Raman Microspectroscopic Analysis of Bioactive in Palm Oil-in-Water Emulsions Stabilised by Dairy Protein

by

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To:

Ajah & Bushra

Mama & Ayah

Umi & Walid

and

My family in Malaysia

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ABSTRACT

The quest for designing a stable encapsulation system for natural bioactive in food products has never stopped. The development and innovation of analytical instruments allows for new factors influencing the system design to be explored. It is believed that the partitioning of bioactive within an emulsion affects the stability of the system, and so understanding it may inform the development of stable formulations as carriers for health promoting bioactives.

This research is focussed on analysing the partitioning characteristics of a bioactive carried in emulsion systems, using a powerful but as yet relatively unexplored technique in food-related studies; *in-situ* confocal Raman microspectroscopy (CRM). Stable food-grade palm oil-in-water (O/W) emulsions were engineered for the encapsulation of natural β-carotene, using whey protein isolate (WPI) as the emulsifier and carrier. High-pressure emulsification was employed for processing and improving the physical stability of the emulsions. This was followed by *in-situ* spectroscopic measurement of changes in the system upon variations in its formulation, storage condition and treatment procedure.

The Raman technique was first validated against a conventional method involving solvent extraction of β-carotene and quantification by visible absorption spectrophotometry. It is shown that CRM enables *in situ* quantification of the β-carotene content of oil droplets within emulsion systems. The Raman image obtained shows that the bioactive molecules were dispersed into both the oil and aqueous phases of the emulsions, but at a much higher concentration in the former.

Next, it was found that increasing the β-carotene concentration (from 0.1 to 0.3 g/kg emulsion) at a fixed gross composition of an emulsion (10% palm olein:2% WPI) reduced the concentration of β-carotene in the oil droplet, whereas increasing the WPI concentration (2–20 g/kg emulsion) had the opposite effect. A storage study was then conducted using these variables to determine the stability and partitioning characteristics of β-carotene throughout 30 d storage at 20 and 40 °C. It was found that a higher composition of β-carotene was in the aqueous phase at the end of the storage period, suggesting that β-carotene oxidation occurred faster in the oil phase and that β-carotene was more protected against oxidation by WPI in the aqueous phase.

Additionally, using only CRM, it has been shown that the use of small molecule Tween 40, as compared to that of the dairy protein (WPI) as the emulsifier, resulted in lesser partitioning

of the bioactive molecules into the aqueous phase of emulsions. In contrast, the partitioning of β-carotene into the aqueous phase increased with the rise in the solid fat content of the lipid phase of the emulsions, achieved by manipulating the ratio between the liquid palm olein and its solid fraction; palm stearin.

Moreover, the variation in partitioning of β-carotene molecules between the phases of emulsions was examined following the use of pre-treated (heat and pressure) WPI as an emulsifier. Generally the partitioning of β-carotene into the aqueous phase of emulsions increased when the protein emulsifier was heat- or high pressure-treated prior to emulsion formation. However, increasing the concentration of high pressure-treated WPI reduced the β-carotene partitioning into the aqueous phase.

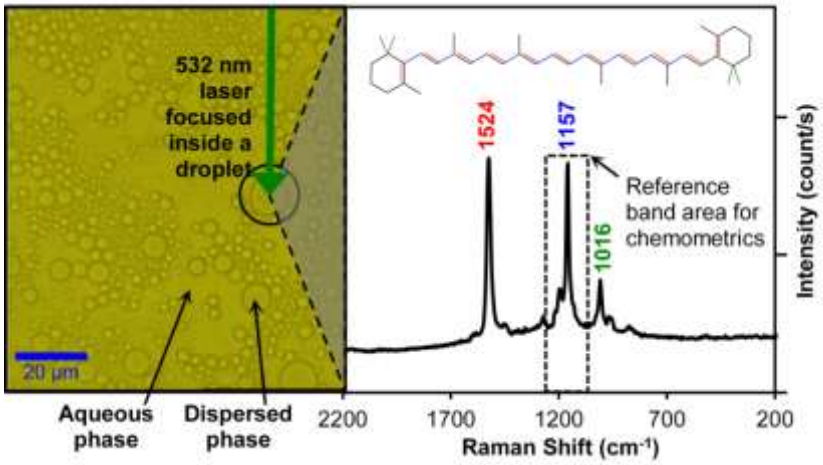

Figure T1. Raman spectrum of β-carotene in a droplet of O/W emulsion, with three significant peaks originating from vibrations of the isoprene units of β-carotene molecules, at 1524, 1157 and 1016 cm^{-1}, representing the double bond C=C stretching mode, ν_s (C=C); the C−C in-plane single bond stretching mode, ν_s (C−C); and the C−H bending mode, ρ (C−CH_3) of β-carotene respectively.

PUBLICATIONS

Articles in refereed journals

Wan Mohamad, W. A. F., Buckow, R., Augustin, M., & McNaughton, D. (2017). *In situ* quantification of β-carotene partitioning in oil-in-water emulsions by confocal Raman microscopy. *Food Chemistry*, 233, page 197–203. **(Published)**

Wan Mohamad, W. A. F., McNaughton, D., Buckow, R., & Augustin, M. (2017). Stability and partitioning of β-carotene in whey protein emulsions during storage. *Food and Function*, 8(11), page 3917-3925. **(Published)**

Wan Mohamad, W. A. F., McNaughton, D., Augustin, M., & Buckow, R. (2018). Characterisation of β-carotene partitioning in protein emulsions: Effects of pre-treatments, solid fat content and emulsifier type. *Food Chemistry*, 257, page 361-367. **(Published)**

Conference/Symposium/Colloquium presentations

Wan Mohamad, W. A. F., McNaughton, D., Augustin, M., & Buckow, R., *Raman microscopy depicts partitioning of encapsulated β-carotene*, 4th Asian Spectroscopy Conference (ASC): 15-18 December 2013, Singapore. **(Poster presentation)**

Wan Mohamad, W. A. F., Augustin, M., McNaughton, D., & Buckow, R., *Confocal Raman microscopy for characterisation of β-carotene-enriched whey protein emulsions*, 2nd International Dairy Foundation (IDF) Symposium: 3-5 March 2014, Melbourne. **(Poster presentation)**

Wan Mohamad, W. A. F., McNaughton, D., Augustin, M., & Buckow, R., *Characterisation of β-carotene-enriched O/W emulsions using confocal Raman microscopy*, Australian Institute of Food Science and Technology (AIFST) Summer School 2015: 28-30 January 2015, Melbourne. **(Oral presentation)**

Wan Mohamad, W. A. F., McNaughton, D., Augustin, M., & Buckow, R., *Raman microspectroscopy for β-carotene partitioning in oil/water protein-based emulsion*, 8th International Conference on Advanced Vibrational Spectroscopy (ICAVS): 12-17 July 2015, Vienna. **(Poster presentation)**

Wan Mohamad, W. A. F., McNaughton, D., Augustin, M., & Buckow, R., *Raman microspectroscopy for β-carotene partitioning in O/W protein emulsions*, Malaysian Post Graduate Colloquium (MPGC) 2015: 19-20 December 2015, Melbourne. **(Oral presentation)**

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes **TWO** original papers published in peer reviewed journals and **ONE** submitted publication. The core theme of the thesis is **"Raman Microspectroscopic Analysis of Bioactive in Palm Oil-in-Water Emulsions Stabilised by Dairy Protein"**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the **Centre for Biospectroscopy, School of Chemistry** under the supervision of **Professor Don McNaughton, Dr. Mary Ann Augustin** and **Dr. Roman Buckow**.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In particular, during the initial stages, instrumental assistance and guidance for sample preparation were sought from **Mr. Finlay Shanks** of the School of Chemistry, Monash University, and **Mr. Lijiang Cheng** of CSIRO Agriculture and Food, whose contributions are acknowledged appropriately in the publications. All other laboratory work in Manuscripts I, II and III in Chapters 5, 6 and 7, respectively, were conducted by myself, the principal author, including samples preparation, spectral measurements and analytical work. All co-authors contributed to the reviewing of the drafted manuscripts.

In the case of Chapters 5, 6 and 7, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and (%) of student contribution	Co-author name(s) Nature and (%) of Co-author's contribution	Co-author(s), Monash student Y/N
5	*In situ* quantification of β-carotene partitioning in oil-in-water emulsions by confocal Raman microscopy	**Published**	Initiation, key ideas, experimental work, data analysis, interpretation of results and manuscript writing (80%)	Don McNaughton – Supervision, advice, proofread and manuscript submission (10%)	**N**
				Mary Ann Augustin – Supervision, advice and proofread (7%)	**N**
				Roman Buckow – Supervision and proofread (3%)	**N**
6	Stability and partitioning of β-carotene in whey protein emulsions during storage	**Published**	Initiation, key ideas, experimental work, data analysis, interpretation of results and manuscript writing (70%)	Mary Ann Augustin – Supervision, advice, proofread and manuscript submission (15%)	**N**
				Don McNaughton – Supervision, advice and proofread (10%)	**N**
				Roman Buckow – Supervision and proofread (5%)	**N**
7	Characterisation of β-carotene partitioning in protein emulsions: Effects of pre-treatments, solid fat content and emulsifier type	**Submitted**	Initiation, key ideas, experimental work, data analysis, interpretation of results and manuscript writing (85%)	Roman Buckow – Supervision, advice, proofread and manuscript submission (8%)	**N**
				Mary Ann Augustin – Supervision, advice and proofread (5%)	**N**
				Don McNaughton – Supervision and proofread (2%)	**N**

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: 23 January 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: Donald McNaughton Digitally signed by Donald McNaughton DN: cn=Donald McNaughton, o=Monash University, ou=School of Chemistry, e[redacted] ash.edu, c=AU Date: 2018.03.28 17:14:31 +11'00' Date: 23 January 2018

Update:

Manuscript III entitled "Characterisation of β-carotene partitioning in protein emulsions: Effects of pre-treatments, solid fat content and emulsifier type" in Chapter 7 has now been published in the journal Food Chemistry, volume 257, page 361-367.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
ANOVA	Analysis of variance
ASCII	American Standard Code for Information Interchange
ATR	Attenuated total reflectance
BC	β-carotene
BHT	Butylated hydroxy-toluene
CCD	Charge coupled device
CPO	Crude palm oil
CRM	Confocal Raman microspectroscopy
DMSO	Dimethyl sulfoxide
EMCCD	Electron multiplying CCD
FTIR	Fourier transform infrared
GI	Gastrointestinal
HPKO	Hydrogenated palm kernel oil
HPP	High pressure processing
HTST	High-temperature-short-time
IR	Infrared
LCT	Long chain triglyceride
LOD	Limit of detection
LSD	Least significant difference
MCT	Medium chain triglyceride
NaCas	Sodium caseinate
NIR	Near infrared
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil
pI	Isoelectric point
PIT	Phase-inversion temperature
PKO	Palm kernel oil
RBD	Refined, bleached and deodorised
REP	Relative error of prediction
RSD	Relative standard deviation
S/N	Signal-to-noise
SCT	Short chain triglyceride
SEM	Scanning electron microscopy
SERS	Surface enhanced Raman spectroscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SIR	Synchrotron infrared
SR	Synchrotron radiation
UV-Vis	Ultraviolet-Visible
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water
WPC	Whey protein concentrate
WPI	Whey protein isolate

LIST OF NOMENCLATURES

A	Interfacial area
C	Concentration
c	Speed of light in vacuum (2.998×10^8 ms^{-1})
C_{SO}*	Saturation concentration of solute in oil
C_{SW}*	Saturation concentration of solute in water
E	Energy of a photon
ΔG	Energy of emulsion formation
h	Planck's constant (6.626×10^{-34} Js)
ha	Hectare(s)
hr	Hour(s)
Hz	Hertz(s)
J	Joule(s)
K_{OW}*	Equilibrium partition coefficient
min	Minute(s)
N	Number of spectra
r^2	Correlation coefficient
S	Entropy of dispersion system
s	Second(s)
$s_{y/x}$	Standard deviation of residuals
T	Temperature
% (w/w)	Weight-to-weight percent
% (w/v)	Weight-to-volume percent
γ	Interfacial tension
η	Viscosity
λ	Wavelength (m or cm)
ν	Bond stretching mode
ν	Frequency (Hz or s^{-1})
$\tilde{\nu}$	Wavenumber (cm^{-1})
ρ	In-plane bending mode
σ	Standard deviation

CHAPTER 1 BACKGROUND

1. Introduction

Palm oil is rich in carotenoids (mainly α and β-carotene) and other phytonutrients (e.g. tocopherols, tocotrienols and Co-enzymeQ_{10}). Phytonutrients are bioactives which have a role in health and nutrition. In traditional palm oil milling, the palm fruit is sterilised, and the palm mesocarp is pressed and clarified to produce a red crude palm oil. Traditionally, this crude palm oil has been refined, bleached and deodorised (RBD) to produce food-grade RBD palm oil. Most of the phytonutrients are removed or degraded during the refining process. RBD palm oil can also be fractionated into a liquid fraction (palm olein) and a more solid fraction (palm stearin). There is also a range of other fractions with intermediate melting points (e.g. palm mid fraction). The availability of fractions with different melting characteristics increases the range of applications of palm oil. Most applications of the fractions have concentrated on the use of the bulk fat in food products. The realisation that the phytochemicals in palm oil have beneficial health effects has led to the development of a food-grade red palm oil product that is rich in palm bioactives, mainly β-carotene. There are also palm lipid bioactives on the market that contain palm carotenoids in more concentrated forms for applications in the pharmaceutical and functional food market.

The various fractions of refined palm oil may be used for the preparation of emulsion-based products, which can also act as a carrier in encapsulation of health-promoting bioactives. Oil-in-water based emulsions stabilised by low molecular weight surfactants (e.g. Tween, lecithin), surface active biopolymers (e.g. milk proteins) and mixtures of these have been used for the preparation of food-grade emulsions and nanoemulsions. The use of milk proteins (e.g. caseins and whey proteins) for the formulation of emulsions has several advantages: (i) they act as emulsifiers for physical stabilisation of the oil droplets in an oil-in-water (O/W) emulsion due to their amphiphilic character and; (ii) they have the potential to bind with small hydrophobic molecules such as phytonutrients and therefore can act as a carrier for those bioactives. The use of an emulsion system to carry a lipophilic bioactive such as β-carotene, into aqueous-based food products has enabled the fortification of a wider range of functional foods.

Various high energy methods (e.g. high pressure valve homogenisation, microfluidisation and high-intensity ultrasonication) or lower energy methods (e.g. spontaneous emulsification, phase inversion and microchannel emulsification) may be used for producing the emulsion. The physical stability of the emulsion is dependent on the formulation, preparation method and quality of the interface (McClements & Rao, 2011). To date, most studies on emulsions containing bioactive have focussed on the physical stability of emulsions and the chemical stability of the bioactive that is carried (Chew et al., 2011; Cheong & Tan, 2010; Mao, Yang, Xu, Yuan, & Gao, 2010).

2. Objectives

A relatively unexplored area of research in the encapsulation of bioactives is the location and partitioning of these bioactives stabilised in protein-based emulsion systems (McClements & Rao, 2011). Knowing the partitioning characteristics of the encapsulated bioactives in emulsion systems is crucial as a guide to design the best formulation for an emulsion product that will serve its purpose efficiently as a carrier of the beneficial bioactives. For example, if an encapsulated bioactive is known to be more stable when it is partitioned into the continuous phase of an O/W emulsion, the emulsion formulation can be designed to reduce the oil loading of the system or to adjust the emulsifier content in the aqueous phase, allowing for more of the bioactive to bind after partitioning into the aqueous phase. Further strategies to protect the aqueous phase carrying the bioactive can then be established so that the whole encapsulation system is able to function as desired. Similarly when the partitioning shows that there is a preference for the oil rather than the aqueous phase and the bioactive remains stable in this phase, strategies such as increasing the bioactive content in the oil prior to emulsification, and adjusting the solid fat content of the oil phase to affect the solubility of bioactives in the oil, may help to improve the overall functionality of the delivery system.

In terms of the integration of such delivery system into food structures, the bioactive compatibility and thus bioavailability is highly important. Different food ingredients and products have different chemical compositions (e.g. ingredients with various functional groups reactive to the bioactives) and pH (e.g. acidic or alkaline). When they are to be fortified with certain bioactives stabilised by emulsion systems, they require different strategies to ensure success in fulfilling the purpose of the functional foods. Therefore determining the location of the bioactives within the emulsion will assist in designing an

encapsulation system with the required protection capability against the degradation of the bioactives due to the food matrices.

Furthermore, considering the ultimate purpose of a functional food enriched with bioactives encapsulated in an emulsion system, which is to give health benefits upon consumption, the knowledge of the partitioning characteristics of bioactives encapsulated in an emulsion is also undeniably relevant. It helps in formulating a carrier system that can withstand the differing types of environment along the gastrointestinal (GI) tract of human, to bring the health-promoting bioactives to their specific location for digestion. This knowledge is also a complement to the previous research on the stability of bioactives via the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) *in vitro* (Thakkar, Maziya-dixon, Dixon, & Failla, 2007; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011; Liu, Hou, Lei, Chang, & Gao, 2012).

In addition, the conventional extraction-spectrophotometric or extraction-chromatographic method for determining the concentration of bioactives in an emulsion is unable to contribute to the quest for understanding the bioactive partitioning behaviour within emulsion systems *in situ*, because the emulsion system is first separated between its aqueous and dispersed phases via ultracentrifugation, in which the emulsion structure will be completely destroyed. Moreover the procedure is very tedious and sometimes hazardous, since it involves laborious multi-stage extraction processes using chemicals such as n-hexane and ethanol, and enzymes, depending on the type of ingredients present in the emulsion.

Nevertheless, advances in new combined imaging and vibrational spectroscopic techniques i.e. microspectroscopy (e.g. Fourier transform infrared (FTIR), Raman, confocal–Raman, scanning electron microspectroscopy (SEM)–Raman, atomic force microspectroscopy (AFM)–Raman) have the potential to enable the study of the location and partitioning of bioactives within emulsions as well as the composition of the emulsions. A recent example is the use of Cryo In-SEM Raman for locating carotene in processed food samples (Lopez-Sanchez, Schumm, Pudney, & Hazekamp, 2011).

Furthermore, it has also been reported that specific interactions between carotenoids and proteins exist (Dufour & Haertlé, 1991; Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). Hence there may as well be a bioactive partitioning between the dispersed oil phase and the aqueous phase containing proteins in oil-in-water emulsions. In addition, the physical

state of the lipid used for formulation of the emulsion affects the stability of the carried carotene (Cornacchia & Roos, 2011a).

This PhD study examines the science that underpins the development of food-grade palm oil-in-water emulsions containing mainly whey protein isolate (WPI) for stabilising β-carotene. It is divided into THREE main exploratory parts, answering the listed research questions:

PART 1: Validating the Raman microspectroscopic technique to quantify the concentration of bioactive molecules in emulsions and locate them.

a) How accurate, precise and sensitive is the Raman method in comparison with the conventional extraction-spectrophotometric method? What are the relative error of prediction (REP), relative standard deviation (RSD) and the limit of detection (LOD) of this method against the reference method?

b) How could Raman microspectroscopy be used to determine β-carotene partitioning between the phases of emulsions?

c) How could the Raman technique locate the β-carotene molecules dispersed in the protein emulsions *in situ*?

PART 2: Understanding the partitioning characteristics of β-carotene in emulsions upon varying the components concentration, in fresh samples and throughout a storage period.

a) What are the β-carotene partitioning characteristics in fresh and stored emulsion samples when:

- varying the bioactive (β-carotene) concentration?
- varying the emulsifier (WPI) concentration?
- varying the storage temperature?

b) How physically stable is the emulsion for encapsulation upon storage?

c) How chemically stable is the encapsulated bioactive to degradation upon storage?

PART 3: Examining the effects of varying the solid fat content, emulsifier types and treatment of emulsifier and emulsion, on the partitioning of β-carotene within the dispersed systems.

a) How does the solid fat content of the lipid phase, varied using the fractions of palm oil, affect the partitioning of the β-carotene between the aqueous and lipid phases?
b) How does the molecular size of emulsifiers affect the β-carotene partitioning?
c) What are the partitioning characteristics of β-carotene when using heat and pressure-treated WPI as emulsifiers?
d) Will the partitioning characteristics be different upon pasteurising the processed emulsions using HTST (high-temperature-short-time) method?

3. Thesis outline

Following the first four preliminary chapters on the background and objectives of the study, literature reviews on emulsions for encapsulation and vibrational spectroscopies, as well as a description of experimental techniques, the results are presented in Chapters 5-7. In these final chapters, the uses of confocal Raman microspectroscopy (CRM) in analysing bioactive encapsulated in protein emulsions are progressively explored to achieve the objectives of the study.

In Chapter 5, a published article examining and validating the ability of CRM to quantify the concentration of β-carotene in protein emulsions is presented. The Raman technique was validated using the conventional extraction-spectrophotometric method. With the confocal microscopic instrument, the Raman image was obtained to locate the bioactive molecules dispersed in the emulsion systems. The effect of varying the concentration of β-carotene on its partitioning in the emulsion samples was analysed *in situ* using CRM for the first time. This study provides the necessary technical background for understanding the partitioning of bioactive within emulsions using only the confocal Raman instrument in the following experiments.

The study is extended in Chapter 6, which presents another published article on the storage study of β-carotene in protein emulsions. The partitioning and stability of the bioactive were analysed upon varying the β-carotene and WPI concentrations in the emulsion samples, throughout 30-day storage at different temperatures. The total β-carotene in the emulsion was

solvent-extracted and quantified using UV-Vis spectroscopy. The β-carotene in oil phase was measured *in situ* using CRM, while the aqueous phase concentration was obtained by difference. The physical stability of the emulsion systems was also monitored throughout the study to provide an informed basis for fabricating stable protein-based emulsions for the β-carotene delivery.

In Chapter 7, *in situ* investigations using CRM for the partitioning characteristics of bioactive molecules in emulsions stabilised by native (untreated) WPI, heat-treated WPI or high pressure-treated WPI prior to emulsion formation are presented in the form of a submitted article for publication. The partitioning of β-carotene within the emulsions stabilised by a low weight emulsifier (Tween 40) is also reported along with the effects of using fats with different solid fat content, namely palm olein (the liquid fraction of palm oil) and palm stearin (the solid fraction of palm oil). In addition, the variation in β-carotene partitioning between the phases of emulsions was examined upon pasteurising the emulsions using the high-temperature-short-time (HTST) method, emulating industrial practices.

Finally, Chapter 8 summarises the conclusions of this study with suggestions for future prospect of research and applications.

Reference

Cheong, J. N., & Tan, C. P. (2010). Palm-based functional lipid nanodispersions: Preparation , characterization and stability evaluation. *European Journal of Lipid Science and Technology*, *112*, 557–564.

Chew, L., Basri, M., Tejo, B. A., Ismail, R., Lau, H., Nang, L., … Yuen, C. (2011). An improved method for the preparations of nanostructured lipid carriers containing heat-sensitive bioactives. *Colloids and Surfaces B: Biointerfaces*, *87*, 180–186.

Cornacchia, L., & Roos, Y. H. (2011). State of dispersed lipid carrier and interface composition as determinants of beta-carotene stability in oil-in-water emulsions. *Journal of Food Science*, *76*, C1211–C1218.

Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta*, *1079*, 316–320.

Liu, Y., Hou, Z., Lei, F., Chang, Y., & Gao, Y. (2012). Investigation into the bioaccessibility and microstructure changes of β-carotene emulsions during in vitro digestion. *Innovative Food Science & Emerging Technologies*, *15*, 86–95.

Lopez-Sanchez, P., Schumm, S., Pudney, P. D. a, & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst*, *136*, 3694–3697.

Mao, L., Yang, J., Xu, D., Yuan, F., & Gao, Y. (2010). Effects of homogenization models and emulsifiers on the physicochemical properties of β-carotene nanoemulsions. *Journal of Dispersion Science and Technology*, *31*, 986–993.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrician*, *51*, 285–330.

Svelander, C. A., Lopez-Sanchez, P., Pudney, P. D. A., Schumm, S., & Alminger, M. A. G. (2011). High pressure homogenization increases the in vitro bioaccessibility of α- and β-carotene in carrot emulsions but not of lycopene in tomato emulsions. *Journal of Food Science*, *76*, H215–H225.

Thakkar, S. K., Maziya-dixon, B., Dixon, A. G. O., & Failla, M. L. (2007). β-carotene micellarization during in vitro digestion and uptake by Caco-2 cells is directly droportional to b-carotene content in different genotypes of Cassava. *The Journal of Nutrition*, *137*, 2229–2233.

Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International*, *42*, 1254–1258.

CHAPTER 2 INTRODUCTION TO β-CAROTENE EMULSION

1. Functional foods

Food science and technology has long been contributing to the needs of society in supplying edible raw materials from nature, or processed yet safe, tasty and healthy foods. Not until the last three decades has the focus been shifted to developing what is called nowadays 'functional foods' (Onwulata, 2013).

The terms 'functional foods' and 'nutraceuticals' are sometimes used interchangeably. These terms refer to foods or food components consumed for health optimisation and body wellness, and relate nutrients to specific health effects that go beyond normal nutrition (Mollet & Rowland, 2002). The study of the interaction between food components, nutritional science, and pharmacology is on the rise, with the science of pharmacology and food nutrition attempting to tag one disease to one nutrient or class of nutrients (Onwulata, 2013). However the distinct difference between medicines formulated to treat, cure or prevent diseases, and foods developed to supply nutrition and maintain or improve health should be well understood.

The main drive for developing functional foods has been to use bioactive nutrients contained in food for mitigating disease conditions, thus enhancing human health through food consumption (Richardson, 2009). Functional foods are also created to address the lack of certain nutrients in the body, or to reduce an excess of nutrients as well as reactive by-products such as free radicals or other species of regulatory concern, using appropriate delivery systems. Besides that, the demands of modern health-conscious consumers interested in foods that improve health and prevent illnesses, have also promoted the development of functional foods (Jacobs & Tapsell, 2007). Hence farmers, food scientists and manufacturers have responded to the increased demand for these healthy foods by making use of innovative technology to formulate new nutrient-enriched produce and products.

Studies in different fields of science are providing the knowledge of food processing tools that preserve bioactives as well as delivery methods that protect them during processing and storage, and also during GI transit up to reaching the desired site in the body after ingestion (Galland, 2005). Protected delivery, namely 'encapsulation', is an important strategy for

delivering food ingredients, at the right time and place (Onwulata, 2013). Encapsulation can increase flavour retention and mask strong flavours, besides stabilising and increasing the bioavailability of food ingredients like bioactive nutrients and enzymes (Versic, 1988). Furthermore encapsulation can also be used for controlled release of flavours and bioactive functionality, such as in chewing gums where encapsulated flavouring agents are released on chewing, and in baking, during which encapsulated leavening agents are released at the elevated baking temperatures (Augustin & Hemar, 2009). Sometimes non-digestible materials are used to entrap live microbes like probiotic bacteria and carry them safely through the harsh gastric environment, to the small intestine and colon where healing effects from digestion problems may take place (Gibson, 2004)

2. Encapsulation

Encapsulation involves the packaging of solid, liquid or gas particles referred to as the core or active, within a secondary material as either matrix or shell, to form small capsules.

Table 2.1: Common encapsulation methods in food industry (Fang & Bhandari, 2012).

Method	Description
Spray drying	Core is dispersed into aqueous encapsulant solution and atomised into a drying chamber
	Most commonly used method due to cost-effective
Spray chilling	Core is dispersed into coating solution and sprayed into a cold environment to solidify the carrier material
	Used for protection of heat-sensitive cores
Extrusion	Emulsion containing the core is passed through a die at high temperature and pressure into a bath for solidification of the particle
	Used for encapsulation of flavours and volatile cores in glassy matrices
Fluidised bed coating	Particles are suspended in air and a coating is applied
	Used for enhancing and achieving finer control over release properties of the core
Inclusion complexation	An inclusion complex is formed between cyclodextrin and the core
	Used for encapsulation of flavours and lipophilic nutrients
Coacervation	Coacervates are formed when two oppositely charged biopolymers associate and phase separate
	Can entrap high loadings of cores such as flavours and many nutrients

Capsules with diameters less than 100 nm are categorised as nanocapsules, while microcapsules are those in the order of microns (Augustin & Hemar, 2009). Based on the formulation and the required final structure of the capsule, the encapsulation technology may be chosen from the list of processing methods summarised in Table 2.1.

In contrast to its well-established role within the pharmaceutical, cosmetic, chemical and agricultural industries, encapsulation is relatively new to the food industry (Oliver & Augustin, 2009). The early applications of microencapsulation in the food industry as mentioned before include concealing of unwanted flavours, regulating the release of desirable flavours, and converting liquid streams into powdered forms for convenience and shelf-life extension (Versic, 1988). Augustin and Sanguansri (2008) have reviewed the history of and developments in the encapsulation of food ingredients and encapsulation technologies. Hitherto, encapsulation strategy is continuously being applied in the food industry for protection and controlled delivery of food ingredients and bioactives (Oliver & Augustin, 2009; Augustin & Sanguansri, 2008; McClements, Decker, & Weiss, 2007).

Examples of food components that are regularly encapsulated are listed in Table 2.2, while the common encapsulants used in food applications are summarised in Table 2.3.

Table 2.2: Common active cores delivered via encapsulation (Oliver & Augustin, 2009).

Type of core	Examples	Role of encapsulation
Oils	Milk fat, omega-3 oils	Improves storage stability and target release
Flavours	Mint, orange oil	Assists in preservation of flavours and controlled release in the mouth
Bioactives	Flavonoids, polyphenols, tocopherols, tocotrienols, carotenoids	Ensures protection from environment and interactions with food matrices, and improves target delivery on digestion
Food additives	Leavening agents	Assists in controlled release during baking
Minerals	Iron salts	Avoids undesirable interactions e.g. catalysing fat oxidation
Probiotics	Bifidobacteria, lactobacilli	Improves survival during storage and upon exposure to stomach acids

Table 2.3: Encapsulants with their characteristics (Oliver & Augustin, 2009).

Encapsulants	Examples	Characteristics
Proteins	Caseins, whey proteins, soy proteins, egg proteins, pea protein, gelatin, hydrolysed protein	Ability to build viscosity, gelling, emulsifying
Sugars and glucose syrups	Mono-, di- and oligo-saccharides, glucose syrups	Low viscosity at high solids, ability to form glassy solids on dehydration
Polysaccharides	Starch, maltodextrins, gums, carboxymethylcellulose, pectins, alginates, chitosan	Gelling, emulsion stabilisation, film forming, ability to form glassy solids on dehydration
Fats and waxes	Animal fats (e.g. milkfat), vegetable fat (e.g. canola oil), waxes (e.g. beeswax)	Solubilisation of lipophilic cores, matrix for embedding cores, water barrier properties, film forming
Surfactants	Mono- and diglycerides, phospholipids (e.g. lecithin), glycolipids, Tweens, Spans	Emulsifying

The followings are attributes for materials to be suitable as encapsulants in the food industry (McClements et al., 2007):

- **Food grade**: The delivery system must be fabricated from permitted food ingredients using processing operations that have regulatory approval.
- **Economic production**: The delivery system should be capable of being economically manufactured from inexpensive ingredients.
- **Protection against chemical degradation**: The delivery system may have to protect an encapsulated bioactive against chemical degradation, for example, oxidation and hydrolysis.
- **Loading capacity and retention**: Ideally, a delivery system should be capable of encapsulating a large amount of bioactive per unit mass of carrier material, and should efficiently retain the encapsulated component until it needs to be released.
- **Delivery mechanism**: The delivery system may have to be designed so that it releases the bioactive lipid at a particular site-of-action, at a controlled rate or in response to a specific environmental stimulus.
- **Food matrix compatibility**: The delivery system should be compatible with the surrounding food matrix, that is, it should not adversely affect the appearance, texture, flavour, or stability of the final product.

- **Bioavailability/bioactivity**: A delivery system should enhance (or at least not adversely affect) the bioavailability/bioactivity of the encapsulated components.

The capsule contents isolated from the surrounding environment are only released in response to a trigger or stimulus such as shear, pH change or enzyme action, thus allowing their controlled and timed delivery to a targeted site (Augustin & Hemar, 2009). Based on the target application, various capsule structures can be designed, which can broadly be categorised into the classical core–shell structures or matrix structures (Figure 2.1). From these basic structures, variations of capsule have been designed including capsules with multiple cores and layers (Kamyshny & Magdassi, 2006).

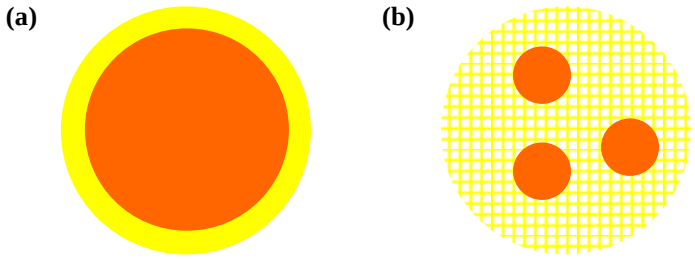

Figure 2.1: Capsules are mainly in the form of (a) core-shell structure, or (b) matrix with embedded core.

Examples of core–shell capsules are conventional oil-in-water (O/W) emulsions, multiple emulsions (W/O/W or O/W/O), solid lipid particles, nanoemulsions and liposomes. On the other hand, hydrogels, lipospheres and biopolymeric micro- and nano-particles with active cores are common encapsulation systems with matrix structures. (Oliver & Augustin, 2009)

For this study, the classical O/W emulsion was used for encapsulation of a lipophilic bioactive (β-carotene), incorporating whey protein isolate (WPI) from milk as the encapsulant and palm oil as the dispersed phase of the emulsion system.

3. Emulsion and emulsification

Emulsion science is a combination of aspects in engineering, chemistry, physics, and biology. Its basic principles were traditionally derived mainly from the polymer and colloid sciences, interfacial chemistry, and fluid mechanics (McClements, 2005). However, various other disciplines such as sensory science and physiology have been associated with the emulsion science when it started to be applied and developed within the food industry. A consequence of this is the establishment of novel interrelationships between perceived mouthfeel (sensory

science and physiology), droplet characteristics (colloidal science), emulsion rheology (fluid mechanics), and interfacial properties (interfacial chemistry) (McClements, 2005).

An emulsion system comprises two immiscible liquids (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other. Therefore emulsions are micro-heterogeneous materials whose composition and properties differ between regions when studied at micro- and nanometre length scales (McClements, 2005). Most food emulsions generally have three distinct regions (Figure 2.2) with respective physicochemical properties. The substance that forms the droplets in an emulsion is called the dispersed, discontinuous, or internal phase, while the substance that makes up the surrounding liquid is referred to as the continuous or external phase. The region between these two phases is termed the interface.

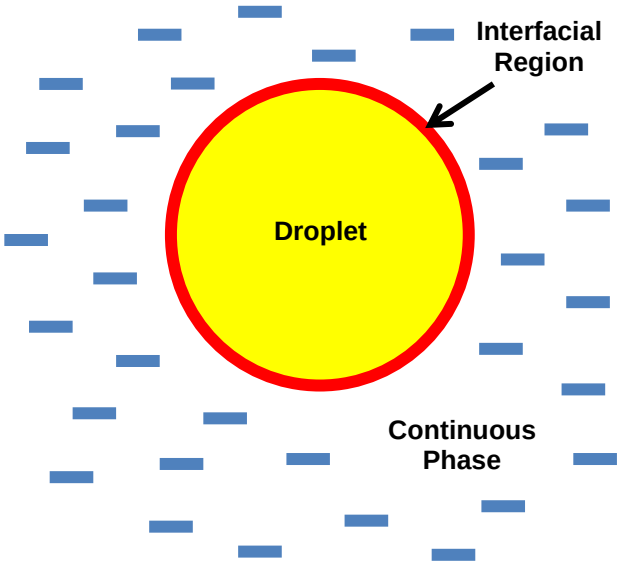

Figure 2.2: The ingredients in an emulsion partition themselves between the oil, water, and interfacial regions according to their concentration and interactions with the local environment.

Emulsions can be classified into two main categories according to their thermodynamic stability, which are macroemulsions and microemulsions (Wilde, 2009). Macroemulsions (commonly simplified as 'emulsions'), as described above, are dispersions of droplets in an immiscible fluid. Since energy is required to overcome the surface tension between the two phases and break up the dispersed phase into droplets, the droplets formed are thermodynamically unstable and will eventually collapse back into their component phases due to various physicochemical phenomena such as gravitational separation, flocculation, coalescence, and/or Ostwald ripening (McClements, 2005; McClements & Rao, 2011). The

droplet diameter for macroemulsions is generally in the range of 0.1–100 μm, which is a typical size range of most food molecules (McClements, 2005).

The ambiguously named ‘nanoemulsion’ is a class of macroemulsions (Mason, Wilking, Meleson, Chang, & Graves, 2006). With the same thermodynamic stability as macroemulsions, nanoemulsions are produced under much higher shear forces by incorporating ultrasound (Kentish et al., 2008), microfluidiser (Mason et al., 2006) or other high-pressure techniques. As a result, much smaller droplets (typically 30–100 nm diameter) are formed, with enhanced functionality such as rheology and flow, and improved delivery because of the much larger surface area in comparison to a conventional emulsion (Kotyla, Kuo, Moolchandani, Wilson, & Nicolosi, 2008).

Microemulsions on the other hand overcome the barrier of thermodynamic instability by using high concentrations of surfactants (and often co-surfactants; mixtures of oils and oil soluble compounds) and thus reducing the interfacial tension to near-zero (Wilde, 2009). As a consequence, when the two phases come into contact, they spontaneously emulsify to form a transparent microemulsion containing very small droplets with diameters less than 30 nm. This type of emulsion is usually restricted to non-food applications due to the high concentration of surfactant required.

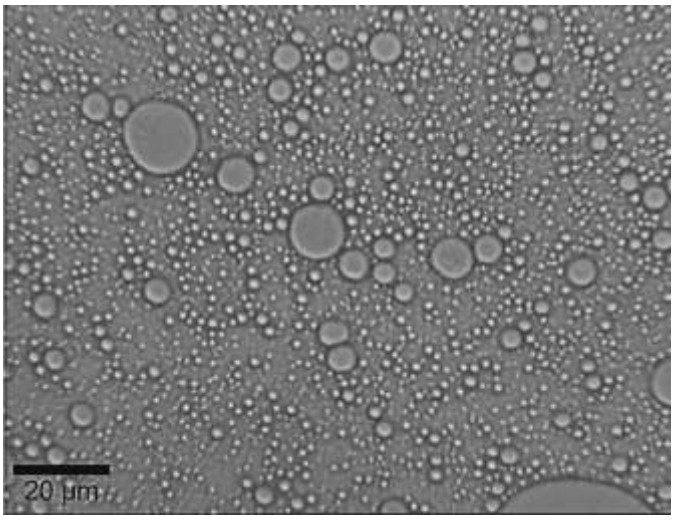

Figure 2.3: A micrograph of a food oil-in-water emulsion containing oil droplets dispersed in an aqueous medium.

Emulsions can also be conveniently categorised based on the relative spatial distribution of the dispersed and continuous phases (McClements, 2005). Figure 2.3 shows a food sample

with oil droplets dispersed in an aqueous phase i.e. O/W emulsion (e.g. milk, dressings, cream, mayonnaise, soups, beverages, and sauces). If water droplets are dispersed in an oil phase, it is then called a water-in-oil or W/O emulsion (e.g. margarine and butter). Nowadays multiple emulsions, oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) may also be made for specific applications (Schramm & Stasiuk, 2006).

In this study, conventional oil-in-water (O/W) emulsions were formulated to carry bioactive in protein-based stabilised system. Due to the droplet diameters being in the range of the wavelength of light i.e. 0.1-100 μm, these systems appear opaque or milky white (McClements, 2010). Conventional O/W emulsions are thermodynamically unstable due to the energetically unfavourable contact of a non-polar phase and a polar medium, and the different density of the two phases. For this reason, they tend to evolve to minimise the surface contact between the two immiscible phases and show typical particle movement in response to the force of gravity (McClements, 2005). However, the presence of emulsifiers that lower the interfacial tension and provide a protective membrane to the dispersed droplets, and small size of the dispersed particles, can ensure a reasonable long-term kinetic stability of the dispersed systems by preventing particles from coalescence and gravitational separation (Dickinson, 2001; McClements et al., 2007).

An emulsion (i.e. macroemulsion) can be made simply by mixing oil into water with sufficient mechanical shear and the aid of at least one emulsifying agent for physical stability. The emulsifying agent will reduce interfacial tension of the system to assist the formation of small droplets. In the classical method of emulsion preparation, the emulsifying agent is dissolved into the phase where it is most soluble (usually the aqueous phase), after which the second phase is added, and the whole mixture is vigorously agitated (Schramm & Stasiuk, 2006). Turbulent agitation is important to produce sufficiently small droplets.

This so called ‘emulsification’ process can also be explained thermodynamically. Equation 2.1 shows the total free energy of formation of an emulsion, ΔG:

$$\Delta G = \Delta A\,\gamma - T\Delta S \qquad \text{(Equation 2.1)}$$

where ΔA is the increase in interfacial area (when the bulk oil with area A_1 produces a large number of droplets with area A_2; $A_2 >> A_1$), γ is the interfacial tension, T is the process temperature and ΔS is the change in entropy of the system. Since γ is positive, the energy to expand the interface, $\Delta A\,\gamma$ is large and positive. This energy term cannot be compensated by

the small entropy of dispersion, $T\Delta S$ (which is also positive) and the total free energy of formation of the emulsion, ΔG is thus positive. Therefore emulsion formation is non-spontaneous and energy is required to produce the droplets (Tadros, Izquierdo, Esquena, & Solans, 2004). The formation of large droplets (few micrometres) as in the case for macroemulsions is fairly easy and hence high-speed stirrers such as the Ultra-Turrax and Silverson mixers are sufficient to produce the emulsions.

Nano- and micro-emulsions however require an extra step to produce, which can be generally categorised as either low- or high-energy methods (McClements & Rao, 2011). Low-energy methods such as phase-inversion and spontaneous-emulsification depend on the spontaneous formation of small oil droplets within mixed oil-water-emulsifier systems when the solution or environmental conditions are changed (Tadros et al., 2004). High-energy methods, e.g., high pressure valve homogenisers, microfluidisers and sonication, on the other hand utilise mechanical devices that can generate intense disruptive forces capable of disrupting and mixing the oil and aqueous phases into tiny oil droplets (Wooster, Golding, & Sanguansri, 2008; Leong, Wooster, Kentish, & Ashokkumar, 2009). Nowadays, the high-energy approaches are more common in the preparation of small sized and physically stable emulsion droplets in food operations due to their establishment in producing conventional emulsions. Besides being capable of large-scale production, they can also be used to fabricate nanoemulsions from different starting materials (McClements & Rao, 2011).

The preparation of pre-emulsions in this study utilised the high-speed stirrers (Ultra-Turrax or Silverson mixer) to yield coarse oil droplets (O/W system), before passing through high-energy homogeniser twice, to get homogenised and stable emulsion systems with controlled droplet sizes.

3.1 Palm oil as dispersed phase

Various non-polar components, including triacylglycerols, flavour oils, essential oils, mineral oils and waxes, can be used to formulate the oil phase of emulsions. The formation, stability, and properties of an emulsion system are greatly influenced by the bulk physicochemical characteristics of the oil phase, e.g. interfacial tension, polarity, refractive index, water-solubility, density, viscosity, phase behaviour, and chemical stability (Tadros et al., 2004; McClements, 2005; Wooster et al., 2008). These characteristics often determine the suitable homogenisation method to produce an emulsion from a particular oil phase.

Table 2.4 summarises how the bulk physicochemical characteristics of the oil phase affect the formation and stability of emulsions (McClements & Rao, 2011).

Table 2.4: Effect of different physicochemical properties of oil phase on the formation and stability of food-grade O/W emulsions.

Property	Formation	Stability
Viscosity, η **- Dispersed, η_D** **- Continuous, η_C**	Viscosity ratio (η_D / η_C) determines efficiency of droplet breakup in high-energy methods	Creaming rate decreases with increasing η_C
Interfacial tension, γ	Low γ promotes droplet breakup in high-energy methods Low γ promotes droplet formation in spontaneous-emulsification and phase-inversion methods	Low γ leads to droplet coalescence Low γ may cause poor emulsifier affinity for droplet surfaces
Solubility	Water-solubility of surfactants and solvents influences droplet formation in solvent displacement and spontaneous emulsification methods	High water-solubility of an oil phase promotes Ostwald ripening
Polarity	-	Governs components partitioning between oil and aqueous phases
Density	-	Creaming rate increases with increasing density contrast

In food manufacturing, preparation of emulsions using triacylglycerol oils (e.g. corn, soybean, sunflower, safflower, olive, flaxseed, algae, fish, and palm oils) is often desirable because of their abundance, low cost, and functional or nutritional attributes. These oils commonly contain long-chain triacylglycerols (LCT). Oils with medium-chain triacylglycerols (MCT) and short-chain triacylglycerols (SCT) are only used in some food applications. Particularly for preparing nanoemulsions, using MCT and LCT oils is quite challenging as a result of their relatively low polarity or high hydrophobicity (Witthayapanyanon, Acosta, Harwell, & Sabatini, 2006), high interfacial tension (McClements & Rao, 2011), and high viscosity (Wooster et al., 2008). Due to their high hydrophobicity for example, it is difficult to produce nanoemulsions from these oils using the phase-inversion temperature (PIT) method. Their high viscosity on the other hand limits droplet breakup when using high-pressure homogenisation methods. In these situations, the use of emulsifying agent(s) is often necessary to fabricate the very small and physically stable droplets required for nanoemulsion production.

In contrast, some edible oils are very effective at preparing emulsions with small droplets, but less effective at stabilising them. For example, flavour and essential oils can form very small droplets under both high pressure homogenisation and PIT methods, since they have a relatively high polarity, low interfacial tension, and low viscosity (McClements & Rao, 2011). However the low interfacial tension of the oil may rupture the emulsion once it is formed as a result of either Ostwald ripening or coalescence. Therefore alternative strategies to enhance the long-term stability of the emulsions prepared using these oils have to be developed, such as by incorporating ripening inhibitors to avoid Ostwald ripening or by using other types of emulsifier to prevent coalescence.

In this study, palm oil products were used as the dispersed phase of our emulsion systems. Palm oil is one of the major edible oil produced in the world. Palm oil and its fractions have wide applications as the neat oil (e.g. frying) or an ingredient in many emulsion-based food products (e.g. ice cream, formulated milk beverages, mayonnaise). Being free of trans-fatty acids, palm oil has gained wide acceptance by the food industry worldwide, especially in the United States after the US Food and Drug Administration's final ruling on trans-fatty acid labelling in 2003 transformed the fat and oil industries.

The oil palm tree, *Elaeis guineesis Jacq.*, originates from the Guinea Coast of Africa and is native to many West African countries. However large-scale plantations have been established in tropical regions of Asia, Africa and Latin America, aiming mainly at the production of palm oil (Wong & Radhakrishnan, 2012). The oil palm is a monocotyledon perennial tree crop, which undergoes 25 years of economic life cycle. Its plantation produces 44 tonnes of dry matter/ha/year, about 18.3 tonnes of dry matter/ha/year higher than that assimilated by a rainforest. The dry matter production stays at peak throughout the 25 years of the plant economic life cycle, after which the crop can be replanted (Gee, 2007). The long economic life cycle, in addition to the closed canopy of the oil palm plantation, supports stable ecosystems. However the biodiversity of its surrounding flora and fauna is inferior to that of a tropical rainforest.

The oil palm produces more oil per area than any other plant. It produces 3.80 tonnes of oil/ha on average, multiple times higher than that by rapeseed and soybean oil; 0.56 and 0.35 tonnes of oil/ha respectively. Palm oil will also be the most efficient oil crop to fill up the foreseeable shortfall of fats and oils as a result of biofuel replacement, since the crop does not

require as much forest land as that sacrificed for the low-oil-yield crops such as rapeseed or soybean (Gee, 2007).

The fruits, comprising two main parts; mesocarp and its kernel or seed, are rich in vegetable oil. The mesocarp produces orange-red crude palm oil (CPO), which contains mainly palmitic and oleic acids, whereas the seed yields brownish yellow crude palm kernel oil (PKO) that is a main source of lauric-based oil (Mortensen, 2005). CPO consists of relatively high concentrations of free fatty acids and low concentrations of phospholipids as compared to PKO. Physical refining of CPO removes undesirable impurities, producing refined, bleached and deodorised (RBD) palm oil.

The palm oil is semi-solid, hence can be fractionated into palm olein (the liquid fraction) and palm stearin (the solid fraction). The process begins with crystallisation at low temperatures, followed by filtration to separate the liquid and solid fractions. For fractionation of CPO, detergent fractionation is used, while dry fractionation is normally used for fractionation of RBD palm oil (Gee, 2007). In order to obtain products with desired characteristics, multiple fractionations can be carried out. The fatty acid compositions of palm oil and its fractions are given in Table 2.5 (Gee, 2007). During fractionation, unsaturated fatty acids are preferentially distributed into the liquid palm olein, together with diacylglycerols, squalene, carotenoids, tocopherol and tocotrienols. On the contrary, saturated fatty acids, monoacylglycerols, sterols and phospholipids acids are preferentially distributed into the palm stearin.

Table 2.5: Mean values of fatty acid composition in palm oil, palm olein and palm stearin.

Fatty acid	Palm oil (%)	Palm olein (%)	Palm stearin (%)
Palmitic	44.14	40.93	56.79
Oleic	39.04	41.51	29.00
Linoleic	10.57	11.64	7.23
Stearic	4.44	4.18	4.93
Myristic	1.11	1.09	1.27
Arachidoic	0.38	0.37	0.24
Lauric	0.24	0.27	0.18
Linolenic	0.37	0.40	0.09

Table 2.6: Characteristics (in mean values) of RBD palm oil and its fractions.

Parameter	Palm oil	Palm olein	Palm stearin
Iodine value	52.07	56.75	37.74
Slip melting point (°C)	36.72	21.45	51.44
Refractive index*	1.4548	1.4589	1.4493
Apparent density (g/ml)*	0.8899	0.8972	0.8822
Solid fat content (%) at			
10 °C	53.7	38.3	76.0
15 °C	39.1	19.9	68.9
20 °C	26.1	5.7	60.2
25 °C	16.3	2.1	50.6
30 °C	10.5		40.4
35 °C	7.9		34.3
40 °C	4.6		28.1
45 °C			22.4
50 °C			12.5
55 °C			0.6

*Refractive index and apparent density for palm oil, palm olein and palm stearin were measured at 50, 40 and 60 °C respectively.

Table 2.6 summarises the chemical and physical properties of palm oil and its single fractionated palm olein and palm stearin (Gee, 2007). The iodine value marks the unsaturation of oils and fats, which can theoretically be calculated from the fatty acid composition. However in practice, the value is commonly determined by titration using the Wijs method. Other characteristics of palm oil and its fractions given in Table 2.6 are parameters that describe physical properties of the palm oil products, especially the fractions which were extensively used throughout this study.

3.2 Milk protein as emulsifier

Milk proteins are natural vehicles, which aim to deliver essential micronutrients (e.g. calcium and phosphate), building blocks (e.g. amino acids) as well as immune system components (e.g. immunoglobulins and lactoferrin), from a mother to her newborn. The recognition that they could be harnessed to deliver additional health-promoting and other bioactive compounds in food and drug applications has led to intensive research and development

efforts over the past few years, resulting in a wide variety of novel applications (Livney, 2010).

The major proteins in milk are caseins and whey proteins, both of which have been incorporated into the formulation of various encapsulation systems. Table 2.7 shows the differences between caseins and whey proteins in many aspects (Oliver & Augustin, 2009).

Table 2.7: Comparison of properties between caseins and whey proteins.

Properties	Caseins	Whey proteins
Isoelectric point, pI	~4.6	~5.2
Conformation	Random coil	Globular structure
Heat sensitivity	Relatively heat stable	Denature at high temperature
Emulsifying properties	Form thin, flexible interface	Form denser interface
Gelling properties	Gels formed in the presence of calcium ions	Denaturation precedes gelation

The properties of these proteins are influenced by their amino acid composition, conformation and charge. Due to their amphiphilic nature, proteins are prone to self-assembly, resulting in aggregation and gelation that allow the proteins to form networks with embedded ingredients. The integrated contribution of hydrogen bonding as well as hydrophobic, electrostatic, and van der Waals' interactions to the development of a three-dimensional network will vary according to conditions manipulated to direct the proteins assembly (Augustin & Hemar, 2009).

The selection of protein for encapsulation systems is dictated by the protein functionality. The functional properties of milk proteins and their modification strategies have been extensively reviewed since 1984 (Kinsella & Morr, 1984). Milk proteins as part of encapsulation systems play various roles; as emulsifying agents, viscosity builders and carriers of the encapsulated materials. Proteins have the ability to assemble at an interface, including that of the oil-water interface of emulsion systems. The proteins used to form an emulsion determine the type and robustness of the interface. Random coiled caseins for example, form an entangled layer of flexible chains, whereas whey proteins with globular structures form strong, dense assemblies at the interface (Augustin & Hemar, 2009). The

emulsion stability is significantly affected by both the structure and the rheological properties of the interface. Therefore the surface-active properties of proteins and their ability to build viscosity have been carefully manipulated in emulsion-based encapsulation systems (McClements et al., 2007).

By definition, an emulsifier is a surface-active compound that can adsorb to droplet surfaces, promote droplet breakup, and prevent the aggregation of droplets (McClements, 2005). In high-energy emulsification methods, droplet breakup within the homogeniser is facilitated by the emulsifier, which reduces the droplet interfacial tension, thus favouring the formation of small droplets. However in low-energy applications, the emulsifier promotes the spontaneous formation of small droplets by lowering the interfacial tension under specific conditions of the environment and the solution (McClements & Rao, 2011).

The type of homogenisation technique to form tiny emulsion droplets is highly dependent on the type of emulsifier used. Small-molecule surfactants are often very effective at forming nanoemulsions using either high- or low-energy method. On the contrary, proteins and polysaccharides are rarely used as emulsifiers to form nanoemulsions using low-energy methods, and are generally less effective than the small-molecule surfactants at forming nanoemulsions using high-energy methods. However, proteins and polysaccharides are often considered 'label-friendly' for being natural ingredients, and this results in the great interest of incorporating them for emulsions fabrication (McClements & Rao, 2011). Furthermore, proteins specifically can form a viscoelastic membrane-like film around oil droplets, which is very useful in the preparation of O/W emulsions. Caseinate, one of the most commonly used surfactants in the food industry, is itself a mixture of interacting proteins of varying surface activity (Stainsby & Dickinson, 1988). The phospholipids can also interact with proteins to form independent vesicles, thus creating an additional dispersed phase.

Apart from the above, proteins are also effective at transporting bioactives because of their ligand binding ability. Taking milk proteins as examples, the casein micelle has high affinity for calcium, while β-lactoglobulin, which belongs to the lipocalin family of whey proteins, is an effective carrier for fatty acids, retinol and other hydrophobic molecules (Augustin & Hemar, 2009).

In addition, previous studies have shown that certain types of food protein emulsifier are able to reduce the oxidation rate of emulsified lipids, for example polyunsaturated oils (Qian,

Decker, Xiao, & McClements, 2012b). It was therefore hypothesised that coating the lipid droplets with a protein layer may improve the chemical stability of an encapsulated bioactive.

Contextually, this study focuses on the use of whey protein (to be exact, whey protein isolate (WPI)) from milk, as the emulsifier and carrier in the emulsion-based encapsulation systems designed. As previously mentioned, whey proteins are effectively all the other soluble, non-casein proteins, which remain upon removal of the precipitated protein or curd fraction in cheese making or casein production. The milk serum contains around 0.7% protein, 5% lactose and some other minor fractions (Smithers, 2008). Whey proteins account for about 20% of the total milk proteins, and contain approximately 50–60% β-lactoglobulin, 15–20% α-lactalbumin, 15% glycomacropeptide (in renneted whey only), and about 15–20% minor protein/peptide components (e.g. immunoglobulins, lactoperoxidase, lactoferrin, serum albumin, and lysozyme), depending on the source and the process (Wilde, 2009).

Commercially, whey proteins are hardly used as individual proteins. Instead, they are more preferable in the form of total whey protein fractions, either whey protein concentrates (WPC) or whey protein isolates (WPI). A range of filtration and separation techniques are used to form WPC or WPI powders by dehydrating and concentrating whey proteins (Smithers, 2008). Typical composition data for some of these products generalised from numerous references and industrial manufacturers are included in Table 2.8 (Jelen, 2009).

Table 2.8: Typical chemical composition (g/100g) of major types of dried whey products.

Product	Protein	Lactose	Minerals
Regular dried powder	12.5	73.5	8.5
Demineralised (70%) whey powder	13.7	75.7	3.5
Demineralised (90%) whey powder	15.0	83.0	1.0
Ultrafiltration permeate powder	1.0	90.0	9.0
WPC 35 (skimmed milk replacer)	35.0	50.0	7.2
WPC	65.0-80.0	4.0-21.0	3.0-5.0
WPI	88.0-92.0	<1	2.0-3.0
Traditional lactalbumin	86.0-90.0	3.5-5.0	1.5-3.0

The production of WPC uses mainly ultrafiltration techniques to yield a whey fraction containing about 30–75% protein, 2–4% fat, and some lactose and salts, depending on the process conditions (Wilde, 2009). WPI however has much higher protein content (≥90% w/w) as a result of the separation process involving ion-exchange or membrane-based filtration, which can effectively separate the protein from the non-protein whey components (Kilara, 2008). Both whey fractions are thought to be less denatured, unless they undergo pasteurisation and drying processes that can significantly cause structural changes. WPC tends to be less effective at forming and stabilising emulsions than WPI, but is much cheaper as an ingredient (Wilde, 2009).

Whey proteins as emulsifiers are not as extensively applied as caseins. It has long been demonstrated that whey proteins adsorb at the interfaces at a slower rate than other proteins like β-casein (Tornberg, 1977). Around their isoelectric point (pI), whey proteins form poor unstable emulsions (Kilara, 2008). However, whey proteins do help in emulsification in infant formula, meal replacement beverages, soups and gravies, and coffee whiteners. They may be used in conjunction with low molecular weight emulsifiers (Kilara, 2008). In one study by Pearce and Kinsella (1978), when the oil phase volume was maintained at a constant 25% and whey protein concentrations were increased from 0.5 to 5% in emulsions, it was observed that the oil droplet size decreased as the protein concentration increased. Cornacchia and Roos (2011b) compared WPI and sodium caseinate (NaCas) as the stabiliser in a hydrogenated palm kernel oil (HPKO)-in-water emulsion, and showed the former has better stabilising properties at lipid to protein ratios of 10:1, 7.5:1, 5:1 and 4:1. This could be explained with the formation of a highly compact protein membrane of WPI which strengthened the resistance of the interfaces to rupture.

It has been shown by Lee and Rosenberg (2001) that composite wall systems made from whey protein and anhydrous milk fat molecules were able to delay the release of water-soluble cores (e.g. theophylline) into simulated GI fluids. Prior to that, they had shown that heat gelation also promoted controlled release of core materials in whey protein-based emulsions, as a result of the microcapsules having limited water-solubility (Lee & Rosenberg, 2000). By employing the same approach, Cho et al. (2005) reported that whey proteins were resistant to degradation by acid and heat, besides displaying improved stability against digestion by pepsin. Similar results were obtained in another study that implemented calcium

alginate coating on whey protein-stabilised O/W emulsions containing paprika oleoresin (Rosenberg & Lee, 2004).

Finally, the fact that β-lactoglobulin and α-lactalbumin are the major constituents of WPI, both of which contain cystyl residues, disulfide bonds and thiol functional groups, plays an important role in inhibiting lipid oxidation by scavenging free radicals (Sun, Gunasekaran, & Richards, 2007). Therefore, WPI can also act as an antioxidant, which probably accounts for the less severe degradation of β-carotene in a WPI stabilised emulsion-based encapsulation system compared to other protein stabilised systems.

3.3 β-carotene as active core

β-carotene belongs to the family of carotenoids without oxygen, besides α-carotene and lycopene. Carotenoids which contain oxygen are known as xanthophylls e.g. lutein and zeaxanthin (McClements et al., 2007). Carotenoids are a class of natural pigments mainly found in fruits and vegetables that typically have 40 carbon molecules and multiple conjugated double bonds (Failla, Huo, & Thakkar, 2007). Carotenoids are widely used as natural colouring agents in food industries and in cosmetic industries. In fact the yellowish to reddish colour of several types of palm oils is due to the presence of this bioactive (Maiani et al., 2009). Carotenes dominate the carotenoid content of palm oil with mainly (more than 90%) α- and β-carotenes (Figure 2.4) (Mortensen, 2005; Chew et al., 2011).

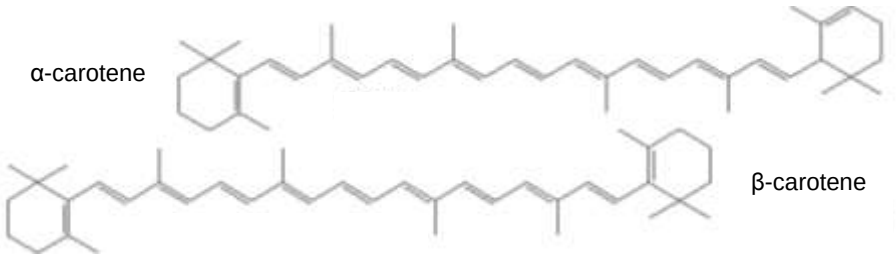

Figure 2.4: The chemical structure of α-carotene and β-carotene.

When consumed in sufficient levels, carotenoids have been claimed to have biological activities that may reduce the risk of certain chronic diseases, such as cancers, cardiovascular disease, age-related macular degeneration and cataracts (Qian, Decker, Xiao, & McClements, 2012a). Many physiological mechanisms have been suggested to account for the health benefits of carotenoids, including preventing oxidative damage, quenching singlet oxygen, altering transcriptional activity, and serving as precursors for vitamin A (Qian et al., 2012b).

Among the carotenoids, β-carotene has the highest pro-vitamin A activity, and is therefore a strong candidate for incorporation into functional foods. It is also a lipid-soluble antioxidant that has free-radical scavenging properties, such as the ability to quench singlet oxygen effectively (Chew et al., 2011). Protection against skin damage from sunlight by nutritional means of β-carotene and other carotenoids has been examined by Stahl and Sies (2012). Likewise, there has been work on the topical application of β-carotene to the skin, making this compound useful as an active component in sun-protection products (Chew et al., 2011).

Nevertheless, the absorption of carotenoids from many natural and processed foods is often inefficient and highly variable. This has been attributed to a number of reasons (Wackerbarth et al., 2009; Chew et al., 2011; Qian et al., 2012b):

(i) entrapment within food matrices,
(ii) low water-solubility,
(iii) high melting point, and
(iv) poor chemical stability e.g. sensitive to oxygen, heat, and light.

Studies have shown that the bioavailability of these bioactives can be improved by incorporating them with digestible lipids in emulsion-based systems (Borel, 2003; Thakkar, Maziya-dixon, Dixon, & Failla, 2007; Gonnet, Lethuaut, & Boury, 2010). Absorption of both emulsified fats and carotenoids into the body occurs upon the lipid hydrolysis by gastric and pancreatic lipases, forming free fatty acids which are combined along with bile acids and phospholipids into mixed micelles that can solubilise and transport the carotenoids to the epithelium cells (Tyssandier, Lyan, & Borel, 2001, Yi, Li, Zhong, & Yokoyama, 2014).

Therefore, food-grade delivery systems from oils can be designed to encapsulate, protect, and release these bioactives into specific body systems, through various encapsulation strategies including emulsification as discussed before. This encapsulation method also protects sensitive bioactives against degradation during processing and storage, prior to their consumption (Yang & McClements, 2013).

Natural β-carotene was chosen as the core of the capsules made from emulsion-based systems in this study. Its numerous health benefits and ease of administration with emulsion-based encapsulation system make it a desirable candidate for the study, in addition to its significant response to the analytical tools used.

While protein-based formulations have been used for the delivery of lipophilic nutrients such as β-carotene, most work has concentrated on a single pure component, the global efficiency of encapsulation and overall degradation of sensitive bioactives in emulsion systems. This study is differentiated from others in that it examined the bioactive location within protein-based emulsion structures and its partitioning characteristics between the aqueous phase and lipid core of the emulsion *in situ*, and used non-destructive spectroscopic methods (Raman) coupled with imaging (i.e. confocal microspectroscopy) to provide insights into the partitioning and relative rates of degradation of the chosen bioactive in various emulsion structures.

4. Partitioning of bioactive

Molecules (e.g. emulsifiers, bioactives) present in the emulsion system will distribute themselves between the three regions of emulsions (see Figure 2.2) according to their concentrations and polarity (McClements, 2005). Polar molecules prefer to site in the aqueous phase, non-polar molecules in the oil phase, and amphiphilic molecules at the interface. Even at equilibrium, molecules continuously move and exchange sites at a rate dependent on their mass transport through the different regions in the system. Besides that, the transit of molecules from one region to another may also occur upon changes in the surrounding conditions of an emulsion, such as a variation in pressure and temperature, or even dilution within the mouth following consumption. The location and movement of the molecules within an emulsion significantly affects the flavour and physicochemical stability of food products (McClements, 2005).

Among other molecules in an emulsion system, the partitioning of the bioactive component, which is usually hydrophobic or lipophilic, encapsulated within the system is particularly important one to be understood. The bioactive partitioning within an emulsion affects the protection available against degradation since the molecular environment influences the chemical stability of the lipophilic component (Cornacchia & Roos, 2011). In other words, the importance of understanding this property can be summarised as:

(i) a guide for designing the best formulation of an emulsion product that will serve its purpose efficiently as a carrier of the beneficial bioactive,
(ii) a reference in designing an encapsulation system with the required protection capability against the degradation of the bioactive due to the food matrices, and

(iii) a factor in formulating a carrier system that can withstand certain types of environment along the gastrointestinal (GI) tract of human upon consumption, to bring the health-promoting bioactive to their specific location for digestion.

Many hydrophobic bioactives within an emulsion-based delivery system have a finite solubility in both the oil and aqueous phases. If they are below their saturation concentration, they may be distributed between the phases as liquids, but if the bioactives are above their saturation concentration, a part of them may be present as solid crystals (McClements, 2012).

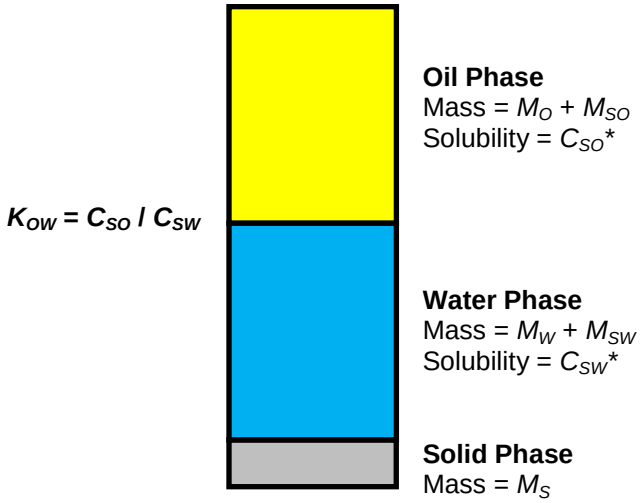

Figure 2.5: The oil-water partition coefficient, K_{OW} of a solute (bioactive) at equilibrium determines the solute partitioning between the oil and water phases of an emulsion. Key: The symbols *M* refer to mass, *C* solubility concentration and *C saturation concentration; the subscripts *S, O* and *W* mean solute, oil and water, respectively.**

At relatively low concentrations, the solute (i.e. hydrophobic component) will be solubilised completely in both the oil and aqueous phases, and its partitioning between the phases is determined by the equilibrium partition coefficient (K_{OW}). However above its saturation concentration in the oil (C_{SO}*) and water (C_{SW}*) phases, the solute will tend to form a separate solid crystalline phase (Figure 2.5) (McClements, 2012).

In practice, it is useful to know the saturation concentrations of a bioactive in an oil–water system at equilibrium, so that the formation of the solid phase can be monitored based on the bioactive actual concentrations in each phase.

In this research, factors influencing the partitioning of bioactive between the different phases of an emulsion were studied. They are the emulsion formulation (i.e. concentration and type

of components) and the treatments undergone by the emulsifier and the emulsion system. These factors lead to changes in the chemical and physical characteristics of the emulsion, which consequently affect the distribution of bioactive molecules within the system.

4.1 Formulation of emulsion

The formulation of an emulsion containing bioactive is expected to affect the bioactive partitioning, and thus its chemical stability. The change in bioactive and emulsifier concentrations will establish a new equilibrium between the different phases of emulsions. Besides, the bioactive partitioning within the emulsions follows the relative solubility of the bioactive in both the oil and aqueous phases, the propensity of the bioactive molecules to assemble at the interface, and their affinity to bind to other types of molecules in the emulsions. In the context of this study, since β-carotene can bind to β-lactoglobulin (Dufour & Haertlé, 1991; Wackerbarth et al., 2009), which is the major component of WPI, the protein dissolved in the aqueous phase of the emulsion samples studied, altering the amount of bioactive molecules in the emulsion systems may result in the change of the bioactive partitioning characteristics.

On the other hand, the emulsifier molecules at the interface of the oil droplet in an emulsion influence particle-particle aggregation and interaction of lipophilic bioactive in the oil droplets with reactive components around them. The protection capacity provided by the interfacial membrane relies on the packing density and the layer thickness of the emulsifier molecules, their chemical composition as well as the electrical charge on the particle surface (Augustin & Sanguansri, 2010; Yucel, Elias, & Coupland, 2012). Therefore varying the emulsifier content of an emulsion may also affect the bioactive partitioning between the phases.

With respect to the type of emulsifiers, both low (e.g. Tweens) and high molecular weight emulsifiers (e.g. proteins) may be used in the formulation of emulsions. The use of these different emulsifiers results in emulsions with different interfacial structures (Mao et al., 2009). In addition, when protein is used, it is possible to modify the whey protein structure through processing, which will influence its functional properties. For example, factors such as pH, temperature and pressure affect whey protein conformation, which in turn changes binding characteristics of these proteins to small molecules (Kühn, Considine, & Singh, 2008).

Furthermore, bioactive partitioning within an emulsion-based delivery system can also be influenced by the nature of the carrier oil used. The chemical composition and physicochemical properties of the carrier oil will determine the oil–water partition coefficient (K_{OW}) and the maximum amount of solute that can be dissolved in the oil phase (C_{SO}*) (McClements, 2012).

4.2 Treatments of emulsifier and emulsion

Besides the ingredients of an emulsion, the pre-treatments applied on the emulsifier will also influence the bioactive partitioning between phases of the emulsion.

Heat treatment is usually applied to alter the functional characteristics of ingredients in food. Industrially, protein-stabilised emulsions undergo thermal processing techniques such as pasteurisation and sterilisation. Above the protein denaturation temperature (e.g. 65 °C for whey proteins), the heat treatment often results in the adsorbed protein to partially unfold and expose some reactive amino acids in the aqueous phase (Peng et al., 2016). Cross-links are formed between denatured adsorbed protein molecules and denatured protein of the aqueous phase, thus leading to a three-dimensional solid-like network which contributes to the aggregation of the protein structure (Anton, Chapleau, Beaumal, & Delepine, 2001). Protein aggregation related to heat treatment is highly dependent on the pH and temperature of the system (Cui, Chen, Kong, Zhang, & Hua, 2014). Dybowska (2008) reported that preheating solutions of milk protein concentrate prior to emulsification increased the emulsion creaming stability. In another study, the freeze-thawing stability of O/W emulsions was improved upon heat treatment of a protein isolate used as the emulsifier (Palazolo, Sobral, & Wagner, 2011). Besides that, thermal treatment of emulsions also significantly alters their viscosity and physical stability. The inter-connections between adsorbed proteins and proteins of the network could modify the viscoelastic properties of interfacial film and alter its resistance towards coalescence, forming gelled-like structure (Dickinson & James, 1998).

High pressure processing (HPP) is an alternative preservation method to thermal processing for some food products. It is known to have a less severe effect on stability and gelation of emulsions made with milk proteins, than thermal treatments like pasteurisation and sterilisation (Anton et al., 2001). It is a non-thermal technology that was first applied in the food industry for extending the shelf life of perishable foods. Interestingly, the high pressure application can be carried out near room temperature, from which the resultant products

usually retain their natural colour, flavour, taste, and texture with little or no loss of vitamins, while being microbiologically stable during subsequent refrigerated storage (Galazka, Dickinson, & Ledward, 2000). For commercial applications, pressures up to 700 MPa and temperatures between 5 and 40 °C are typically used (Heinz & Buckow, 2010).

HPP of food can result in starch gelatinisation and protein denaturation, besides influencing interactions between food components (Devi, Buckow, Hemar, & Kasapis, 2014). The effect of HPP on modifying the macromolecular structure and functional properties of proteins is well recognised (Dickinson & James, 1998). Whey proteins, in particular, are susceptible to HPP, which unfolds them and allows both non-covalent and disulfide intermolecular interactions to occur (Dickinson, 2010). Studies have reported the effect of HPP on protein-flavour/bioactive interactions (Liu, Powers, Swanson, Hill, & Clark, 2005; J. Yang et al., 2003). They indicated that HPP of whey proteins can decrease the binding affinity for flavour and bioactive compounds or has no effect on the interactions, depending on the nature of the solute compound.

Under the conditions of neutral pH and high oil/protein ratio, the pressure-induced unfolding and aggregation of the protein structure is related to a reduction in emulsifying capacity of the milk protein (Galazka, Dickinson, & Ledward, 1996; Galazka, Ledward, Dickinson, & Langley, 1995). Structural studies of pressure-treated β-lactoglobulin, the main component of whey proteins, have shown that intermolecular disulfide bond is responsible for the irreversible formation of protein aggregates and that the extent of protein aggregation is highly dependent on protein concentration (Iametti et al., 1997). The polymerisation of partly unfolded monomers into stable aggregates, as a result of exposure to high pressure, appears in many respects to resemble thermal denaturation of β-lactoglobulin (Dickinson & James, 1998).

Comparing the effect of pressure treatment before and after emulsion formation, emulsions prepared with pressure treated whey protein solutions had considerably larger droplets than those made with the native protein, while there was less effect on the mean droplet size and creaming behaviour of the emulsions when pressure treatment was applied after emulsification (Galazka et al., 1996, 1995). The HPP on the protein solutions had caused modification of the protein structure, leading to protein aggregation hence the loss of emulsifying efficiency, despite an increase in surface hydrophobicity. On the other hand, the pressure treatment applied after emulsion formation probably partially unfolded the protein at

the interface, and subsequent pressure treatment caused no further conformational change. However, a significant increase in the viscosity and formation of gel-like structure of the sterilised emulsions was observed following the pressure treatment (Galazka et al., 2000).

Since the treatments substantially affect the conformation of proteins used as the emulsifiers and thus the physical properties of the emulsion systems, there can also be a new equilibrium established in the partitioning of the bioactive carried by the system, as more binding sites on the proteins are exposed for the bioactive molecules to attach (Dufour & Haertlé, 1991; Wackerbarth et al., 2009).

Reference

Anton, M., Chapleau, N., Beaumal, V., & Delepine, S. (2001). Effect of high-pressure treatment on rheology of oil-in-water emulsions prepared with hen egg yolk. *Innovative Food Science & Emerging Technologies*, 9–21.

Augustin, M. A., & Hemar, Y. (2009). Nano- and micro-structured assemblies for encapsulation of food ingredients. *Chemical Society Reviews*, *38*, 902–912.

Augustin, M. A., & Sanguansri, L. (2010). Use of encapsulation to inhibit oxidation of lipid ingredients in foods. In A. D. Eric, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in Foods and Beverages and Antioxidant Applications* (pp. 479–495). United Kingdom: Woodhead Publishing Limited.

Augustin, M. A., & Sanguansri, P. (2008). Chapter 24 Encapsulation of Bioactives. In J. M. Aguilera & P. J. Lillford (Eds.), *Food Materials Science – Principles and Practice* (pp. 577–601). Springer, New York.

Borel, P. (2003). Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols). *Clinical Chemistry and Laboratory Medicine*, *41*, 979–994.

Chew, L., Basri, M., Tejo, B. A., Ismail, R., Lau, H., Nang, L., Yuen, C. (2011). An improved method for the preparations of nanostructured lipid carriers containing heat-sensitive bioactives. *Colloids and Surfaces B: Biointerfaces*, *87*, 180–186.

Cho, Y. H., Lee, J. J., Park, I. B., Huh, C. S., Baek, Y. J., & Park, J. (2005). Protective effect of microencapsulation consisting of multiple emulsification and heat gelation processes on immunoglobulin in yolk. *Journal of Food Science*, *70*, 148–151.

Cornacchia, L., & Roos, Y. H. (2011a). Solid-liquid transitions and stability of HPKO-in-water systems emulsified by dairy proteins. *Food Biophysics*, *6*, 288–294.

Cornacchia, L., & Roos, Y. H. (2011b). State of dispersed lipid carrier and interface composition as determinants of beta-carotene stability in oil-in-water emulsions. *Journal of Food Science*, *76*, C1211–C1218.

Cui, Z., Chen, Y., Kong, X., Zhang, C., & Hua, Y. (2014). Emulsifying properties and oil/water (O/W) interface adsorption behavior of heated soy proteins: Effects of heating concentration, homogenizer rotating speed, and salt addition level. *Journal of Agricultural and Food Chemistry*, *62*, 1634–1642.

Devi, A. F., Buckow, R., Hemar, Y., & Kasapis, S. (2014). Modification of the structural and rheological properties of whey protein/gelatin mixtures through high pressure processing. *Food Chemistry*, *156*, 243–249.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces*, *20*, 197–210.

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces*, *81*, 130–140.

Dickinson, E., & James, J. D. (1998). Rheology and flocculation of high-pressure-treated B-lactoglobulin-stabilized emulsions : Comparison with thermal treatment. *Journal of Agricultural and Food Chemistry*, *46*, 2565–2571.

Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta*, *1079*, 316–320.

Dybowska, B. E. (2008). Properties of milk protein concentrate stabilized oil-in-water emulsions. *Journal of Food Engineering*, *88*, 507–513.

Fang, Z., & Bhandari, B. (2012). Encapsulation techniques for food ingredient systems. In B. Bhandari & Y. H. Roos (Eds.), *Food Materials Science and Engineering* (pp. 320–

348). Blackwell Publishing Ltd.

Galazka, V. B., Dickinson, E., & Ledward, D. a. (1996). Effect of high pressure on the emulsifying behaviour of β-lactoglobulin. *Food Hydrocolloids*, *10*, 213–219.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000). Influence of high pressure processing on protein solutions and emulsions. *Current Opinion in Colloid & Interface Science*, *5*, 182–187.

Galazka, V. B., Ledward, D. A., Dickinson, E., & Langley, K. R. (1995). High Pressure Effects on Emulsifying Behavior of Whey Protein Concentrate. *Journal of Food Science*, *60*, 1341–1343.

Galland, L. (2005). Functional foods: Health effects and clinical applications regulatory aspects. In B. Caballero (Ed.), *Encyclopedia of Human Nutrition* (pp. 360–366). Elsevier Ltd.

Gee, P. T. (2007). Analytical characteristics of crude and refined palm oil and fractions. *European Journal of Lipid Science and Technology*, *109*, 373–379.

Gibson, G. R. (2004). From probiotics to prebiotics and a healthy digestive system. *Food Microbiology and Safety*, *69*, 141–143.

Gonnet, M., Lethuaut, L., & Boury, F. (2010). New trends in encapsulation of liposoluble vitamins. *Journal of Controlled Release*, *146*, 276–290.

Heinz, V., & Buckow, R. (2010). Food preservation by high pressure. *Journal Für Verbraucherschutz Und Lebensmittelsicherheit*, *5*, 73–81.

Iametti, S., Transidico, P., Bonomi, F., Vecchio, G., Pittia, P., Rovere, P., & DallAglio, G. (1997). Molecular modifications of beta-lactoglobulin upon exposure to high pressure. *Journal of Agricultural and Food Chemistry*, *45*, 23–29.

Jacobs, D. R., & Tapsell, L. C. (2007). Food, not nutrients, is the fundamental unit in nutrition. *Nutrition Reviews*, *65*, 439–450.

Jelen, P. (2009). Dried Whey, Whey Proteins, Lactose and Lactose Derivative Products. In A. Y. Tamime (Ed.), *Dairy Powders and Concentrated Products* (pp. 256–267). Oxford, UK: Wiley-Blackwell.

Kamyshny, A., & Magdassi, S. (2006). Microencapsulation. In P. Somasundaran (Ed.), *Encyclopedia of Surface and Colloid Science* (pp. 3957–3969). Taylor & Francis.

Kentish, S., Wooster, T. J., Ashokkumar, M., Balachandran, S., Mawson, R., & Simons, L. (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative*, *9*, 170–175.

Kilara, A. (2008). Whey and Whey Products. In R. C. Chandan, A. Kilara, & N. P. Shah (Eds.), *Dairy Processing and Quality Assurance* (pp. 337–355). John Wiley and Sons.

Kinsella, J. E., & Morr, C. V. (1984). Milk proteins: Physicochemical and functional properties. *Critical Reviews in Food Science and Nutrition*, *21*, 197–262.

Kotyla, T., Kuo, F., Moolchandani, V., Wilson, T., & Nicolosi, R. (2008). Increased bioavailability of a transdermal application of a nano-sized emulsion preparation. *International Journal of Pharmaceutics*, *347*, 144–148.

Kühn, J., Considine, T., & Singh, H. (2008). Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. *Journal of Agricultural and Food Chemistry*, *56*, 10218–10224.

Lee, S. J., & Rosenberg, M. (2000). Whey protein-based microcapsules prepared by double emulsification and heat gelation. *Lebensmittel-Wissenschaft & Technologie*, *33*, 80–88.

Lee, S. J., & Rosenberg, M. (2001). Microencapsulation of theophylline in composite wall system consisting of whey proteins and lipids. *Journal of Microencapsulation*, *18*, 309–321.

Leong, T. S. H., Wooster, T. J., Kentish, S. E., & Ashokkumar, M. (2009). Ultrasonics sonochemistry minimising oil droplet size using ultrasonic emulsification. *Ultrasonics - Sonochemistry*, *16*, 721–727.

Liu, X., Powers, J. R., Swanson, B. G., Hill, H. H., & Clark, S. (2005). High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *Journal of Food Science*, *70*, C581–C585.

Livney, Y. D. (2010). Milk proteins as vehicles for bioactives. *Current Opinion in Colloid & Interface Science*, *15*, 73–83.

Maiani, G., Castón, M. J. P., Catasta, G., Toti, E., Cambrodón, I. G., Bysted, A., … Schlemmer, U. (2009). Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Molecular Nutrition and Food Research*, *53*, S194–S218.

Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y., & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of b-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology*, *9862*, 336–342.

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., & Graves, S. M. (2006). Nanoemulsions: Formation, structure, and physical properties. *Journal of Physics Condensed Matter*, *18*, 635–666.

McClements, D. J. (2005). Context and background. In D. J. Mcclements (Ed.), *Food Emulsions: Principles, Practices, and Techniques* (Third, pp. 1–26). CRC Press.

McClements, D. J. (2010). Emulsion design to improve the delivery of functional lipophilic components. *Annual Review of Food Science and Technology*, *1*, 241–269.

McClements, D. J. (2012). Crystals and crystallization in oil-in-water emulsions: implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science*, *174*, 1–30.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science*, *72*, R109–R124.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrician*, *51*, 285–330.

Mollet, B., & Rowland, I. (2002). Functional foods: At the frontier between food and pharma. *Food Biotechnology*, *13*, 483–485.

Mortensen, a. (2005). Analysis of a complex mixture of carotenes from oil palm (Elaeis guineensis) fruit extract. *Food Research International*, *38*, 847–853.

Oliver, C. M., & Augustin, M. A. (2009). Using dairy ingredients for encapsulation. In M. Corredig (Ed.), *Dairy Derived Ingredients-Food and Nutraceutical Uses* (pp. 565–588). United Kingdom: Woodhead Publishing Limited.

Onwulata, C. I. (2013). Microencapsulation and Functional Bioactive Foods. *Journal of Food Processing and Preservation*, *37*, 510–532.

Palazolo, G. G., Sobral, P. A., & Wagner, J. R. (2011). Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates. *Food Hydrocolloids*, *25*, 398–409.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, *26*, 716–723.

Peng, W., Kong, X., Chen, Y., Zhang, C., Yang, Y., & Hua, Y. (2016). Effects of heat treatment on the emulsifying properties of pea proteins. *Food Hydrocolloids*, *52*, 301–310.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012a). Nanoemulsion delivery systems: influence of carrier oil on β-carotene bioaccessibility. *Food Chemistry*, *135*, 1440–1447.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012b). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, *132*, 1221–1229.

Richardson, D. P. (2009). Health claims on foods: Strength of the evidence and problems of proof. *Food Science and Technology Bulletin: Functional Foods*, *6*, 1–3.

Rosenberg, M., & Lee, S. J. (2004). Water-insoluble, whey protein-based microspheres prepared by an all-aqueous process. *Journal of Food Science*, *69*, 50–58.

Schramm, L. L., & Stasiuk, E. N. (2006). Emulsions : Overview. In A. M. Spasic & J.-P. Hsu (Eds.), *Finely Dispersed Particles: Micro-, Nano-, and Atto-Engineering* (pp. 79–112). Taylor & Francis.

Smithers, G. W. (2008). Whey and whey proteins: From " gutter-to-gold ." *International Dairy Journal*, *18*, 695–704.

Stahl, W., & Sies, H. (2012). β-Carotene and other carotenoids in protection from sunlight. *American Journal of Clinical Nutrition*, *96*, 1179–1184.

Sun, C., Gunasekaran, S., & Richards, M. P. (2007). Effect of xanthan gum on physicochemical properties of whey protein isolate stabilized oil-in-water emulsions. *Food Hydrocolloids*, *21*, 555–564.

Tadros, T., Izquierdo, P., Esquena, J., & Solans, C. (2004). Formation and stability of nano-emulsions. *Advances in Colloid and Interface Science*, *108–109*, 303–18.

Thakkar, S. K., Maziya-dixon, B., Dixon, A. G. O., & Failla, M. L. (2007). β-carotene micellarization during in vitro digestion and uptake by Caco-2 cells is directly droportional to b-carotene content in different genotypes of Cassava. *The Journal of Nutrition*, *137*, 2229–2233.

Tornberg, E. (1977). Functional characterization of protein stabilized emulsions: Effect of processing. *Journal of Food Science*, *42*, 468–472.

Tyssandier, V., Lyan, B., & Borel, P. (2001). Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles. *Biochemica et Biophysica Acta*, *1533*, 285–292.

Versic, R. J. (1988). Flavor encapsulation: An overview. In S. Risch & G. A. Reineccius (Eds.), *Flavor Encapsulation* (pp. 1–6). American Chemical Society.

Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International*, *42*, 1254–1258.

Wilde, P. J. (2009). Emulsions and nanoemulsions using dairy ingredients. In M. Corredig (Ed.), *Dairy Derived Ingredients- Food and Nutraceutical Uses* (pp. 539–564). United Kingdom: Woodhead Publishing Limited.

Witthayapanyanon, A., Acosta, E. J., Harwell, J. H., & Sabatini, D. A. (2006). Formulation of ultraflow interfacial tension systems using extended surfactants. *Journal of Surfactants and Detergents*, *9*, 331–339.

Wong, R. S. Y., & Radhakrishnan, A. K. (2012). Tocotrienol research: Past into present. *Nutrition Reviews*, *70*, 483–90.

Wooster, T. J., Golding, M., & Sanguansri, P. (2008). Impact of oil type on nanoemulsion formation and Ostwald ripening stability. *Langmuir*, *24*, 12758–12765.

Yang, J., Yang, J., Powers, J. R., Powers, J. R., Clark, S., Clark, S., … Swanson, B. G. (2003). Ligand and flavor binding functional properties of beta-lactoglobulin in the molten globule state induced by high pressure. *Journal of Food Science*, *68*, 444–452.

Yang, Y., & Mcclements, D. J. (2013). Encapsulation of vitamin E in edible emulsions fabricated using a natural surfactant. *Food Hydrocolloids*, *30*, 712–720.

Yi, J., Li, Y., Zhong, F., & Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloids*, *35*, 19–27.

Yucel, U., Elias, R. J., & Coupland, J. N. (2012). Emulsions , Nanoemulsions and Solid Lipid Nanoparticles as Delivery Systems in Foods. In N. T. Dunford (Ed.), *Food and Industrial Bioproducts and Bioprocessing* (pp. 167–184). John Wiley and Sons, Inc.

CHAPTER 3 INTRODUCTION TO RAMAN MICROSPECTROSCOPY

1. Spectroscopy

Fundamentally, spectroscopy refers to the study of interactions between electromagnetic radiation and matter. Electromagnetic radiation is comprised of waves of oscillating electric and magnetic fields that travel in discrete quanta known as photons.

Matter and its constituent crystal structure, molecules, atoms and electrons can interact with photons of different energies categorised in separate regions of the electromagnetic spectrum (Figure 3.1). Lower energy photons in the infrared and microwave regions can cause increased molecular vibrations and rotations respectively, and high energy radiation such as γ-rays can displace an electron resulting in ionisation and/or radical production, which can be damaging for biomaterials. Meanwhile, electrons and their transitions between valence atomic or molecular orbitals can be probed using UV-Visible light (in UV-Vis spectrometer), while those between inner (core) orbitals can be probed by hard UV and higher energy photons towards the X-Ray region.

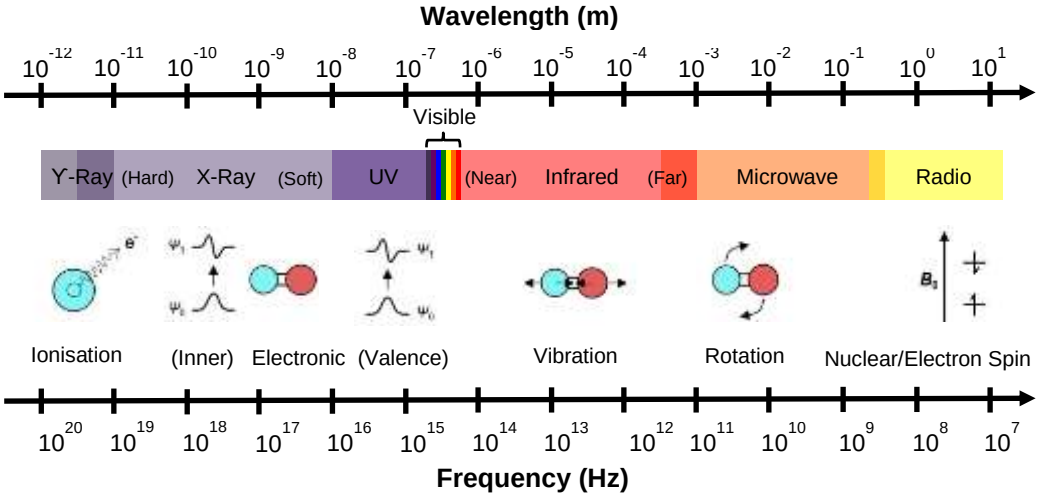

Figure 3.1: The electromagnetic spectrum, regions and interactions with matter.

The energy, *E*, in Joules (J) (SI unit: kg m^2 s^{-2}), of a photon is determined by the radiation frequency as given in the following equation:

$$E = h\,\nu = \frac{h\,c}{\lambda} \quad \text{(Equation 3.1)}$$

where h = 6.626 × 10^{-34} J s is Planck's constant, c = 2.998 × 10^{8} m s^{-1} is the speed of light, ν is the frequency (Hz or s^{-1}) and λ is the wavelength (m). In spectroscopy, the wavenumber, $\tilde{\nu}$ (cm^{-1}), is commonly used as it is proportional to the energy:

$$\bar{\nu} = \frac{1}{\lambda} = \frac{E}{h\,c} \quad \text{(Equation 3.2)}$$

where λ is the wavelength in centimetres (cm). Typical wavenumber values in vibrational spectroscopy range from 400–4000 cm^{-1} (mid-IR), but can extend in either direction in related spectroscopies (near-IR and far-IR).

2. Vibrational Spectroscopy

Vibrational spectroscopy has been a central tool for chemical analysis for decades, and it is nowadays widely used in various research and technology for quality inspection as well as for quantification. Of the traditional methods for vibrational analysis; mid- and near-infrared (IR), as well as Raman spectroscopy, the IR techniques have become by far the most common. The evolution of low-cost and compact equipment has led to IR techniques often being utilised outside the traditional analytical laboratory environment. For Raman spectroscopy to approach the widespread applicability of IR techniques, the methodology must become more flexible, more affordable and more easily adapted to on-site and *in situ* analysis (Clarke, Londhe, Premasiri, & Womble, 2000). Thanks to the continuous research and technology advancement, the Raman techniques are nowadays almost as widely used as IR. Furthermore, these two spectroscopy techniques are complementary to each other in providing data on transitions of the molecular vibration in a system (Gotter, Faubel, & Neubert, 2010).

Matter absorbs electromagnetic radiation of the IR region when the IR frequency aligns with the frequency of a molecular vibration, thus enabling manipulation of the molecular dipole moment (Gotter et al., 2010). Traditionally, an IR spectrum is obtained by passing IR radiation through a sample and measuring the absorbed fraction of the incident radiation. The versatile acquisition modes of IR spectroscopy (transmission, attenuated total reflection

(ATR), diffuse reflectance (DRIFTS), micro- and macro sampling) have allowed the technique to proliferate in industry and in research. However, one of the disadvantages of IR spectroscopy is that it is highly sensitive to water; a ubiquitous solvent, which places limitations on certain applications such as the study of biological samples.

The use of Fourier transform IR (FTIR) for data acquisition has enabled a generally rapid (1–2 min) quantitative analysis, besides allowing the process to be automated and reducing the needs for solvents and toxic reagents (Che Man, Ammawath, & Mirghani, 2005). Particularly the FTIR-ATR spectroscopy, it has emerged as one of the standard techniques for analyses as it is more easily automated and the sampling is much simpler than other IR acquisition modes. In the food area, this technique has been used to obtain structural information for vegetable oils such as soy, olive and sunflower (Herrero, Carmona, Pintado, Jiménez-Colmenero, & Ruíz-Capillas, 2011a). The roles of proteins and lipids in emulsion formation have also been shown in oil model systems emulsified with proteins (α-lactalbumin and β-lactoglobulin) via FTIR spectra (Fang & Dalgleish, 1997; 1998). The same analytical tool was also used to study emulsions formulated with olive oil stabilised either with NaCas or with mixtures of NaCas and trans-glutaminase (Herrero et al., 2011a; 2011b). In short, the use of FTIR enables a deeper understanding of the molecular behaviour within emulsion systems prepared with different emulsification strategies, and thereby helps to improve the preparation of foods in which the emulsion ingredients may influence the structural and physicochemical properties of the emulsion (e.g. texture, fat and water binding properties).

Detection of Raman scattering radiation on the other hand results from a laser-induced excitation of a sample. Figure 3.2 depicts several types of light scattering involved in a Raman experiment. While Rayleigh scattering does not affect the wavelength of individual photons, the Raman effect results in the energy change of some photons upon inelastic collision of incident light with molecules. Following the excited state of the vibration interacting with the photon, the scattered radiation may lose or gain energy. The loss of energy when the interaction is with the vibrational ground level is called Stokes Raman scattering. However the scattered photon will be of higher energy if the interaction is with an excited vibrational state, and termed anti-Stokes. Recording the Stokes Raman spectrum only is adequate in most data analyses (Gotter et al., 2010).

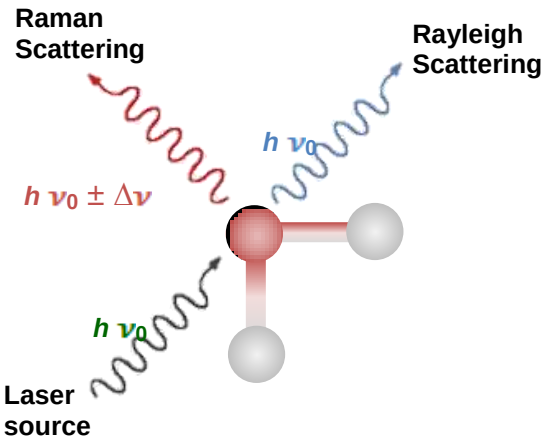

Figure 3.2: A simple layout of light scattering processes with a simple molecule: Rayleigh scattering (blue) and Raman scattering (red).

The various energy processes involved in vibrational spectroscopy are contrasted and shown in Figure 3.3. Both IR and Raman spectroscopies are non-destructive and minimally or even non-invasive techniques, supplying massive chemical information on the subject of interest.

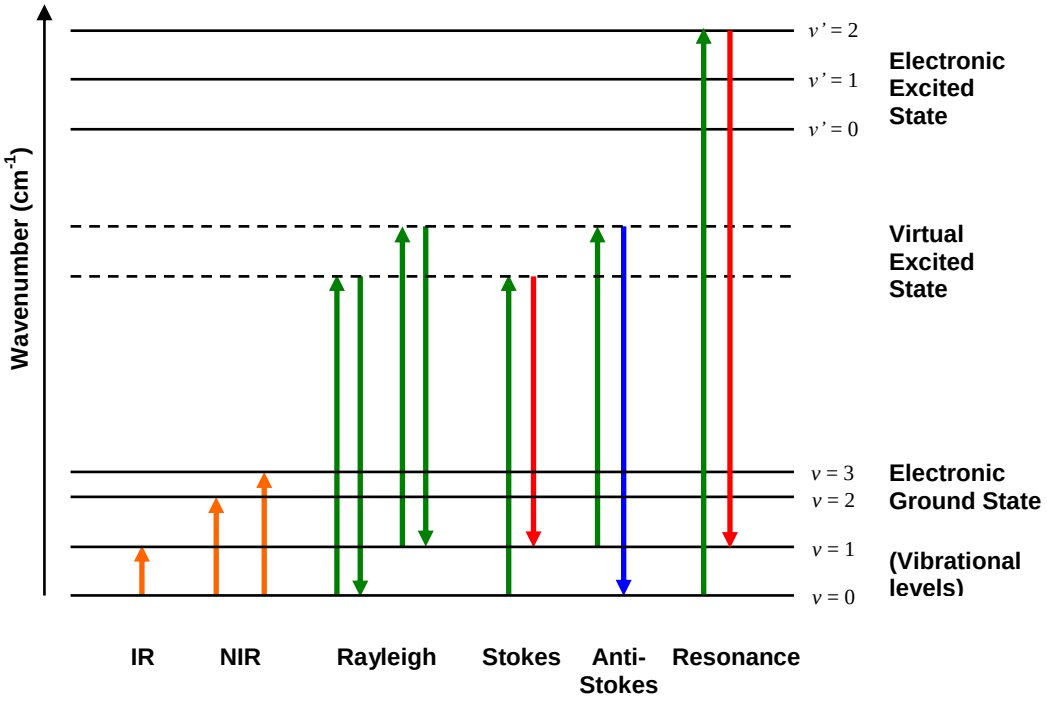

Figure 3.3: An energy level diagram showing transitions by infrared (IR) and near-infrared (NIR) absorptions, and by different scattering processes e.g. Rayleigh, Stokes Raman, anti-Stokes Raman and resonance Raman. The upward arrow indicates absorption of incident photons and the downward arrows indicate emission of scattered photons. The numbers represent different vibrational levels within each electronic state.

Combining IR and Raman spectrometers with microscopes (becoming vibrational microspectroscopy) marks a significant development in the field of vibrational spectroscopy especially in life sciences (Mantsch, Choo-smith, & Shaw, 2002). The innovation does not only provide chemical information on bulk samples, but also enables chemical based photography of the sample, thus allowing derivation of chemical information at high spatial resolution.

3. Raman History

In 1928, the discovery of "A New Type of Secondary Radiation" from molecules in dust-free liquids or gases of "degraded frequency" was reported by (Sir) Chandrasekhara Venkata Raman, a professor from Calcutta University in India (Raman & Krishnan, 1928). Inspired by Arthur Holly Compton's breakthrough of inelastic scattering of X-rays by electrons 5 years prior (Compton, 1923), Raman innovatively performed the experiment merely using natural light with complementary filters. A focussed beam of sunlight was passed through a dust-free media, from which a diffuse glow emanated and showed the beam path. This glow could be extinguished by placing two complementary filters between the media and the source. However it reappeared when one of the filters was moved and placed between the media and the observer.

This observation was realised to be a proof of the change in photon frequency, and an evident that the inelastic scattering was from the atoms or molecules within, not from electrons. The phenomenon was later named the "Raman effect", in recognition of its founder who immediately received international acknowledgement such as the bestowment of the Nobel Prize in Physics in 1930 (Nobel Media, 2014) for this discovery and later a knighthood.

However, Raman himself realised that the intensity of the Raman scattered light is "feeble" or weak, and it required "powerful illumination" to be observed (Raman & Krishnan, 1928). Consequently in the late 1940's, infrared spectroscopy overtook Raman spectroscopy, which heavily relied on atomic emission lines from arc lights (such as the 435 nm line from a mercury arc) or filtered sunlight as a source (Gordon & Fraser-Miller, 2016). It was not until the 1960's, when the laser became available as a source capable of producing intense, monochromatic light, that the Raman field developed further. Later on, tunable lasers were introduced, enhancing the Raman scattering by molecular resonance, together with the

invention of the charge coupled device (CCD) detector, which greatly improved the signal to noise ratios and allowed the advancement in the field of microspectroscopy and imaging.

The year 2018 will mark the 90th anniversary of the publication by Sir C.V. Raman on the revolutionary Raman effect. Raman instrumentation has seen remarkable advances hitherto. Today, it is a near-ubiquitous tool, which is almost insensitive to water, thus advantageous in many fields including medical and biological. It is a well-established tool in mineralogy and geology, where Raman sensitivity to solid-state structure is utilised together with electron microscopy techniques. The potential of Raman has also been demonstrated in the field of art, archaeology and palaeontology. Although it is relatively new in the field of food and beverages (Li & Church, 2014; Yang & Ying, 2011), the continuous development of technology and an increasing number of studies promises much in the high-demand food sector.

4. Raman Overview

The development of Raman spectroscopy techniques in the past decades has given rise to a number of new applications. For example, Gordon and Fraser-Miller (2016) have reviewed applications of Raman spectroscopy and imaging in the pharmaceutical industry, whereas studies on chemical heterogeneity of emulsion systems has been reported by Andrew, Browne, Hancewicz and Millichope (1998).

Raman spectrometers can be coupled to a microscope, in a so-called micro-Raman or Raman microspectroscopic configuration, where the excitation and collection of Raman spectra are acquired through a microscope objective. There are several advantages of a micro-Raman setup over conventional (macro-) Raman spectrometers (Table 3.1), the most distinguished of which is superior spatial (x-y) resolution.

Raman microspectroscopy can be used to detect and quantify the molecular composition of microscopic samples; molecular information can be obtained at a spatial resolution comparable to optical microscopy. In this technique, the spatial resolution is defined by the laser profile used to excite a particular part of the specimen. Functional groups are identified by their specific wavenumber positions in the Raman spectrum, and the relative numbers of these groups are determined by the magnitude of intensity at each wavenumber. Samples can be measured directly in air or water, at room temperature and pressure (i.e., wet or dry) without destroying the samples.

Table 3.1: Comparison of macro- and micro-Raman spectrometer configurations

Property	Macro-Raman	Micro-Raman	Consequence of micro-Raman
Spatial resolution	Low	High	Spectra may not be representative of entire sample
Photon flux	Low	High	Higher signal intensity Possible sample damage Shorter acquisition time
Sampling volume	High	Low	Higher confocality enabling depth profiling Surface layer sensitive Requires less sample
Cost and size	Small	Larger	Expensive instrumentation Lack of portability

The capability of performing spatially resolved chemical analyses of microscopic regions of samples *in situ* has been of huge interest in both the material and biological sciences. Raman microspectroscopy is a brilliant tool for investigating the chemistry of these regions due to its independency from homogenisation, extraction or dilution, but rather each structure is analysed *in situ* (Wang & Spencer, 2002).

The introduction of confocal Raman microspectroscopy (CRM) further improves both the microstructural examination of a specimen and its molecular information (Lopez-Sanchez et al., 2011). CRM gives a high spatial and spectral resolution; the laser is pointed to a small region within the sample and the Raman scattered light is collected only from that focal point (Williams et al., 1994). This technique is relatively slow for imaging areas as the focal spot must be rastered through the entire sample in order to create a three-dimensional map of the molecular distribution (Arikan, Sands, Rodway, & Batchelder, 2002).

Since CRM enables depth-resolved measurements, this method has been applied to observe drug penetration into human skin (Pudney, Melot, Caspers, Van Der Pol, & Puppels, 2007) and to analyse the hydration levels in certain layers of human epidermis (Chrit et al., 2006). The application of CRM in the study of foods and beverages is now well established (Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011). It has been used to determine the concentration of components within a food structure on the micron length scale (Pudney, Hancewicz, Cunningham, & Gray, 2003). Similarly, Svelander et al. (2011) have

reported the use of CRM to quantify the distribution of carotenoids within tomato cells, from which the Raman spectra were also shown to be sensitive to the physical state of the carotenoids (solid against solvated). Concentrations of carotenoids within living cells have been estimated by comparing Raman band intensities to those of concentration standards (Arikan et al., 2002). This method can, however, produce misleading results as it was assumed that the incident or scattered light was unaffected by passing through surrounding biological material. Although the information collected from a confocal spectrometer comes from a small volume, the incident laser light may be partially absorbed and scattered before reaching this region; the Raman light may also be absorbed and scattered before being collected by the objective lens (Arikan et al., 2002).

In general, the use of both FTIR and Raman microspectroscopies is quite common for the analysis of WPI (Kher, Udabage, McKinnon, McNaughton, & Augustin, 2007; Fang & Dalgleish, 1997; Fang & Dalgleish, 1998; Blanpain-Avet et al., 2012; Li et al., 2011), but only Raman is frequently used for analysing carotenes (De Oliveira, Castro, Edwards, & De Oliveira, 2009; Arikan et al., 2002), while FTIR has been applied to palm oil and its fractions (Muppaneni et al., 2012; Rohman & Man, 2010).

In the context of this study, CRM was used to analyse the location and partitioning of β-carotene within the milk protein-stabilised dispersion systems. Zheng, Gordon and Everett (2013) have done similar study to measure physical partitioning of volatile organic compounds in dairy food matrices using CRM. Our study employed a confocal Raman system to determine the bioactive concentration in oil droplets of emulsion samples, and compared the results to that in their respective whole emulsion system acquired from the conventional extraction-spectrophotometric process. Background studies on the Raman signals of each compound involved in this research especially that of the bioactive, had been conducted. This is essential for the following analysis of the emulsion samples in order (i) to know the compatibility of the microspectroscopy with the type of compounds used in this study (i.e. does the compound give fluorescent signals when analysed under the microscopy), (ii) to control and manipulate the parameters of the instrument to give the highest signal-to-noise ratio (e.g. resolution, type and intensity of Raman laser, integration time etc.), and (iii) to assist in the assignment of signals and spectra to their respective chemical configurations and compounds.

Reference

Andrew, J. J., Browne, I. E., Hancewicz, T. M., & Millichope, A. J. (1998). Raman imaging of emulsion systems. *Applied Spectroscopy*, *52*, 790–796.

Arikan, S., Sands, H. S., Rodway, R. G., & Batchelder, D. N. (2002). Raman spectroscopy and imaging of beta-carotene in live corpus luteum cells. *Animal Reproduction Science*, *71*, 249–266.

Blanpain-Avet, P., Hédoux, a., Guinet, Y., Paccou, L., Petit, J., Six, T., & Delaplace, G. (2012). Analysis by Raman spectroscopy of the conformational structure of whey proteins constituting fouling deposits during the processing in a heat exchanger. *Journal of Food Engineering*, *110*, 86–94.

Che Man, Y. B., Ammawath, W., & Mirghani, M. (2005). Determining α-tocopherol in refined bleached and deodorized palm olein by Fourier transform infrared spectroscopy. *Food Chemistry*, *90*, 323–327.

Chrit, L., Bastien, P., Sockalingum, G. D., Batisse, D., Leroy, F., Manfait, M., & Hadjur, C. (2006). An in vivo randomized study of human skin moisturization by a new confocal Raman fiber-optic microprobe: Assessment of a glycerol-based hydration cream. *Skin Pharmacology and Physiology*, *19*, 207–215.

Clarke, R. H., Londhe, S., Premasiri, W. R., & Womble, M. E. (2000). Low-resolution Raman Spectroscopy: Instrumentation and applications in chemical analysis. *Journal of Raman Spectroscopy*, *832*, 827–832.

Compton, A. H. (1923). A Quantum Theory of the Scattering of X-rays by Light Elements. *Physical Review*, *21*, 483–502.

De Oliveira, V. E., Castro, H. V., Edwards, H. G. M., & De Oliveira, L. F. C. (2009). Carotenes and carotenoids in natural biological samples: a Raman spectroscopic analysis. *Journal of Raman Spectroscopy*, *41*, 642–650.

Fang, Y., & Dalgleish, D. G. (1997). Conformation of β-lactoglobulin studied by FTIR: Effect of pH, temperature, and adsorption to the oil-water interface. *Journal of Colloid and Interface Science*, *298*, 292–298.

Fang, Y., & Dalgleish, D. G. (1998). The conformation of α-lactalbumin as a function of pH, heat treatment and adsorption at hydrophobic surfaces studied by FTIR. *Food Hydrocolloids*, *12*, 121–126.

Gordon, K. C., & Fraser-Miller, S. J. (2016). Raman Spectroscopy. In A. Müllertz, Y. Perrie, & T. Rades (Eds.), *Analytical Techniques in the Pharmaceutical Sciences* (pp. 139–169). New York: Springer.

Gotter, B., Faubel, W., & Neubert, R. H. H. (2010). FTIR microscopy and confocal Raman microscopy for studying lateral drug diffusion from a semisolid formulation. *European Journal of Pharmaceutics and Biopharmaceutics*, *74*, 14–20.

Herrero, A. M., Carmona, P., Pintado, T., Jiménez-Colmenero, F., & Ruíz-Capillas, C. (2011a). Infrared spectroscopic analysis of structural features and interactions in olive oil-in-water emulsions stabilized with soy protein. *Food Research International*, *44*, 360–366.

Herrero, A. M., Carmona, P., Pintado, T., Jiménez-Colmenero, F., & Ruíz-Capillas, C. (2011b). Olive oil-in-water emulsions stabilized with caseinate: Elucidation of protein–lipid interactions by infrared spectroscopy. *Food Hydrocolloids*, *25*, 12–18.

Kher, A., Udabage, P., McKinnon, I., McNaughton, D., & Augustin, M. A. (2007). FTIR investigation of spray-dried milk protein concentrate powders. *Vibrational Spectroscopy*, *44*, 375–381.

Li, Y.-S., & Church, J. S. (2014). Raman spectroscopy in the analysis of food and pharmaceutical nanomaterials. *Journal of Food and Drug Analysis*, *22*, 29–48.

Li, Y., Jiang, Y., Liu, F., Ren, F., Zhao, G., & Leng, X. (2011). Fabrication and characterization of TiO2/whey protein isolate nanocomposite film. *Food Hydrocolloids*, *25*, 1098–1104.

Lopez-Sanchez, P., Schumm, S., Pudney, P. D. a, & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst*, *136*, 3694–3697.

Mantsch, H. H., Choo-smith, L., & Shaw, R. A. (2002). Vibrational spectroscopy and medicine: An alliance in the making. *Vibrational Spectroscopy*, *30*, 31–41.

Marcelli, A., Cricenti, A., Kwiatek, W. M., & Petibois, C. (2012). Biological applications of synchrotron radiation infrared spectromicroscopy. *Biotechnology Advances*, *30*, 1390–1404.

Muppaneni, T., Reddy, H. K., Ponnusamy, S., Patil, P. D., Sun, Y., Dailey, P., & Deng, S. (2013). Optimization of biodiesel production from palm oil under supercritical ethanol conditions using hexane as co-solvent: A response surface methodology approach. *Fuel*, *107*, 633–640.

Nobel Media, A. B. (2014). The Nobel Prize in Physics 1930. Retrieved November 30, 2017, from https://www.nobelprize.org/nobel_prizes/physics/laureates/1930/

Pudney, P. D. A., Hancewicz, T. M., Cunningham, D. G., & Gray, C. (2003). A novel method for measuring concentrations of phase separated biopolymers: The use of confocal Raman spectroscopy with self-modelling curve resolution. *Food Hydrocolloids*, *17*, 345–353.

Pudney, P. D. A., Melot, M., Caspers, P. J., Van Der Pol, A., & Puppels, G. J. (2007). An in vivo confocal Raman study of the delivery of trans-retinol to the skin. *Applied Spectroscopy*, *61*, 804–811.

Raman, C. V., & Krishnan, K. S. (1928). A new type of secondary radiation. *Nature*, *121*, 501–502.

Rohman, A., & Che Man, Y. B. (2010). Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. *Food Research International*, *43*, 886–892.

Svelander, C. A., Lopez-Sanchez, P., Pudney, P. D. A., Schumm, S., & Alminger, M. A. G. (2011). High pressure homogenization increases the in vitro bioaccessibility of α- and β-carotene in carrot emulsions but not of lycopene in tomato emulsions. *Journal of Food Science*, *76*, H215–H225.

Wang, Y., & Spencer, P. (2002). Quantifying adhesive penetration in adhesive/dentin interface using confocal Raman microspectroscopy. *Journal of Biomedical Materials Research*, *59*, 46–55.

Yang, D., & Ying, Y. (2011). Applications of raman spectroscopy in agricultural products and food analysis: A review. *Applied Spectroscopy Reviews*, *46*, 539–560.

Zheng, H., Gordon, K. C., & Everett, D. W. (2013). Innovative application of confocal Raman microscopy to investigate the interaction between trans-2-hexenal and bovine milk fat globules. *International Dairy Journal*, *32*, 68–70.

CHAPTER 4 INSTRUMENTATION AND EXPERIMENTAL TECHNIQUES

1. Pre-experimentation procedures

1.1 Materials

As described in Chapter 2, the emulsion systems fabricated in this entire study incorporated the following main ingredients:

- Palm olein and/or palm stearin as the dispersed phase
- Whey protein isolate (WPI) as the emulsifier
- β-carotene as the carried bioactive

Since the study is focussed on exploring Raman microspectroscopy as an alternative method to the current extraction-spectrophotometric technique to determine the content of bioactive carried in an emulsion system, the β-carotene was first analysed under the Raman system to observe its compatibility and sensitivity to the microscopic system. A series of measurement were conducted to compare the Raman signals of the bioactive. Two Raman lasers (the 532 nm green and 785 nm NIR) with two objective lenses (20× and 50×) were used and compared to determine the optimum excitation source and microscopic lens for the entire study.

The bioactive used was β-carotene suspension in medium chain triglyceride (MCT) (30% natural micro-algal based β-carotene), kindly provided by Cognis Australia Pty. Ltd (Melbourne, Victoria, Australia).

The NIR system presented stronger signals with low fluorescence for both objectives, while the green gave strong but fluorescent signals under the 20×, and weak but less fluorescent signals under the 50× objective lens. Even though the NIR system seemed favourable, the power required by the system was up to 30 mW, which was much higher than that by the green; maximum 5 mW. Further tests were carried out, and it was found that even at the 5 mW power under the green system, the photosensitive β-carotene was severely degraded over time, and that the power as low as 0.5 mW was sufficient to give clear signals of β-carotene without sample degradation. Hence it was eventually decided that the green laser at 0.5 mW power and under the 50× objective lens was preferred over the other configurations.

All the Raman spectra of β-carotene in oil are consistent and show three significant peaks at 1524, 1157 and 1016 cm^{-1} (Figure 4.1), representing the double bond C=C stretching mode, $v_1(\nu_s C{=}C)$; the C−C in-plane single bond stretching mode, $v_2(\nu_s C{-}C)$; and the C−H bending mode, $v_3(\rho C{-}CH_3)$ of β-carotene respectively (Lopez-Sanchez et al., 2011).

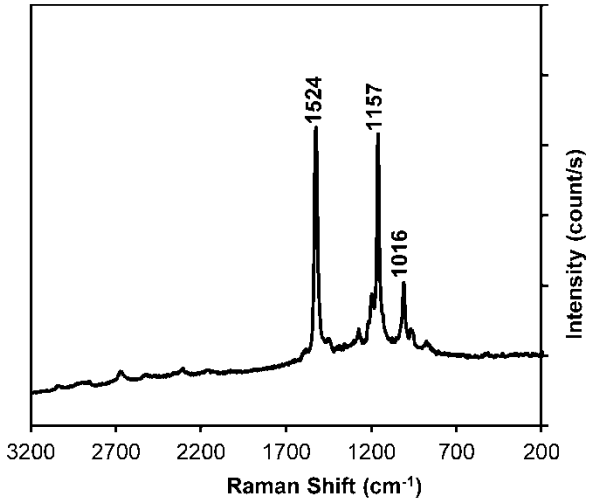

Figure 4.1: Raman spectrum of β-carotene in oil, dominated only by the β-carotene bands.

In general, molecules with easily polarised π-electrons lead to stronger Raman scattering than those with tightly held σ-bonded. Since encapsulants such as oil and protein are σ-bonded and active cores such as β-carotene comprise π electrons, the Raman spectra obtained throughout this study are dominated by the β-carotene bands, instead of a mixture of signals from other ingredients of the emulsions. Besides, long conjugated analytes like that of β-carotene give strong visible absorbance spectra, which become more intense when the wavelength of the laser is approaching (pre-resonance Raman) or coincident (resonance Raman) with the electronic absorption energy of the analytes. In β-carotene, pre-resonance Raman occurs when using the 532 nm laser as the strong band at 450 nm tails off through the visible region (Saito, Tasumi, & Eugster, 1983; Thrash, Fang, & Leroi, 1977). This accounts for the high intensity of the β-carotene bands.

The preliminary studies also aimed to specify the formulation of emulsion samples for analyses. As previously mentioned, the main emulsifier used in this study was the WPI, which was obtained as powder (91% protein, 5% moisture, 3% ash, and 1% fat) from Fonterra (Darnum, Victoria, Australia). Cornacchia and Roos (2011c) in their study had found the optimum concentration of 8 g/kg for WPI in a stable emulsion system with oil loading of 100 g/kg, containing 0.5 g/kg β-carotene in oil. In this study, the same oil loading

was used. A range of WPI concentrations (1–20 g/kg) was used to examine the effect of WPI concentration on the partitioning of β-carotene in the emulsion phases.

Meanwhile, a range of bioactive concentrations within emulsion systems is recorded in the previous literature, from as low as 0.5 up to 5 g/kg β-carotene in the oil phase (Cornacchia & Roos, 2011c; Qian, Decker, Xiao, & McClements, 2012b; Qian, Decker, Xiao, & McClements, 2012c). Therefore this study incorporated bioactive with concentrations in the above range, as long as the amounts were significant enough for detection and quantification by the measuring instruments.

For the emulsion-based encapsulation system in this study, the dispersed phase was either refined, bleached and deodorised (RBD) palm olein, or a mixture of RBD palm olein and RBD palm stearin. The latter allowed the examination for the effects of the solid fat content on emulsion systems containing bioactive. The palm olein and palm stearin were provided by Wilmar Trading Pty. Ltd (Pasir Gudang, Johor, Malaysia). Double-distilled water was used to prepare all solutions and emulsions.

Besides the emulsion ingredients, diluted sodium azide (~0.02% w/w) from Sigma-Aldrich (Castle Hill, NSW, Australia) was also added to preserve samples for stability tests.

To analyse emulsion samples under the Raman microspectrometer, the sample slides were coated by poly-L-lysine (0.1% (w/v) in water, with 0.01% of thimerosal added as preservative) from Sigma-Aldrich (Castle Hill, NSW, Australia) , to immobilise oil droplets deposited on the surface.

Chemicals used for β-carotene extraction were dimethyl sulfoxide (DMSO), n-hexane, and ethanol, purchased from Merck Pty. Ltd (Kilsyth, Victoria, Australia). Butylated hydroxyl toluene (BHT) used as an anti-oxidant, was supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). All the reagents and chemicals used were of analytical grade.

1.2 Sample preparation

There are several important sample attributes that had to be considered in preparing the emulsion samples in this study:

(i) a complete hydration of WPI powder for the aqueous phase of emulsions,
(ii) a complete solubilisation of bioactive in oil prior to formation of the emulsions, and

(iii) consistent emulsification and homogenisation procedures to produce constant and suitable sizes of emulsion droplets for measurement and analysis.

According to Cornacchia and Roos (2011a; 2011b; 2011c), WPI powder at 5% (w/w) is completely hydrated and dispersed after continuous stirring for ~3 hours with a magnetic stirrer at 50 °C. Other studies show different stirring periods for a complete hydration of WPI (1–5% w/w); the fastest being 1 hour (Singh, Tamehana, Hemar, & Munro, 2003), then 2 hours (Demetriades & McClements, 1998), 6 hours (Sun & Gunasekaran, 2009; Sun, Gunasekaran, & Richards, 2007) and up to a whole night (Liu, Hou, Lei, Chang, & Gao, 2012). In the preliminary studies, a water bath (at 50 °C) and a high-speed mixer were used to accelerate the hydration process. So for this work, WPI was stirred continuously for 1 hour at a constant speed. Further action was taken upon the formation of a foam layer on top of the protein solution. The foam was left to settle and dissolve in the solution overnight, before being used for emulsification. The solution was also reweighed, and an amount of water equivalent to the amount of evaporated water (during heating and stirring) was added.

A complete solubilisation of bioactive in the oil phase prior to emulsification depends on the type and initial condition (e.g. powder or in oil suspensions) of the bioactive. The bioactive in this study came naturally in oil suspensions. Yuan et al. (2008) has suggested in his paper that stirring β-carotene in oil suspensions with the lipid phase of the emulsions at 140 °C in dark will dissolve the carotene. Following this method, the preliminary studies had obtained homogeneous particle-free dispersions for the dispersed phase in the next emulsification step (Figure 4.2).

Figure 4.2: Different concentrations of β-carotene-in-olein solutions (0.5, 1, 2, 3, 5 g/kg) after stirring and heating at 140 °C for 30 min.

There are various methods of emulsification and homogenisation applied in the production of stable and functional emulsion systems. During the preliminary studies, the emulsification process was performed using a Silverson rotor-stator mixer at a constant speed for 2 min. This configuration was sufficient to form a coarse emulsion with droplet sizes around 10 μm. To stabilise this emulsion, it was then passed through a single-pass high-pressure homogeniser twice, first at 500 bar and then at 100 bar. The former pressure was to break the droplets up into smaller ones, by which a higher value gives smaller droplets, while the latter was to break up clusters formed during high pressure homogenisation in order to stabilise the fine emulsion system. At the end of this process, an emulsion with droplet sizes of 1–5 μm was produced, which was in the range that suits the use of CRM with a small spatial resolution. Smaller droplet sizes via a higher pressure in the homogeniser, or by using a microfluidiser would cause a difficulty in measurement and analysis under the microspectroscopic system. So homogenisation by the high-pressure homogeniser at 500 bar, followed by another pass at 100 bar was finalised as the method to prepare a stable, fine emulsion to be analysed in this study.

For sample preservation in stability studies, a small concentration of sodium azide (~0.02% w/w) was added later to the cooled emulsion system. Each formulation of fine emulsion is sub-sampled into two (duplicate) 1.5 ml screw-capped glass vials for storage (approximately 30 days).

1.3 Calibrations

For Raman measurements, a calibration curve (concentration vs. Raman intensity) was plotted in order to determine the concentration of β-carotene within the emulsion droplets. The calibration samples were prepared by the same procedures for solubilising the bioactive at required concentrations in the palm oil as the dispersed phase of an emulsion for measurement under the Raman system. The instrument was first calibrated with a Si wafer, and the Si signal intensity was recorded as the integrated area under the most significant peak (band integration at 520 cm^{-1}) as the reference for all measurements in the study.

A drop of a known concentration of β-carotene-in-olein sample was mounted on a slide before being covered with a coverslip. Based on the preliminary studies, the Raman signal of the sample was then obtained under 0.5 mW green laser (532 nm wavelength), with 0.5 s integration time for 10 scans, through a 50× objective lens. The integrated area (excluding

background contributions) of the most significant band of β-carotene, 1524 cm^{-1} was determined between 1480 and 1570 cm^{-1}. This Raman intensity was recorded with its corresponding known bioactive concentration. The procedures were repeated using different known concentrations of bioactive-in-oil samples. The graph of Raman signal intensity vs. bioactive concentration was plotted as a calibration curve.

1.4 Synchrotron IR

As a preliminary study to examine the conformational changes in the protein structure upon partitioning of the bioactive into the aqueous phase of emulsions, synchrotron infrared (SIR) microspectroscopy was used.

The major restriction when using laboratory based IR microspectroscopy for following macromolecular changes in any biological or food sample is a reduced signal-to-noise (S/N) ratio, when the microscope aperture is used to target areas below approximately 40 μm in diameter. Below this spot size, the photon flux passing through the masking aperture is considerably reduced and the throughput advantage of FTIR spectroscopy is lost. The use of IR microscopes coupled to a synchrotron radiation (SR) source with its inherent brightness gives outstanding results and overcomes this limitation by allowing all the radiation to pass through the microscope masking aperture thus making spectroscopy possible down to the diffraction limit with good S/N ratio (Marcelli, Cricenti, Kwiatek, & Petibois, 2012).

SIR radiation also has other distinctive features that benefit its multi-disciplinary users (McNaughton & Wood, 2012):

(i) a pulsed beam typically in the range between ~50 and 500 ps with repetition rates anywhere between 50 and 500 MHz allowing for time resolved spectroscopy;
(ii) linearly, circularly or elliptically polarised light, depending on how it is extracted from the synchrotron ring, which provides a source of bright broadband polarised light for dichroism, grazing angle reflectance and ellipsometry experiments;
(iii) high photon flux in the far IR region which enables terahertz (THz) spectroscopy and high resolution far infrared studies of gases.

Consequently, experiments running at SIR beamlines are the most demanding, such as high-pressure studies, high-resolution microspectroscopy and time resolved experiments (Marcelli

et al., 2012). Microspectroscopy and spectral imaging are two research areas with most benefits deriving from the availability of SIR facilities.

In this study, the aqueous phase of emulsion samples with β-carotene (100 g/kg palm olein, 20 g/kg WPI, 0.2 g/kg β-carotene) was analysed *in situ* using SIR microspectrometer, and its signal was compared to that without β-carotene (100 g/kg palm olein, 20 g/kg WPI). It was expected that there would be conformational changes in the WPI structure when β-carotene was present in the aqueous phase of emulsions upon partitioning, since it was previously found that β-carotene binds to the β-lactoglobulin, the major component of WPI (Dufour & Haertlé, 1991; Wackerbarth et al., 2009). The materials and sample preparation procedures were as previously described. Two independent replicates were prepared for each sample and duplicated spectral measurement was carried out for every formulation, from which five random points in the aqueous phase region were targeted for spectral quantification before the average for each duplicate was recorded.

Table 4.1: Band assignment for ingredients of emulsions analysed under SIR microspectrometer.

Ingredient	Wavenumber (cm^{-1})	Assignment	Reference
Protein	1600-1700	Amide I	Blanpain-Avet et al. (2012); Yi, Lam, Yokoyama, Cheng, & Zhong (2015)
	1510-1550	Amide II	
	1320	Amide III (alpha-helices)	
	1240	Amide III (beta-sheets)	
	1395	C–H (bending)	
Oil	2925	CH_2 (asymmetric)	Herrero et al., (2011a); Rohman & Che Man (2010)
	2854	CH_2 (symmetric)	
	2952	CH_3 (symmetric)	
	3027	Cis C=CH	
	1743	Ester carbonyl of triglycerides	
	1465	CH_2 (bending), CH_3 aliphatic	
	1377	CH_2 (bending)	
	1236-1160	C–O (stretching)	
	1117-1098	C–O ester (stretching)	
	962	C–H trans-olefin (bending)	
Carotene	966	C=CH trans-conjugated (out-of-plane)	Yi, Lam, Yokoyama, Cheng, & Zhong (2015)

Since the area of interest is the aqueous environment of the emulsions, the effect of water bands on the overall IR signal obtained was inevitable. However using the vector projection technique through Matlab, water-subtracted spectra of the two sample types were obtained and each band of the spectra was individually assigned to the ingredients of the emulsion samples (Table 4.1).

In addition, every ingredient was also measured under the SIR microspectroscopy to obtain their spectra independent from each other. The treated spectra of the emulsion samples were then qualitatively analysed to determine any difference between the protein bands of the samples with and without β-carotene, thus identifying any conformational change of the protein structure upon β-carotene partitioning into the aqueous phase.

Based on the results depicted in Figure 4.3, there are two small differences in the protein bands between the two spectra; (i) the amide II band at 1550.57 cm^{-1} seems to reduce in intensity after the addition of β-carotene, and (ii) the band at 1238.14 cm^{-1} which is believed to account for amide III (beta-sheets) of the protein appears upon β-carotene addition to the emulsion sample. There are slight band shifts identified, but no significant changes in bands directly related to protein.

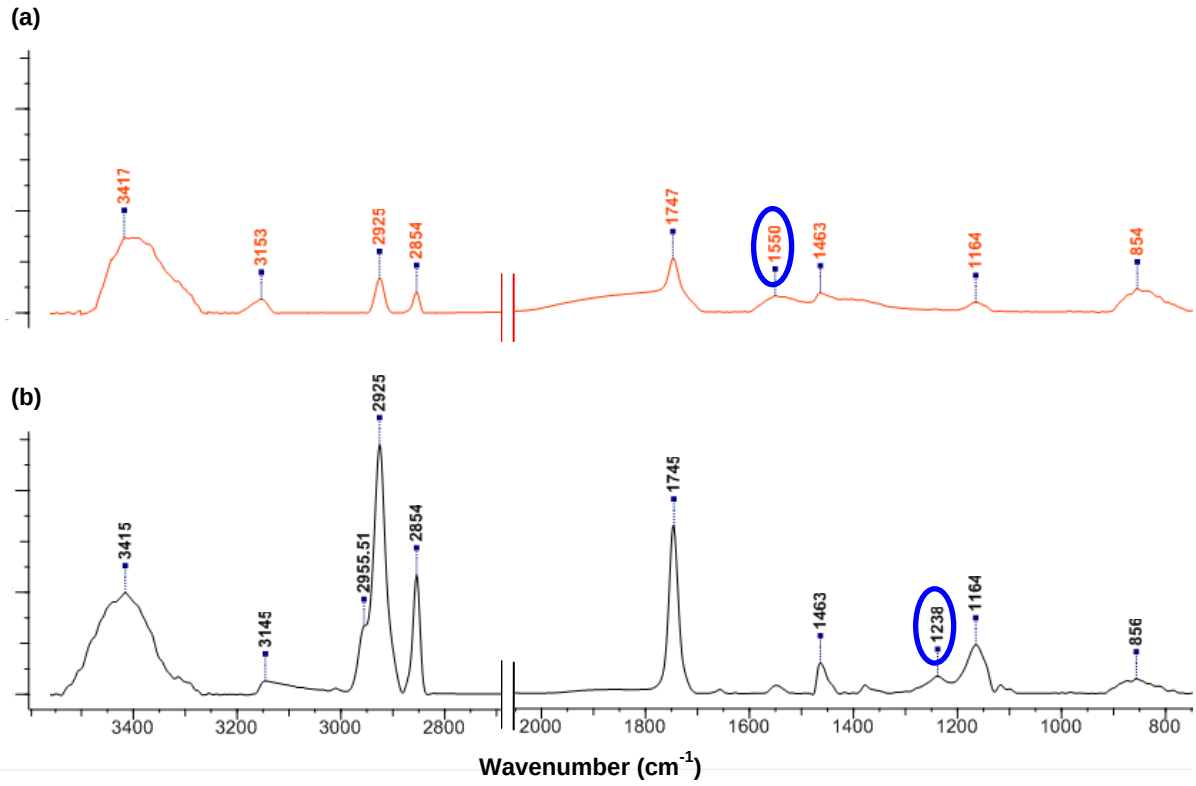

Figure 4.3: Water-subtracted SIR spectra of emulsion samples (a) without β-carotene (100 g/kg palm olein, 20 g/kg WPI) and (b) with β-carotene (100 g/kg palm olein, 20 g/kg WPI, 0.2 g/kg β-carotene). The circled bands show differences in the protein bands between the two samples.

This observation is probably due to some conformational changes of the protein structure upon binding with β-carotene molecules partitioned into the aqueous phase, as previously hypothesised. However since the variables were limited to one concentration of WPI and β-

carotene, and the concentrations were relatively low in relation to the effect of water bands on the emulsion spectra, this results need to be further improved to confidently prove the hypothesis and relate it to the factors of the bioactive partitioning between the phases of emulsion samples later in this study. Suggestions for future experimentation procedures are briefly discussed in Chapter 8.

2. Analytical methods

2.1 Confocal Raman microspectroscopy (CRM)

CRM was introduced in the study as a potential analytical instrument to determine some characteristics of emulsion systems. So the preliminary studies began with the process of determining the suitable equipment and configurations of the system that would make such analysis possible. Different types of Raman microspectrometer and laser source at various power values were tested first on the ingredients of the emulsion system using several configurations, then on the emulsion samples prepared via methods described in the previous section.

Figure 4.4: The WITec alpha300 R confocal Raman microspectroscopy in the spectroscopy laboratory of School of Chemistry, Monash University.

Eventually, a WITec alpha300 R (WITec, Ulm, Germany) confocal Raman imaging microscope (Figure 4.4) was chosen as the most appropriate for the entire study. The Raman scattering spectra were collected *in situ* onto a 600 groove/mm grating in the backscattering geometry, giving a spectral resolution of approximately 3 cm^{-1}, as well as a high spatial resolution down to ~0.4 µm and confocality with ca. 0.8 µm depth.

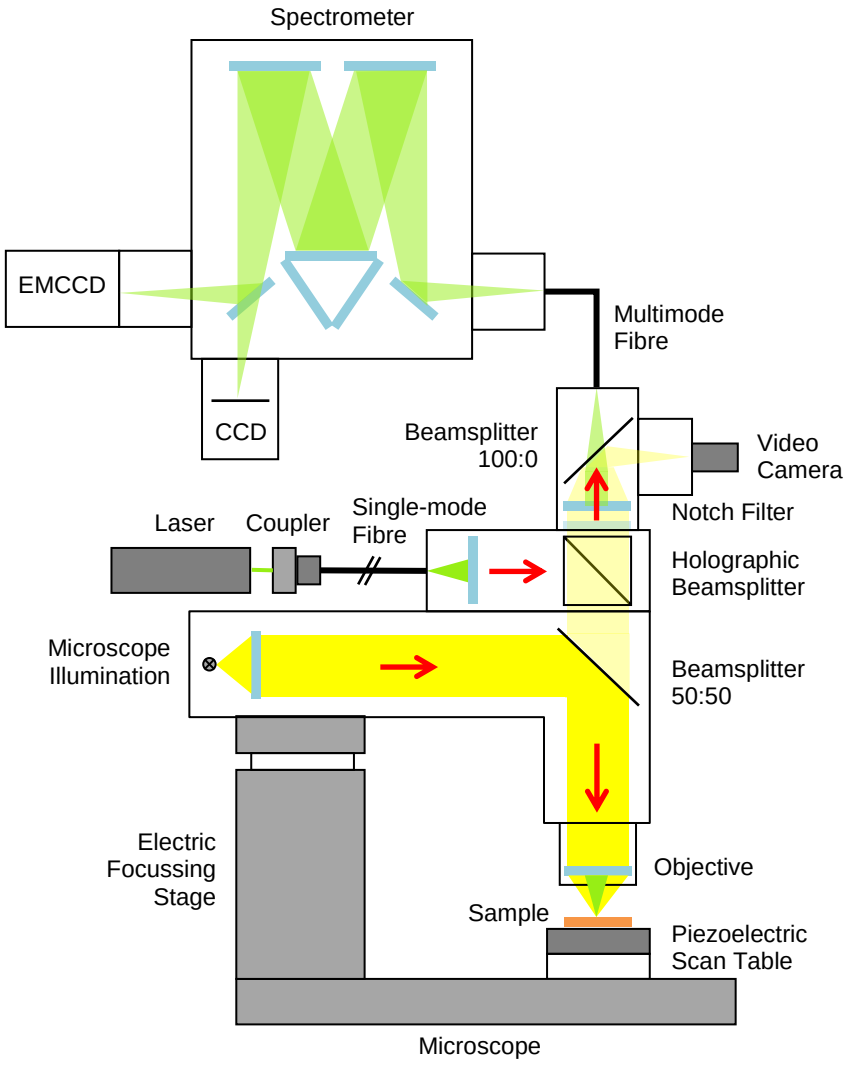

Figure 4.5: Beam path for a WITec confocal Raman microscopy

The WITec confocal Raman microspectrometer was equipped with the following main components (Figure 4.5):

- Raman excitation lasers: 532 nm (diode, solid) and 785 nm (diode, NIR)
- Microscope unit with 10×, 20×, 50× and 100× objectives
- Visible image capture device (camera)

- Piezoelectric x-y-z motorised sample scan table
- Electric z motorised focussing stage
- Newton Peltier cooled charge coupled device (CCD) detector
- Electron multiplying CCD (EMCCD) detector

The excitation laser was coupled to the microscope with a single-mode optical fibre, which provided a Gaussian beam profile that could be focussed to a diffraction-limited spot size. The laser beam was reflected by a holographic beamsplitter and focussed onto the sample with the microscope objective lens, before being scanned on the piezoelectric scan table. The same lens collected the Raman-scattered light, and a holographic notch filter or an extremely steep edge filter suppressed reflected laser and Rayleigh-scattered light.

The scattered light was then focussed into a multimode optical fibre in which the fibre core acted as a pinhole for the confocal microscopy. The multimode fibre was coupled to a spectrometer with two output ports; one equipped with a CCD camera for Raman spectral imaging when the 785 nm laser was used, and the other with an EMCCD detector for enhanced sensitivity of Raman spectral imaging using the 532 nm laser. The core of the multimode fibre also acted as an entrance slit for the spectrometer.

Based on the preliminary study, the 532 nm green laser was found to be optimum for the following experiments, when operated at 0.5 mW power (measured at the sample), under the 50× objective lens.

There were however some difficulties faced in measuring and analysing the aqueous emulsion samples under the microscopic system during the preliminary studies:

(i) to immobilise the moving droplets between the microscopic slide and the coverslip that contained the sample,
(ii) to focus at the right plane within the sample before measurement, and
(iii) to stabilise the fluctuating laser intensity at a constant power value.

The first problem was resolved with the use of poly-L-lysine, which is commonly applied in biological analysis to coat tissue cultureware e.g. Petri dishes and microscopic slides, to provide a surface that improves cell adherence. A drop of this amino-acid solution was dried on a microscopic slide using a hairdryer, before the emulsion sample was layered above it, and covered with a coverslip. To investigate whether poly-L-lysine might possibly affect the

spectra of an emulsion sample, a study was conducted involving the emulsion of canola oil stabilised by sodium caseinate (NaCas), in which the spectra of poly-L-lysine and each ingredient of the emulsion were obtained, followed by the spectra of the dispersed and aqueous phases of the emulsion with and without the application of poly-L-lysine on the slide. It was concluded that poly-L-lysine did not affect the spectrum of the emulsion sample.

The second problem arose from the varied amount of emulsion samples dropped on the slide each time, which caused inconsistent thickness of the sample layer between the slide and the coverslip. This resulted in difficulty in focussing the laser axially in the right plane within a droplet. This was experimentally resolved by first focussing the light microscope within an emulsion droplet with the guide from the live video in the computer, until the interface of the droplet was clearly observed. Then the Raman laser was turned on and shone on the focal point, giving live spectra under the oscilloscope function, from which the focal plane was adjusted until the strongest signal was obtained. Finally the single-measurement function was selected, replacing the oscilloscope, to give the required spectral data of the sample.

The last issue to be solved involved a slow drift of the signal intensity over time. However in the preliminary studies, the error caused by this problem was controlled and monitored by recording the spectral intensity of the Si wafer (used in the calibration of the system) before and after measuring the emulsion samples. If there was a difference between the initial and final reading of the Si chip, the spectral data of the emulsion samples recorded in between was discarded. It was also eventually determined that an overhead light source above the instrument provided enough warmth to change the temperature of the objective and hence cause the drift. Therefore in the later studies turning the light off gave a stable system.

For the determination of β-carotene concentration in the dispersed phase of an emulsion, the point-microspectroscopic mode was used by coadding 10 scans of 0.5 s integration time with the 0.5 mW laser power. This configuration was exactly the same as that used for plotting the calibration curve as described earlier in this chapter. The laser light was pointed within an oil droplet containing the bioactive and the corresponding bioactive concentration was determined univariately using the calibration curve, based on the most significant band of the bioactive spectra. Five random droplets of 5–10 μm diameter were targeted for duplicated spectral quantification and the average for each duplicate was recorded.

On the other hand, to map the location of β-carotene in the WPI emulsions, Raman images were constructed using the point mapping technique. A sample prepared as above was brought into focus (i.e. the circumference of the biggest emulsion droplet in view was sharp and clearly separated the phases) under the 50× objective to select an area comprising both the dispersed and aqueous phases. Using 0.5 mW laser power, the image scan function was set up to coadd 10 × 0.5 s scans on each point in the area. The number of points per line and the number of lines per image would determine the total mapping time. A Raman image was then produced and colour-coded using the filter manager function of WITec Process version 6.5, based on the intensity of the selected band obtained from the sample. The brightness (yellow) of a region in the Raman image accounts for greater concentration of β-carotene in that region.

For the chemical stability study of β-carotene within the dispersed phase, emulsions with different formulations were stored for approximately 30 days at a constant temperature. This was to determine the effects of varying the formulations on the chemical stability to degradation. For the storage-temperature study, emulsion samples were stored for 30 days at two different temperatures; 25 and 40 °C. Each sample was duplicated for error study. Every time the sample was taken out from the storage, the bioactive concentration within the oil droplets was determined and recorded using Raman analysis as described previously. The concentration of bioactive in the whole emulsion of each sample was also determined via the conventional extraction-spectrophotometric method (described in the next section), so that the partitioning characteristic of the stored sample could be studied under the different storage temperatures. Graphs of the bioactive concentration vs. time were plotted to analyse the degradation pattern of the bioactive encapsulated within the oil phase of the emulsion systems.

2.2 Extraction followed by spectrophotometric analysis

To determine the β-carotene concentration within the emulsion droplets via the traditional method for comparison with the Raman method, the WPI emulsion was first ultracentrifuged at 30,000 g and 20 °C for 1 hour (Beckman Coulter Optima L-90K Ultracentrifuge). Under these conditions, the emulsion separated into a top cream layer (the lipid phase with bioactives bound to it) and a transparent aqueous layer, which contained most of the whey protein and any bound bioactive molecules.

The concentrations of bioactive in the whole emulsions, as well as in the aqueous layer of the emulsions after centrifugation, were determined using this destructive analysis, from which the concentration of bioactive in the oil phase could be calculated and compared to that obtained using the Raman method. Direct determination of bioactive concentration in the oil phase via this traditional method was quite difficult since the cream layer representing the oil phase of emulsions after centrifugation was not homogeneous, thus sampling a part of it may not be representative of the bioactive concentration in that phase.

The extraction procedure began by constructing a standard curve using 3 g/kg β-carotene in MCT. A series of weighed β-carotene solutions were added to 10 mL flasks, which were then filled with n-hexane up to the mark, and mixed well using a vortex. The samples were scanned spectrophotometrically using Shimadzu UV-Vis spectrophotometer UV-1700 from 300 to 550 nm, and the corresponding ABS readings of the maximum peaks for all the different β-carotene concentrations were recorded at 450 nm (λ_{max} of β-carotene).

For quantification of total β-carotene in an emulsion, 200 mg of the emulsion was weighed into a 30 mL glass tube, before 4 mL DMSO was added and hand-shakenly mixed. The tube was placed in a water bath at 75 °C for 5 min to dissolve the sample in the solvent, and then cooled again to the room temperature for extraction.

The non-polar β-carotene in the emulsion was extracted using 4 mL of n-hexane containing 0.1% (w/v) BHT, added to the tube and vortex-mixed for 10 s and left for 30 min. A few drops of ethanol were also added to prevent emulsion formation during the process.

Using a glass Pasteur pipette, the top layer formed was carefully removed and transferred into a 10 mL volumetric flask. The extraction procedures were repeated for 2 more times, and the extracts were combined in the flask, which was eventually filled with n-hexane up to the mark.

This extract was then analysed spectrophotometrically at 450 nm to obtain its corresponding absorbance reading. The concentration of β-carotene in the whole emulsion sample was finally quantified using the standard curve plotted previously using the same bioactive and solvent. All measurements were repeated three times.

The same procedure was carried out for determining β-carotene concentration within the aqueous phase. After centrifugation, about 200 mg of the clear aqueous layer was carefully

removed using a glass Pasteur pipette from the bottom of the centrifugal tube, and the followings steps were the same as those described for the whole emulsion before.

The procedures were repeated for other WPI emulsions with different formulations or process conditions to quantify the resulting bioactive concentration in the whole emulsions and their aqueous phase.

Alternatively, the solvent extraction procedure could have been performed by incorporating a saponification process to separate the bioactive from the lipid (Cornacchia and Roos, 2011b) and simply using a mixture of ethanol and n-hexane (volume fraction 2:3) (Xu et al., 2013; Yuan, Gao, Zhao, & Mao, 2008) or methanol and methylene chloride (volume fraction 1:2) (Qian, Decker, Xiao, & McClements, 2012b).

2.3 Light-scattering droplet sizer

The characterisation of emulsified systems was done by measuring the particle size distribution and the average diameter of the emulsion droplets with a laser diffraction particle size analyser (Malvern MasterSizer MSE, Malvern Instruments Ltd., Malvern, Worcestershire, UK) as described by Cornacchia and Roos (2011). The refractive indices of the emulsion droplets and the dispersion medium were 1.456 (for food oils) and 1.33 (for water) respectively. The absorbance value of the emulsion droplets was 0.1. Droplet size measurements were reported as particle size distributions, volume-weighted mean diameters D[4,3] and as the surface-weighted or Sauter mean diameters, D[3,2]. Triplicate readings were taken for accuracy. The graph of droplet size vs. time was then plotted in the case of the storage stability study.

Reference

Blanpain-Avet, P., Hédoux, a., Guinet, Y., Paccou, L., Petit, J., Six, T., & Delaplace, G. (2012). Analysis by Raman spectroscopy of the conformational structure of whey proteins constituting fouling deposits during the processing in a heat exchanger. *Journal of Food Engineering*, *110*, 86–94.

Cornacchia, L., & Roos, Y. H. (2011a). Solid-liquid transitions and stability of HPKO-in-water systems emulsified by dairy proteins. *Food Biophysics*, *6*, 288–294.

Cornacchia, L., & Roos, Y. H. (2011b). Stability of β-carotene in protein-stabilized oil-in-water delivery systems. *Journal of Agricultural and Food Chemistry*, *59*, 7013–7020.

Demetriades, K., & McClements, D. J. (1998). Influence of pH and Heating on Physicochemical Properties of Whey Protein-Stabilized Emulsions Containing a Nonionic Surfactant. *Journal of Agricultural and Food Chemistry*, *46*, 3936–3942.

Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta*, *1079*, 316–320.

Herrero, A. M., Carmona, P., Pintado, T., Jiménez-Colmenero, F., & Ruíz-Capillas, C. (2011). Infrared spectroscopic analysis of structural features and interactions in olive oil-in-water emulsions stabilized with soy protein. *Food Research International*, *44*, 360–366.

Liu, Y., Hou, Z., Lei, F., Chang, Y., & Gao, Y. (2012). Investigation into the bioaccessibility and microstructure changes of β-carotene emulsions during in vitro digestion. *Innovative Food Science & Emerging Technologies*, *15*, 86–95.

Lopez-Sanchez, P., Schumm, S., Pudney, P. D. a, & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst*, *136*, 3694–3697.

Marcelli, A., Cricenti, A., Kwiatek, W. M., & Petibois, C. (2012). Biological applications of synchrotron radiation infrared spectromicroscopy. *Biotechnology Advances*, *30*, 1390–1404.

McNaughton, D., & Wood, B. R. (2012). Synchrotron Infrared Spectroscopy of Cells and Tissue. *Australian Journal of Chemistry*, *65*, 218–228.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Inhibition of β-carotene degradation in oil-in-water nanoemulsions: influence of oil-soluble and water-soluble antioxidants. *Food Chemistry*, *135*, 1036–1043.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Nanoemulsion delivery systems: influence of carrier oil on β-carotene bioaccessibility. *Food Chemistry*, *135*, 1440–1447.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, *132*, 1221–1229.

Rohman, A., & Che Man, Y. B. (2010). Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. *Food Research International*, *43*, 886–892.

Saito, S., Tasumi, M., & Eugster, C. H. (1983). Resonance Raman spectra (5800–40 cm^{-1}) of All-trans and 15-cis isomers of β-carotene in the solid state and in solution. Measurements with various laser lines from ultraviolet to red. *Journal of Raman Spectroscopy*, *14*, 299–309.

Singh, H., Tamehana, M., Hemar, Y., & Munro, P. A. (2003). Interfacial compositions, microstructure and stability of oil-in-water emulsions formed with mixtures of milk proteins and κ-carrageenan: 2. Whey protein isolate (WPI). *Food Hydrocolloids*, *17*, 549–561.

Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, *23*, 165–174.

Sun, C., Gunasekaran, S., & Richards, M. P. (2007). Effect of xanthan gum on physicochemical properties of whey protein isolate stabilized oil-in-water emulsions. *Food Hydrocolloids*, *21*, 555–564.

Thrash, R. J., Fang, H. L. B., & Leroi, G. E. (1977). The Raman excitation profile spectrum of β-carotene in the preresonance region: Evidence for a low-lying singlet state. *The Journal of Chemical Physics*, *67*, 5930.

Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International*, *42*, 1254–1258.

Xu, D., Wang, X., Jiang, J., Yuan, F., Decker, E. A., & Gao, Y. (2013). Influence of pH, EDTA, a-tocopherol, and WPI oxidation on the degradation of b-carotene in WPI-stabilized oil-in-water emulsion. *Food Science and Technology*, *54*, 236–241.

Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., & Zhong, F. (2015). Beta-carotene encapsulated in food protein nanoparticles reduces peroxyl radical oxidation in Caco-2 cells. *Food Hydrocolloids*, *43*, 31–40.

Yuan, Y., Gao, Y., Zhao, J., & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International*, *41*, 61–68.

CHAPTER 5 VALIDATION OF RAMAN TECHNIQUE TO QUANTIFY β-CAROTENE IN WHEY PROTEIN EMULSIONS

Overview

In Chapter 5, the ability of CRM to quantify the concentration of β-carotene in protein emulsions is examined and validated. The Raman technique was validated using the conventional extraction-spectrophotometric method. With the confocal microscopic instrument, the Raman image was obtained to locate the bioactive molecules dispersed in the emulsion systems. The effect of varying the concentration of β-carotene on its partitioning in the emulsion samples was analysed *in situ* using CRM for the first time. This study provides the necessary technical background for understanding the partitioning of bioactive within emulsions using only the confocal Raman instrument in the following experiments.

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In situ quantification of β-carotene partitioning in oil-in-water emulsions by confocal Raman microscopy

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ABSTRACT

Confocal Raman microscopy (CRM) was able to quantify the β-carotene concentration in oil droplets and determine the partitioning characteristics of β-carotene within the emulsion system *in situ*. The results were validated by a conventional method involving solvent extraction of β-carotene separately from the total emulsion as well as the aqueous phase separated by centrifugation, and quantification by absorption spectrophotometry. CRM also enabled the localization of β-carotene in an emulsion. From the Raman image, the β-carotene partitioning between the aqueous and oil phases of palm olein-in-water emulsions stabilized by whey protein isolate (WPI) was observed. Increasing the concentration of β-carotene in an emulsion (from 0.1 to 0.3 g/kg emulsion) with a fixed gross composition (10% palm olein:2% WPI) decreased the concentration of β-carotene in the oil droplet. CRM is a powerful tool for *in situ* analyses of components in heterogeneous systems such as emulsions.

1. Introduction

Emulsions are commonly used for the stabilization and delivery of bioactives (Augustin & Sanguansri, 2014; McClements, 2015). The stability and the release of the encapsulated bioactive are dependent on its partitioning characteristics in the emulsion. However, determining the location and partitioning characteristics of bioactives stabilized in emulsion systems is a relatively unexplored area of research in the field of food encapsulation (McClements & Rao, 2011). It is anticipated that this knowledge will be useful for designing emulsion formulations for the stabilization and controlled delivery of bioactives.

Carotene, like most lipophilic bioactives, may be delivered in an oil-in-water (O/W) emulsion (Oliver & Augustin, 2009). The conventional methods of determining the partitioning characteristics of carotene in an emulsion involve the separation of the fat and aqueous fractions by centrifugation and the use of solvents for extracting the carotene, followed by measurement of the extracted carotene. Antioxidants (e.g., butylated hydroxytoluene or ascorbic acid) may be added to the system during extraction to prevent oxidation of sensitive bioactives. The quantification of carotene may then be carried out using UV–Vis spectrophotometry (Cornacchia & Roos, 2011a, 2011b).

Although centrifugation can be used to separate different phases of an emulsion prior to quantification of a bioactive in each phase, it is tedious and involves meticulous procedures. Hence a more rapid and *in situ* method of bioactive quantification for emulsions that allows the determination of partitioning characteristics of the bioactive in the systems is highly desirable. An example of where partitioning of components in emulsions was examined *in situ* is the use of front face fluorescence spectroscopy for determination of protein in the aqueous phase and the interface of emulsions (Granger, Barey, Toutain, & Cansell, 2005). In this work we examine the potential of confocal Raman microscopy (CRM) as a technique for real time measurement of partitioning of β-carotene in emulsions *in situ*.

CRM has been widely used to determine the composition and structure of a target material, and specifically to probe the chemistry and reactivity of food (Cherney & Harris, 2010; Li-Chan, 1996; Zheng, Gordon, & Everett, 2013). The Raman spectrum of a molecule is essentially a fingerprint that can be used for identification, whilst the intensity of the Raman scatter (photon count) is a direct measure of the concentration of the molecule. It is contactless, non-destructive, minimally or even non-invasive, and provides chemical information about the sample of interest. CRM has become an accepted tool in food and biological analysis due to the weak Raman scattering from water, allowing *in situ* study

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of samples in aqueous media; its rapid analysis with no alteration of the sample's macro-structure; and no requirement for chemical labelling as a prerequisite (Li-Chan, 1996; Zheng et al., 2013).

The coupling of Raman spectroscopy with microscopy has made chemical visualisation possible. CRM can be used to detect and quantify the molecular nature of microscopic samples, as well as for mapping the distribution of components within samples. In its most basic form, there are three modes for acquiring spontaneous Raman spectra: 1) point microspectroscopy with no scanning or imaging, 2) spectral (point and line) mapping, also known as series imaging that requires image reconstruction, and 3) global imaging, which is referred to as direct imaging that results in the immediate production of a complete two-dimensional image at a chosen wavenumber shift.

The application of CRM and imaging to study chemical heterogeneity of emulsion systems has been reported (Andrew, Browne, Hancewicz, & Millichope, 1998). Samples can be analysed directly in either air or water, at a range of temperatures and pressures and without destroying the sample. Spatially resolved chemical analyses of microscopic regions of samples *in situ* have been applied in both material and biological sciences. CRM is hence an exceptional tool for investigating the chemistry of emulsion phases because it does not rely on homogenization, extraction, or dilution, but rather the molecular nature of each phase is analysed *in situ* (Wang & Spencer, 2002). It allows spatial resolution down to the diffraction limit, spectral resolution down to 1 cm^{-1} or better and is confocal with the laser focused to a small spot within the sample and the Raman scattered light collected only from that point (Williams, Pitt, Batchelder, & Kip, 1994). The confocality of CRM further enhances the microstructural molecular evaluation of a specimen (Lopez-Sanchez, Schumm, Pudney, & Hazekamp, 2011).

Since CRM provides depth-resolved measurements, the technique has frequently been used to monitor drug penetration into human skin (Pudney, Melot, Caspers, Van Der Pol, & Puppels, 2007) and to study the hydration levels of the distinct layers of the human epidermis (Chrit et al., 2006). The use of CRM in food analysis is growing and an example pertinent to this work is its use to measure the distribution of carotenoids within tomato cells in which the Raman spectra were shown to be sensitive to the physical state of the carotenoid (Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011).

The main objective of the present study was to validate the use of CRM to quantify the amount of β-carotene in emulsion droplets stabilized by WPI using single point-confocal microspectroscopic mode. A comparison of the CRM results with those of a conventional method involving centrifugation, extraction and spectrophotometry has been carried out to validate the use of CRM. Moreover it has been shown that there are specific interactions between carotenoids and proteins (Dufour & Haertlé, 1991; Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). Therefore in protein-based emulsions with β-carotene, there may well be a partitioning of the bioactive between the bulk (aqueous protein phase) and the oil phase of the emulsions upon emulsification. The ability of CRM to locate β-carotene and follow its partitioning within a heterogeneous system using point mapping followed by Raman image reconstruction has also been assessed. The partitioning characteristic of carotene in WPI emulsions upon varying the concentrations of the β-carotene was also assessed once the method was validated.

2. Experimental

2.1. Materials

The dispersed phase for the emulsion-based encapsulation system was refined, bleached and deodorized (RBD) palm olein from Wilmar Trading Pty. Ltd. (Pasir Gudang, Johor, Malaysia). The active core was micro-algal based natural β-carotene suspended (30%) in medium-chain triglyceride oil, kindly donated by Cognis Australia Pty. Ltd. (Melbourne, Victoria, Australia), while the carrier of the active ingredient was the high molecular weight WPI (91% protein, 5% moisture, 3% ash, and 1% fat), which was purchased from Fonterra (Darnum, Victoria, Australia). Double-distilled water was used to prepare all solutions and emulsions.

For the emulsion analyses using the CRM, poly-L-lysine (0.1% (w/v) in water, with 0.01% of thimerosal added as preservative) from Sigma-Aldrich (Castle Hill, NSW, Australia) was used to coat the sample slides and immobilize oil droplets deposited on the surface.

The traditional extraction-spectrophotometric method used the following chemicals from Merck Pty. Ltd. (Kilsyth, Victoria, Australia): dimethyl sulfoxide (DMSO), *n*-hexane, and ethanol. Butylated hydroxytoluene (BHT) from Sigma-Aldrich (Castle Hill, NSW, Australia) was also used as an anti-oxidant. All the chemicals used were of analytical grade.

2.2. Preparation of the β-carotene O/W emulsions

Homogenization of lipid phase with aqueous phase yields oil-in-water (O/W) emulsions. WPI powder was hydrated in double-distilled water by constant stirring by a mixer in a water bath at 50 °C for 1 h to give the aqueous phase (2 g/kg). β-Carotene, at levels of 1, 2 or 3 g/kg, was solubilized in the lipid phase by constant stirring and heating at 140 °C for 30 min on a magnetic stirrer in the dark, giving a completely transparent red oil with no evidence of crystals (Mao et al., 2009). The aqueous (90% (w/w)) and lipid (10% (w/w)) phases were mixed using a high-speed rotor-stator mixer (L2R, Silverson Machines Ltd., Chesham, UK) with a constant speed for 2 min to form a pre-emulsion at room temperature (~22 °C). This pre-emulsion was homogenized twice through a high-pressure single-pass valve homogenizer (EmulsiFlex, Avestin Inc., Ottawa, Canada) at 500/100 bar, and the fine emulsion samples collected for analyses. Two independent replicates were made for each sample.

2.3. Raman microscopic method

Raman scattering spectra were collected *in situ* with a WITec alpha300 R (WITec, Ulm, Germany) confocal Raman imaging microscope equipped with a Newton Peltier cooled charge coupled device (CCD) detector and a 532 nm solid state diode laser excitation source. The scattered light from the sample was collected in the backscattering geometry onto a 600 groove/mm grating, providing a spectral resolution of approximately 3 cm^{-1}. Each sample was analysed using a 50x objective lens. This Raman microscope setup results in a high spatial resolution down to ~0.4 μm and confocality with ca. 0.8 μm depth.

2.3.1. Point microspectroscopy for partitioning determination

To quantify and determine the partitioning of β-carotene in the emulsion samples, each sample was analysed with the point-microspectroscopic mode; coadding 10 scans of 0.5 s integration time with 0.5 mW laser power.

A calibration curve (Raman intensity of β-carotene band at 1524 cm^{-1} against known concentrations of β-carotene in oil) was first plotted using β-carotene-in-oil samples (0.5–5 g/kg) prepared by the same procedure used for solubilizing the β-carotene in the palm olein to make the dispersed phase of an emulsion (Fig. 1).

For the determination of β-carotene concentrations in the emulsion droplets, a drop of poly-L-lysine was dried on a glass slide to provide a thin coating of poly-L-lysine, after which a drop of emulsion sample was deposited above it. This sample was then carefully

covered with a coverslip. The laser light was then focused within an oil droplet (the dispersed phase) containing β-carotene and the Raman intensity of the β-carotene band at 1524 cm^{-1} was used to determine the corresponding β-carotene concentration univariately using the calibration curve. This measurement was carried out in duplicate for every formulation, from which five random droplets of 5–10 μm diameter were targeted for spectral quantification and the average for each duplicate was recorded.

2.3.2. Point mapping for localization

The point mapping technique was also used to define the location of β-carotene in the WPI emulsions, through the construction of Raman images. A sample prepared as above was brought into focus (i.e. the circumference of the biggest emulsion droplets in view was sharp and clearly separates the phases) under the microscope to select an area comprised of both the dispersed and aqueous phases. The image scan function was set up to coadd 10 × 0.5 s scans on each point in the area, using 0.5 mW laser power. The total mapping time was dependent on the number of points per line, and number of lines per image. Then using the filter manager function of WITec Process version 6.5, a Raman image was produced and colour-coded based on the intensity of the 1524 cm^{-1} band obtained from the sample. The brightness (yellow) of a region in the Raman image represents greater concentration of β-carotene in that region.

2.4. Conventional extraction-spectrophotometric method

The concentrations of β-carotene in the whole emulsions, as well as in the aqueous layer of the emulsions after centrifugation, were determined using the method proposed by Ying et al. (2015) with some modifications, from which the concentration of β-carotene in the oil phase was calculated by difference.

A 3 g/kg solution of β-carotene in oil was used to make up the standard β-carotene curve. A series of concentrations of β-carotene (0–1 mg/100 mL) in *n*-hexane were prepared in 10 mL flasks and mixed well using a vortex. UV–Vis spectra were collected from 300 to 550 nm using a Shimadzu UV-1700 spectrophotometer (Rowville, Victoria, Australia), and the corresponding absorbance readings for all β-carotene concentrations were recorded at λ_{max} of 450 nm.

For quantification of β-carotene in a whole emulsion, about 200 mg of the emulsion containing β-carotene was weighed into a 30 mL glass tube, 4 mL of DMSO added and the solutions mixed by hand shaking. The tube was placed in a water bath at 75 °C for 5 min to ensure dissolution of the sample in the solvent, and then cooled again to room temperature (~22 °C) for extraction. The β-carotene was extracted with 4 mL of *n*-hexane containing 0.1% (w/v) BHT, which was added to the tube prior to vortex mixing for 10 s and leaving for 30 min. A few drops of 100% ethanol were added to precipitate any protein in the *n*-hexane phase. The top layer formed was carefully removed using a glass Pasteur pipette, and transferred into a 10 mL volumetric flask. The extraction procedure was repeated two more times, each using 3 mL of *n*-Hexane, and the extracts combined in a flask, which was then filled with *n*-hexane up to the 10 mL mark. This extract was analysed spectrophotometrically at λ_{max} = 450 nm. The concentration of β-carotene in the whole emulsion sample was finally quantified using the standard curve plotted previously.

To determine the β-carotene concentration within the aqueous phase, the emulsion containing β-carotene was first ultracentrifuged at 30,000*g* at 20 °C for 1 h (Beckman Coulter Optima L-90K Ultracentrifuge). Under these conditions, the emulsion separated into a top cream layer and a clear aqueous layer. Approximately 200 mg of the aqueous layer was weighed and extracted with *n*-hexane as previously described for the whole emulsion.

The analysis was performed in duplicate on each replicated fresh emulsion.

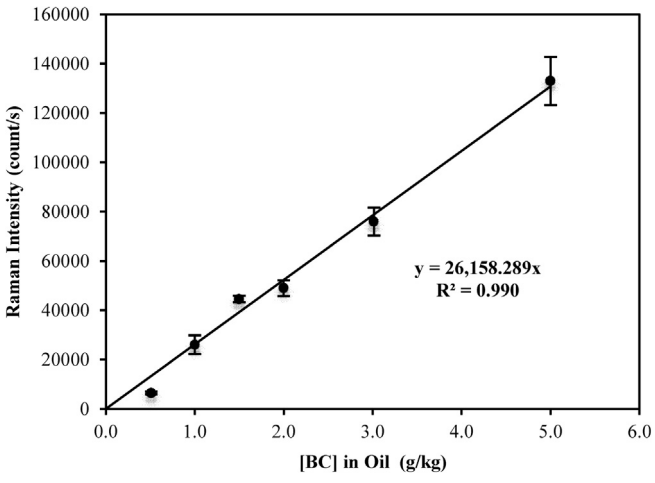

Fig. 1. Calibration curve of the Raman method plotted using β-carotene-in-oil samples (0.5–5 g/kg) for univariate quantification of β-carotene concentration in the oil phase of emulsion samples. Data are mean ± standard deviations (n = 3).

2.5. Analysis

The raw Raman data of the samples were directly analysed using the filter manager of WITec Process version 6.5 in which the integrated area (excluding background contributions) of the 1524 cm^{-1} band was determined between 1480 and 1570 cm^{-1}. The UV–Vis absorbance values of samples were directly obtained at λ_{max} = 450. All spectral data were exported in ASCII format to Microsoft Excel for univariate analyses. The integrated area of the 1524 cm^{-1} band from the Raman data and the absorbance values at λ_{max} = 450 nm from the UV–Vis spectrophotometer were plotted separately against the known concentrations of β-carotene in olein, giving the linear regression model of both the Raman and conventional methods respectively. The equation of the regression line for each plot ($y = m\,x$) was determined along with the regression correlation value (r^2). Using the gradient value (m) of the regression line equations, the corresponding β-carotene concentration of the samples can be predicted either using the Raman or conventional methods.

In order to illustrate the capability of CRM to provide quantitative measurements of β-carotene in emulsion systems, another linear regression model was plotted comparing the β-carotene concentrations determined by CRM and those by the conventional method as the reference. All quantitative data were obtained from two replicated samples, analysed in duplicates, and expressed as mean ± standard deviation. Therefore the model was developed using 12 spectra (3 samples with 4 spectra per sample).

Besides looking at the linearity and the regression correlation value of the model, three figures of merit; relative error of prediction (REP), relative standard deviation (RSD) and limit of detection (LOD), were used to validate the use of CRM against the reference method (Dingari, Horowitz, Kang, & Dasari, 2012). The values of REP and RSD correlate directly with the accuracy and precision of the Raman method, respectively. The LOD, as defined by IUPAC (1997), was computed from the best fit line obtained between predicted concentrations by Raman and the reference concentrations. The calculations were based on the following equations:

(i) Average relative error of prediction (REP):

$$\text{REP}\ (\%) = \frac{100}{N}\sum_{i=1}^{N}\left|\frac{c'_i - c_i}{c_i}\right| \quad (1)$$

where N is the number of spectra in the dataset, c_i is the reference concentration and c'_i is the predicted Raman concentration.

(ii) Average relative standard deviation of predicted concentrations (RSD):

$$\text{RSD}\ (\%) = \frac{100}{N_c}\sum_{k=1}^{N_c}\frac{\sigma_{c_k}}{c_k} \text{ where } \sigma^2_{c_k} = \sum_{i=1}^{p}\frac{(c'_k - c_k)^2}{p-1} \quad (2)$$

where N_c is the number of distinct concentrations in the dataset, p is the number of spectra per concentration and σ_{ck} is the standard deviation obtained at concentration c_k.

(iii) Limit of detection (LOD):

$$\text{LOD}\ (\text{g/kg}) = 3\frac{s_{y/x}}{\text{slope}} \text{ where } s_{y/x} = \left[\frac{\sum_i (c'_i - c_i)^2}{N-2}\right]^{1/2} \quad (3)$$

where $s_{y/x}$ is the standard deviation of residuals and is a measure of the average deviation of the predicted values from the regression line.

Statistical differences of the experimental results were determined by two-way analysis of variance (ANOVA) in Microsoft Excel, in which $p < 0.05$ was considered to be statistically significant. Significant differences of means between the different types of sample were further determined by the Fisher's Least Significant Difference (LSD) test.

The Raman results from the point mapping were only reliable for qualitative analyses since the calibration curve used as the reference was valid only for the dispersed phase (oil) regions.

3. Results and discussion

3.1. Validation of Raman method

The concentrations of β-carotene in the emulsion systems measured by both the conventional and Raman methods are shown in Table 1. From this we could compare the concentration of β-carotene remaining in oil droplets using CRM with the difference in concentrations of β-carotene in the whole emulsion and the aqueous phase obtained using the conventional method. The β-carotene concentration in the oil phase for each sample obtained using CRM was not significantly different ($p = 0.98$) to that obtained using the conventional method.

Fig. 2 further depicts the validation model of the Raman method to quantify β-carotene concentrations in the oil droplets of emulsion samples, where the reference concentrations obtained from the conventional method and the Raman predicted concentrations are given along the x- and y-axis, respectively. The solid line represents y = x, illustrating explicitly the linearity of the response. From the figure, it is evident that the predicted values show excellent agreement with the reference concentrations ($r^2 = 0.999$), thus validating the Raman method for determining the concentration of β-carotene in the oil droplets of emulsion systems.

In addition, the relative error of prediction (REP) was calculated to be 4.02% (Eq. (1)), showing thereby that the model provides highly accurate predictions of the β-carotene concentrations in the oil droplets using CRM, over the entire concentration range of 0.1–0.3 g/kg. On the other hand, the precision of our measurements for the entire concentration range was also good, as shown by the relative standard deviation (RSD) metric determined at 1.06% (Eq. (2)).

Finally for the method validation, the limit of detection (LOD) for the Raman method was computed using the standard deviation of the residuals and the slope of the regression line (Eq. (3)), giving a concentration of 0.036 g/kg β-carotene in the oil droplets of emulsions. This LOD value is lower than all the sample concentrations (0.1–0.3 g/kg), reassuring the Raman system's sensitivity in measuring very low concentrations of β-carotene within the dispersed phase of emulsions.

Therefore CRM was proven to be accurate, precise and sensitive enough to quantify the β-carotene content in emulsion systems. The method is powerful yet simple due to the contactless, non-destructive, and chemical-free nature of the technology, which overcomes the limitations and laborious procedures in the conventional approach, and opens wider opportunities for *in situ* analyses with minimal sampling and preparation time.

3.2. Raman spectra of β-carotene in the oil phase

All the Raman spectra of the single spots within the oil droplets are consistent and show three significant peaks at 1524, 1157 and 1016 cm^{-1} (Fig. 3), representing the double bond C═C stretching mode, $\nu_1(\nu_s C{=}C)$; the C—C in-plane single bond stretching mode, $\nu_2(\nu_s C{-}C)$; and the C—H bending mode, $\nu_3(\rho C{-}CH_3)$ of β-carotene respectively (Lopez-Sanchez et al., 2011). Fig. 3 also depicts the increasing band intensity from 1 to 3 g/kg for β-carotene in oil prior to emulsification.

The Raman effect leads to a change in energy of a small number of photons due to inelastic collisions of incident light with molecules. The intensity of Raman transitions varies greatly with the analyte material and with the excitation laser used. The intrinsic intensity of a Raman spectrum depends on a change of polarisability upon vibration and is proportional to the square of the induced dipole moment. Generally, molecules with π-electrons that are easy to polarise will give stronger Raman scattering than those held tightly i.e. σ-bonded. This has an important implication for the study of encapsulation of bioactive components for food applications since encapsulants such as protein and starch, which are commonly used are sigma-bonded molecules and active cores such as beta-carotene contain π electrons. Consequently the Raman signal obtained throughout these experiments is dominated by the β-carotene bands (Fig. 3), instead of a mixture of spectra from other ingredients of the emulsions.

Table 1
Quantification of β-carotene concentration, [BC] in the oil phase of oil-in-water emulsions (10% palm olein, 2% WPI).[a]

Theoretical	Extraction and Quantification by Absorbance (Conventional Method)[b]			*In situ* Raman Method[c]
[BC] in Emulsion ($\times 10^{-3}$ g/kg)	[BC] in Emulsion ($\times 10^{-3}$ g/kg)	[BC] in Aqueous ($\times 10^{-3}$ g/kg)	[BC] in Oil[c] ($\times 10^{-3}$ g/kg)	[BC] in Oil[d] ($\times 10^{-3}$ g/kg)
100	100.0 ± 1.1	6.6 ± 0.3	93.4 ± 1.1^{I}	92.7 ± 3.0^{I}
200	204.4 ± 2.2	16.3 ± 1.4	188.0 ± 2.6^{II}	181.4 ± 8.8^{II}
300	317.5 ± 4.4	55.5 ± 1.5	262.0 ± 4.6^{III}	254.0 ± 15.8^{III}

$^{I, II, III}$Mean data followed by the same superscript in each row show that there is no significant difference ($p < 0.05$).

[a] β-carotene (BC) was added into the oil phase prior to combining with the aqueous phase and emulsification.

[b] Average of at least duplicate sets of measurements for each replicated sample, with standard deviation ($n = 4$).

[c] Calculated from that of whole emulsion and aqueous phase by difference.

[d] Determined from calibration curve of Raman intensity against β-carotene concentration.

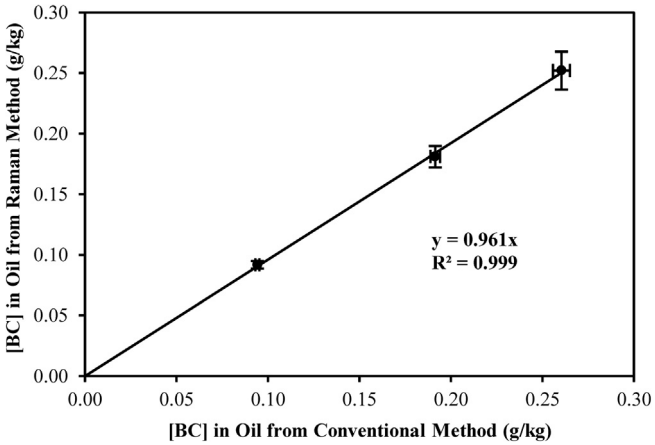

Fig. 2. Correlation between the β-carotene concentrations in oil droplets of emulsions (10% palm olein, 2% WPI) determined by Raman method and those determined by conventional method involving extraction and quantification by absorbance. Data are mean ± standard deviations (n = 4).

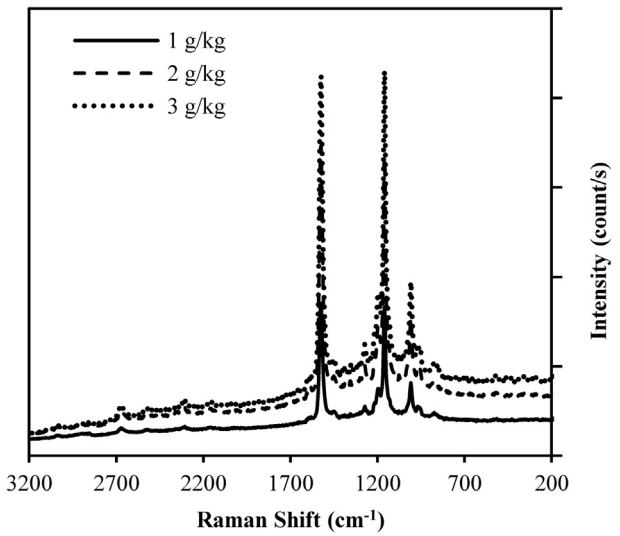

Fig. 3. Raman spectra for β-carotene in palm olein at different concentrations; 1, 2 and 3 g/kg. Three significant β-carotene bands are observed at 1524, 1157 and 1016 cm^{-1}. The integrated area of the band 1524 cm^{-1} was used to represent the β-carotene concentration of the system.

Moreover, long conjugated systems like that of β-carotene give strong visible absorbance spectra and the signal becomes more intense when the laser wavelength is approaching (pre-resonance Raman) or coincident (resonance Raman) with the electronic absorption energy of the analyte. In β-carotene where the strong band at 450 nm tails off through the visible region, pre-resonance Raman occurs when using the 532 nm laser and this accounts for the high intensity of the bands of β-carotene. The limit of detection quoted above is only valid for the laser wavelength, laser power and integration times used in this work which were optimised to eliminate laser damage. Using a shorter wavelength, increasing the laser power or increasing the integration time may result in lower limits of detection but given that all of these could result in laser damage we have not fully explored these factors.

3.3. Partitioning of β-carotene within emulsions

The integrated intensity of the strong 1524 cm^{-1} peak was used in the univariate analysis for determining the concentration of β-carotene within the oil droplets. From the results in Table 1, the concentrations of β-carotene in the oil phase were lower than those before emulsification. Migration of β-carotene molecules from the dispersed phase to the aqueous phase once the emulsions were formed, resulted in partitioning of the β-carotene between the two phases. Based on the calculated percentage of β-carotene in the oil droplets (Fig. 4), it is apparent that increasing the β-carotene concentration results in a decrease of the β-carotene percentage concentration in oil droplets. This indicates that more β-carotene molecules are partitioned into the aqueous phase at a higher β-carotene concentration. Although there was no significant difference between the partitioning concentration of β-carotene between emulsions containing 0.1 g/kg and 0.2 g/kg emulsion, increasing the β-carotene concentration to 0.3 g/kg emulsion resulted in a significant decrease in the β-carotene partitioning in the oil phase. This trend of increasing β-carotene partitioning (%) in the aqueous phase of the emulsion with increasing total β-carotene in the whole emulsion was unexpected but explainable. As the concentration of total β-carotene in the whole emulsion is changed, there is likely to be a new equilibrium in the partitioning of the carotene between the various phases of the emulsion.

The partitioning of the bioactive between the various phases of emulsions is governed by the relative solubility of the β-carotene in the oil and aqueous phases of the emulsion, their propensity to assemble at the interface and their affinity to bind to components in the emulsions. It is known that β-carotene binds to the β-lactoglobulin, the major component of WPI in the aqueous phase of the emulsion samples (Dufour & Haertlé, 1991; Wackerbarth et al., 2009). Therefore, the higher the concentration of β-carotene in the system, the more likely β-carotene is to partition out from the oil phase to bind with the protein molecules in the aqueous phase.

The Raman images (Fig. 5b) reconstructed from the point mapping of selected areas within the emulsion samples also qualitatively depict the location and partitioning of β-carotene molecules. Based on the 1524 cm^{-1} band of a sample, the colour-coded image in Fig. 5b is brighter in the droplet regions, corresponding to a higher concentration of β-carotene in the lipid phase as compared to that in the aqueous phase. For the largest (5–10 μm) emulsion droplets there is a distinguishable sharp circumference line because the microscope was easily focused down to the centre of the droplets, which then became the confocal plane of spectral acquisition for the Raman mapping. The intensity across the large droplets is consistent except for very close to the edges.

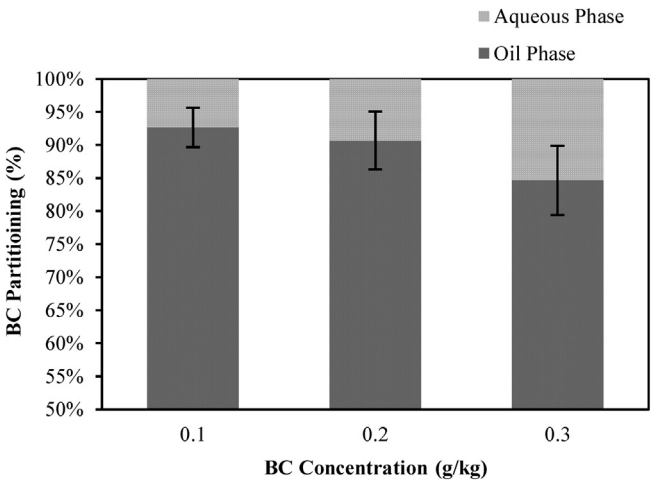

Fig. 4. Partitioning of β-carotene (BC) between the oil and aqueous phases of three emulsions (10% palm olein, 2% WPI) with different β-carotene concentrations; 0.1, 0.2 and 0.3 g/kg, determined by Raman microspectroscopic method. Data are mean ± standard deviations (n = 4).

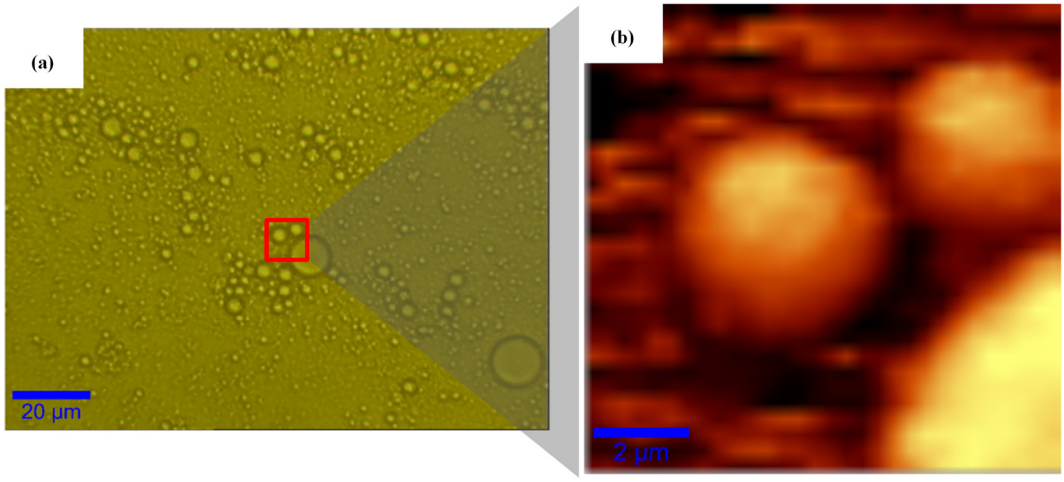

Fig. 5. (a) Micrograph of oil droplets in emulsion (10% palm olein, 2% WPI) containing 0.3 g/kg β-carotene, under 50x objective lens, (b) The corresponding Raman image based on the 1524 cm^{-1} β-carotene band, from the mapping of the area highlighted in red box (20 points per line, 20 lines per image, 0.5 s integration time for 10 scans, using 0.5 mW laser intensity).

This further illustrates that β-carotene within the droplet is homogeneous, while the decrease of intensity at the edges is most likely due to the smaller confocal volume sampled at the edges, instead of representing a lower β-carotene concentration at the droplet surface. For the smaller droplets in the Raman image, there appears to be some heterogeneity, which may be a result of part of the confocal volume of the instrument being outside the droplet and possibly of the confocal plane going through the top surface and not through the middle.

This point mapping approach was made possible by the WITec CRM used in this study, which gives a high spatial resolution down to 0.4 μm, allowing the laser to be focused on a small spot within the 5–10 μm oil droplets chosen for mapping. However it is important to note that the choices of integration time, the number of coadded scans for each point, the laser power and the overall mapping area are crucial since they affect the total scanning time of the sample containing β-carotene, which is both photo- and heat-sensitive, thus easily degraded by time upon the laser illumination. Therefore the integration time of 0.5 s, 10 scans for each point and the laser power of 0.5 mW were carefully chosen so that the β-carotene spectra were identical at the start and finish of Raman mapping.

4. Conclusion

This work has shown that Raman microspectroscopic methods enabled *in situ* quantification of the β-carotene content of oil droplets and determination of the partitioning characteristics of β-carotene within the emulsion system. The results have been validated by a conventional method involving centrifugation, extraction and spectrophotometry to determine the β-carotene concentration of the whole emulsion and its aqueous phase. The major advantages of the Raman method over the conventional method are much greater speed of analysis, simplicity, no need for centrifugation and extraction and no need for chemicals. A higher percentage of β-carotene molecules were partitioned out to the aqueous phase of emulsion upon emulsification at higher concentrations of the β-carotene in the total emulsion likely due to increased binding of β-carotene to protein. Point mapping to generate images that depict the location and distribution of the β-carotene bioactive in the emulsion system have been demonstrated. Although based on β-carotene this study shows also that CRM can be a powerful tool for the analysis of any strong Raman scattering bioactive in a heterogeneous system *in situ*.

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References

Augustin, M. A., & Sanguansri, L. (2014). Challenges and solutions to incorporation of nutraceuticals in foods. *Annual Review of Food Science and Technology, 6*, 463–477.
Cherney, D. P., & Harris, J. M. (2010). Confocal Raman microscopy of optical-trapped particles in liquids. *Annual Review of Analytical Chemistry, 3*, 277–297.
Chrit, L., Bastien, P., Biatry, B., Simonnet, J., Potter, A., Minondo, A. M., ... Hadjur, C. (2006). In vitro and in vivo confocal Raman study of human skin hydration: Assessment of a new moisturizing agent, pMPC. *Biopolymers, 85*(4), 1–4.
Cornacchia, L., & Roos, Y. H. (2011a). Stability of β-carotene in protein-stabilized oil-in-water delivery systems. *Journal of Agricultural and Food Chemistry, 59*, 7013–7020.
Cornacchia, L., & Roos, Y. H. (2011b). State of dispersed lipid carrier and interface composition as determinants of beta-carotene stability in oil-in-water emulsions. *Journal of Food Science, 76*(8), C1211–C1218.
Dingari, N. C., Horowitz, G. L., Kang, J. W., & Dasari, R. R. (2012). Raman spectroscopy provides a powerful diagnostic tool for accurate determination of albumin glycation. *PLoS ONE, 7*(2).
Dufour, E., & Haertlé, T. (1991). Binding of retinoids and b-carotene to b-1actoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta, 1079*, 316–320.
Granger, C., Barey, P., Toutain, J., & Cansell, M. (2005). Direct quantification of protein partitioning in oil-in-water emulsion by front-face fluorescence: Avoiding the need for centrifugation. *Colloids and Surfaces B, 43*, 158–162.
IUPAC (1997). Limit of detection in analysis. In A. D. McNaught & A. Wilkinson (Eds.), *IUPAC compendium of chemical terminology* (2nd ed.. Oxford: Blackwell Scientific Publications.
Li-Chan, E. C. Y. (1996). The applications of Raman spectroscopy in food science. *Trends in Food Science & Technology, 7*(November), 361–370.
Lopez-Sanchez, P., Schumm, S., Pudney, P. D. A, & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst, 136*(18), 3694–3697.
Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y., & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of b-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology, 9862*(3), 336–342.
McClements, D. J. (2015). Encapsulation, protection, and release of hydrophilic active components: Potential and limitations of colloidal delivery systems. *Advances in Colloid and Interface Science, 219*, 27–53.
McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrition, 51*(4), 285–330.

Oliver, C. M., & Augustin, M. A. (2009). Using dairy ingredients for encapsulation. In M. Corredig (Ed.), *Dairy derived ingredients – Food and nutraceutical uses*. Woodhead Pub.

Andrew, J. J., Browne, I. E., Hancewicz, T. M., & Millichope, A. J. (1998). Raman imaging of emulsion systems. *Applied Spectroscopy, 52*(6), 790–796.

Pudney, P. D. A., Melot, M., Caspers, P. J., Van Der Pol, A., & Puppels, G. J. (2007). An in vivo confocal Raman study of the delivery of trans-retinol to the skin. *Applied Spectroscopy, 61*(8), 804–811.

Svelander, C. A., Lopez-Sanchez, P., Pudney, P. D. A., Schumm, S., & Alminger, M. A. G. (2011). High pressure homogenization increases the in vitro bioaccessibility of α- and β-carotene in carrot emulsions but not of lycopene in tomato emulsions. *Journal of Food Science, 76*(9), H215–H225.

Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International, 42*(9), 1254–1258.

Wang, Y., & Spencer, P. (2002). Quantifying adhesive penetration in adhesive/dentin interface using confocal Raman microspectroscopy. *Journal of Biomedical Materials Research, 59*(1), 46–55.

Williams, K. P. J., Pitt, G. D., Batchelder, D. N., & Kip, B. J. (1994). Confocal Raman microspectroscopy using a stigmatic spectrograph and CCD detector. *Applied Spectroscopy, 48*(2), 232–235.

Ying, D., Cheng, L. J., Chibracq, G., Sanguansri, L., Oiseth, S. K., & Augustin, M. A. (2015). The format of β-carotene delivery affects its stability during extrusion. *LWT – Food Science and Technology, 60*(1), 1–7.

Zheng, H., Gordon, K. C., & Everett, D. W. (2013). Innovative application of confocal Raman microscopy to investigate the interaction between trans-2-hexenal and bovine milk fat globules. *International Dairy Journal, 32*(2), 68–70.

CHAPTER 6 STABILITY AND PARTITIONING OF β-CAROTENE IN WHEY PROTEIN EMULSIONS DURING STORAGE

Overview

In this chapter, the storage study of β-carotene in protein emulsions is presented. It is the extension of the previous experiments reported in Chapter 5. The partitioning and stability of the bioactive were analysed upon varying the β-carotene and WPI concentrations in the emulsion samples, throughout 30-day storage at different temperatures. The total β-carotene in the emulsions was extracted with a solvent and quantified using UV-Vis spectroscopy. The β-carotene in oil phase was measured *in situ* using CRM, while that in the aqueous phase was obtained by difference. The physical stability of the emulsion systems was also monitored throughout the study, which provides an informed basis for developing stable protein-based emulsions for the delivery of β-carotene.

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Stability and partitioning of β-carotene in whey protein emulsions during storage

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Varying the β-carotene (0.1–0.3 g kg^{-1}) and whey protein isolate (WPI) (2–20 g kg^{-1}) concentrations in an oil-in-water (O/W) emulsion influenced the partitioning and stability of β-carotene upon 30 d storage at 25 and 40 °C. The total β-carotene in the emulsion was extracted with a solvent and quantified using UV/visible spectroscopy. The β-carotene in oil phase was obtained using *in situ* Raman micro-spectroscopy. The β-carotene in the aqueous phase was obtained by difference. Increasing β-carotene concentration resulted in increased partitioning of β-carotene into the aqueous phase whereas increasing WPI concentration had the opposite effect. With all freshly made emulsions, there was a higher proportion of β-carotene found in the oil phase. At the end of the storage period, the higher proportion and concentration of β-carotene was in the aqueous phase. This suggested that oxidation of β-carotene occurred faster in the oil phase and that WPI in the aqueous phase protected β-carotene against oxidation. This work informs the formulation of protein-based emulsions for the delivery of β-carotene.

Introduction

The incorporation of bioactives and healthy nutrients into functional foods is a major sector in the food industry.[1–4] Many bioactives are unstable after being isolated from their natural source. The design of formulations which stabilise sensitive bioactives and enable their delivery to the desired site after ingestion has the potential to increase the health benefits of the added bioactives. Lipophilic bioactives such as carotenoids can be directly added to oils when the continuous phase of the food is oil (*e.g.* margarine and butter). However, when lipophilic bioactives need to be delivered into aqueous based foods or where water is the continuous phase in emulsion based foods, the lipophilic components are usually delivered in oil-in-water (O/W) based formulations[3] (*e.g.* homogenised milk and cream).

Carotenoids are prone to degradation, with isomerization, fragmentation and oxidation being some of the degradation pathways for carotenoids. Carotenoid degradation by oxidation is caused by the interaction with oxygen-active species, singlet oxygen, superoxides, peroxides, hydroxyl radicals, and transition metals; which are catalysed in the presence of heat and light.[5,6]

The low bioavailability of carotenoids from natural sources is related to their occurrence either as crystals or within protein complexes of fruits and vegetables, which are not bioavailable in the gastrointestinal tract after consumption.[6] There is interest in developing effective delivery systems that improve the bioavailability, and stability of carotenoids in foods.[7–9] Emulsion-based systems are suitable for delivering lipophilic bioactive components such as lycopene, astaxanthin, β-carotene, and lutein.[10–14]

For the manufacture of lipophilic bioactive containing emulsions, the lipophilic bioactives are solubilised into the oil phase before being added to the aqueous phase containing an emulsifier, and homogenised to form an oil-in-water (O/W) emulsion. The oil phase should remain liquid during the homogenisation process for efficient homogenisation. Many β-carotene enriched O/W emulsions are stabilised by small molecule surfactants (*e.g.* Tween 20 and decaglycerol monolaurate) or biopolymers (*e.g.* WPI and modified starch with emulsifying properties).[7–9,15] The lipophilic bioactive in an O/W emulsion is partitioned between the oil and aqueous phases of the emulsion system.[16]

The partitioning of the bioactive within an emulsion influences the protection afforded against degradation,[9] because the molecular environment influences the chemical stability of the lipophilic components.[16] The formulation of the emulsion (*i.e.* concentration and type of components) also affects the bioactive partitioning, and consequently its chemical stability. The surfactants at interface of the oil droplet in an O/W emulsion influence particle–particle aggregation and interaction of

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lipophilic bioactives in the oil droplet with reactive species in the surroundings. The extent of protection provided by the interfacial membrane depends on the chemical composition, the packing density as well as the thickness of the interfacial layer and the electrical charge on the particle surface.[9,17,18]

Many factors can compromise the stability of bioactive molecules within the emulsion, causing degradation and losses over time. The stability of lipophilic bioactives has often been assessed after the extraction with organic solvents, and quantification of the extracted bioactive using a spectrophotometric method.[8,12,15,19] In order to determine the partitioning of bioactive molecules and their stability in the oil and aqueous phases, the different phases may be separated by centrifugation prior to extraction of the bioactive from each phase. This is a laborious process. The advent and innovation of spectroscopy methods, particularly Raman micro-spectroscopy, has allowed for *in situ* examination for localisation of bioactives in food emulsions and biological samples.[20,21]

In general, molecules with π-electrons that are easily polarised will give stronger Raman scattering than those held tightly, *i.e.* σ-bonded. Raman spectra of β-carotene are dominated by three bands originating from vibrations of the isoprene units building the backbone of the molecule. The peaks are at 1524, 1157 and 1016 cm^{-1} (Fig. 1), representing the double bond C=C stretching mode, ν_s(C=C); the C–C in-plane single bond stretching mode, ν_s(C–C); and the C–H bending mode, ρ(C–CH_3) of β-carotene respectively. Raman micro-spectroscopy has been used to microscopically locate β-carotene in a food matrix in SEM samples.[21] Killeen *et al.* have used macro-Raman spectroscopy to develop a chemometric method to quantify carotenoids and polyacetylenes in carrot extracts.[22] The fact that most bioactive ingredients, including β-carotene, contain π-electrons results in the Raman signal in a complex matrix being dominated by the β-carotene bands, instead of a mixture of spectra from other ingredients of the emulsions. The isomers of β-carotene that can be produced at high temperatures also have completely different Raman signatures so their presence can be monitored simultaneously.[23] Hence, Raman micro-spectroscopy lends itself to the quantification of β-carotene in emulsion systems *in situ* as we have recently reported.[24]

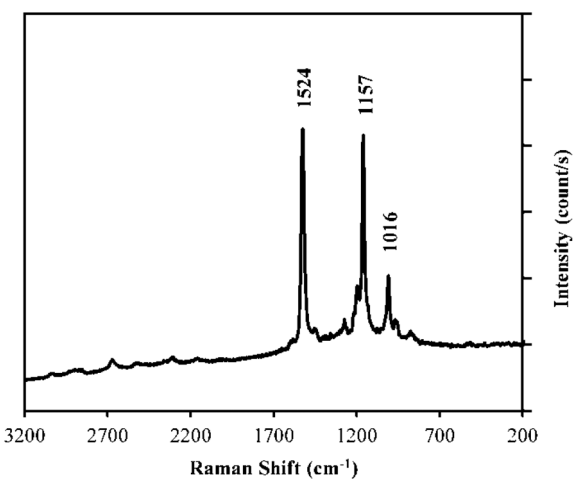

Fig. 1 Raman spectra of β-carotene with three significant peaks at 1524, 1157 and 1016 cm^{-1}, representing the double bond C=C stretching mode, ν_s(C=C); the C–C in-plane single bond stretching mode, ν_s(C–C); and the C–H bending mode, ρ(C–CH_3) of β-carotene.

In this study, β-carotene was incorporated into emulsions stabilised by whey protein isolate (WPI). Amphiphilic dairy proteins such as WPI and caseins are widely used as ingredients in emulsions, because they can easily adsorb at the O/W interface and stabilise the dispersed systems.[14,25,26] These food proteins are effective at reducing the oxidation rate of emulsified lipids, such as polyunsaturated oils.[15,17] Previous research has also shown that the binding of β-carotene to macromolecules such as proteins protects it against degradation. Hattori *et al.* found that β-lactoglobulin protects β-carotene from degradation by heating, oxidation and irradiation,[27] while Chang *et al.* concluded that β-carotene bound to bovine serum albumin is protected against photobleaching.[28] Liu *et al.* used β-lactoglobulin, sodium caseinate, lactalbumin and lactoferrin to stabilise β-carotene emulsions to explore the bioavailability of β-carotene using an *in vitro* release model.[29] We investigated the changes in partitioning of β-carotene molecules between the oil and aqueous phases of freshly made WPI emulsions, and throughout their storage at 25 or 40 °C. The physical and chemical stability of the β-carotene containing emulsions were determined.

Experimental

Materials

The β-carotene suspension in medium chain triglyceride (MCT) (30% natural β-carotene) was kindly donated by Cognis Australia Pty. Ltd (Melbourne, Victoria, Australia). The refined, bleached and deodorized (RBD) palm olein was provided by Wilmar Trading Pty. Ltd (Pasir Gudang, Johor, Malaysia). Whey protein isolate (WPI) (91% protein, 5% moisture, 3% ash, and 1% fat) was obtained from Fonterra (Darnum, Victoria, Australia). Sodium azide was from by Sigma-Aldrich (Castle Hill, NSW, Australia). Double-distilled water was used to prepare all solutions and emulsions.

The poly-L-lysine (0.1% (w/v) in water, with 0.01% of thimerosal added as preservative) for confocal Raman microscopy (CRM) analysis was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Chemicals used for β-carotene extraction purchased from Merck Pty. Ltd (Kilsyth, Victoria, Australia) were dimethyl sulfoxide (DMSO), *n*-hexane, and ethanol. Butylated hydroxytoluene (BHT) used as an anti-oxidant, was from Sigma-Aldrich (Castle Hill, NSW, Australia). All the chemicals and reagents used were of analytical grade.

Preparation of β-carotene O/W emulsions

Anhydrous WPI powder was dispersed in double distilled water by constant stirring of a mixer in a water bath at 50 °C

for 1 hour to obtain the aqueous phase. β-Carotene was dissolved in refined bleached and deodorised palm olein by magnetic stirring for approximately 30 min at 140 °C in the dark, giving a homogeneous particle-free lipid phase. Coarse pre-emulsions were formed by mixing the aqueous (90% (w/w)) and lipid (10% (w/w)) phases with a high-speed rotor-stator mixer (L2R, Silverson Machines Ltd, Chesham, UK) at a constant speed for 2 min at ambient temperature (~22 °C). The pre-emulsions containing 0.1, 0.2 or 0.3 g kg^{-1} β-carotene at a constant 20 g kg^{-1} WPI, and 2, 10 or 20 g kg^{-1} WPI at a constant 0.2 g kg^{-1} β-carotene were then homogenized twice through a high-pressure single-pass valve homogenizer (EmulsiFlex, Avestin Inc., Ottawa, Canada) at 500/100 bar, cooled to room temperature and ~0.02% (w/w) sodium azide was added as a preservative.

The freshly prepared emulsions were immediately transferred into 1.5 mL screw-capped amber glass vials. Each system was analysed for the particle size and β-carotene concentration within one day after preparation. Samples were wrapped in Al foil and stored in a 25 °C incubator and a 40 °C oven for 30 d. Aliquots were subsequently removed periodically for the same analyses.

Particle size analysis

Characterisation of oil droplets in the emulsions was carried out by measuring the particle size distribution and the average particle diameter using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). The emulsions were sampled and added to recirculating water in the measuring cell (GydroSM, Worcestershire, UK), until more than 10% of the incident light was absorbed. The refractive index of the emulsion droplets chosen was 1.456, while the refractive index of the dispersion medium was 1.33. The absorbance value of the emulsion droplets was 0.1. Droplet size measurements are reported as particle size distribution and the surface-weighted, or Sauter mean diameter, $D[3,2]$.

β-Carotene content

β-Carotene content of the whole emulsion. The concentration of β-carotene in the whole emulsions was determined using the method proposed by Ying *et al.* with some modifications.[30]

Briefly, the emulsion containing β-carotene (~200 mg) was accurately weighed into a 30 mL glass tube, before 4 mL DMSO was added and the mixture shaken. The tube was placed in a water bath at 75 °C for 5 min to disperse the sample, and then cooled to room temperature (~22 °C) for extraction. Four mL *n*-hexane containing 0.1% (w/v) BHT was added to the tube and the contents were vortexed for 10 s and left for 30 min. A few drops of 100% ethanol were added to precipitate the protein in the *n*-hexane phase. The top layer formed was carefully removed using a glass Pasteur pipette, and transferred into a 10 mL volumetric flask. The extraction procedures were repeated twice more, each using 3 mL of *n*-hexane. The extracts were combined in the flask, and made up to the 10 mL mark with *n*-hexane. This extract was then analyzed using a Shimadzu UV-Vis UV-1700 spectrophotometer (Rowville, Victoria, Australia) at the maximum absorbance wavelength of β-carotene, λ_{max} = 450 nm. The β-carotene concentration in the solution was calculated against a standard curve, which was obtained using known concentrations of β-carotene (0 to 1 mg per 100 mL) in *n*-hexane. Separate spiking experiments showed that the extraction method recovered 100 ± 2% of the β-carotene.

The loss of β-carotene in the whole emulsion over storage was calculated $[(C/C_0) \times 100\%]$ (where C_0 is the initial concentration of β-carotene and C is the concentration of β-carotene at each time point) and gives the residual β-carotene over time.

Partitioning of β-carotene content between oil and aqueous phase. The concentrations of β-carotene in the oil phase were determined from the univariate analysis of Raman data, while those in the aqueous phase were calculated by difference from the spectrophotometric data of β-carotene concentrations in whole emulsion and its corresponding concentrations in the oil phase from Raman analysis.

β-Carotene content of oil phase. To quantify β-carotene concentrations in the oil phase and determine its partitioning characteristic within the emulsions, each sample was analysed *in situ* with a confocal WITec alpha300 R (WITec, Ulm, Germany) Raman microspectrometer equipped with a thermo-electrically (Peltier) cooled electron multiplier charge coupled device (EMCCD) detector, through a 50× objective lens. The point-microspectroscopic mode was applied; using the single-measurement function with 0.5 s integration time for 10 scans under 0.5 mW green (532 nm) laser.

A calibration curve (concentration *vs.* Raman intensity of β-carotene band at 1524 cm^{-1}) was first plotted based on the β-carotene-in-oil samples prepared by the same procedures for solubilizing the β-carotene in the palm olein used to make the dispersed phase of an emulsion.

A drop of poly-L-lysine was dried on a slide before mounting a drop of emulsion sample on it, and covering with a coverslip. The poly-L-lysine layer acted to keep the emulsion droplet precisely located. A point was microscopically focused within an oil droplet (the dispersed phase) containing the β-carotene. The Raman signal of the focal point was retrieved, from which the intensity of β-carotene band at 1524 cm^{-1} was recorded to determine the corresponding β-carotene concentration using the calibration curve univariately. The measurement was done in duplicate for every formulation, in which at least five droplets with a consistent intensity value, were chosen for documentation.

β-Carotene content of aqueous phase. The concentrations of β-carotene in the aqueous phase were calculated by difference from the spectrophotometric data of β-carotene concentrations in the whole emulsion and its corresponding concentrations in the lipid phase from Raman analysis. This method was previously validated.[24]

Statistical analysis

Construction of the univariate regression models for calibration was performed in Microsoft Excel by exporting the

spectral data as ASCII files from the spectroscopies. The highest peak intensity at 450 nm wavenumber from the spectrophotometer data and the integrated area (sum of all the intensities) of the 1524 cm^{-1} band from the Raman data, were plotted separately against the known concentrations of β-carotene in olein. A linear regression model of known concentration of β-carotene in olein *vs.* spectral intensity was calculated. The equation of the regression line was determined along with regression correlation values. Using this equation, the corresponding ratios of bands from emulsion spectra could be applied to predict the concentration of β-carotene within the oil droplets. These procedures were adapted from the work by Whelan *et al.*[31]

The experimental data were analyzed by two-way analysis of variance (ANOVA) in VassarStats website (http://vassarstats.net/), where $p < 0.05$ was considered to be statistically significant. Significant differences of means between the different types of sample were further determined by the Fisher's Least Significant Difference (LSD) test.

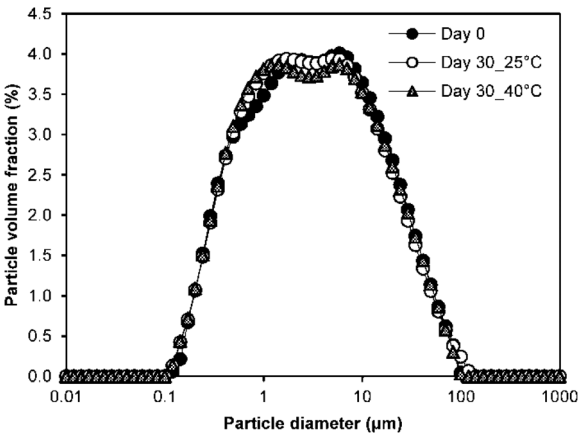

Fig. 2 Particle size distribution of β-carotene enriched emulsion stabilised by the least concentration of WPI, measured after 0 and 30 d storage at 25 and 40 °C (2 g kg^{-1} WPI, 0.2 g kg^{-1} β-carotene).

There were two replicates of every formulated emulsion system. Each replicate was divided into two aliquots for all analyses, giving a duplicate measurement at each time point. All data are reported as mean ± standard deviation.

Results and discussion

Particle size distribution and physical stability

A bimodal particle size distribution (peaks at ~0.2 and 1.0 μm) was obtained immediately after homogenisation regardless of the β-carotene and WPI concentrations (Fig. 2). Table 1 shows that freshly made emulsions containing 0.1, 0.2 or 0.3 g kg^{-1} β-carotene had mean droplet size of 1.05 ± 0.01, 1.04 ± 0.01 and 1.01 ± 0.01 μm respectively, showing a trend towards smaller particle size with increasing β-carotene concentration in emulsions at a fixed WPI concentration (20 g kg^{-1}). This may be due to the β-carotene acting as a co-surfactant in the emulsified systems, which lowered the interfacial tension between the phases and further stabilised the emulsion.[19,32] On the other hand, the mean particle size in emulsions containing 2, 10 and 20 g kg^{-1} WPI with the same β-carotene concentration (0.2 g kg^{-1}) were 1.22 ± 0.02, 1.07 ± 0.01 and 1.04 ± 0.01 μm respectively. Even at the lowest concentration of WPI (2 g kg^{-1}), there was sufficient protein to stabilise the oil droplets. At the higher concentrations, the protein possibly has a more compact conformation at the surface, and excess protein chains could extend into the continuous phase, preventing flocculation by steric and electrostatic repulsion.[33]

The mean particle radius and particle size distribution did not change appreciably after the emulsions were stored at 25 and 40 °C for 30 d, indicating that they were stable to particle aggregation under these conditions (Fig. 2). This can be associated with the high activation energy resulting from electrostatic repulsion between the emulsion particles. At neutral pH, oil droplets coated by whey proteins are negatively charged, promoting electrostatic repulsion between the droplets.[15] It is expected that there would be some macromolecular rearrange-

Table 1 Effects of varying β-carotene (BC) and WPI concentrations on the droplet size, *D*[3,2] and percentage loss of β-carotene in the whole emulsions[a] upon 30 d storage at 25 and 40 °C. Data are mean ± standard deviations ($n = 4$)

T (°C)	[BC][b] (g kg^{-1})	[WPI][c] (g kg^{-1})	D[3.2] initial (μm)	D[3,2] final (μm)	[BC] initial (g kg^{-1})	[BC] final (g kg^{-1})	[BC] Loss (%)
25	0.1	20	1.05 ± 0.03^{A}	1.08 ± 0.03^{A}	0.099 ± 0.005^{D}	0.076 ± 0.002^{G}	23.1
	0.2	20	1.04 ± 0.01^{A}	1.08 ± 0.01^{A}	0.197 ± 0.001^{E}	0.153 ± 0.001^{H}	22.6
	0.3	20	1.01 ± 0.01^{B}	1.02 ± 0.03^{B}	0.295 ± 0.001^{F}	0.234 ± 0.002^{I}	20.8
	0.2	2	1.22 ± 0.02^{C}	1.23 ± 0.02^{C}	0.197 ± 0.002^{E}	0.140 ± 0.002^{J}	28.8
	0.2	10	1.07 ± 0.01^{A}	1.08 ± 0.01^{A}	0.198 ± 0.002^{E}	0.159 ± 0.001^{K}	20.0
40	0.1	20	1.05 ± 0.01^{A}	1.10 ± 0.02^{A}	0.099 ± 0.005^{D}	0.052 ± 0.001^{L}	47.8
	0.2	20	1.04 ± 0.01^{A}	1.07 ± 0.01^{A}	0.197 ± 0.001^{E}	0.096 ± 0.001^{D}	51.6
	0.3	20	1.01 ± 0.01^{B}	1.03 ± 0.02^{B}	0.295 ± 0.001^{F}	0.156 ± 0.002^{M}	47.2
	0.2	2	1.22 ± 0.02^{C}	1.24 ± 0.02^{C}	0.197 ± 0.002^{E}	0.017 ± 0.001^{N}	91.6
	0.2	10	1.07 ± 0.01^{A}	1.09 ± 0.01^{A}	0.198 ± 0.002^{E}	0.081 ± 0.001^{O}	59.1

[A–C] *D*[3,2] values, data followed by different capital superscript letters are significantly different ($p < 0.05$). [D–O] [BC] values, data followed by different capital superscript letters are significantly different ($p < 0.05$). [a] The emulsions were prepared at room temperature (~22 °C) and homogenisation pressure of 500/100 bar. [b] [BC], concentration of β-carotene in emulsion. [c] [WPI], concentration of WPI in emulsion.

ment and partial unfolding of structured globular whey proteins upon adsorption at the O/W interface. The partial denaturation of the structure reveals the nonpolar groups, thiol and disulfide groups, which increases protein–protein hydrophobic interactions and cross-linking (*via* intermolecular disulfide bonding), causing polymerization of the adsorbed protein layer. The interfacial layer isolates the bioactive in the oil phase from the other components in the emulsion.[8]

Particle size affects the degradation rate of the bioactive compounds, but there are different effects reported in the literature. For example, increasing droplet size was found to increase the degradation rate of β-carotene and α-tocopherol in emulsions.[9,34] In contrast, it was observed that the oxidation of docosahexaenoic acid increases in emulsion systems with a small particle size.[35]

Partitioning of β-carotene in freshly made emulsions

The intensity of the strong 1524 cm^{-1} peak of β-carotene was used in the univariate analysis for determining the concentration of β-carotene within the oil droplets. From the results of integrating the area under the selected Raman peak for all samples, the calculated percentages of β-carotene in the oil droplets represented in Fig. 3(a) show that increasing the β-carotene concentration results in a decrease of the β-carotene percentage concentration in oil droplets. This is consistent with the results of our previous study,[24] which confirms a trend of increasing β-carotene partitioning (%) in the aqueous phase of emulsion with increasing total β-carotene in the whole emulsion. As the concentration of total β-carotene in the whole emulsion is changed, there is a new equilibrium established in the partitioning of the β-carotene between the various phases of the emulsion. It is known that β-carotene binds to the β-lactoglobulin, the major component of WPI in the aqueous phase of the emulsion samples.[36,37] Therefore, with the higher the concentration of β-carotene molecules in the system, there is an expected increase in β-carotene partitioned into the aqueous phase due to the increased binding of β-carotene to WPI at higher β-carotene concentrations.

Fig. 3(b) in contrast illustrates the increase in β-carotene concentration in oil droplets with increasing WPI concentration. The partitioning of a bioactive between the various phases of emulsions is governed by (i) the relative solubility of the bioactive in the oil and aqueous phases of the emulsion, (ii) its affinity to bind to components in the emulsions and (iii) its propensity to assemble at the interface. It might have been expected that an increased concentration of WPI will increase the availability of sites for β-carotene adsorption if all other factors remained constant. Hence the observation that less β-carotene is partitioned into the aqueous phase is counter to expectations. It is possible that there was incomplete hydration at high WPI concentrations or changes in affinity between protein binding sites and β-carotene, resulting in reduced affinity in systems with higher protein concentrations. This could be because there is a change in the protein conformation or interaction of the proteins, whether at the interface or in the aqueous phase. Additionally, the density of packing and the thickness of the interfacial layer that regulates diffusion of molecules through the interface, may also be a contributory factor.[6]

β-Carotene stability in whole emulsions at different temperatures

As expected, emulsion formulations stored at 25 °C had higher β-carotene stability than those at 40 °C (Table 1). The β-carotene concentration was reduced by 20–29% after storage at 25 °C, and generally by 44–92% at 40 °C after 30 d, depending on the formulation. Previous studies have also found a rapid loss of β-carotene in emulsions stored at elevated temperatures due to its thermal-sensitive nature.[15,38]

The total β-carotene degradation was largely independent of concentration at fixed WPI concentration, being 20.8–23.1% and 47.2–51.6% for emulsions containing 20 g kg^{-1} WPI stored at 25 or 40 °C, respectively (Table 1, Fig. 4). Varying the WPI concentration at a fixed β-carotene concentration

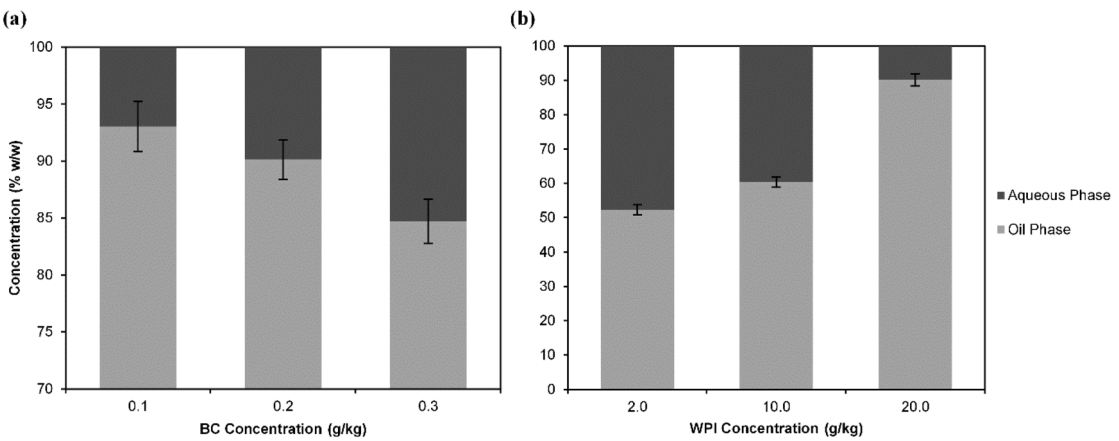

Fig. 3 Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with: (a) different β-carotene concentrations (0.1, 0.2 and 0.3 g kg^{-1}) at 20 g kg^{-1} WPI, and (b) different WPI concentrations (2, 10 and 20 g kg^{-1}) at 0.2 g kg^{-1} β-carotene. Data are mean ± standard deviations (n = 4).

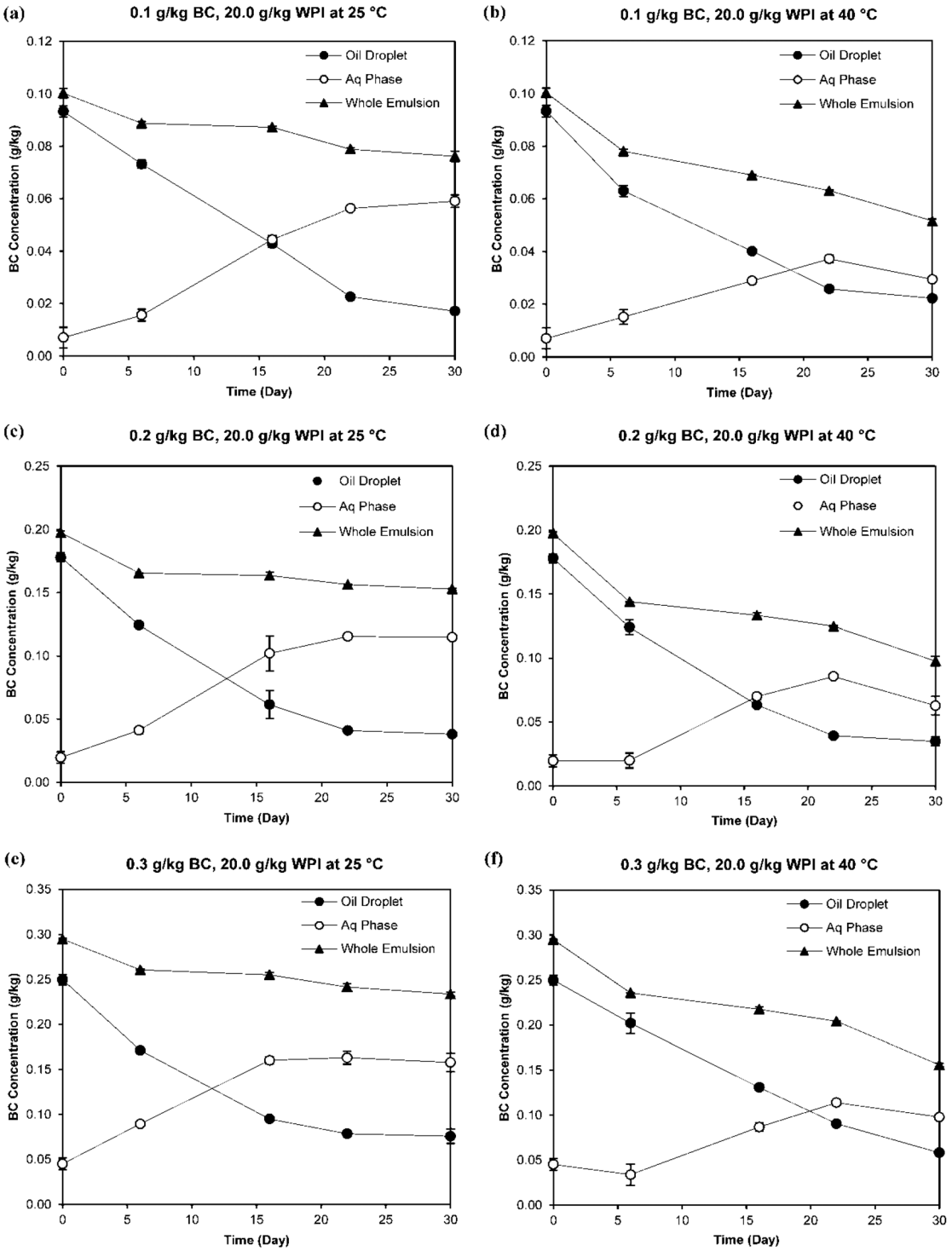

Fig. 4 Degradation of β-carotene (BC) in whole emulsions and its partitioning between the oil and aqueous phases of emulsions with different β-carotene concentrations (0.1, 0.2 and 0.3 g kg^{-1}), during 30 d storage at (a, c, e) 25 °C and (b, d, f) 40 °C. Data are mean ± standard deviations (n = 4).

(0.2 g kg^{-1}) had a larger effect. For formulations containing 0.2 g kg^{-1} β-carotene, lowering the WPI concentration generally resulted in greater β-carotene degradation at each of the storage temperatures, with the highest loss across all formulations being 91.6% for the emulsion containing the lowest WPI concentration (2 g kg^{-1}) at 40 °C (Table 1, Fig. 5). Others have also found a decrease in secondary lipid oxidation in O/W emulsions with increased WPI concentration.[39]

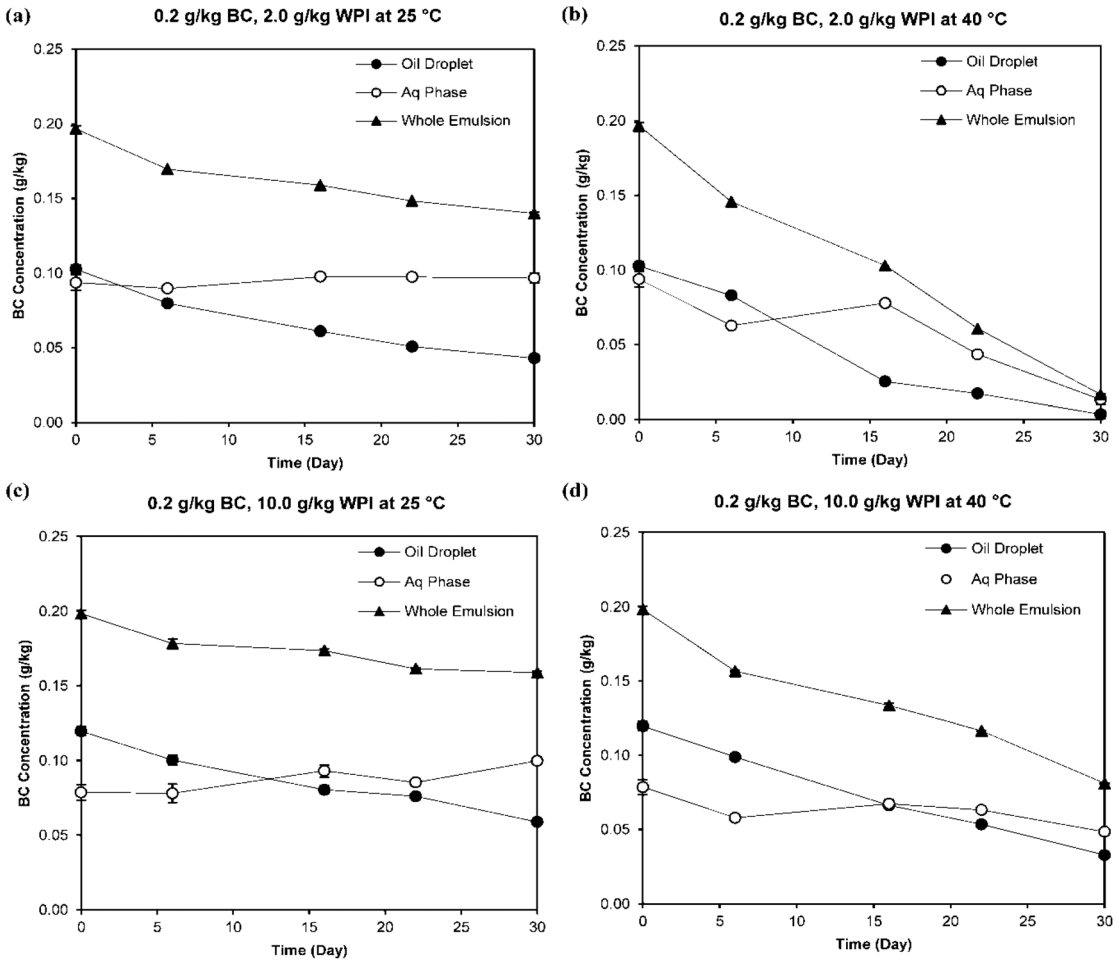

Fig. 5 Degradation of β-carotene (BC) in whole emulsions and its partitioning between the oil and aqueous phases of emulsions with different WPI concentrations (2 and 10 g kg^{-1}), during 30 d storage at (a, c) 25 °C and (b, d) 40 °C. Data are mean ± standard deviations (n = 4).

Increasing WPI concentration promotes the formation of a thicker barrier and an increased scavenging action against oxidative species. Adsorbed protein may reduce the accessibility of metal ions or other pro-oxidants to reactive unsaturated fatty acids and β-carotene within the emulsion.[33] Liu, Chen and Mao reported that β-lactoglobulin shows a strong antioxidant activity, enabling the WPI to buffer the oxidising capacity of the system beyond a critical concentration.[40]

Partitioning of β-carotene upon storage at different temperatures

Fig. 4 and 5 depict the partitioning of β-carotene between the aqueous and lipid phases of emulsions stabilised by WPI during 30 d at 25 and 40 °C.

In freshly made solutions, β-carotene was predominantly located in the oil phase (Fig. 3) but by the end of the storage period, there was a higher proportion of β-carotene in the aqueous phase (Fig. 4 & 5). This trend in partitioning of undegraded β-carotene was more pronounced in emulsions containing 0.1–0.3 g kg^{-1} β-carotene stabilised by the highest concentration of WPI (20 g kg^{-1}) stored at both 25 and 40 °C (Fig. 4) compared to those with 0.2 g kg^{-1} β-carotene stabilised by lower levels of WPI (2 and 10 g kg^{-1}) (Fig. 5). While the absolute concentration of β-carotene in the total emulsion decreased over time for all emulsions, the concentration of β-carotene in the aqueous phase increased by the end of the storage period for emulsions containing the highest concentration of WPI (20.0 g kg^{-1}) (Fig. 4) but either slightly decreased or did not change over time for emulsions stabilised by lower concentrations of WPI (2 or 10 g kg^{-1}) (Fig. 5). The results suggest that β-carotene was less prone to degradation in the aqueous phase compared to when it was present in the lipid phase.

The trend of increasing absolute concentration of β-carotene in the aqueous phase (Fig. 4) was counter to expectations, suggesting a preferential re-partitioning of β-carotene into the aqueous phase over time. One possible explanation is that when a high concentration of WPI (20 g kg^{-1}) was used in the formulation, there may have been incomplete hydration of WPI in freshly prepared solutions with the dispersion procedure used. As WPI becomes more fully hydrated, it becomes more functional, driving an increased interaction between

β-carotene and the protein. When bound to protein, the β-carotene is stabilised.

Conclusions

This study has shown that β-carotene can be effectively delivered within food-grade emulsions stabilised by WPI. Varying the β-carotene concentration at fixed WPI concentration had little effect on particle size or β-carotene stability. However, increasing WPI at fixed β-carotene concentration reduced the lipid droplet size and improved β-carotene stability in the whole emulsion. With all freshly made emulsions: (i) a higher proportion of β-carotene was found in the oil phase, (ii) increasing β-carotene concentration resulted in increased partitioning of β-carotene into the aqueous phase whereas increasing WPI concentration had the opposite effect. At the end of the storage period, the higher proportion and concentration of β-carotene was in the aqueous phase, suggesting that β-carotene was stabilised by the WPI in the aqueous phase. The results show the usefulness of *in situ* Raman microspectroscopy for quantitative analysis and are useful for informing the design of effective emulsion delivery systems to encapsulate and stabilise β-carotene for application within food (*e.g.* healthy oils, spreads, sauces and dips), beverage (*e.g.* multivitamin drinks, dairy beverages and vitamin-enriched juices), and pharmaceutical products (*e.g.* supplements, creams and ointments).

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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References

1 L. Galland, in *Encyclopedia of Human Nutrition*, ed. B. Caballero, Elsevier Ltd., 2005, pp. 360–366.
2 D. P. Richardson, *Food Sci. Technol. Bull. Funct. Foods*, 2009, **6**, 1–3.
3 L. Sanguansri and M. A. Augustin, in *Functional Food Product Development*, ed. J. Smith and E. Charter, Wiley-Blackwell Publishing Ltd., 2010, pp. 3–23.
4 C. I. Onwulata, *J. Food Process. Preserv.*, 2013, **37**, 510–532.
5 G. Britton, *FASEB J.*, 1995, **9**, 1551–1558.
6 C. S. Boon, D. J. McClements, J. Weiss and E. A. Decker, *Crit. Rev. Food Sci. Nutr.*, 2010, **50**, 515–532.
7 L. Mao, J. Yang, D. Xu, F. Yuan and Y. Gao, *J. Dispersion Sci. Technol.*, 2010, **31**, 986–993.
8 L. Cornacchia and Y. H. Roos, *J. Agric. Food Chem.*, 2011, **59**, 7013–7020.
9 L. Cornacchia and Y. H. Roos, *J. Food Sci.*, 2011, **76**, C1211–C1218.
10 H. S. Ribeiro, K. Ax and H. Schubert, *J. Food Sci.*, 2003, **68**, 2730–2734.
11 K. Ax, E. Mayer-Miebach, B. Link, H. Schuchmann and H. Schubert, *Eng. Life Sci.*, 2003, **3**, 199–201.
12 Y. Yuan, Y. Gao, J. Zhao and L. Mao, *Food Res. Int.*, 2008, **41**, 61–68.
13 J. Losso, A. Khachatryan, M. Ogawa, J. Godber and F. Shih, *Food Chem.*, 2005, **92**, 737–744.
14 D. J. McClements, E. A. Decker and J. Weiss, *J. Food Sci.*, 2007, **72**, R109–R124.
15 C. Qian, E. A. Decker, H. Xiao and D. J. McClements, *Food Chem.*, 2012, **132**, 1221–1229.
16 D. J. McClements, *Adv. Colloid Interface Sci.*, 2012, **174**, 1–30.
17 M. A. Augustin and L. Sanguansri, in *Oxidation in Foods and Beverages and Antioxidant Applications*, ed. A. D. Eric, R. J. Elias and D. J. McClements, Woodhead Publishing Limited, United Kingdom, 2010, pp. 479–495.
18 U. Yucel, R. J. Elias and J. N. Coupland, in *Food and Industrial Bioproducts and Bioprocessing*, ed. N. T. Dunford, John Wiley and Sons, Inc., 2012, pp. 167–184.
19 A. Trentin, S. De Lamo, C. Güell, F. López and M. Ferrando, *J. Food Eng.*, 2011, **106**, 267–274.
20 J. R. Beattie, C. Maguire, S. Gilchrist, L. J. Barrett, C. E. Cross, F. Possmayer, M. Ennis, J. S. Elborn, W. J. Curry, J. J. McGarvey and B. C. Schock, *FASEB J.*, 2007, **21**, 766–776.
21 P. Lopez-Sanchez, S. Schumm, P. D. a. Pudney and J. Hazekamp, *Analyst*, 2011, **136**, 3694–3697.
22 D. P. Killeen, C. E. Sansom, R. E. Lill, J. R. Eason, K. C. Gordon and N. B. Perry, *J. Agric. Food Chem.*, 2013, **61**, 2701–2708.
23 M. Tasumi, *J. Raman Spectrosc.*, 1988, **19**, 37–49.
24 W. A. F. Wan Mohamad, R. Buckow, M. Augustin and D. McNaughton, *Food Chem.*, 2017, **233**, 197–203.
25 C. M. Oliver and M. A. Augustin, in *Dairy Derived Ingredients- Food and Nutraceutical Uses*, ed. M. Corredig, Woodhead Publishing Limited, United Kingdom, 2009, pp. 565–588.
26 P. J. Wilde, in *Dairy Derived Ingredients- Food and Nutraceutical Uses*, ed. M. Corredig, Woodhead Publishing Limited, United Kingdom, 2009, pp. 539–564.
27 M. Hattori, A. Watabe and K. Takahashi, *Biosci., Biotechnol., Biochem.*, 1995, **59**, 2295–2297.
28 H. Chang, H. Cheng, R. Han, J. Zhang and L. H. Skibsted, *J. Agric. Food Chem.*, 2016, **64**, 5951–5957.
29 Y. Liu, F. Lei, F. Yuan and Y. Gao, *Food Funct.*, 2014, **5**, 2940–2947.

30 D. Ying, L. J. Cheng, G. Chibracq, L. Sanguansri, S. K. Oiseth and M. A. Augustin, *LWT–Food Sci. Technol.*, 2015, **60**, 1–7.
31 D. R. Whelan, K. R. Bambery, L. Puskar, D. McNaughton and B. R. Wood, *J. Biophotonics*, 2013, **6**, 775–784.
32 H. Salminen, C. Gömmel, B. H. Leuenberger and J. Weiss, *Food Chem.*, 2016, **190**, 928–937.
33 J. Yi, Y. Li, F. Zhong and W. Yokoyama, *Food Hydrocolloids*, 2014, **35**, 19–27.
34 P. Relkin, J.-M. Yung, D. Kalnin and M. Ollivon, *Food Biophys.*, 2008, **3**, 163–168.
35 S. Gohtani, M. Sirendi, N. Yamamoto, K. Kajikawa and Y. Yamano, *J. Dispersion Sci. Technol.*, 1999, **20**, 1319–1325.
36 E. Dufour and T. Haertlé, *Biochim. Biophys. Acta*, 1991, **1079**, 316–320.
37 H. Wackerbarth, T. Stoll, S. Gebken, C. Pelters and U. Bindrich, *Food Res. Int.*, 2009, **42**, 1254–1258.
38 L. Mao, D. Xu, J. Yang, F. Yuan, Y. Gao and J. Zhao, *Food Technol. Biotechnol.*, 2009, **9862**, 336–342.
39 C. Sun and S. Gunasekaran, *Food Hydrocolloids*, 2009, **23**, 165–174.
40 H. C. Liu, W. L. Chen and S. J. T. Mao, *J. Dairy Sci.*, 2007, **90**, 547–555.

CHAPTER 7 PARTITIONING CHARACTERISTICS OF β-CAROTENE IN EMULSIONS OF VARIOUS FORMULATIONS AND TREATMENTS

Overview

In Chapter 7, *in situ* investigations using CRM for the partitioning characteristics of bioactive molecules in emulsions stabilised by native (untreated) WPI, heat-treated WPI or high pressure-treated WPI prior to emulsion formation are presented. The partitioning of β-carotene within the emulsions stabilised by a low weight emulsifier (Tween 40) is also reported along with the effects of varying the solid fat content of the dispersed phase with different ratios of palm olein (the liquid fraction of palm oil) and palm stearin (the solid fraction of palm oil).

Manuscript III entitled "**Characterisation of β-carotene partitioning in protein emulsions: Effects of pre-treatments, solid fat content and emulsifier type**" by W. A. Fahmi Wan Mohamad, Don McNaughton, Mary Ann Augustin and Roman Buckow was submitted to the journal Food Chemistry (FOODCHEM-D-17-05611) on 6 October 2017.

Note: At the time of submission, Manuscript III was under review but has now been published in the journal Food Chemistry volume 257, page 361-367, since 9 March 2018. The published version of Manuscript III is presented at the end of this chapter.

In addition, the variation in partitioning of β-carotene molecules between the phases of emulsions was also examined upon pasteurising the emulsions using the high-temperature-short-time (HTST) method, emulating industrial practices. However the results were not incorporated in the submitted manuscript due to insignificant characteristic differences between the untreated and the pasteurised samples. The materials and preparation method of samples are as described in Manuscript III. The analytical methods and subsequent results are nevertheless presented as follows.

The thermal treatment was carried out for the emulsion sample stabilised by 20 g/kg of untreated WPI, at a set temperature of 70 °C for 1 min. Figure 7.1 (c) shows the temperature-time profile for the HTST treatment, together with the profiles for other heat treatment processes reported in Manuscript III.

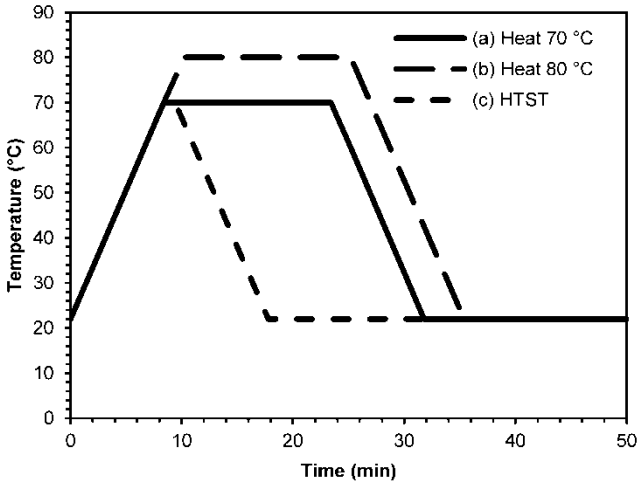

Figure 7.1: Individual temperature-time profiles for heat treatment processes of WPI solutions at (a) 70 °C for 15 min, and (b) 80 °C for 15 min; and of WPI emulsion at (c) 70 °C for 1 min (HTST).

Measuring the average particle diameter using a laser diffraction particle analyser, Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK), it was found that the mean droplet size did not significantly change when heat treatment was applied after emulsion formation (Table 7.1). During homogenisation, the protein probably became partially unfolded at the interface, and subsequent heat treatment caused no further conformational change (Galazka et al., 2000).

Table 7.1: The difference in mean droplet size of heat-treated (HTST) and untreated emulsions. Data are mean ± standard deviations (*n* = 4).

Type of Emulsifier	[BC][a] (g/kg)	[Emulsifier][b] (g/kg)	Olein : Stearin Ratio	Droplet Size, D[3,2] (μm)	Droplet Size, D[4,3] (μm)
WPI (HTST)[c]	0.2	20	100 : 0	0.43 ± 0.02^{A}	1.08 ± 0.02^{H}
WPI	0.2	20	100 : 0	0.41 ± 0.01^{A}	1.05 ± 0.03^{H}

[a] [BC], concentration of β-carotene.
[b] [WPI], concentration of WPI.
[c] The whole emulsion was treated by high-temperature-short-time (HTST) method at 70 ºC for 1 min.

Upon integrating the area under the 1524 cm^{-1} peak of Raman spectra, it is shown in Figure 7.2 that the partitioning characteristic of β-carotene between the oil and aqueous phases of the emulsion with untreated WPI is similar to that treated under the HTST method. Again the treatment upon emulsification and homogenisation did not result in subsequent structural

changes in the protein conformation (Galazka et al., 2000), thus causing no further partitioning of β-carotene molecules into the aqueous phase. These results are useful to the extent that they demonstrate that pasteurisation of the emulsion does not alter the emulsion properties.

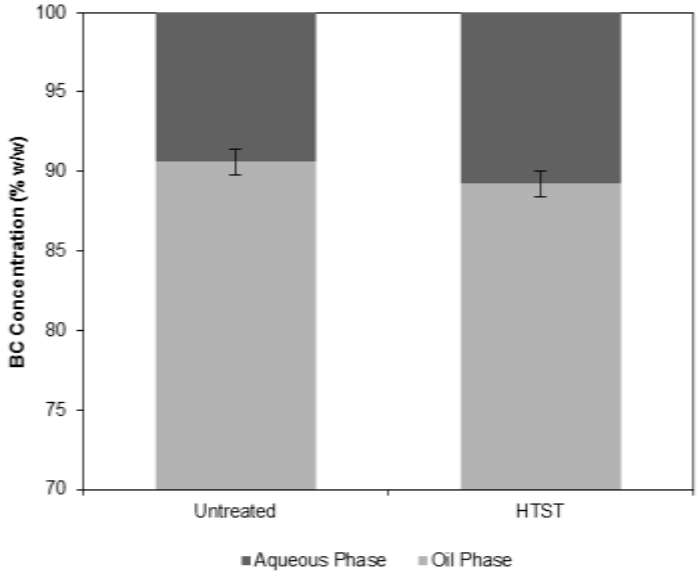

Figure 7.2: Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with: (a) untreated WPI and (b) HTST treatment at 70 °C for 1 min. Both samples contained the same concentrations of palm olein, emulsifier and β-carotene at 100 g/kg, 20 g/kg and 0.2 g/kg respectively. Data are mean ± standard deviations (*n* = 4).

CHARACTERISATION OF β-CAROTENE PARTITIONING IN PROTEIN EMULSIONS: EFFECTS OF PRE-TREATMENTS, SOLID FAT CONTENT AND EMULSIFIER TYPE

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Abstract

Understanding the bioactive partitioning between the phases of an emulsion system underpins strategies for improving the efficiency of bioactive protection against degradation. We analysed *in-situ* partitioning of β-carotene in emulsions with various formulations using confocal Raman microscopy (CRM). The partitioning of β-carotene into the aqueous phase of emulsions increased when whey protein isolate (WPI) was heat- or high pressure-treated prior to emulsion formation. However, increasing the concentration of high pressure-treated WPI reduced the β-carotene partitioning into the aqueous phase. Increasing the solid fat content in the carrier oil favoured the migration of β-carotene into the aqueous phase. The use of WPI as the emulsifier resulted in a greater partitioning of β-carotene into the aqueous phase compared to when Tween 40 was the emulsifier. This study demonstrates that partitioning of β-carotene between the aqueous and oil phase is dependent on the characteristics of the oil phase, emulsifier type and processing.

Keywords:

Emulsion; Raman microscopy; partitioning; β-carotene; heat treatment; high pressure processing

1. Introduction

Carotenoids are natural pigments mainly found in fruits and vegetables. They are commonly categorised into two groups: (i) carotenes comprised entirely of carbon and hydrogen (e.g. α-carotene, β-carotene, and lycopene) and (ii) xanthophylls comprised of carbon, hydrogen, and oxygen (e.g. lutein and zeaxanthin) (Qian et al., 2012b). The most significant pro-vitamin A carotenoid used in foods and beverages is β-carotene (Boon, McClements, Weiss, & Decker, 2010; Rao & Rao, 2007). Epidemiological studies suggest that regular consumption of β-carotene in foods may lower the risk of certain chronic illnesses such as macular degeneration, cataracts, cardiovascular disease as well as some cancers (Maiani et al., 2009; Miller & Snyder, 2012).

A lipophilic bioactive such as β-carotene is often delivered using emulsions (McClements, Decker, & Weiss, 2007). Carotenoid-enriched, emulsion-based delivery systems can be produced by dissolving the β-carotene within carrier oil, and then homogenising this oil phase with an aqueous phase containing a water-soluble emulsifier such as small molecule surfactants (e.g. Tween 20 and decaglycerol monolaurate) or biopolymers (e.g. WPI and modified starch with emulsifying properties) (Cornacchia & Roos, 2011, 2011; Mao et al., 2010; Qian et al., 2012b). The effects of droplet size, carrier oil type and emulsifier type on the chemical stability and bioavailability of β-carotene have been examined (McClements & Rao, 2011), but less attention has been paid to the partitioning of the bioactive within the system. β-carotene is likely to be distributed and partitioned between the oil and aqueous phases of the emulsion system (McClements, 2012). The compartmentalisation of the bioactive within the system might be critical since the protection efficacy against degradation and bioavailability may be dependent on it (Cornacchia & Roos, 2011b).

The ingredients that are used in an emulsion with the bioactive are expected to influence the partitioning of a bioactive in an emulsion. With respect to emulsifiers, both low (e.g. Tweens) and high molecular weight emulsifiers (e.g. proteins) may be used in the formulation of emulsions. The use of these different emulsifiers results in emulsions with different interfacial structures (Mao et al., 2009). In addition, when protein is used it is possible to modify the whey protein structure through processing, which will influence its functional properties. For example, factors such as pH and temperature influence whey protein conformation, which in turn changes binding characteristics of these proteins to small molecules (Kühn et al., 2008). High pressure processing (HPP) is an alternative preservation

method to thermal processing for some food products. HPP of food can result in protein denaturation, starch gelatinisation, or influence interactions between food ingredients (Devi et al., 2014). Whey proteins, in particular, are susceptible to HPP, which unfolds them and allows both non-covalent and disulfide intermolecular interactions to occur (Dickinson, 2010). Furthermore, the nature of the carrier oil used within an emulsion-based delivery system can also influence the bioactive partitioning. The chemical composition and physicochemical properties of the carrier oil may influence the maximum amount of solute that can be solubilised in the oil phase (C_{SO}*) as well as the oil–water partition coefficient (K_{OW}) (McClements, 2012).

We have recently reported that confocal Raman microscopy (CRM) can be used to quantify the partitioning of β-carotene in whey protein-based emulsions (Wan Mohamad, Buckow, Augustin, & McNaughton, 2017). The Raman signal of the emulsion matrix is dominated by the β-carotene distinctive signal comprising three distinctive bands at 1524 (C=C stretching mode), 1157 (C−C in-plane stretching mode) and 1016 cm^{-1} (C−H bending mode) (Lopez-Sanchez et al., 2011).

In this study, we investigated the partitioning of β-carotene molecules between the aqueous and oil phases of emulsions stabilised by native (untreated) WPI, heat-treated WPI or high pressure-treated WPI prior to emulsion formation. We also compared the partitioning of β-carotene within the emulsions when WPI was replaced by a low weight emulsifier (Tween 40) as well as the effects of using fats with different solid fat content, namely palm oil (liquid fraction of palm oil) and palm stearin (solid fraction of palm oil). The particle sizes of the emulsions were also measured.

2. Materials and Methods

2.1 Materials

The refined, bleached and deodorised (RBD) palm olein (I.V: 56 Wijs, M.Pt: 24 °C) and palm stearin (I.V: 48 Wijs, M.Pt: 44 °C) was supplied by Wilmar Trading Pte. Ltd. (Pasir Gudang, Johor, Malaysia). The micro-algal based β-carotene suspended (30%) in medium-chain triglyceride oil, was kindly provided by Cognis Australia Pty. Ltd. (Melbourne, Victoria, Australia). WPI (91% protein, 5% moisture, 3% ash, and 1% fat) was purchased from Fonterra (Darnum, Victoria, Australia), while polyoxyethylenesorbitan monopalmitate

(Tween 40) was from Sigma-Aldrich (Castle Hill, NSW, Australia). All solutions and emulsions were prepared using double-distilled water.

The poly-L-lysine (0.1% (w/v) in water, with 0.01% of thimerosal added as preservative) from Sigma-Aldrich (Castle Hill, NSW, Australia) was used to immobilise the oil droplets for Raman analysis.

2.2 Preparation of protein dispersion

A stock solution of native WPI (50 g/kg) was prepared by continuous stirring of the protein powder in double distilled water using a mixer. The process was maintained at 50 °C for 1 h in a water bath. This was followed by overnight storage at 5 °C to allow complete hydration. The protein solutions were re-stirred for 1 min on the next day to ensure homogeneity prior to direct emulsification with oil, or undergoing high pressure or thermal treatments before being used to make the aqueous phase of emulsions. Sodium azide (0.1 g/kg) was added to the stock solutions to prevent microbial growth.

2.2.1 Heat treatment

Heat treatment was performed by heating the WPI solutions at 20 g/kg, in a closed water bath, where the temperature was monitored every 10 s by a thermometer. The solutions were separately heated up to set temperatures of 70 °C or 80 °C for 15 min. After each thermal process, the protein solutions were immediately cooled to ~20 °C using an ice bath, before being used to make emulsion samples.

2.2.2 High pressure processing

Prior to high pressure treatment, 50 ml of the WPI stock solution (50 g/kg) was transferred into screw-capped polyethylene bottles (PET TB 410 Clear 384, Synergy Packaging Pty Ltd, Tullamarine, VIC, Australia,). HPP was performed at 600 MPa for 10 min in a QUINTUS® Food Press (QFP 35 L-600-S Food Press, Avure Technologies AB, Västerås, Sweden) conditioned at 20 °C. The recorded temperature profile during HPP showed that during compression to 600 MPa the temperature increased by approximately 20 °C (from 20 to nearly 40 °C). Following the specified holding time, pressure was released instantaneously. The compression and decompression rate was standardised at approximately 4 and 100 MPa/s, respectively.

2.3 Preparation of emulsions

β-carotene, at a concentration 2 g/kg, was dissolved in palm olein and/or palm stearin on a magnetic stirrer at 140 °C for 30 min in the dark, until a transparent red oil without any sign of crystals was obtained (Mao et al., 2009). A high-speed rotor-stator mixer (L2R, Silverson Machines Ltd., Chesham, UK) was used to mix the aqueous (90% (w/w)) and lipid (10% (w/w)) phases and form pre-emulsions at a constant speed for 2 min at room temperature (~22 ºC). The solutions were passed through a high-pressure single-pass valve homogeniser (EmulsiFlex, Avestin Inc., Ottawa, Canada) twice at 500/100 bar, before the samples of fine emulsions were collected. The procedures were repeated to give two independent replicates for each sample.

2.3.1 Emulsions with different emulsifiers

Untreated WPI, heat-treated, high pressure-treated protein dispersions or Tween 40 were used as emulsifiers for formation of emulsions containing 100 g/kg lipid (palm olein) and 0.2 g/kg β-carotene. The stock solution of native WPI was diluted to give 20 g/kg of untreated WPI in emulsion. The high presssure-treated WPI solutions were diluted from their stock concentration of 50 g/kg, and used to give the aqueous phase at 2, 10 and 20 g/kg of emulsions. The heat-treated WPI solutions were weighed to give only 20 g/kg concentration of emulsifier in emulsion. Emulsions containing 20 g/kg Tween 40 was also prepared.

2.3.2 Emulsions with different lipid phase

The solid fat content of the phase was manipulated through a mix of palm olein and stearin at the following ratios: 1:0, 2:1, 1:1, 1:2 and 0:1, at 20 g/kg untreated WPI and 0.2 g/kg β-carotene in emulsions.

2.4 Analysis of β-carotene content of oil phase

The concentration of β-carotene in the oil phase of our emulsion samples was measured using a confocal Raman micro-spectroscopic method, following the steps outlined in our previous work (Wan Mohamad et al., 2017). *In-situ* collection of Raman scattering spectra was carried out using the confocal WITec alpha300 R (WITec, Ulm, Germany), a Raman microspectrometer equipped with a thermo-electrically cooled electron multiplier charge coupled device (EMCCD) detector. The excitation source was the 532 nm solid state green

laser diode. All samples were microscopically analysed through a 50 × objective lens. This setup yields a high spatial resolution down to ca. 0.4 μm and confocality with ca. 0.8 μm depth. To quantify and determine The partitioning of β-carotene between the phases of the emulsion samples was quantified under the point-microspectroscopic mode; using the single-measurement function with 0.5 s integration time for 10 scans under 0.5 mW laser power (measured at the laser output).

A point was microscopically focussed within an oil droplet (immobilised by a layer of poly-L-lysine on the slide) containing the β-carotene, before the laser targeted the focal point to retrieve a Raman signal, from which the intensity of β-carotene band at 1524 cm^{-1} was recorded to calculate the corresponding β-carotene concentration univariately using the calibration curve (concentration vs. Raman intensity of β-carotene band at 1524 cm^{-1}).

From the initial concentration of β-carotene in whole emulsion, the percentage concentration of β-carotene in both the oil and aqueous phases were determined based on our previous work validating this method to the conventional extraction-spectrophotometric analysis (Wan Mohamad et al., 2017). Duplicated measurements were carried out for every formulation, where five random droplets (5-10 μm diameter) were chosen for quantification and the average recorded.

2.5 Particle size analysis

A laser diffraction particle analyser, Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) was used to characterise the oil droplets by measuring the particle size distribution and the average particle diameter as outlined previously (Wan Mohamad, McNaughton, Buckow, & Augustin, 2017). All measurements were recorded as particle size distribution, surface-weighted, or Sauter mean diameter, D[3,2] and surface-volume diameter, D[4,3].

2.6 Statistical analysis

The spectral data were exported as ASCII files and analysed using the univariate regression models constructed in Microsoft Excel. The integrated area (sum of all the intensities) of the 1524 cm^{-1} band from the Raman data was plotted against the known concentrations of β-carotene in olein, from which the equation of the regression line ($y = m\ x$) and correlation values (r^2) of known concentration of β-carotene in olein vs. spectral intensity were

determined. From this equation, the corresponding ratios of bands from emulsion spectra were used to predict the concentration of β-carotene within the oil droplets. These procedures follow the work by Whelan et al. (2013).

The experimental data were statistically analysed by two-way analysis of variance (ANOVA) in Microsoft Excel, where $p < 0.05$ was considered to be significant. Fisher's Least Significant Difference (LSD) test was applied to further determine the significant differences of means between the different types of sample.

Every formulated emulsion system was prepared in two replicates. Each replicate was divided into two aliquots for all analyses, giving a duplicate measurement for each sample. All data are reported as means ± standard deviation.

3. Results and Discussion

3.1 Particle size distribution and mean droplet size

Morphological characterisation of the various emulsion systems was necessary to determine the dimensions and structure of the carrier hosting the bioactive compound. Particle size distribution and mean droplet size of the emulsion droplets were the two parameters considered. Figure 7.3 shows that bimodal particle size distributions were obtained immediately after homogenisation in all formulations.

Figure 7.3 (b) further shows that increasing the treated WPI concentrations resulted in more distinct dual peaks with a greater proportion of larger particle diameter particles. The pattern of clearer distinction between the two peaks is seen with the increase of solid fat content in the oil phase of emulsions (Figure 7.3 (c)), and with the use of WPI as compared to using Tween 40 (Figure 7.3 (d)). This indicates a higher level of flocculation in comparison with the broad shoulder on the distribution for the emulsion with untreated WPI as its emulsifier (Dickinson & James, 1998).

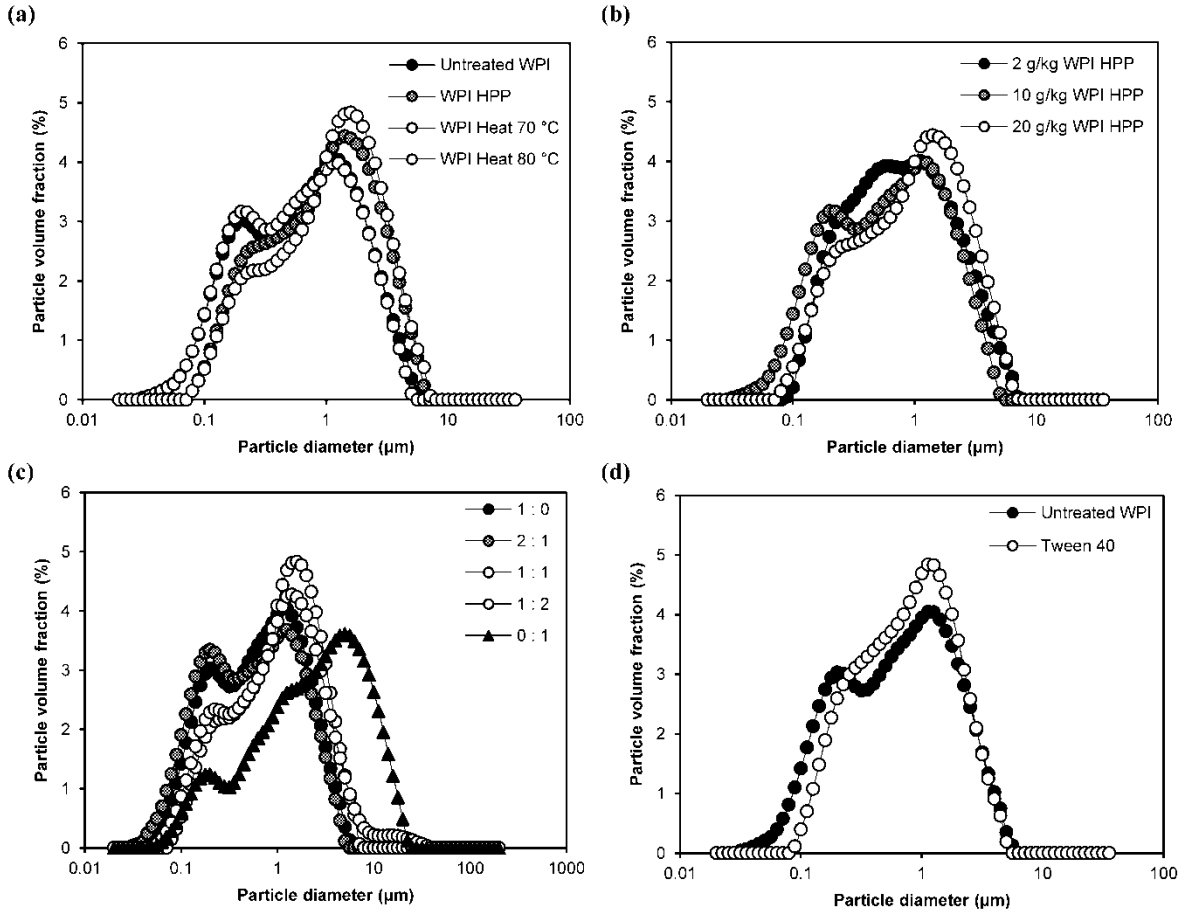

Figure 7.3: Particle size distribution of emulsions with: (a) untreated and treated WPI (heating at 70 or 80 °C for 15 min, or HPP at 600 MPa for 10 min at 20 °C) (100 g/kg palm olein, 20 g/kg treated or untreated WPI, 0.2 g/kg β-carotene), (b) different concentrations of high pressure-treated (HPP) WPI (100 g/kg palm olein, 0.2 g/kg β-carotene, 2-20 g/kg HPP WPI), (c) different solid fat content (100 g/kg palm olein and/or palm stearin at different ratios, 0.2 g/kg β-carotene, 20 g/kg untreated WPI), and (d) different types of emulsifier; untreated WPI and Tween 40 (100 g/kg palm olein, 20 g/kg untreated WPI or Tween 20, 0.2 g/kg β-carotene), measured immediately after preparation.

Table 7.2 summarises the effect of varying the emulsion formulations and pre-treatmet conditions on the mean droplet sizes. Emulsions prepared with high pressure or heat treated WPI solutions as the emulsifier, generally have larger droplets than those made with the native protein. Increasing the heating temperature from 70 to 80 °C further increased the droplet size of emulsions stabilised by the thermal-treated proteins. The results indicate further modification of protein structure following the higher heat treatment leading to the loss of emulsifying efficiency owing to protein aggregation, despite an increase in surface hydrophobicity. McClements, Monahan and Kinsella (1993) suggest that the flocs of whey protein-coated droplets are initially formed by non-covalent bonding or bridging flocculation,

which are further stabilised by disulfide bonds arising from the reaction of free sulfhydryl groups exposed on adsorbed protein layers.

Table 7.2: The change in emulsion mean droplet size in response to the variation in emulsion formulations and pre-treatment conditions. Data are mean ± standard deviations (*n* = 4).

Type of Emulsifier	[BC][a] (g/kg)	[Emulsifier][b] (g/kg)	Olein : Stearin Ratio	Droplet Size, D[3,2] (μm)	Droplet Size, D[4,3] (μm)
WPI (HTST)[c]	0.2	20	100 : 0	0.43 ± 0.02^{A}	1.08 ± 0.02^{H}
WPI_Heat 70 °C 15 min	0.2	20	100 : 0	0.57 ± 0.01^{B}	1.35 ± 0.02^{I}
WPI_Heat 80 °C 15 min	0.2	20	100 : 0	0.68 ± 0.03^{C}	1.79 ± 0.04^{J}
WPI_HPP[d]	0.2	2	100 : 0	0.71 ± 0.02^{C}	1.82 ± 0.02^{J}
WPI_HPP	0.2	10	100 : 0	0.60 ± 0.02^{B}	1.39 ± 0.03^{I}
WPI_HPP	0.2	20	100 : 0	0.57 ± 0.02^{B}	1.36 ± 0.02^{I}
WPI	0.2	20	100 : 0	0.41 ± 0.01^{A}	1.05 ± 0.03^{H}
WPI	0.2	20	67 : 33	0.51 ± 0.01^{D}	1.25 ± 0.01^{K}
WPI	0.2	20	50 : 50	0.60 ± 0.01^{E}	1.55 ± 0.01^{L}
WPI	0.2	20	33 : 67	0.72 ± 0.01^{C}	1.85 ± 0.02^{J}
WPI	0.2	20	0 : 100	0.82 ± 0.01^{F}	4.16 ± 0.06^{M}
Tween 40	0.2	20	100 : 0	0.32 ± 0.01^{G}	0.92 ± 0.01^{N}

[A-G] D[3,2] values, data followed by different capital superscript letters are significantly different ($p < 0.05$)
[H-N] D[4,3] values, data followed by different capital superscript letters are significantly different ($p < 0.05$)
[a] [BC], concentration of β-carotene.
[b] [WPI], concentration of WPI.
[c] The whole emulsion was treated by high-temperature-short-time (HTST) method at 70 ºC for 1 min.
[d] The WPI solutions were high pressure-treated at 600 MPa for 10 min at 20 ºC, before being used as emulsifiers.

A significant variation in droplet sizes is obvious when varying the high pressure-treated WPI concentration of the emulsion systems. At 2 g/kg of treated WPI using in the emulsion, the mean droplet diameter, D[4,3] was higher at 1.82 ± 0.02 μm, compared to those with 10 and 20 g/kg treated WPI, which were not significantly different (1.39 ± 0.03 and 1.36 ± 0.02 um respectively). At the higher concentrations, the protein possibly has a more compact

conformation at the surface, and excess protein chains could extend into the continuous phase, preventing flocculation by steric and electrostatic repulsion. (Yi et al., 2014)

Table 7.2 also depicts an overall increase in the mean droplet size, D[4,3] from 1.05 ± 0.03 to 4.16 ± 0.06 μm upon increasing the solid fat content in the oil phase of emulsions. This shows that palm stearin, the solid fraction of the oil phase, contributed to the formation of larger droplets, probably by preventing or reducing further breakup of emulsion droplets into smaller ones during the homogenisation process.

The droplets of emulsions with WPI show larger mean droplet sizes than those with Tween 40 as the emulsifier (Table 7.2). Interfacial behaviour of the two emulsifiers may contribute to the difference in droplet sizes. Trentin, De Lamo, Güell, López and Ferrando (2011) reported that the interfacial tension between oil and water in emulsions using Tween 20 was lower than that using whey protein as the emulsifier. The surface-active properties of the emulsifier determine the droplet size of the final emulsion because they play a double role of reducing the interfacial tension between oil and water and stabilising the droplets against aggregation and/ or coalescence.

3.2 Effects of pre-treatments on β-carotene partitioning

The concentration of β-carotene within the oil droplets was determined using the intensity of the strong 1524 cm^{-1} peak of β-carotene in the univariate analysis. Figure 7.4 shows that pre-treating the WPI used as emulsifier at a higher temperature (80 °C instead of 70 °C) did not significantly change the partitioning of β-carotene between the phases of the emulsions. Although further denaturation might have occurred at the elevated temperature, the conformational change of protein structure did not significantly influence the protein affinity to further attract β-carotene molecules towards the aqueous phase.

However compared to that when untreated WPI was used, the heat-treatment of WPI at both 70 and 80 °C for 15 min, resulted in an overall increase in β-carotene partitioning into the aqueous phase of the emulsion although to a lesser degree than that using the same concentration of high pressure-treated WPI (20 g/kg). Obviously, there can be a new equilibrium established in the partitioning of β-carotene between the different phases of the emulsion samples as the protein structure is altered during the pre-treatments, exposing more binding sites for β-carotene to attach (Dufour & Haertlé, 1991; Wackerbarth et al., 2009).

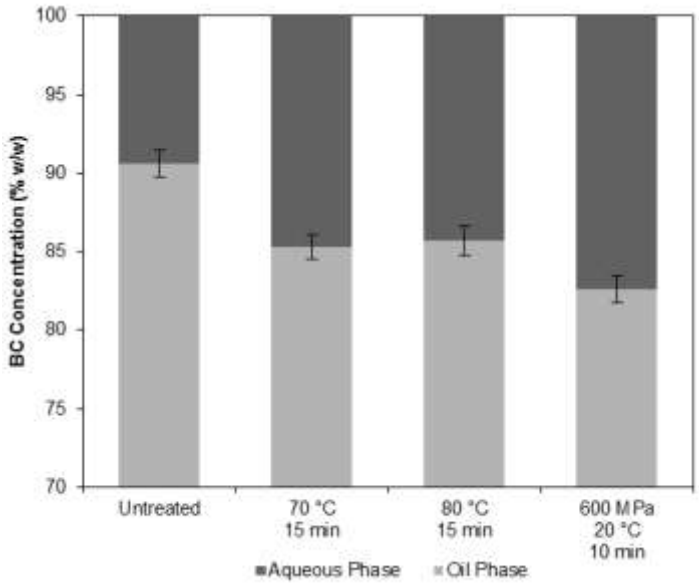

Figure 7.4: Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with: (a) untreated WPI, (b) heat-treated WPI at 70 °C for 15 min, (c) heat-treated WPI at 80 °C for 15 min, and (d) high pressure-treated (HPP) WPI at 600 MPa for 15 min at 20 °C. All samples contained the same concentrations of palm olein, emulsifier and β-carotene at 100 g/kg, 20 g/kg and 0.2 g/kg respectively. Data are mean ± standard deviations (*n* = 4).

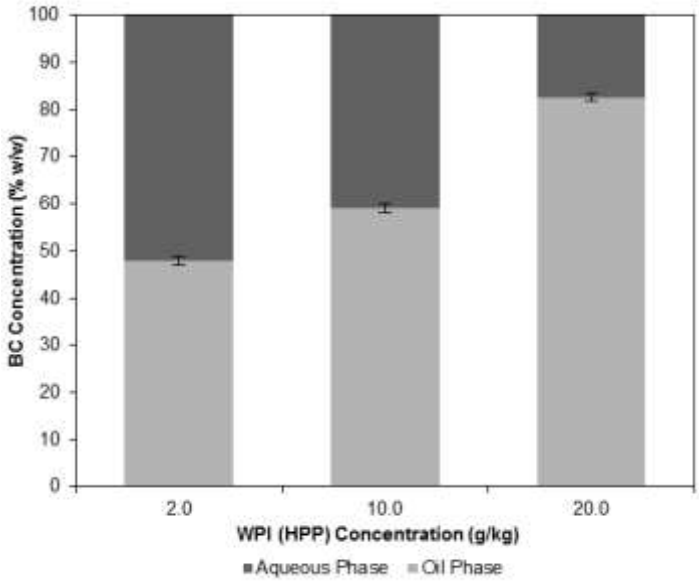

Figure 7.5: Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with different high pressure-treated (HPP) WPI concentrations (2, 10 and 20 g/kg) at 100 g/kg palm olein and 0.2 g/kg β-carotene. The WPI solutions were high pressure processed at 600 MPa for 10 min at 20 ºC, before being used as emulsifiers. Data are mean ± standard deviations (n = 4).

Increasing the concentration of high pressure-treated WPI as the emulsifier resulted in the reduction of β-carotene in the aqueous phase (Figure 7.5). This observation is consistent with our work using native, untreated WPI as emulsifiers (Wan Mohamad, McNaughton, Buckow, & Augustin, 2017).

However, this is counter to expectations that an increased concentration of WPI does provide more sites for β-carotene adsorption, provided that all other factors such as the relative solubility of the bioactive in the oil and aqueous phase, as well as its propensity to assemble at the interface remained constant. Another possible contributory factor may be the thickness of the interfacial layer and the packing density of protein that regulates diffusion of bioactive molecules through the interface (Boon et al., 2010). The pre-treatments further thicken the interfacial layer as the result of protein denaturation.

3.3 Effects of solid fat content on β-carotene partitioning

The partitioning of bioactive in an emulsion-based delivery system can also be influenced by solid fat content of the carrier oil. In this study, palm stearin i.e. the solid fraction of palm oil, was mixed in an increasing ratio to the liquid palm olein, forming increasingly crystalline oil phase of emulsions.

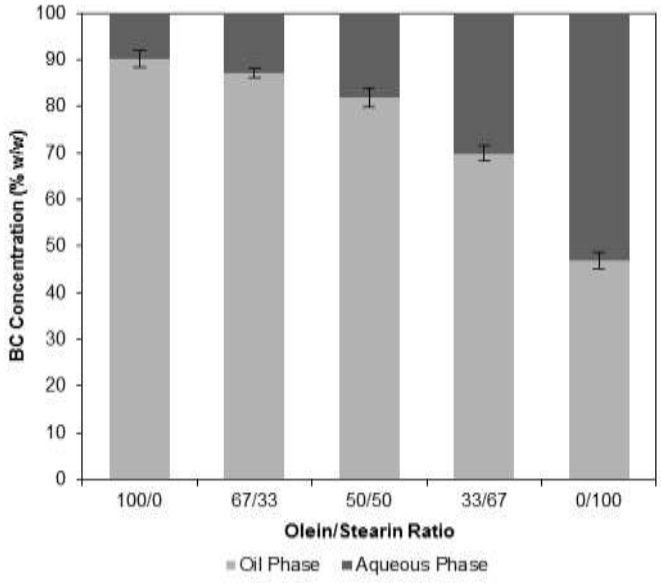

Figure 7.6: Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with different solid fat content (100 g/kg palm olein and/or palm stearin at different ratios, 0.2 g/kg β-carotene, 20 g/kg WPI). Data are mean ± standard deviations (*n* = 4).

It has been postulated that highly hydrophobic components such as β-carotene can be trapped within colloidal crystalline matrices (the oil phase with higher solid fat content), which slows down their diffusion and that of any reactant that might promote instability e.g. oxygen, so as to improve their chemical stability during storage and control their release kinetics (Joshi & Müller, 2009). However practically, it is usually found that the solute or bioactive molecules are expelled once a carrier lipid crystallises because they cannot easily be incorporated into the highly ordered structure of the crystalline phase. Several studies have reported this phenomenon experimentally, for example in partitioning of drugs (Joshi & Müller, 2009), unsaturated oils (Okuda, McClements, & Decker, 2005), aroma compounds (Ghosh, Peterson, & Coupland, 2007) and flavour compounds (Longyuan et al., 2010). This might also explain the partitioning of β-carotene into the aqueous phase apparent in Figure 7.6 that escalates from 9.9 ± 1.7 to 53.0 ± 1.8% w/w, with the increase in the solid fat content of emulsions, from manipulating the ratio between the liquid palm olein and its solid fraction; palm stearin.

3.4 Effects of emulsifier type on β-carotene partitioning

In the emulsion field, the emulsifier is an essential part of the system, and it can generally be classified into two groups: small molecule emulsifiers, like polysorbate and glycerol esters, which generally have strong emulsifying properties; and large molecule emulsifiers (or surface-active polymers), like milk proteins and modified starches, which have both emulsifying and stabilising properties. Here, we also report the difference in partitioning characteristic of β-carotene in oil-in-water emulsions using Tween 40 as the emulsifier, as compared to that using WPI.

Figure 7.7 illustrates that the use of Tween 40 results in less partitioning of β-carotene into the aqueous phase of emulsions (6.0 ± 1.1% w/w) than in WPI emulsions (9.9 ± 1.7% w/w). The fact that partitioning was observed in both types of sample indicates interactions between both emulsifiers and β-carotene in the aqueous phase. However, the two interactions happened to different degrees, since the molecular structures and conformations of WPI and Tween 40 may give rise to different distributions of polar/non-polar groups as the binding sites (McClements, 2004).

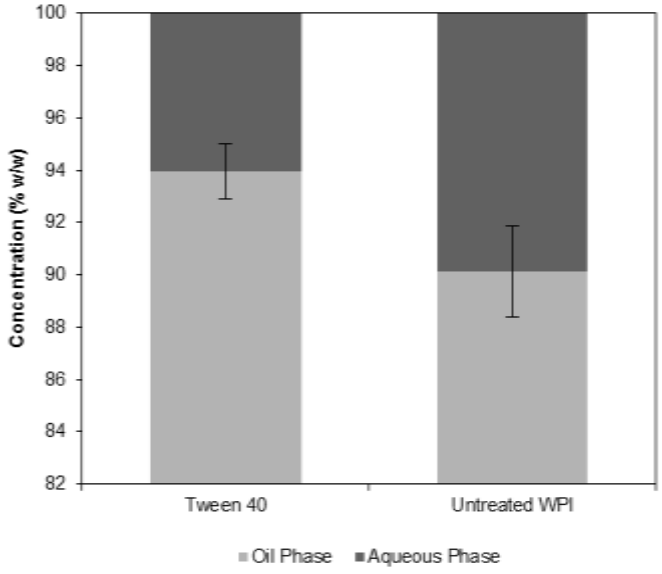

Figure 7.7: Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with two types of emulsifier; WPI and Tween 40 (100 g/kg palm olein, 20 g/kg WPI or Tween 40, 0.2 g/kg β-carotene). Data are mean ± standard deviations (*n* = 4).

Figure 7.7 suggests that there were more β-carotene-WPI than β-carotene-Tween 40 interactions, due to the binding of β-carotene to the protein. Several studies have experimentally shown that β-carotene is able to bind with proteins, particularly β-lactoglobulin, the major component of WPI, to form stable protein–carotenoid complexes with surface–active properties that stabilise the emulsions (Dufour & Haertlé, 1991; Wackerbarth et al., 2009).

4. Conclusion

In-situ investigations, using the CRM technique, of the partitioning characteristics of β-carotene in emulsions with various formulations and pre-treatment conditions have been reported. The partitioning of β-carotene into the aqueous phase of emulsions increased when using heat and high pressure-treated WPI as emulsifier in comparison to the use of untreated WPI due to the new equilibrium of β-carotene between the different phases of the emulsions as a result of the protein structure modification during the pre-treatment processes. Similarly, partitioning of β-carotene into the aqueous phase can be increased by increasing the solid fat content of emulsions. This could be a result of more solute molecules being expelled with the increase of ordered crystalline structure in the carrier lipid. The use of Tween 40 as compared to that of WPI as the emulsifiers resulted in less partitioning of the bioactive molecules into

the aqueous phase of emulsions because the hydrophobic interactions between β-carotene and the two emulsifiers is dependent on the availability of binding sites. The results of this study will be beneficial in designing delivery systems for encapsulating β-carotene to effectively incorporate into aqueous-based food, beverage and pharmaceutical products while sustaining its oral bioavailability.

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Reference

Boon, C. S., McClements, D. J., Weiss, J., & Decker, E. A. (2010). Factors influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition*, *50*, 515–532.

Cornacchia, L., & Roos, Y. H. (2011a). Stability of β-carotene in protein-stabilized oil-in-water delivery systems. *Journal of Agricultural and Food Chemistry*, *59*, 7013–7020.

Cornacchia, L., & Roos, Y. H. (2011b). State of dispersed lipid carrier and interface composition as determinants of beta-carotene stability in oil-in-water emulsions. *Journal of Food Science*, *76*, C1211–C1218.

Devi, A. F., Buckow, R., Hemar, Y., & Kasapis, S. (2014). Modification of the structural and rheological properties of whey protein/gelatin mixtures through high pressure processing. *Food Chemistry*, *156*, 243–249.

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces*, *81*, 130–140.

Dickinson, E., & James, J. D. (1998). Rheology and flocculation of high-pressure-treated B-lactoglobulin-stabilized emulsions : Comparison with thermal treatment. *Journal of Agricultural and Food Chemistry*, *46*, 2565–2571.

Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta*, *1079*, 316–320.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000). Influence of high pressure processing on protein solutions and emulsions. *Current Opinion in Colloid & Interface Science*, *5*, 182–187.

Ghosh, S., Peterson, D. G., & Coupland, J. N. (2007). Aroma release from solid droplet emulsions: Effect of lipid type. *JAOCS, Journal of the American Oil Chemists' Society*, *84*, 1001–1014.

Joshi, M. D., & Müller, R. H. (2009). Lipid nanoparticles for parenteral delivery of actives. *European Journal of Pharmaceutics and Biopharmaceutics*, *71*, 161–172.

Kühn, J., Considine, T., & Singh, H. (2008). Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. *Journal of Agricultural and Food Chemistry*, *56*, 10218–10224.

Longyuan, M., Seung, J. C., Jean, A., Lulu, H., Michael Popplewell, Mcclements, D. J., & Decker, E. a. (2010). Citral stability in oil-in-water emulsions with solid or liquid octadecane. *Journal of Agricultural and Food Chemistry*, *58*, 533–536.

Lopez-Sanchez, P., Schumm, S., Pudney, P. D. a, & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst*, *136*, 3694–3697.

Maiani, G., Castón, M. J. P., Catasta, G., Toti, E., Cambrodón, I. G., Bysted, A., … Schlemmer, U. (2009). Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Molecular Nutrition and Food Research*, *53*, S194–S218.

Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y., & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of b-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology*, *9862*, 336–342.

Mao, L., Yang, J., Xu, D., Yuan, F., & Gao, Y. (2010). Effects of homogenization models and emulsifiers on the physicochemical properties of β-carotene nanoemulsions. *Journal of Dispersion Science and Technology*, *31*, 986–993.

McClements, D. J. (2012). Crystals and crystallization in oil-in-water emulsions: implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science*, *174*, 1–30.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science*, *72*, R109–R124.

McClements, D. J., Monahan, F. J., & Kinsella, J. E. (1993). Disulfide bond formation affects stability of whey protein isolate emulsions. *Journal of Food Science*, *58*, 1036–1039.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrician*, *51*, 285–330.

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

Okuda, S., McClements, D. J., & Decker, E. a. (2005). Impact of lipid physical state on the oxidation of methyl linolenate in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *53*, 9624–9628.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, *132*, 1221–1229.

Rao, A. V., & Rao, L. G. (2007). Carotenoids and human health. *Pharmacological Research*, *55*, 207–216.

Trentin, A., De Lamo, S., Güell, C., López, F., & Ferrando, M. (2011). Protein-stabilized emulsions containing beta-carotene produced by premix membrane emulsification. *Journal of Food Engineering*, *106*, 267–274.

Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International*, *42*, 1254–1258.

Wan Mohamad, W. A. F., Buckow, R., Augustin, M., & Mcnaughton, D. (2017). In situ quantification of β-carotene partitioning in oil-in-water emulsions by confocal Raman microscopy. *Food Chemistry*, *233*, 197–203.

Wan Mohamad, W. F., McNaughton, D., Buckow, R., & Augustin, M. (2017). Stability and partitioning of β-carotene in whey protein emulsions during storage. *Food Function*, *8*, 3917-3925.

Whelan, D. R., Bambery, K. R., Puskar, L., McNaughton, D., & Wood, B. R. (2013). Quantification of DNA in simple eukaryotic cells using Fourier transform infrared spectroscopy. *Journal of Biophotonics*, *6*, 775–84.

Yi, J., Li, Y., Zhong, F., & Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloids*, *35*, 19–27.

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Characterisation of β-carotene partitioning in protein emulsions: Effects of pre-treatments, solid fat content and emulsifier type

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ABSTRACT

Understanding the bioactive partitioning between the phases of an emulsion system underpins strategies for improving the efficiency of bioactive protection against degradation. We analysed partitioning of β-carotene in emulsions with various formulations *in-situ* using confocal Raman microscopy (CRM). The partitioning of β-carotene into the aqueous phase of emulsions increased when whey protein isolate (WPI) was heat or high pressure-treated prior to emulsion formation. However, increasing the concentration of high pressure-treated WPI reduced the β-carotene partitioning into the aqueous phase. Increasing the solid fat content in the carrier oil favoured the migration of β-carotene into the aqueous phase. The use of WPI as the emulsifier resulted in a greater partitioning of β-carotene into the aqueous phase compared to when Tween 40 was the emulsifier. This study demonstrates that partitioning of β-carotene between the aqueous and oil phase is dependent on the characteristics of the oil phase, emulsifier type and processing.

1. Introduction

Carotenoids are natural pigments mainly found in fruits and vegetables. They are commonly categorised into two groups: (i) carotenes comprised entirely of carbon and hydrogen (e.g. α-carotene, β-carotene, and lycopene) and (ii) xanthophylls comprised of carbon, hydrogen, and oxygen (e.g. lutein and zeaxanthin) (Qian, Decker, Xiao, & McClements, 2012). The most significant pro-vitamin A carotenoid used in foods and beverages is β-carotene (Boon, McClements, Weiss, & Decker, 2010; Rao & Rao, 2007). Epidemiological studies suggest that regular consumption of β-carotene in foods may lower the risk of certain chronic illnesses such as macular degeneration, cataracts, cardiovascular disease as well as some cancers (Maiani et al., 2009; Miller & Snyder, 2012).

A lipophilic bioactive such as β-carotene is often delivered using emulsions (McClements, Decker, & Weiss, 2007). Carotenoid-enriched, emulsion-based delivery systems can be produced by dissolving the β-carotene within carrier oil, and then homogenizing this oil phase with an aqueous phase containing a water-soluble emulsifier such as small molecule surfactants (e.g. Tween 20 or decaglycerol monolaurate) or biopolymers (e.g. WPI or modified starch with emulsifying properties) (Cornacchia & Roos, 2011a, 2011b; Mao, Yang, Xu, Yuan, & Gao, 2010; Qian et al., 2012). The effects of droplet size, carrier oil type and emulsifier type on the chemical stability and bioavailability of β-carotene have been examined (McClements & Rao, 2011), but less attention has been paid to the partitioning of the bioactive within the system. β-carotene is likely to be distributed and partitioned between the oil and aqueous phases of the emulsion system (McClements, 2012). The compartmentalization of the bioactive within the system might be critical since the protection efficacy against degradation and bioavailability may be dependent on it (Cornacchia & Roos, 2011b).

The ingredients that are used in an emulsion with the bioactive are expected to influence the partitioning of a bioactive in an emulsion. With respect to emulsifiers, both low (e.g. Tweens) and high molecular weight emulsifiers (e.g. proteins) may be used in the formulation of emulsions. The use of these different emulsifiers results in emulsions with different interfacial structures (Mao et al., 2009). In addition, when protein is used, it is possible to modify the whey protein structure through processing, which will influence its functional properties. For example, factors such as pH and temperature influence whey protein conformation, which in turn changes binding characteristics of these proteins to small molecules (Kühn, Considine, & Singh, 2008). High pressure processing (HPP) is an alternative preservation method to thermal processing for some food products. HPP of food can result in protein denaturation, starch gelatinisation, or influence interactions between food ingredients (Devi, Buckow, Hemar, & Kasapis, 2014).

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Whey proteins, in particular, are susceptible to HPP, which unfolds them and allows both non-covalent and disulfide intermolecular interactions to occur (Dickinson, 2010). Furthermore, the nature of the carrier oil used within an emulsion-based delivery system can also influence the bioactive partitioning. The chemical composition and physicochemical properties of the carrier oil may influence the maximum amount of solute that can be solubilized in the oil phase (C_{SO}^{*}) as well as the oil–water partition coefficient (K_{OW}) (McClements, 2012).

We have recently validated that confocal Raman microscopy (CRM) can be used to quantify the partitioning of β-carotene in whey protein-based emulsions (Wan Mohamad, Buckow, Augustin, & McNaughton, 2017). The Raman signal of the emulsion matrix is dominated by the β-carotene distinctive signal comprising three distinctive bands at 1524 (C═C stretching mode), 1157 (C−C in-plane stretching mode) and 1016 cm^{-1} (C−H bending mode) (Lopez-Sanchez, Schumm, Pudney, & Hazekamp, 2011).

In this study, we investigated the partitioning of β-carotene molecules between the aqueous and oil phases of emulsions stabilised by native (untreated) WPI, heat-treated WPI or high pressure-treated WPI prior to emulsion formation. We also compared the partitioning of β-carotene within the emulsions when WPI was replaced by a low weight emulsifier (Tween 40) as well as the effects of using fats with different solid fat content, namely palm oil (liquid fraction of palm oil) and palm stearin (solid fraction of palm oil). The particle sizes of the emulsions were also measured.

2. Materials and methods

2.1. Materials

The refined, bleached and deodorized (RBD) palm olein (I.V: 56 Wijs, melting point (MP): 24 °C) and palm stearin (I.V: 48 Wijs, MP: 44 °C) was supplied by Wilmar Trading Pte. Ltd. (Pasir Gudang, Johor, Malaysia). The micro-algal based β-carotene suspended (30%) in medium-chain triglyceride oil, was kindly provided by Cognis Australia Pty. Ltd. (Melbourne, Victoria, Australia). WPI (91% protein, 5% moisture, 3% ash, and 1% fat) was purchased from Fonterra (Darnum, Victoria, Australia), while polyoxyethylenesorbitan monopalmitate (Tween 40) was from Sigma-Aldrich (Castle Hill, NSW, Australia). All solutions and emulsions were prepared using double-distilled water.

The poly-L-lysine (0.1% (w/v) in water, with 0.01% of thimerosal added as preservative) from Sigma-Aldrich (Castle Hill, NSW, Australia) was used to immobilize the oil droplets for Raman analysis.

2.2. Preparation of protein dispersion

A stock solution of native WPI (50 g/kg) was prepared by continuous stirring of the protein powder in double distilled water using a mixer. The process was maintained at 50 °C for 1 h in a water bath. This was followed by overnight storage at 5 °C to facilitate complete hydration. The protein solutions were re-stirred for 1 min on the next day to ensure homogeneity prior to direct emulsification with oil, or undergoing high pressure or thermal treatments before being used to make the aqueous phase of emulsions. Sodium azide (0.1 g/kg) was added to the stock solutions to prevent microbial growth.

2.2.1. Heat treatment

Heat treatment was performed by heating the WPI solutions at 20 g/kg, in a closed water bath, where the temperature was monitored every 10 s by a thermometer. The solutions were separately heated up to set temperatures of 70 °C or 80 °C for 15 min. After each thermal process, the protein solutions were immediately cooled to ~20 °C using an ice bath, before being used to make emulsion samples.

2.2.2. High pressure processing

Prior to high pressure treatment, 50 ml of the WPI stock solution (50 g/kg) was transferred into screw-capped polyethylene bottles (PET TB 410 Clear 384, Synergy Packaging Pty. Ltd., Tullamarine, VIC, Australia). HPP was performed at 600 MPa for 10 min in a QUINTUS® Food Press (QFP 35 L-600-S Food Press, Avure Technologies AB, Västerås, Sweden) conditioned at 20 °C. The recorded temperature profile during HPP showed that during compression to 600 MPa the temperature increased by approximately 20 °C (from 20 to nearly 40 °C). Following the specified holding time, pressure was released instantaneously. The compression and decompression rate was standardised at approximately 4 and 100 MPa/s, respectively.

2.3. Preparation of emulsions

β-carotene, at a concentration 2 g/kg, was dissolved in palm olein and/or palm stearin on a magnetic stirrer at 140 °C for 30 min in the dark, until a transparent red oil without any sign of crystals was obtained (Mao et al., 2009). The β-carotene concentration used in this study was much lower than its saturation concentration in the oil phase, ensuring no β-carotene crystals form in the consequent emulsion samples (Wan Mohamad, Buckow, et al., 2017a). A high-speed rotor–stator mixer (L2R, Silverson Machines Ltd., Chesham, UK) was used to mix the aqueous (90% (w/w)) and lipid (10% (w/w)) phases and form pre-emulsions at a constant speed for 2 min at room temperature (~22 °C). The solutions were passed through a high-pressure single-pass valve homogenizer (EmulsiFlex, Avestin Inc., Ottawa, Canada) twice at 500/100 bar, before the samples of fine emulsions were collected. The procedures were repeated to give two independent replicates for each sample.

2.3.1. Emulsions with different emulsifiers

Untreated WPI, heat-treated, high pressure-treated protein dispersions or Tween 40 were used as emulsifiers for formation of emulsions containing 100 g/kg lipid (palm olein) and 0.2 g/kg β-carotene. The stock solution of native WPI was diluted to give 20 g/kg of untreated WPI in emulsion. The high pressure-treated WPI solutions were diluted from their stock concentration of 50 g/kg, and used to give the aqueous phase at 2, 10 and 20 g/kg of emulsions. The heat-treated WPI solutions were weighed to give only 20 g/kg concentration of emulsifier in emulsion. Emulsions containing 20 g/kg Tween 40 was also prepared.

2.3.2. Emulsions with different lipid phase

The solid fat content of the phase was manipulated through a mix of palm olein and stearin at the following ratios: 1:0, 2:1, 1:1, 1:2 and 0:1, at 20 g/kg untreated WPI and 0.2 g/kg β-carotene in emulsions.

2.4. Analysis of β-carotene content of oil phase

The concentration of β-carotene in the oil phase of our emulsion samples was measured using the confocal Raman micro-spectroscopic method, following the steps outlined in our previous work (Wan Mohamad, Buckow, et al., 2017). Partitioning of bioactive at the time of emulsion preparation is a spontaneous process, which occurs upon droplet breakups during homogenisation. Sample measurement under Raman was carried out on the same day of the emulsion fabrication to avoid re-partitioning during storage. *In-situ* collection of Raman scattering spectra was carried out using the confocal WITec alpha300 R (WITec, Ulm, Germany), a Raman microspectrometer equipped with a thermo-electrically cooled electron multiplier charge coupled device (EMCCD) detector. The excitation source was the 532 nm solid state green laser diode. All samples were microscopically analysed through a 50× objective lens. This setup yields a high spatial resolution down to ca. 0.4 μm and confocality with ca. 0.8 μm depth.

To quantify and determine the partitioning of β-carotene between the phases of the emulsion samples, the point-microspectroscopic mode was used; applying the single-measurement function with 0.5 s integration time for 10 scans under 0.5 mW laser power (measured at the

laser output). A point was microscopically focused within an oil droplet (immobilised by a layer of poly-L-lysine on the slide) containing the β-carotene, before the laser targeted the focal point to retrieve a Raman signal, from which the intensity of β-carotene band at 1524 cm^{-1} was recorded to calculate the corresponding β-carotene concentration univariately using the calibration curve (concentration vs. Raman intensity of β-carotene band at 1524 cm^{-1}).

From the initial concentration of β-carotene in whole emulsion, the percentage concentration of β-carotene in both the oil and aqueous phases were determined according to the calculation used in our previous work, which validated this method to the conventional extraction-spectrophotometric analysis (Wan Mohamad, Buckow, et al., 2017). Duplicated measurements were carried out for every formulation, where five droplets in the range of 5–10 μm in diameter were chosen for quantification and the average recorded. The method used in this study required a minimum droplet size of 5 μm to accurately focus the laser for Raman quantification. Droplets with sizes bigger than 10 μm were not representative of the average droplet size of the emulsions.

2.5. Particle size analysis

A laser diffraction particle analyser, Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) was used to characterise the oil droplets by measuring the particle size distribution and the average particle diameter as outlined previously (Wan Mohamad, McNaughton, Buckow, & Augustin, 2017). All measurements were recorded as particle size distribution, surface-weighted, or Sauter mean diameter, D[3,2] and surface-volume diameter, D[4,3].

2.6. Statistical analysis

The spectral data were exported as ASCII files and analysed using the univariate regression models constructed in Microsoft Excel. The integrated area (sum of all the intensities) of the 1524 cm^{-1} band from the Raman data was plotted against the known concentrations of β-carotene in olein, from which the equation of the regression line ($y = m x$) and correlation values (r^2) of known concentration of β-carotene in olein vs. spectral intensity were determined. From this equation, the corresponding ratios of bands from emulsion spectra were used to predict the concentration of β-carotene within the oil droplets. These procedures follow the work by Whelan, Bambery, Puskar, McNaughton, and Wood (2013).

The experimental data were statistically analysed by two-way analysis of variance (ANOVA) in Microsoft Excel, where $p < 0.05$ was considered to be significant. Fisher's Least Significant Difference (LSD) test was applied to further determine the significant differences of means between the different types of sample.

Every formulated emulsion system was prepared in two replicates. Each replicate was divided into two aliquots for all analyses, giving a duplicate measurement for each sample. All data are reported as means ± standard deviation.

3. Results and discussion

3.1. Particle size distribution and mean droplet size

Morphological characterization of the various emulsion systems was necessary to determine the dimensions and structure of the carrier hosting the bioactive compound. Particle size distribution and mean droplet size of the emulsion droplets were the two parameters considered. Fig. 1 shows that bimodal particle size distributions were obtained immediately after homogenisation in all formulations.

Fig. 1(b) further shows that increasing the treated WPI concentrations resulted in more distinct dual peaks with a greater proportion of larger particle diameter particles. The pattern of clearer distinction between the two peaks is seen with the increase of solid fat content in the oil phase of emulsions (Fig. 1(c)), and with the use of WPI as compared to using Tween 40 (Fig. 1(d)). This indicates a higher level of flocculation in comparison with the broad shoulder on the distribution for the emulsion with untreated WPI as its emulsifier (Dickinson & James, 1998).

Table 1 summarizes the effect of varying the emulsion formulations and pre-treatment conditions on the mean droplet sizes. Emulsions prepared with high pressure or heat treated WPI solutions as the emulsifier, generally have larger droplets than those made with the native protein. Increasing the heating temperature from 70 to 80 °C further increased the droplet size of emulsions stabilised by the thermal-treated proteins. The results indicate further modification of protein structure following the higher heat treatment leading to the loss of emulsifying efficiency owing to protein aggregation, despite an increase in surface hydrophobicity. McClements, Monahan and Kinsella (1993) suggest that the flocs of whey protein-coated droplets are initially formed by non-covalent bonding or bridging flocculation, which are further stabilised by disulfide bonds arising from the reaction of free sulfhydryl groups exposed on adsorbed protein layers.

A significant variation in droplet sizes is obvious when varying the high pressure-treated WPI concentration of the emulsion systems. At 2 g/kg of treated WPI using in the emulsion, the mean droplet diameter, D[4,3] was higher at 1.82 ± 0.02 μm, compared to those with 10 and 20 g/kg treated WPI, which were not significantly different (1.39 ± 0.03 and 1.36 ± 0.02 um respectively). At the higher concentrations, the protein possibly has a more compact conformation at the surface, and excess protein chains could extend into the continuous phase, preventing flocculation by steric and electrostatic repulsion. (Yi, Li, Zhong, & Yokoyama, 2014)

Table 1 also depicts an overall increase in the mean droplet size, D[4,3] from 1.05 ± 0.03 to 4.16 ± 0.06 μm upon increasing the solid fat content in the oil phase of emulsions. This shows that palm stearin, the solid fraction of the oil phase, contributed to the formation of larger droplets, probably by preventing or reducing further breakup of emulsion droplets into smaller ones during the homogenisation process.

The droplets of emulsions with WPI show larger mean droplet sizes than those with Tween 40 as the emulsifier (Table 1). Interfacial behaviour of the two emulsifiers may contribute to the difference in droplet sizes. Trentin, De Lamo, Güell, López and Ferrando (2011) reported that the interfacial tension between oil and water in emulsions using Tween 20 was lower than that using whey protein as the emulsifier. The surface-active properties of the emulsifier determine the droplet size of the final emulsion because they play a double role of reducing the interfacial tension between oil and water and stabilizing the droplets against aggregation and/ or coalescence.

3.2. Effects of pre-treatments on β-carotene partitioning

The concentration of β-carotene within the oil droplets was determined using the intensity of the strong 1524 cm^{-1} peak of β-carotene in the univariate analysis. Fig. 2 shows that pre-treating the WPI used as emulsifier at a higher temperature (80 °C instead of 70 °C) did not significantly change the partitioning of β-carotene between the phases of the emulsions. Although further denaturation might have occurred at the elevated temperature, the conformational change of protein structure did not significantly influence the protein affinity to further attract β-carotene molecules towards the aqueous phase. However compared to that when untreated WPI was used, the heat-treatment of WPI at both 70 and 80 °C for 15 min, resulted in an overall increase in β-carotene partitioning into the aqueous phase of the emulsion although to a lesser degree than that using the same concentration of high pressure-treated WPI (20 g/kg). Obviously, there can be a new equilibrium established in the partitioning of β-carotene between the different phases of the emulsion samples as the protein structure is altered during the pre-treatments, exposing more binding

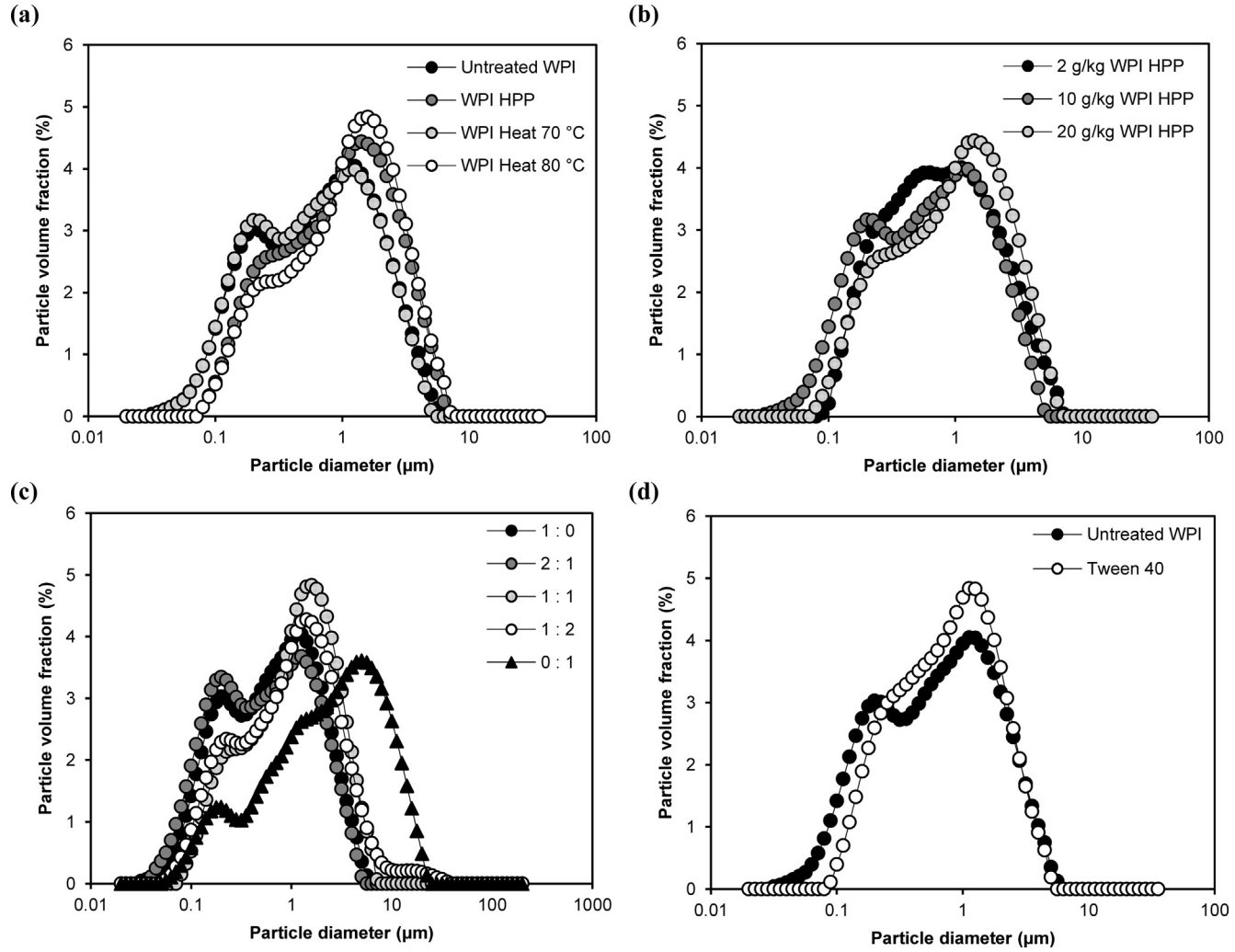

Fig. 1. Particle size distribution of emulsions with: (a) untreated and treated WPI (heating at 70 or 80 °C for 15 min, or HPP at 600 MPa for 10 min at 20 °C) (100 g/kg palm olein, 20 g/kg treated or untreated WPI, 0.2 g/kg β-carotene), (b) different concentrations of high pressure-treated (HPP) WPI (100 g/kg palm olein, 0.2 g/kg β-carotene, 2–20 g/kg HPP WPI), (c) different solid fat content (100 g/kg palm olein and/or palm stearin at different ratios, 0.2 g/kg β-carotene, 20 g/kg untreated WPI), and (d) different types of emulsifier; untreated WPI and Tween 40 (100 g/kg palm olein, 20 g/kg untreated WPI or Tween 20, 0.2 g/kg β-carotene), measured immediately after preparation.

Table 1
The change in emulsion mean droplet size in response to the variation in emulsion formulations and pre-treatment conditions. Data are mean ± standard deviations (n = 4).

Type of Emulsifier	[BC][a] (g/kg)	[Emulsifier][b] (g/kg)	Olein: Stearin Ratio	Droplet Size, D[3,2] (μm)	Droplet Size, D[4,3] (μm)
WPI (HTST)[c]	0.2	20	100: 0	0.43 ± 0.02^{A}	1.08 ± 0.02^{H}
WPI_Heat 70 °C 15 min	0.2	20	100: 0	0.57 ± 0.01^{B}	1.35 ± 0.02^{I}
WPI_Heat 80 °C 15 min	0.2	20	100: 0	0.68 ± 0.03^{C}	1.79 ± 0.04^{J}
WPI_HPP[d]	0.2	2	100: 0	0.71 ± 0.02^{C}	1.82 ± 0.02^{J}
WPI_HPP	0.2	10	100: 0	0.60 ± 0.02^{B}	1.39 ± 0.03^{I}
WPI_HPP	0.2	20	100: 0	0.57 ± 0.02^{B}	1.36 ± 0.02^{I}
WPI	0.2	20	100: 0	0.41 ± 0.01^{A}	1.05 ± 0.03^{H}
WPI	0.2	20	67: 33	0.51 ± 0.01^{D}	1.25 ± 0.01^{K}
WPI	0.2	20	50: 50	0.60 ± 0.01^{E}	1.55 ± 0.01^{L}
WPI	0.2	20	33: 67	0.72 ± 0.01^{C}	1.85 ± 0.02^{J}
WPI	0.2	20	0: 100	0.82 ± 0.01^{F}	4.16 ± 0.06^{M}
Tween 40	0.2	20	100: 0	0.32 ± 0.01^{G}	0.92 ± 0.01^{N}

$^{A–G}$D[3,2] values, data followed by different capital superscript letters are significantly different ($p < 0.05$).
$^{H–N}$D[4,3] values, data followed by different capital superscript letters are significantly different ($p < 0.05$).
[a] [BC], concentration of β-carotene.
[b] [WPI], concentration of WPI.
[c] The whole emulsion was treated by high-temperature-short-time (HTST) method at 70 °C for 1 min.
[d] The WPI solutions were high pressure-treated at 600 MPa for 10 min at 20 °C, before being used as emulsifiers.

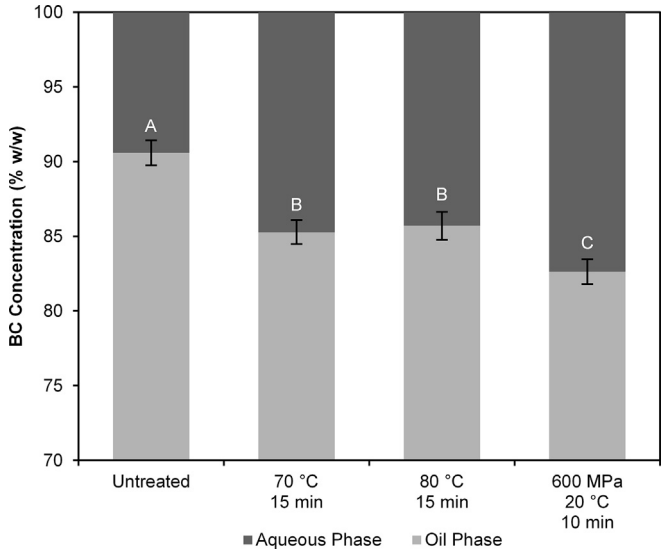

Fig. 2. Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with: (a) untreated WPI, (b) heat-treated WPI at 70 °C for 15 min, (c) heat-treated WPI at 80 °C for 15 min, and (d) high pressure-treated (HPP) WPI at 600 MPa for 15 min at 20 °C. All samples contained the same concentrations of palm olein, emulsifier and β-carotene at 100 g/kg, 20 g/kg and 0.2 g/kg respectively. Data are mean ± standard deviations (n = 4). Data with different capital letters (A–C) are significantly different ($p < 0.05$).

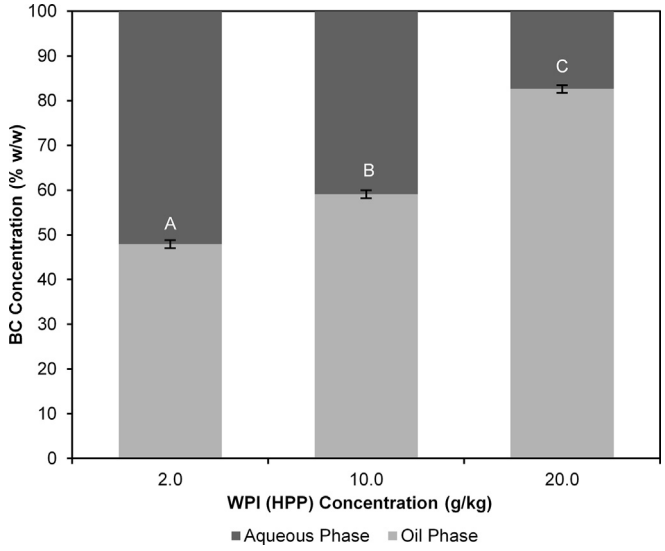

Fig. 3. Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with different high pressure-treated (HPP) WPI concentrations (2, 10 and 20 g/kg) at 100 g/kg palm olein and 0.2 g/kg β-carotene. The WPI solutions were high pressure processed at 600 MPa for 10 min at 20 °C, before being used as emulsifiers. Data are mean ± standard deviations (n = 4). Data with different capital letters (A–C) are significantly different ($p < 0.05$).

sites for β-carotene to attach (Dufour & Haertlé, 1991; Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009).

Increasing the concentration of high pressure-treated WPI as the emulsifier resulted in the reduction of β-carotene in the aqueous phase (Fig. 3). This observation is consistent with our work using native, untreated WPI as emulsifiers (Wan Mohamad, McNaughton, et al., 2017). However, this is counter to expectations that an increased concentration of WPI does provide more sites for β-carotene adsorption, provided that all other factors such as the relative solubility of the bioactive in the oil and aqueous phase, as well as its propensity to assemble at the interface remained constant. Another possible contributory factor may be the thickness of the interfacial layer and the packing density of protein that regulates diffusion of bioactive molecules through the interface (Boon et al., 2010). The pre-treatments further thicken the interfacial layer as the result of protein denaturation.

3.3. Effects of solid fat content on β-carotene partitioning

The partitioning of bioactive in an emulsion-based delivery system can also be influenced by solid fat content of the carrier oil. In this study, palm stearin i.e. the solid fraction of palm oil, was mixed in an increasing ratio to the liquid palm olein, forming increasingly crystalline oil phase of emulsions.

It has been postulated that highly hydrophobic components such as β-carotene can be trapped within colloidal crystalline matrices (the oil phase with higher solid fat content), which slows down their diffusion and that of any reactant that might promote instability e.g. oxygen, so as to improve their chemical stability during storage and control their release kinetics (Joshi & Müller, 2009). However practically, it is usually found that the solute or bioactive molecules are expelled once a carrier lipid crystallises because they cannot easily be incorporated into the highly ordered structure of the crystalline phase. Several studies have reported this phenomenon experimentally, for example in partitioning of drugs (Joshi & Müller, 2009), unsaturated oils (Okuda, McClements, & Decker, 2005), aroma compounds (Ghosh, Peterson, & Coupland, 2007) and flavour compounds (Longyuan et al., 2010). This might also explain the partitioning of β-carotene into the aqueous phase apparent in Fig. 4 that escalates from 9.9 ± 1.7 to 53.0 ± 1.8% w/w, with the increase in the solid fat content of emulsions, from manipulating the ratio between the liquid palm olein and its solid fraction; palm stearin.

3.4. Effects of emulsifier type on β-carotene partitioning

In the emulsion field, the emulsifier is an essential part of the system, and it can generally be classified into two groups: small molecule emulsifiers, like polysorbate and glycerol esters, which generally have strong emulsifying properties; and large molecule emulsifiers (or surface-active polymers), like milk proteins and modified starches, which have both emulsifying and stabilizing properties. Here, we also report the difference in partitioning characteristic of β-carotene in oil-in-water emulsions using Tween 40 as the emulsifier, as compared to

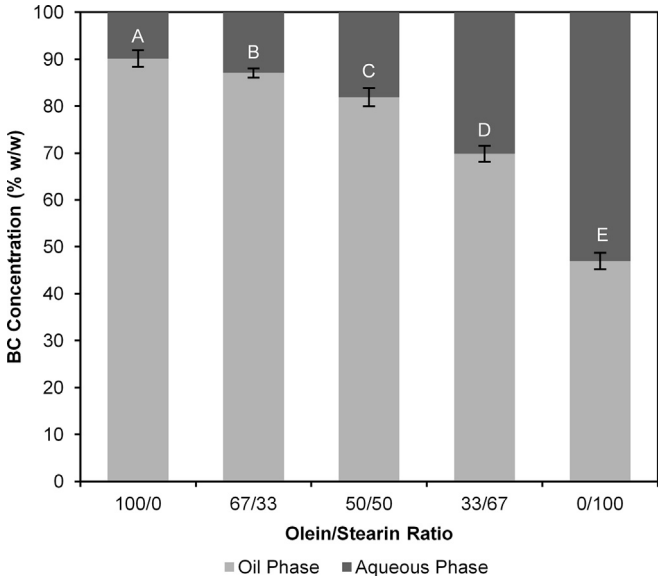

Fig. 4. Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with different solid fat content (100 g/kg palm olein and/or palm stearin at different ratios, 0.2 g/kg β-carotene, 20 g/kg WPI). Data are mean ± standard deviations (n = 4). Data with different capital letters (A–E) are significantly different ($p < 0.05$).

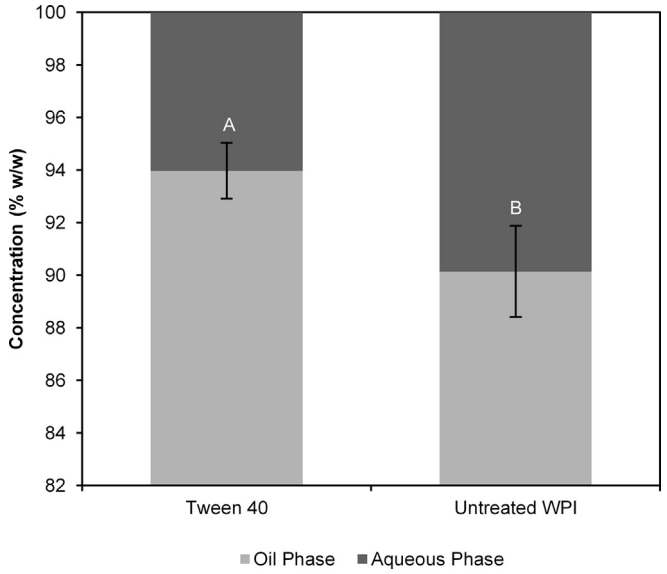

Fig. 5. Partitioning of β-carotene (BC) between the oil and aqueous phases of emulsions with two types of emulsifier; WPI and Tween 40 (100 g/kg palm olein, 20 g/kg WPI or Tween 40, 0.2 g/kg β-carotene). Data are mean ± standard deviations ($n = 4$). Data with different capital letters (A–B) are significantly different ($p < 0.05$).

that using WPI.

Fig. 5 illustrates that the use of Tween 40 results in less partitioning of β-carotene into the aqueous phase of emulsions (6.0 ± 1.1% w/w) than in WPI emulsions (9.9 ± 1.7% w/w). The fact that partitioning was observed in both types of sample indicates interactions between both emulsifiers and β-carotene in the aqueous phase. However, the two interactions happened to different degrees, since the molecular structures and conformations of WPI and Tween 40 may give rise to different distributions of polar/non polar groups as the binding sites (McClements, 2004).

Fig. 5 suggests that there were more β-carotene-WPI than β-carotene-Tween 40 interactions, due to the binding of β-carotene to the protein. Several studies have experimentally shown that β-carotene is able to bind with proteins, particularly β-lactoglobulin, the major component of WPI, to form stable protein–carotenoid complexes with surface–active properties that stabilise the emulsions (Dufour & Haertlé, 1991; Wackerbarth et al., 2009).

Our observations indicate that the β-carotene concentration within an individual droplet and among the different droplets was very similar. This can be seen from the error bars on each column (representing one duplicated system) in Figs. 2–5. Similar insignificant variability was also reported in our previous studies suggesting that β-carotene was uniformly solubilised within the oil phase of all emulsion samples (Wan Mohamad, Buckow, et al., 2017; Wan Mohamad, McNaughton, et al., 2017). According to McClements (2012), the equilibrium solubility of a material in a spherical particle increases as the size of the particle decreases. Thus, it can be assumed that also droplets > 5 μm (not examined in this study) contained fully solubilised β-carotene.

4. Conclusion

In-situ investigations, using the CRM technique, of the partitioning characteristics of β-carotene in emulsions with various formulations and pre-treatment conditions have been reported. The partitioning of β-carotene into the aqueous phase of emulsions increased when using heat and high pressure-treated WPI as emulsifier in comparison to the use of untreated WPI due to the new equilibrium of β-carotene between the different phases of the emulsions as a result of the protein structure modification during the pre-treatment processes. Similarly, partitioning of β-carotene into the aqueous phase can be increased by increasing the solid fat content of emulsions. This could be a result of more solute molecules being expelled with the increase of ordered crystalline structure in the carrier lipid. The use of Tween 40 as compared to that of WPI as the emulsifiers resulted in less partitioning of the bioactive molecules into the aqueous phase of emulsions because the hydrophobic interactions between β-carotene and the two emulsifiers is dependent on the availability of binding sites. The results of this study will be beneficial in designing delivery systems for encapsulating β-carotene to effectively incorporate into aqueous-based food, beverage and pharmaceutical products while sustaining its oral bioavailability.

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References

Boon, C. S., McClements, D. J., Weiss, J., & Decker, E. A. (2010). Factors influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition, 50*, 515–532.
Cornacchia, L., & Roos, Y. H. (2011a). Stability of β-carotene in protein-stabilized oil-in-water delivery systems. *Journal of Agricultural and Food Chemistry, 59*, 7013–7020.
Cornacchia, L., & Roos, Y. H. (2011b). State of dispersed lipid carrier and interface composition as determinants of beta-carotene stability in oil-in-water emulsions. *Journal of Food Science, 76*, C1211–C1218.
Devi, A. F., Buckow, R., Hemar, Y., & Kasapis, S. (2014). Modification of the structural and rheological properties of whey protein/gelatin mixtures through high pressure processing. *Food Chemistry, 156*, 243–249.
Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces, 81*, 130–140.
Dickinson, E., & James, J. D. (1998). Rheology and flocculation of high-pressure-treated B-lactoglobulin-stabilized emulsions: Comparison with thermal treatment. *Journal of Agricultural and Food Chemistry, 46*, 2565–2571.
Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin: Influence of protein modifications. *Biochemica et Biophysica Acta, 1079*, 316–320.
Ghosh, S., Peterson, D. G., & Coupland, J. N. (2007). Aroma release from solid droplet emulsions: Effect of lipid type. *JAOCS, Journal of the American Oil Chemists' Society, 84*, 1001–1014.
Joshi, M. D., & Müller, R. H. (2009). Lipid nanoparticles for parenteral delivery of actives. *European Journal of Pharmaceutics and Biopharmaceutics, 71*, 161–172.
Kühn, J., Considine, T., & Singh, H. (2008). Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. *Journal of Agricultural and Food Chemistry, 56*, 10218–10224.
Longyuan, M., Seung, J. C., Jean, A., Lulu, H., Popplewell, Michael, Mcclements, D. J., & Decker, E.a. (2010). Citral stability in oil-in-water emulsions with solid or liquid octadecane. *Journal of Agricultural and Food Chemistry, 58*, 533–536.
Lopez-Sanchez, P., Schumm, S., Pudney, P. D. A., & Hazekamp, J. (2011). Carotene location in processed food samples measured by cryo In-SEM Raman. *The Analyst, 136*, 3694–3697.
Maiani, G., Castón, M. J. P., Catasta, G., Toti, E., Cambrodón, I. G., Bysted, A., ... Schlemmer, U. (2009). Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Molecular Nutrition and Food Research, 53*, S194–S218.
Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y., & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of b-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology, 9862*, 336–342.
Mao, L., Yang, J., Xu, D., Yuan, F., & Gao, Y. (2010). Effects of homogenization models and emulsifiers on the physicochemical properties of β-carotene nanoemulsions. *Journal of Dispersion Science and Technology, 31*, 986–993.
McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5), 305–313.
McClements, D. J. (2012). Crystals and crystallization in oil-in-water emulsions: Implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science, 174*, 1–30.
McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science, 72*, R109–R124.
McClements, D. J., Monahan, F. J., & Kinsella, J. E. (1993). Disulfide bond formation affects stability of whey protein isolate emulsions. *Journal of Food Science, 58*, 1036–1039.
McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrician, 51*, 285–330.
Miller, P. E., & Snyder, D. C. (2012). Phytochemicals and cancer risk: A review of the

epidemiological evidence. *Nutrition in Clinical Practice, 27*, 599–612.
Okuda, S., McClements, D. J., & Decker, E. A. (2005). Impact of lipid physical state on the oxidation of methyl linolenate in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 53*, 9624–9628.
Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry, 132*, 1221–1229.
Rao, A. V., & Rao, L. G. (2007). Carotenoids and human health. *Pharmacological Research, 55*, 207–216.
Trentin, A., De Lamo, S., Güell, C., López, F., & Ferrando, M. (2011). Protein-stabilized emulsions containing beta-carotene produced by premix membrane emulsification. *Journal of Food Engineering, 106*, 267–274.
Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein interaction as an approach for the formulation of functional food emulsions. *Food Research International, 42*, 1254–1258.
Wan Mohamad, W. A. F., Buckow, R., Augustin, M., & McNaughton, D. (2017). In situ quantification of β-carotene partitioning in oil-in-water emulsions by confocal Raman microscopy. *Food Chemistry, 233*, 197–203.
Wan Mohamad, W. A. F., McNaughton, D., Buckow, R., & Augustin, M. (2017). Stability and partitioning of β-carotene in whey protein emulsions during storage. *Food & Function, 8*, 3917–3925.
Whelan, D. R., Bambery, K. R., Puskar, L., McNaughton, D., & Wood, B. R. (2013). Quantification of DNA in simple eukaryotic cells using Fourier transform infrared spectroscopy. *Journal of Biophotonics, 6*, 775–784.
Yi, J., Li, Y., Zhong, F., & Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloids, 35*, 19–27.

CHAPTER 8 CONCLUSIONS

In general, this research presents successful applications of confocal Raman microspectroscopy (CRM) in food-related studies, particularly in analysing the partitioning characteristics of bioactive encapsulated in emulsion systems. Palm oil-in-water (O/W) emulsions stabilised by whey protein isolate (WPI) were fabricated to encapsulate β-carotene, a natural bioactive with abundant health benefits. The ability of the Raman technique to quantify the bioactive concentration in the dispersed phase of emulsions *in situ* was first validated against the conventional extraction-spectrophotometric method. Knowing the β-carotene concentration in the oil droplets and that formulated in the whole emulsion allows for understanding of the bioactive partitioning within the dispersed system, this time more easily and safely. The CRM was then used to determine the partitioning characteristics of β-carotene in various formulations of fresh and stored WPI emulsion, which results and future recommendations (written in *italics*) are concluded as follows.

1. CRM validation and mapping ability

In Chapter 5, it has been shown that CRM enables *in situ* quantification of β-carotene content in the oil droplets of emulsion systems accurately and precisely. From the validation model, the concentration values from the Raman method show excellent agreement with the reference concentrations obtained from the conventional extraction followed by spectrophotometric analysis. The relative error of prediction (REP) and the relative standard deviation (RSD) values further emphasise the high accuracy and precision of the Raman measurements, respectively. The CRM's sensitivity in measuring very low concentrations of bioactive in the oil droplets is shown by the limit of detection (LOD) value, which is much lower than the concentration range of the samples used in the whole study.

Apart from the spectrophotometric method, high-performance liquid chromatography (HPLC) can also be used to validate the ability of CRM to quantify the bioactive concentration within emulsion samples. This established chromatographic technique, although being more tedious as well as time and cost intensive (Biehler, Mayer, Hoffmann, Krause, & Bohn, 2010), is widely used for bioactive quantification (Che Man et al., 2005; Qiu, Chen, & Li, 2009; Thakkar et al., 2007; Y. Yang & McClements, 2013; Yi et al., 2015) and paired with spectroscopic analyses including Raman (Arikan et al., 2002; Beattie et al.,

2007), as it allows for highly accurate detection and quantification of individual bioactive (Ferruzzi, Sander, Rock, & Schwartz, 1998).

Additionally using CRM, Raman images from the point mapping configuration qualitatively define the location of β-carotene in the emulsion system. It was identified that the bioactive molecules were partitioned more into the oil phase than the aqueous, as quantitatively measured via Raman point microspectroscopic and spectrophotometric methods. The Raman images also illustrate the homogeneity of β-carotene within the oil droplets *in situ*, thus revealing the effectiveness of the bioactive solubilisation technique in oil, and that the bioactive partitioning into the aqueous phase does not affect its homogeneity in the dispersed phase.

Due to the confocal arrangement of the CRM used in this study i.e. WITec alph300 R, not only 2-D colour-coded Raman images can be produced, depth profiling and 3-D mapping are even possible (Fischer, Ibach, Dampel, & Sanchen, 2009). The latest spectroscopic detector technology combined with a high-throughput confocal microscope should allow the acquisition time for a single Raman spectrum to be reduced to 0.7 ms. This ultrafast confocal Raman imaging capability can thus be used to depth-profile the β-carotene emulsion and analyse its components distribution three dimensionally from 3-D images created using a 3-D reconstruction software. Nevertheless, the idea is challenging as it is interesting. Although the mapping is considered ultrafast, the total exposure time of the sample to the laser is quite long for the encapsulated photosensitive β-carotene. Degradation of the bioactive during mapping will result in false representation of the bioactive content and distribution within the heterogeneous system. Therefore further measures such as limiting the mapping area to a very small square, reducing the laser power and choosing the appropriate size of droplets to scan, must be considered for a successful 3-D imaging of the delicate system.

2. Partitioning of β-carotene in various emulsion formulations

Chapter 5 also presents the *in situ* observation using CRM only, on β-carotene partitioning between the phases of emulsions with varying β-carotene concentrations. The results are consistent in the following study reported in Chapter 6, in which the protein (WPI) concentrations were also varied. It was found that increasing the β-carotene concentration of fresh emulsions at a fixed gross reduced that in the oil droplet, whereas increasing the WPI concentration had the opposite effect, which was unexpected. Upon increasing the bioactive

concentration in an emulsion, a new equilibrium is established in the β-carotene partitioning between the various phases, hence causing more bioactive molecules to partition into the aqueous phase and bind to WPI therewith. However when the WPI concentration is increased, the thickness and packing density of the protein at the interface may have also increased and reduced the affinity between protein binding sites and β-carotene molecules. Another possibility is that there might also be incomplete hydration of WPI at high concentrations during emulsion formation, resulting in reduced affinity in systems with higher protein concentrations.

Chapter 7 on the other hand reports that the use of small molecule Tween 40, as compared to that of the dairy protein, as the emulsifier, resulted in lesser partitioning of the bioactive molecules into the aqueous phase of emulsions. This is evident to the stronger β-carotene-protein affinity in comparison to the attraction between β-carotene and Tween 40 molecules. It is also presented in Chapter 7 that the partitioning of β-carotene into the aqueous phase increased with the rise in the solid fat content of emulsions, achieved by manipulating the ratio between the liquid palm olein and its solid fraction; palm stearin. This illustrates the expulsion of more bioactive molecules from the increasingly ordered structure of the crystalline oil phase, as previously reported in literatures.

There are many other properties of the emulsion formulation that can be varied to see their effects on the bioactive partitioning. Different bioactive will partition differently due to different chemical structure and thus affinity to other molecules in the emulsion system. Tocols (e.g. tocopherols and tocotrienols) for example are interesting phytonutrients to consider mainly due to their distinguished signals under Raman (Beattie et al., 2007; Beattie & Schock, 2009), besides being an excellent source of Vitamin E for human consumption (Brigelius-flohe, 1999; Mayer, Weiss, & McClements, 2013). In addition, the type of oil (e.g. LCT, MCT or SCT) and its loading can also influence the maximum amount of dissolved bioactive in the oil phase as well as the oil–water partition coefficient (McClements, 2012). Other than that, the addition of other ingredients as stabiliser or co-surfactant such as sucrose can also affect the partitioning of bioactive in an emulsion, most likely the same way it affects the physical stability of the system as well as chemical stability of the bioactive (Cornacchia & Roos, 2011). Therefore further studies should be conducted to examine the different factors of bioactive partitioning with regards to the emulsion formulation.

3. Partitioning of β-carotene during storage

In Chapter 6, a storage study was also conducted to determine the partitioning characteristics and stability of β-carotene when varying the bioactive and WPI concentrations separately, throughout 30-day storage at 25 and 40 °C. Both the spectrophotometric and Raman techniques were applied to determine the β-carotene content in each phase of the emulsion samples. At the end of the storage period, the higher proportion and concentration of β-carotene was in the aqueous phase, suggesting that oxidation of β-carotene occurred faster in the oil phase and that WPI in the aqueous phase protected β-carotene against oxidation, regardless of the emulsion formulation and storage temperature. The results indicate a preferential re-partitioning of β-carotene into the aqueous phase over time, may be due to the continuous hydration of WPI during storage, as a result of incomplete hydration of the protein during emulsion preparation.

For future experimentation on the storage study, complete hydration of the protein has to be examined via standard procedures such as that reported by Sirotkin and Khadiullina (2011). It is expected that upon complete hydration of the protein in freshly prepared emulsions, there should not be any re-partitioning of the bioactive throughout the storage period, hence the bioactive content in each phase should only decrease by time due to natural degradation. Alternatively, using other types of protein as emulsifier, such as NaCas, bovine serum albumin, ovalbumin and soy protein isolate to name a few, should also influence the bioactive partitioning differently from each other, in the fresh and stored emulsion samples. Moreover, storing the emulsion samples at freezing temperatures should significantly affect the bioactive partitioning within the system, as the properties of each ingredient will change and affect the equilibrium of the carried bioactive between the various phases.

4. Partitioning of β-carotene upon treatments

In Chapter 7, the variation in partitioning of β-carotene molecules between the phases of emulsions was also examined following the use of pre-treated (heat and pressure) WPI as an emulsifier, and upon pasteurising the emulsions using the high-temperature-short-time (HTST) method, exampling industrial practices on processed foods. In general, the partitioning of β-carotene into the aqueous phase of emulsions increased when the protein emulsifier (WPI) was heat- or high pressure-treated prior to emulsion formation. Since the protein conformation is changed during the pre-treatments, more binding sites may be

available for β-carotene to attach. However, increasing the concentration of high pressure-treated WPI reduced the β-carotene partitioning into the aqueous phase, as seen when using untreated WPI as emulsifier. Treating the emulsions with the HTST method on the other hand did not significantly affect the β-carotene partitioning between the emulsion phases, as such treatment causes no further changes in the protein conformation.

Incorporating CRM to the study of emulsion should also allow for in situ observation of changes in bioactive partitioning during a thermal treatment and high pressure processing (HPP) of an emulsion sample. This can be carried out by coupling a diamond anvil cell (DAC) to the microscopic system of the CRM (Goncharov & Crowhurst, 2005). Emulsion sample is loaded into the DAC, before the hydrothermal function is turned on for in situ thermal treatment measurement, or the screws spaced along a platen that causes the anvils to press against a gasket containing the sample are manually adjusted for pressure treatment (Bassett, Shen, Bucknum, & Chou, 1993). During measurements, the working distance of the microscope and the numerical aperture of the lenses can be important as summarised by Takemura, Shimomura and Sawada (1989). Many of the key findings obtained with DAC have been qualitative in terms of direct observation of phenomena under extreme conditions and this is one of the greatest strengths of the apparatus (Smith & Fang, 2009). Therefore the effects of varying temperature and pressure on the location and partitioning of bioactive molecules in the emulsion systems can be recorded qualitatively in situ, before being related to the factors contributing to such results.

Besides thermal and pressure treatment, pH treatment of a protein used as the emulsifier can also influence the bioactive partitioning in the emulsion system. Around their isoelectric point (pI), whey proteins for instance, form poor unstable emulsions (Kilara, 2008), which surely affect the distribution of bioactive within the systems.

5. Binding of β-carotene to WPI in aqueous phase

An exploratory attempt to monitor the structural changes upon β-carotene partitioning into the aqueous phase was also made using synchrotron infrared (SIR) microspectrometer, in order to reveal the conformational changes in the protein molecules and observe its binding structure with the partitioned bioactive molecules. There seems to be small differences in the protein bands upon β-carotene addition to the emulsion system, but the results may not

necessarily be due to the change in protein conformation upon binding to bioactive molecules.

In the future beamtime with the SIR microspectroscopy, a few improvements in the sample formulation and measurement procedures are required for better results and outcomes. Samples prepared with water (H_2O) exhibit strong absorption of water with a maximum between 1650 and 1640 cm^{-1}. Therefore more concentrated protein solutions with higher bioactive concentration should help to obtain more useful IR spectra of the protein with clearer band shifts if any, while reducing the effect of H_2O bands on the overall spectra of the sample. Alternatively, deuterated emulsion samples can also be prepared to obviate the water band problem in the protein structure analysis by treating them with heavy water (D_2O) (Herrero, Carmona, Pintado, Jiménez-Colmenero, & Ruíz-Capillas, 2011a, 2011b). However care must be taken to prevent the deuterated samples turning back to its original H_2O base upon exposure to the air. Hence it is best to equip the SIR spectrometer with a high sensitivity deuterated triglycine sulphate detector to monitor the deuteration by means of the OH stretching band at a spectral resolution of 2 cm^{-1} over a range of 4000-800 cm^{-1}.

This study is differentiated from others in that it (i) uses natural extracts containing the bioactive, (ii) examines the bioactive location within protein-based emulsion structures and its partitioning characteristics between the aqueous phase and lipid core of the emulsion, (iii) investigates the influence of different emulsion formulations and emulsification conditions on the stability of the encapsulated bioactives to degradation, and (iv) uses non-destructive spectroscopic methods (Raman/IR) coupled with imaging (i.e. confocal microspectroscopy) to provide insights into the partitioning and stability of the bioactive *in situ*.

Reference

Arikan, S., Sands, H. S., Rodway, R. G., & Batchelder, D. N. (2002). Raman spectroscopy and imaging of beta-carotene in live corpus luteum cells. *Animal Reproduction Science*, *71*, 249–66.

Bassett, W. A., Shen, A. H., Bucknum, M., & Chou, I. M. (1993). Hydrothermal studies in a new diamond anvil cell up to 10GPa and from -190 °C to 1200 °C. *Pure and Applied Geophysics*, *141*, 487–495.

Beattie, J. R., Maguire, C., Gilchrist, S., Barrett, L. J., Cross, C. E., Possmayer, F., … Schock, B. C. (2007). The use of Raman microscopy to determine and localize vitamin E in biological samples. *The FASEB Journal*, *21*, 766–776.

Beattie, J. R., & Schock, B. C. (2009). Identifying the spatial distribution of vitamin E, pulmonary surfactant and membrane lipids in cells and tissue by confocal Raman microscopy. In D. Armstrong (Ed.), *Lipodimics* (Vol. 579, pp. 513–535). Totowa, NJ: Humana Press.

Biehler, E., Mayer, F., Hoffmann, L., Krause, E., & Bohn, T. (2010). Comparison of 3 spectrophotometric methods for carotenoid determination in frequently consumed fruits and vegetables. *Journal of Food Science*, *75*, C55–C61.

Brigelius-flohe, R. (1999). Vitamin E: Function and metabolism. *The FASEB Journal*, *13*, 1145–1155.

Che Man, Y. B., Ammawath, W., & Mirghani, M. (2005). Determining α-tocopherol in refined bleached and deodorized palm olein by Fourier transform infrared spectroscopy. *Food Chemistry*, *90*, 323–327.

Cornacchia, L., & Roos, Y. H. (2011). Solid-liquid transitions and stability of HPKO-in-water systems emulsified by dairy proteins. *Food Biophysics*, *6*, 288–294.

Ferruzzi, M. G., Sander, L. C., Rock, C. L., & Schwartz, S. J. (1998). Carotenoid determination in biological microsamples using liquid chromatography with a coulometric electrochemical array detector. *Analytical Biochemistry*, *256*, 74–81.

Fischer, H., Ibach, W., Dampel, H., & Sanchen, D. (2009). A Confocal Raman Imaging Study on Emulsions. *Imaging & Microscopy*, *11*, 35–38.

Goncharov, A. F., & Crowhurst, J. C. (2005). Pulsed laser Raman spectroscopy in the laser-heated diamond anvil cell. *Review of Scientific Instruments*, *76*, 1–5.

Herrero, A. M., Carmona, P., Pintado, T., Jiménez-Colmenero, F., & Ruíz-Capillas, C. (2011a). Infrared spectroscopic analysis of structural features and interactions in olive oil-in-water emulsions stabilized with soy protein. *Food Research International*, *44*, 360–366.

Herrero, A. M., Carmona, P., Pintado, T., Jiménez-Colmenero, F., & Ruíz-Capillas, C. (2011b). Olive oil-in-water emulsions stabilized with caseinate: Elucidation of protein–lipid interactions by infrared spectroscopy. *Food Hydrocolloids*, *25*, 12–18.

Kilara, A. (2008). Whey and Whey Products. In R. C. Chandan, A. Kilara, & N. P. Shah (Eds.), *Dairy Processing and Quality Assurance* (pp. 337–355). John Wiley and Sons.

Mayer, S., Weiss, J., & McClements, D. J. (2013). Behavior of vitamin E acetate delivery systems under simulated gastrointestinal conditions: lipid digestion and bioaccessibility of low-energy nanoemulsions. *Journal of Colloid and Interface Science*, *404*, 215–22.

McClements, D. J. (2012). Crystals and crystallization in oil-in-water emulsions: implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science*, *174*, 1–30.

Qiu, D., Chen, Z.-R., & Li, H.-R. (2009). Effect of heating on solid β-carotene. *Food Chemistry*, *112*, 344–349.

Sirotkin, V. A., & Khadiullina, A. V. (2011). Hydration of proteins: Excess partial enthalpies of water and proteins. *Journal of Physical Chemistry B*, *115*, 15110–15118.

Takemura, K., Shimomura, O., & Sawada, T. (1989). A diamond anvil cell for advanced microscopic observations and its application to the study of crystal growth under pressure. *Review of Scientific Instruments*, *60*, 3783.

Thakkar, S. K., Maziya-dixon, B., Dixon, A. G. O., & Failla, M. L. (2007). β-carotene micellarization during in vitro digestion and uptake by Caco-2 cells is directly droportional to b-carotene content in different genotypes of Cassava. *The Journal of Nutrition*, *137*, 2229–2233.

Yang, Y., & McClements, D. J. (2013). Vitamin E bioaccessibility: influence of carrier oil type on digestion and release of emulsified α-tocopherol acetate. *Food Chemistry*, *141*, 473–481.

Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., & Zhong, F. (2015). Beta-carotene encapsulated in food protein nanoparticles reduces peroxyl radical oxidation in Caco-2 cells. *Food Hydrocolloids*, *43*, 31–40.