80 to 74% when raising E171 from 0 to 0.5 wt%; the final bioaccessibility of Fe decreased from 59% without E171 to 53% with 0.2 wt% E171. Moreover, we further explored the mechanisms of repressive effect of both E171 and PS NPs on food digestion process and nutraceuticals or minerals released from corresponding foodstuff.

We summarized that three possible major pathways on the inhibitory effect on food digestion to be considered in the future experiments: 1) the interaction between food-grade particles/NPs and macronutrients (i.e., lipid, protein, starch), the adsorption of tiny particles on these components may restrain the enzyme access to their surfaces; 2) decreased activities of digestive enzymes (e.g., amylase, pepsin, pancreatin, lipase) by food-grade particles or NPs via adsorption and modification in secondary structure of these enzymes; 3) the possible adsorption of released micronutrients from digested food onto food-grade particles or NPs, for instance, Fe released from spinach leaves could
adsorb on to E171 in this research. Taken together, these results suggested the adverse effect of both E171 and PS NPs on nutrient assimilation and human gastrointestinal tract. Also, our study provided an useful information for future investigation on the effect of E171 or NPs on the human gastrointestinal tract \textit{in vivo} studies.

5.2 Perspectives

In the future, a few points need to be considered in further experiments. Firstly, numerous \textit{in vitro} or \textit{in vivo} studies on investigating the toxicities and potential risks of engineered particles, MPs/NPs conducted commonly in high dosage within short periods, which may be excessive and unrealistic related to the environment and humans in real life. In addition, considering the long-term accumulation of E171, MPs/NPs in human via ingestion, or dermal contact, it is more realistic to apply time-series studies to display cumulative effects over weeks. Therefore, further studies are needed to examine potential adverse effect of low concentration or environmental concentrations and chronic exposure to engineered particles, MPs or NPs.

Secondly, various type and properties of engineered particles, MPs or NPs should be involved in further experiments to obtain multiple results rather than simplex results. For example, besides E171 as additives in food products, SiO$_2$ referred to E551 is also an additive in foodstuffs. Many studies on the evaluation of toxicity and risk of MPs or NPs were frequently carried out using PS. This may result in misunderstanding toxicity of MPs or NPs to the ecosystems or human. In the environments, a various type of plastic pollution can be detected, i.e., PS, PE and PP. The different constituents of these particles may show distinct effects or mechanism as their interaction with biological components or organisms differs. Also, engineered particles, MPs or NPs have different physical and chemical properties, including size, shape, and surface properties.
Thirdly, in the realistic conditions, E171 cooperated in food as additives is often mixed with proteins, lipid or other components rather particles alone. For instance, E171 as whiten colorant in salad dressing, which also contains lipid or protein, it is necessary to consider co-effect of E171 with lipid or protein on the bioaccessibilities of minerals released from green leafy vegetables. For MPs or NPs, these tiny plastics in the environment are often derived from the degradation of large waste plastics and the direct release of commercial products. The properties of these plastics, particularly NPs, from the two origins are relatively distinct. It would be more realistically and accurately assess the environmental effects of these plastics which are derived from the corresponding origins. Additionally, as plastic waste releases into environmental, i.e., ocean, river, or soil, they undergo environmental weathering processes that may lead to changes in physicochemical properties. For instance, the formation of functional group (-OH, -COOH) on the surface of these plastics cause various surface properties.\(^\text{187}\) Importantly, because of complicated environmental conditions, plastic debris can adsorb other contaminants in the environments, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxin-like chemicals, polybrominated diphenyl ethers (PBDEs), toxic metals, then, MPs and NPs with adsorbed chemicals could enter digestive system of organisms or human.\(^\text{207}\) Therefore, the toxic effect of these contaminant or leachates with plastics should be considered or distinguished in future investigations.

Bohn et al. showed that although in vitro gastrointestinal models are simple, they are often useful and valuable in predicting results of the digestion \textit{in vivo}; however, this has depends on the complexity of \textit{in vitro} models and resolving specific questions relevant to human digestive physiology.\(^\text{48}\) Typically, these models have been applied to investigate the ingestion of related foods under the same conditions, to study the digestion process of pure substances, or to unravel
the interactions between components at the molecular level. Also, static in vitro digestion models ignore the dynamic features of the digestive process. Finally, in the future, the impact of engineered particles/MPs/NPs on nutrient assimilation, their interaction with digestive enzymes (i.e., amylase, pepsin, lipase, and pancreatin) could be performed on *in vivo* studies for further studies and improvements.


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