Digital Photo Analysis for Tissue Carotenoid Status Assessment

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October 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

ABSTRACT

Background: Carotenoids are yellow-red plant pigments obtained from fruits and vegetables (FV) that are largely deposited into skin and serve as a biomarker for FV intake. Current methods of carotenoid assessment (dietary assessment, HPLC blood analysis, skin spectrophotometry) can be biased, time consuming, complex, and expensive.

Objective: We aimed to assess the feasibility of digital image skin color analysis for skin carotenoid content estimation against spectrophotometric analysis and study the impact of carotenoid-mediated facial skin coloration on measures of perceived health and attractiveness.

Methods: Twenty-five participants completed a two-week open label carrot-based juice supplementation trial with bi-weekly follow up measurements for four weeks. Images of three skin regions (palm, top hand, cheek) were taken under various lighting conditions (standardized, device-flash, incandescent) and with different devices (Canon camera, and two smartphones) along with dietary (FFQ & diet diary) and spectrophotometric measurements. Images were rated for perceived health and attractiveness by trained evaluators that exhibited high inter-rater reliability.

Results: Palm carotenoid-mediated skin coloration increased significantly following supplementation (Week 2) and throughout follow up (Weeks 4 & 6). Digital image skin color analysis of the palm correlated strongly with spectrophotometer changes in palm skin coloration, with the strongest correlation noted for images taken with a device's flash. No significant changes were noted for perceived health and attractiveness ratings.

Conclusion: We conclude that digital image analysis can serve as a rapid estimate for skin carotenoid content when images are taken with the device's flash, but changes in perceived health and attractiveness may require more than two weeks of carotenoid supplementation.

RÉSUMÉ

Contexte: Les caroténoïdes sont des pigments végétaux jaune-rouge obtenus à partir de fruits et de légumes (FL), qui se déposent en grande partie dans la peau et servent de biomarqueur à l'apport en FL. Les méthodes actuelles d'évaluation des caroténoïdes (évaluation du régime alimentaire, analyse sanguine par CLHP, spectrophotométrie cutanée) peuvent être biaisées, longues, complexes et coûteuses.

Objectif: Nous avions pour objectif d'évaluer la faisabilité de l'analyse numérique de la couleur de la peau contre l'analyse spectrophotométrique pour l'estimation du contenu en caroténoïdes de la peau et d'étudier l'impact de la coloration de la peau du visage induite par les caroténoïdes sur les mesures de la santé perçue et de l'attractivité physique.

Méthodes: Un essai en ouvert de deux semaines de supplémentation en jus à base de carottes a été mené auprès de vingt-cinq participants, avec mesures de suivi toutes les deux semaines pendant quatre semaines. Des images de trois régions de la peau (paume, revers de la main, joue) ont été prises sous différentes conditions d'éclairage (standard, flash, incandescent) et avec différents appareils (appareil photo Canon et deux téléphones intelligents) ainsi qu'une évaluation alimentaire (questionnaire de fréquence de consommation alimentaire et journal alimentaire) et mesures spectrophotométriques.

Résultats: La coloration de la peau de la paume médiée par les caroténoïdes a considérablement augmenté suivant la supplémentation (semaine 2) et pendant le suivi (semaines 4 et 6). L'analyse numérique de la couleur de la peau de la paume est étroitement corrélée aux changements de coloration évalués par spectrophotométrie, la corrélation la plus forte étant observée pour les images prises avec un flash. Aucun changement significatif n'a été noté pour les évaluations de la santé perçue et de l'attractivité physique.

Conclusion: Nous concluons que l'analyse numérique des images peut servir d'estimation rapide du contenu en caroténoïdes de la peau lorsque les images sont prises avec le flash de l'appareil, mais des modifications de la santé perçue et de l'attractivité pourraient nécessiter plus de deux semaines de supplémentation en caroténoïdes.

ACKNOWLEDGEMENTS

I want to thank Dr. Stan Kubow for allowing me to jump on what was first mentioned to me as a ‘secret project’ and giving me the freedom to explore his idea. Dr. Kubow acted as a role model and guide, through his ideas and discussions over nutrition topics. I want to thank him for fostering a flexible and enriching research environment for me to explore, all while leaving crumbs of knowledge to nudge me in the right direction. I also wish to thank my committee members Dr. Elena Gheorgiu, Dr. Danielle Donnelly, and Dr. Luis B. Agellon for their helpful insight and advice.

I want to thank Ben Jennings for his tireless support of the in-house Matlab program used to analyze the photos taken. Countless meetings and emails troubleshooting and re-coding the program are a testament to his support.

I want to thank Brian Lee and Travis Bell from cold-pressed juice brand Buda Juice for making this study possible by donating the juice for the intervention. The juice tasted great and the participants were very pleased.

I want to thank Dr. Michele Iskandar for pushing me to see Dr. Kubow to explore any opportunities that aligned with my interests, knowing about the ‘secret project’. I want to thank her for support in the many miscellaneous aspects of work, from study design and logistics to statistics and brainstorming. I want to thank Kebba Sabbally for his constant support on protocol development, first with the juice and skin carotenoid extraction, and then with the HPLC and LC-MS protocols.

I want to thank Baasir Gaisawat for his constant support, from motivation to photography to setup of some aspects of the study. I want to thank Alana Cohen, Dr. John Lydon’s lab coordinator, for organizing and orchestrating the perception trial. I also want to thank Catherine Vanstone for facilitating my work at the Mary Emily Research Unit.

Finally, I want to thank my friends and family for supporting me throughout the journey. Last but not least, I thank my girlfriend for taking me away from my work, allowing me to come back fresher and more focused.

CONTRIBUTION OF AUTHORS

Lucas Roldos (MSc Candidate) was mainly responsible for study design and implementation, development of protocols, ethical approval, coordination of juice donation and delivery, sample recruitment and coordination, data collection (weight, height, spectrophotometer readings, skin samples, photo capture), experiments and analyzing data. The candidate wrote this thesis and prepared all the figures and tables.

Dr. Stan Kubow (Supervisor of Candidate, Associate Professor, School of Human Nutrition, McGill University): Provided the research direction, guidance and feedback for this study, its ethics application, and thesis. Dr. Kubow provided extensive edits on the thesis.

Dr. Elena Gheorghiu (Committee member; Department of Psychology, Sterling University): Guided data analysis, study design and thesis presentation, and helped edit the thesis.

Dr. Danielle Donnelly (Committee member; Department of Plant Science, McGill University): Pushed for an intervention trial, provided guidance throughout with a concentration on thesis presentation and thesis edits.

Dr. Louis Agellon (Committee member; School of Human Nutrition, McGill University): Provided ideas for study design, data analysis and thesis presentation and helped edit the thesis.

Dr. Fred Kingdom (McGill Vision Research Center): Provided guidance for color and image processing.

Dr. Ben Jennings (Lecturer of Psychology; Department of Life Sciences; Brunel University) Designed and edited in-house Matlab image processing software and advised on lighting and color control for image capture conditions.

Dr. John Lydon (Professor and Department Chair; Department of Psychology, McGill University): Provided guidance and design for the perception trial.

Alana Cohen (Lydon Lab Coordinator): Organized and led the perception trial in the Department of Psychology and communicated results and procedures.

Dr. Michele Iskandar (Postdoctoral Fellow; School of Dietetics and Human Nutrition, McGill University): Guided protocol development, study design and data analysis.

Dr. Kebba Sabally (Research Associate; School of Human Nutrition, McGill University): Provided training and testing of juice samples with HPLC and LC-MS.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2'-hydroxyguanosine
8-iso-PGF2	8-isoprostane F2α
AMD	Age-related Macular Degeneration
AUC	Area Under the Curve
BCO1	β-Carotene Oxygenase 1
BCO2	β-Carotene Oxygenase 2
BCVA	Best Corrected Visual Acuity
BP	Blood pressure
CAT	Contrast Acuity Threshold
CCD	Charge Coupled Device
CCHS	Canadian Community Health Survey
CD36	Cluster Determinant 36
CHD	Coronary Heart Disease
CIE	Commission Internationale de l'Eclairage
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DALY	Disability Adjusted Life Years
DASH	Dietary Approaches to Stop Hypertension
EPIC	European Prospective Investigation into Cancer and nutrition
FFQ	Food Frequency Questionnaire
FV	Fruit(s) and Vegetable(s)
GBD	Global Burden of Disease
HPLC	High-Performance Liquid Chromatography
hsCRP	High-sensitivity C-Reactive protein
IR	Insulin Resistance
LCAT	Lecithin Cholesterol Acyltransferase
LC-MS	Liquid Chromatography tandem Mass Spectrometry
LDL	Low-Density Lipoprotein
MPOD	Macular Pigment Optical Density
NCD	Non-communicable Disease
PON-1	ParaoxoNase-1
PURE	Prospective Urban Rural Epidemiology
RCT	Randomized Controlled Trial
ROS	Reactive Oxygen Species
RRS	Resonance Raman Spectrophotometry
SEM	Standard Error Mean
sICAM1	Soluble Intercellular Adhesion Molecule-1
SNP	Single-nucleotide Polymorphism
SR-B1	Scavenger Receptor class B type 1
UV	Ultraviolet
WHO	World Health Organization

Chapter 1

Introduction and Objectives

1.1 Rationale and Statement of Purpose

Almost 80% of the global population consumes less than the recommended intake of five fruit and vegetable (FV) servings per day (Hall et al., 2009). In Canada, about 70% of the population consumes inadequate amounts of FVs (Statistics Canada, 2017) and only 1 in 10 adolescents (ages 11-17 years) consumes adequate amounts (Minaker & Hammond, 2016). This dietary trend has grave consequences as FV consumption is inversely associated with non-communicable diseases (NCDs) such as coronary heart disease, stroke, cancer, diabetes, and more (Boeing et al., 2012); the above-mentioned diseases being within the top ten causes of mortality worldwide (Xu et al., 2016).

Carotenoids are yellow-red plant pigments with provitamin A, antioxidant (Maiani et al., 2009), anti-inflammatory (Watzl et al., 2005), and anti-cancer (Donaldson, 2011) properties that can only be obtained from the diet, primarily from FVs (Namitha & Negi, 2010). These compounds are thought to be one of the driving factors for the inverse relationship between FV intake and chronic disease (Vargas-Murga et al., 2016), exhibiting a positive dose response relationship between serum carotenoid concentrations and NCD risk prevention (Donaldson, 2011). A major limitation towards primary disease prevention is that patient screening for carotenoid status is not a routine practice due to invasive sample-based measures or biased self-reported methods (Maiani et al., 2009). While the prevalence of vitamin A deficiency is decreasing across low-income countries thanks to large vitamin A supplementation programs (UNICIEF, 2018), deficiency is

still of grave concern for most of South Asia and Sub-Saharan Africa (Stevens et al., 2017). Thus, there is a need for an accessible, non-invasive pro-vitamin A carotenoid screening tool.

Tissue carotenoid content has emerged as a biomarker for FV intake and non-invasive, objective spectroscopic methods have been validated for carotenoid and FV intake assessment (Ermakov & Gellerman, 2015; Mayne et al., 2010). Since carotenoids reach the systemic circulation and are ultimately stored in the liver, adipose tissue, and skin (Kiokias et al., 2016), modern optical methods focus on skin yellowing for rapid yet reliable measurement of carotenoid status (Pezdirc et al., 2016; Tan et al., 2015). A validated optical approach involves spectroscopy that can detect the carotenoid associated yellow coloration by utilizing a component of the device independent and perceptually uniform Commission International d'Eclairage (CIE) L*a*b* color space where L* represents lightness, and positive values of a* and b* represent red and yellow color saturation, respectively (Alaluf et al., 2002).

Recently, digital image analysis for CIELab color space coloration using a calibrated Nikon camera was successfully used to identify changes in skin yellowing (b* value) associated with increased carotenoid supplementation (Foo et al., 2017). While the study used various measures of control for image capture in terms of standardized lighting conditions, other modulating imaging variables such as different levels of control or standardization, how much standardization may be necessary and for which parameters, and whether digital imaging using smartphones is feasible, has not been established. Additionally, to our knowledge, digital imaging has not been assessed in relationship to skin spectroscopic measurement of skin coloration.

Skin yellowness, based on the presence of plant pigments in the skin, is associated with greater perceived health and attractiveness (Foo et al., 2017), regardless of race (Whitehead et al., 2012a) or melanin levels (i.e., skin tanning from sun exposure) and surpasses perceived health and attractiveness provided by a skin tan (Stephen et al., 2011; Lefevre & Perrett, 2015). Therefore,

monitoring yellow saturation of the skin could generate motivation to increase FV consumption, by appealing not only to a desire for overall health improvement but also to vanity (Whitehead et al., 2012b). Variations in perceived health and attractiveness have not been assessed over time within individuals over a short duration intervention using a carotenoid-rich food supplementation.

In summary, worldwide FV intake is inadequate and associated with increasing prevalence of NCDs. Carotenoids are plant pigments with antioxidant, anticancer, and anti-inflammatory properties that contribute to the inverse relationship between FV intake and NCDs and serve as a biomarker for FV intake. Recent advances in spectroscopic carotenoid assessment methods have demonstrated skin yellowing from carotenoid supplementation. An increase in perceived health and attractiveness has been linked to the carotenoid associated skin yellowing, regardless of race and melanin content. To date, variations in digital image capture conditions, usage of different devices, and validation against optical methods, as well as intra-individual improvements in perceived health and attractiveness, have not been investigated in a carotenoid-rich food supplement intervention trial.

1.2 Thesis Objective

To investigate whether daily 250 mL supplementation of carrot-based FV juice in healthy male and female young adult subjects over a two-week period is associated with:

a) increased carotenoid status as assessed by skin yellowness “b” values via: (a) Minolta spectrophotometric analysis; (b) digital skin image analysis with a calibrated Canon camera; and (c) digital skin image analysis with two different types of smartphone cameras;
b) higher ratings of perceived health and attractiveness.

Chapter 2

Literature Review

2.1 Fruit and Vegetable (FV) Intake

In 2010, only 0.4% of a sample representing 87% of the world's adult population met United States Department of Agriculture (USDA) dietary guidelines of 4 servings (2 cups) of fruit and 5 servings (2 ½ cups) of vegetables per day, according to a systematic analysis of 204 surveys in 109 countries (Micha et al., 2015). In 2003, according the analysis of the World Health Survey (52 countries), almost 80% of the global population did not meet the World Health Organizations (WHO) minimum recommendation of 5 servings (400 g, 80 g/serving) per day (Hall et al., 2009).

Low FV consumption has been considered as one of the ten most important risk factors for global mortality, contributing to 1.7 million deaths and 16 million disability-adjusted life years worldwide (WHO, 2017). Most prominent dietary risks attributed to the global burden of disease are low FV and high sodium intake (Lim et al., 2012). In that regard, the Dietary Approaches to Stop Hypertension (DASH) program has demonstrated disease risk factors can be diminished with a focus on unprocessed foods, especially FVs (Harnden et al., 2010). Moreover, malnutrition and hunger can arise from micronutrient deficiencies associated with low FV intake, contributing to a lower quality of life; "dietary adjustments may not only influence present health, but may determine whether or not an individual will develop such diseases as cancer, cardiovascular disease, and diabetes much later in life" (WHO, 2003).

Although FV consumption is lower among developing countries, primarily due to the association between adherence to dietary guidelines and economic stability, North America is still subject to the epidemic of low FV intake (Mazzocchi et al., 2007). In the United States, average

FV intake was 2.5 cups/day according to the data from the What We Eat in America 24-hour recalls and the National Health and Nutrition Examination Survey of 2009–2012 (Hoy et al., 2016). These surveys have shown that Americans appear to meet the minimum requirement by the WHO but are consuming under the 4.5 cup/day recommendation of the USDA (Hoy et al., 2016). In Canada, higher levels of education and income are associated with increased FV intake (Azagba & Sharaf, 2011; Dehghan et al., 2011). It is estimated, however, that 70% of the Canadian population consumes inadequate amounts of FVs according to the Canadian Community Health Survey (CCHS) of 2015 (Statistics Canada, 2017), and only 1 in 10 Canadian adolescents (ages 11-17 y) consumes adequate levels (Minaker and Hammond, 2016).

In the 2004 CCHS, about 44% of the Canadian population consumed inadequate amounts of FV's, which signifies that FV intake has reduced over the last decade (Black & Billette, 2011). This latter situation has occurred despite the 2007 Canadian Food Guide recommendation to consume 8-10 and 7-8 servings/day for adult men and women, respectively (Black & Billette, 2011). According to the 2014 CCHS data, the economic burden of inadequate FV intake in Canada is estimated to be $3.3 billion CAD annually, with direct health-care costs for chronic disease accounting for 30.5% and indirect productivity loss costs accounting for 69.5% (Ekwaru et al., 2017). For the past two decades, researchers have warned of a "nutrition transition", pertaining to trends toward increased consumption of sugar and fat from processed foods with decreased intake of FV, along with an accompanying epidemic of non-communicable diseases (NCDs) (Popkin, 2006). There has been increasing research attention directed towards novel dietary and behavioral approaches to counteract this "nutrition transition".

2.2 FV Intake and Non-Communicable Disease (NCD)

A suboptimal diet is one of the leading preventable risk factors for NCDs, with inadequate FV intake accounting for 41% of all dietary risk associated deaths in 2015 (Forouzanfar et al., 2016). While the attributable disease burden of a suboptimal diet in terms of micronutrient deficiencies (i.e., vitamin A, zinc, iron), undernutrition, and suboptimal breastfeeding has declined since 1990, the attributable disease burden for other dietary components have increased, particularly low fruit and low vegetable intake that has accounted for more than 1% of total disability adjust life years (DALYs) (Forouzanfar et al., 2016). DALYs represent the number of years of life lost due to living with disability and premature mortality, generally attributed to NCDs (Kassebaum et al., 2016). Data from the European Prospective Investigation into Cancer and Nutrition (EPIC) study, a multicenter prospective cohort study with 519,978 participants, has suggested decreased mortality with increased FV consumption due to its inverse association with circulatory, respiratory, and digestive diseases (Leenders et al., 2014).

Cardiovascular diseases (CVDs) are the number one cause of global mortality, accounting for 31% of all deaths in 2015, and cancer is the second leading cause of global mortality (WHO, 2017). According to the 2015 Global Burden of Disease (GBD) study, numerous prospective studies demonstrate a dose-response of FV intake against various cancers, CVDs, and diabetes mellitus (Forouzanfar et al., 2016). A recent systematic review of 95 prospective studies demonstrated a dose-response of FV intake and decreased relative risk for cardiovascular diseases (CHD, CVD, and stroke), cancer, and all-cause mortality (Aune et al., 2017). There is also evidence supporting such an inverse association pertaining to eye diseases, osteoporosis, chronic obstructive pulmonary disease, rheumatoid arthritis, dementia, and asthma, according to a comprehensive critical literature review (Boeing et al., 2012). NCDs are a growing global problem

with large socioeconomic and health implications and so they need to be addressed more aggressively (WHO, 2015).

2.2.1 Cardiovascular Diseases (CVDs)

As the primary cause of death, CVD and its related risk factors have received considerable attention for primary prevention through dietary changes. A significant inverse relationship between FV and hypertension was established in a meta-analysis of 25 observational studies where blood pressure (BP) was measured by trained investigators (Li et al., 2016). The favorable effects of increased FV consumption on CVD were confirmed in a Cochrane review, which partially attributed FV intake to decreases in BP and low-density lipoprotein (LDL) (Hartley et al., 2013). While some studies argue that there is a dose-response of FV intake and decreased risk of CVD (Aune et al., 2017; Wang et al., 2014), the Prospective Urban Rural Epidemiological (PURE) study, a study surveying 18 low-, middle-, and high-income countries, has provided evidence to indicate for a maximum plateau of FV intake (500 g/d) towards benefit against the development of CVD (Miller et al., 2017).

2.2.2 Cancer

Progress in some primary prevention efforts and biomedical treatment approaches have led to decreased death from several types of cancers, but the NCD incidence related to cancer is still expected to rise partly due to inadequate primary prevention efforts in some areas such as healthy diet (Fitzmaurice et al., 2017). A review of four meta-analysis studies that analyzed for various dietary components and their influence on different cancers, indicated that fiber, micronutrients (vitamins and minerals), and the antioxidant capacity of the FV aspect of the DASH diet was significantly associated with decreased prevalence of various cancers (Ovani et al., 2015). A dose-

response between FV intake and decreased cancer risk was demonstrated in two separate meta-analysis studies, with an optimal daily FV dosage ranging from 400 g (Wang et al., 2014) to 600 g (dose-response meta-analysis of 95 prospective studies) (Aune et al., 2017).

Not all population studies, however, have demonstrated an inverse association between FV intake and cancer risk. A recent pooled analysis of four Japanese cohort studies demonstrated no association between FV intake and cancer risk (Takachi et al., 2017) and a prospective study on 14,198 Korean males (40-59 y) demonstrated an inverse association with cancer risk with only vegetables (Choi et al., 2015). The European Prospective Investigation into Cancer (EPIC) study, a prospective cohort study of 23 centers in 10 different European countries, revealed only small reductions in prostate cancer risk (Perez-Cornago et al., 2017), and overall cancer risk (Bofetta et al., 2010) with FV intake. Such inconsistencies may result from the method of FV intake assessment, as food frequency questionnaires are subject to random measurement errors (Choi et al., 2015), different biases (social, recall, etc.) (Wrieden et al., 2003), and may lack follow-up to account for dietary changes (Perez-Cornago et al., 2017), potentially reducing the effect of an association.

2.2.3 Type II Diabetes Mellitus

Diabetes mellitus, another NCD in the list of top ten causes of global mortality (WHO, 2017), has shown a decreased incidence associated with consumption of specific groups of FVs (Takahashi et al., 2012; Wang et al., 2016). When weight was held constant, greater FV intake up to 7 servings/day for 12 weeks was not associated with an effect on insulin resistance (IR) in 92 overweight individuals at high risk for CVD (Wallace et al., 2013). Conversely, a sample of 417 type 2 diabetic males consuming >150 g daily of FVs over 11 months, especially green FVs, exhibited significant decreases in hemoglobin A1C (Takahashi et al., 2012). A meta-analysis on

10 prospective cohort studies showed a significant decrease in type 2 diabetes risk associated with higher fruit or leafy green vegetable intake (Li et al., 2014). Another meta-analysis, on type 2 diabetes and high or low FV intake in 23 prospective cohort studies, found a similar decreased risk of type 2 diabetes associated with higher consumption of total fruits (especially berries), and green leafy, yellow, and cruciferous vegetables, or FV fibers (Wang et al., 2016). Although plant fiber has been suggested to be a major disease risk-reducing component of FV intake, micronutrients and phytochemicals, such as carotenoids, from FVs may act synergistically to combat risk of disease outcomes (Dahl & Stewart, 2015).

2.2.4 Other Disease Conditions

Greater FV intake is significantly associated with decreased odds of depression in the Canadian population, although the response was attenuated when adjusted for other health-related factors (McMartin et al., 2013; Kingsbury et al., 2015). There is evidence for neuroprotective benefits of polyphenolic compounds from FVs for their antioxidant properties (Parletta et al., 2013). In terms of hip-fracture in older adults, among 5 cohorts from Europe and the United States, a low FV intake (≤1 serving) was associated with a 39% increased risk (Benetou et al., 2016). Servings above recommendations (>5), however, were not associated with decreased risk. Also, a 16-week randomized controlled trial (RCT) demonstrated no influence of FV intake on fasting serum bone markers (Neville et al., 2014).

FV associations with NCD reduction may be partly attributable to the anti-inflammatory properties associated with various phytochemicals. Chronic low-grade inflammation has been associated with an increased chronic disease risk and has been estimated to lead to the development of approximately 20% of all cancer cases (Aran et al., 2016; Mantovani et al., 2008). Plant-based diets, or diets with an emphasis on FV, grains, and legumes, (in the realm of the DASH,

Mediterranean, and Nordic diets) have been associated with decreased levels of chronic low-grade inflammatory biomarkers (Eichelmann et al., 2016; Welty et al., 2016).

2.3 FV Phytochemicals

While FV intake can prevent several micronutrient deficiency diseases such as scurvy (vitamin C) or birth defects (folate) (Parletta et al., 2013), several other phytochemicals have been attributed to the NCD risk reduction potential of FVs (Shashirekha et al., 2015). Glucosinolates, present in cruciferous vegetables, influence regulation of inflammation and carcinogenesis associated signaling pathways to act as chemopreventative agents (Rodriguez-Casado et al., 2016; Traka et al., 2008). Polyphenolics, such as flavonoids and chlorogenic acid, have been shown to inhibit lipid oxidation, prevent the formation of mutagenic and carcinogenic compounds, exhibit anti-inflammatory properties, and modulate signaling pathways (Shashirekha et al., 2015). In addition to having demonstrated antioxidant (Maiani et al., 2009), anti-inflammatory (Watzl et al., 2005), and anticancer properties (Donaldson, 2011; Gloria et al., 2014), carotenoids exhibit provitamin A activity to prevent irreversible blindness (Sommer et al., 2008) and have been suggested as the best biological markers for FV consumption (National Academy of Sciences, 2000).

2.4 Carotenoids

Carotenoids are yellow, orange, and red, fat soluble plant pigments that mammals cannot synthesize de novo and must obtain from the diet (Namitha & Negi, 2010; Vargas-Murga et al., 2016). The pigments are primarily found in yellow-orange FVs and dark green vegetables, where the chlorophyll masks the carotenoid color, present in the plant cell membrane and plasma vacuoles for photooxidative protection (Namitha & Negi, 2010; Maiani et al., 2009). There are six

carotenoids commonly noted in human plasma: α-carotene and β-carotene, from yellow-orange FVs such as carrots and peppers; β-cryptoxanthin, predominantly found in citrus fruits; lutein and zeaxanthin, found in deep green vegetables; and lycopene, primarily found in red FVs such as tomato and grapefruit (Burrows et al., 2015; Mainani et al., 2009). Carotenoid content of FVs can be influenced by the type of cultivar, season, geographical and cultivation variation, maturity, and storage (Maiani et al., 2009).

The pigmentation of carotenoids confers photooxidative protection to photosynthetic tissues in plants by absorbing blue/green light, while also acting as a precursor for a stress and developmental modulator (Hounsome et al., 2008). The coloration also serves as a biological signal to attract consumers of fruits and flowers for dispersion of their seeds or pollen (Blount et al., 2009). In animals, the accumulation of carotenoids serves to warn of toxins or sickness, act as camouflage or recognition stimuli, signal status or nutrition within species, and may even serve to attract a mate (Blount et al., 2009; Whitehead et al., 2012c). Some theories suggest carotenoid signaling may have evolved as a result of its nutritional cost of production, communicating the health of the animal, displaying deep and bright colors when carotenoids are in excess (García-de et al., 2015).

Carotenoids are a class of hydrocarbons with 40 carbons in an eight-isoprenoid unit structure (Saini et al., 2015). The characteristic conjugated double bond structure contributes to the capacity of carotenoids for isomerization, singlet oxygen quenching, and electron transfer as a part of their antioxidant properties (Namitha & Negi, 2010; Shmarakov et al., 2013). Some carotenoids can be enzymatically cleaved to produce retinol, servings as a source of provitamin A (Maiani et al., 2009; Shmarakov et al., 2013). There are two groups of carotenoids, the hydrocarbons with an oxygenated functional group known as xanthophylls (β-cryptoxanthin, lutein, and zeaxanthin), and the simple hydrocarbons known as carotenes (α-carotene, β-carotene,

and lycopene) (Saini et al., 2015; Shmarakov et al., 2013). Due to their hydrocarbon structure, carotenoids are lipophilic (Butnariu, 2017), which contributes to their low bioavailability and complex metabolism (Saini et al., 2015).

2.4.1 Metabolism and Absorption

When FV are consumed, carotenoids are released from the food matrix in the stomach and travel through the duodenum, where they are incorporated into the lipid phase of the meal and are transferred into mixed micelles (Tyssandier et al., 2003). Carotenoid-containing micelles are taken up by enterocytes and then combined with apolipoproteins and incorporated into chylomicrons for transport throughout the body (Nagao, 2014; Saini et al., 2015). Micelle uptake by enterocytes occurs through passive as well as facilitated diffusion. The former method of diffusion acts through a concentration gradient, while the latter is mediated by the same proteins used in cholesterol transport: scavenger receptor class B type 1 (SR-BI) and cluster determinant 36 (CD36) (Bennekum et al., 2005).

Inside the enterocyte, the carotenoids with provitamin A activity (α-carotene, β-carotene, and β-cryptoxanthin) can be processed into various forms of vitamin A through enzymatic cleavage and are packaged into chylomicrons for transport in the serum along with the other carotenoids (Nagao, 2014; Shmarakov et al., 2013). Vitamin A supports the immune system, hormone synthesis, cell growth and differentiation, and plays a role in vision and prevents night blindness (Hounsome et al., 2008). Once absorbed into the serum, carotenoids, along with the cleavage products of provitamin A carotenoids, are transported throughout the body. Absorption, distribution and storage of carotenoids has been suggested to follow the triage concept: transport via serum to cover immediate needs, such as reactive oxygen species (ROS) neutralization or vitamin A replenishment, while the carotenoid excess undergoes storage in the liver, adipose

tissue, retina (retinal pigment epithelium), and skin to maintain these body systems (Donaldson, 2011; Kiokias et al., 2016; Shmarakov et al., 2013).

The lipophilicity of carotenoids and their incorporation with the lipid phase suggests facilitated carotenoid absorption that is enhanced with dietary fat consumption. Salad consumption with full fat dressing resulted in greater chylomicron carotenoid content than low-fat or fat-free dressed salad consumption (Brown et al., 2004). The increase in carotenoid bioavailability associated with dietary lipids has been attributed primarily to long chain fatty acids since medium chain fatty acids do not induce secretion of the carotenoid-containing chylomicrons (Borel et al., 1998). In addition, the overall interaction of components within both the carotenoid containing food matrix and other dietary components consumed concurrently may also affect carotenoid bioavailability (Faulks & Southon, 2005). For example, lutein was noted to be better absorbed from eggs as compared to from either spinach and lutein supplements when matched for a dosage of 6 mg of lutein/day for nine days in 10 healthy men partaking in a crossover intervention study. This observation was likely due to effects of the food matrix of egg and its accompanying lipid and cholesterol content that could enhance carotenoid bioavailability (Chung et al., 2004).

2.4.2 Bioavailability and Inter-individuality

Differences among plant food matrices in terms of chemical interactions between carotenoids and other food components such as proteins and fiber may affect carotenoid bioavailability and absorption (Maiani et al, 2009). There is a low bioavailability of carotenoids from plant foods, varying from 10% in mangos to <1% in carrots for β-carotene and attributed to the form of β-carotene, whether in liquid crystalline (as in mango and papaya) or solid crystalline form (as in carrots and tomato) (Donhowe & Kong, 2014). Low bioavailability of β-carotene from FVs is also thought to come from the carotene-protein complex and plant cell wall resistance to

digestion and degradation (Rein et al., 2013). Presence of fiber can inhibit absorption, while presences of fats, as well as cooking and processing methods, can increase bioavailability (Maiani et al., 2009). Generally identified by yellow-orange skin discoloration and defined as carotenemia, an excess intake of carotenoids appears to be benign, but high serum carotenoid levels leading to carotenemia can result from diseases linked to changes in carotenoid metabolism in some rare cases (Haught et al., 2007).

When compared to omnivores, vegetarians have 15% greater levels of plasma carotenoids due to their plant-rich diet (Haldar et al., 2007), but greater F&V consumption might not translate into definitive carotenoid status improvement. A great range of inter-individual variation appears to exist for changes in carotenoid status from carotenoid intake, not simply due to dietary habits. A range of no response to an eight-fold increase in serum carotenoids were reported following a six-week supplementation of three forms of a 12 mg dosage of β-carotene, regardless of the source (raw carrots, carrot juice, and purified β-carotene capsules) (Törrönen et al., 1996). In a double tracer study using D_6 β-carotene, the area under the curve (AUC) for plasma carotenoid concentration over time varied from 0.01 to 30.00 μmol h/L (Hickenbottom et al., 2002). In a large observational study on 901 male and female adult subjects over four years, inter- and intra-individual variances in carotenoid concentrations were in the range of 61-70% and 20-35%, respectively (Forman et al., 2009). This inter-individual response regarding carotenoid absorption, distribution, metabolism, and excretion has been demonstrated in some observational studies and proposed to be influenced by several factors: age, alcohol and smoking, asthma, body mass index (BMI), gender, nutrition status, disease, drug intake, physical activity, and race/ethnicity (Bohn et al., 2017).

In support of the sex differences accounting for inter-individual variability, the EPIC study showed that women had higher plasma carotenoid levels than men following investigation of the

quantities of the six carotenoids commonly found in human plasma of 3,043 males and females (Al-Delaimy et al., 2004). Another study reported greater plasma carotenoids in women when compared to men as observed in a series of six different, fully controlled, dietary interventions on a total of 155 men and 109 women (Couillard et al., 2016). On the other hand, studies using accelerated mass spectrometry with labeled doses of β-carotene reported inter-individual differences in absorption, but not differences between females and males (Tang, 2013). Some of the discrepancies may simply be due to imprecise or differing measurement methods or could be dependent on the dosage and metabolism kinetics (Faulks & Southon, 2005).

Carotenoid absorption inter-individuality not only appears to be influenced by sex, but also disease, lifestyle habits, and genetic variation (Bohn et al., 2017). There are a variety of aspects that appear to affect the processes of carotenoid digestion ranging from mastication, decreasing particle size, genes or diseases related to digestive enzymes or bile production (i.e., Crohn's disease) that could influence carotenoid bioaccessibility (i.e., release from food matrix and availability for absorption) (Bohn et al., 2017). Following absorption and digestive enzyme processing, several transport proteins found on the apical membrane of the enterocytes (SR-B1, CD36, NPC1L1) and carotenoid cleavage proteins in the enterocytes (BCO1 and BCO2) have single nucleotide polymorphisms (SNPs) that have been reported to explain upwards of 50% of the absorption variability for various carotenoids (Bohn et al., 2017). Nutritional factors, such as host vitamin A status, are thought to serve as a negative feedback loop (Lobo et al., 2013). Differences in iron, zinc, and protein status have also demonstrated to lead to variability in β-carotene status (Bohn et al., 2017).

One of the more recent explanations for the variability in carotenoid absorption has been related to inter-individual variations in the diversity of gut microbiota, which could affect carotenoid absorption. It has been estimated that, depending on the food matrix, a range of 5-50%

of carotenoids are absorbed in the small intestine with the remaining carotenoids accessing the colon (Bohn et al., 2017; Saura-Calixto et al., 2007). A bioaccessibility model involving *in-vitro* enzymatic digestion and colonic fermentation of plant foods using rat cecal content reported an increase in lycopene and β-carotene release following colonic fermentation, which could allow for enhanced carotenoid bioavailability (Goñi et al., 2009). A recent analysis of colonic biopsies from 76 healthy subjects at risk of colon cancer demonstrated higher bacterial diversity together with a reduction in Firmicutes and increase in Proteobacteria was associated with higher plasma carotenoid concentrations (Djuric et al., 2018). Firmicutes are generally associated with the high-fat Western-diet and obesity (Musso et al., 2010). Thus, the suggestion has been put forward that greater bacterial diversity with a gut microbiome profile associated with health promoting properties also leads to improved bioavailability of carotenoids.

The gut microbiome relationship with carotenoid absorption has been related to phytoene dehydrogenase (phytoene desaturase), which catalyzes a consecutive reaction to the rate limiting step of carotenoid biosynthesis (Tian, 2016). Phytoene dehydrogenase is an enzyme found in thousands of plants and bacteria that is responsible for the conjugated double bond structure of carotenoids, with the number of desaturation steps completed upon the precursor, phytoene, designating the color of the carotenoid produced (Umeno, Tobias, & Arnold, 2005). It has therefore been suggested that the presence of phytoene desaturase enzyme manifesting bacteria would increase carotenoid microbial biosynthesis. A study assessing the gut metagenome of patients with symptomatic atherosclerosis reported elevated plasma β-carotene concentrations and enriched phytoene dehydrogenase in age and gender matched healthy controls (Karlsson et al., 2012). It was suggested that greater abundance of the phytoene dehydrogenase gene sequence in the gut microbe metagenome of healthy controls may indicate greater production and bioavailability of β-carotene by the gut microbiota (Karlsson et al., 2012).

2.4.3 A Valid Biomarker of FV

Since up to 90% of the carotenoids are obtained through consumption of FV, serum concentrations of these plant pigments are an indicator of FV intake (Maiani et al., 2009). A systematic review of FV biomarkers in 96 human intervention studies demonstrated that carotenoids and vitamin C were the most common and most consistently responsive FV biomarkers (Baldrick et al., 2011). A dose-dependent increase of plasma carotenoid status was demonstrated when 63 nonsmoking males underwent an RCT where they consumed a low FV diet (2 servings/day) for four weeks, and then were randomized into a low, medium (5 servings/day) and high (8 servings/day) FV diet for four more weeks (Watzl et al., 2005). A systematic review of validation studies on plasma carotenoid levels as biomarkers for dietary carotenoid consumption concluded that despite weak (0.26) to moderate (0.47) correlations, the correlations need to be considered with respect to adiposity, infection, behavioral aspects (i.e., smoking) and differences in absorption and digestion (Burrows et al., 2015). The authors suggested that skin carotenoid concentrations were a potentially better measure of overall carotenoid status.

Albeit delayed by at least two weeks after carotenoid consumption, skin carotenoid concentrations are positively correlated with serum concentrations (Jahns et al., 2014; Stahl et al., 1998). Carotenoids are deposited at higher concentrations in skin regions with higher density of sweat glands, such as the palms, soles of the feet, and forehead, because they are transported to the epidermis through sweat and reabsorbed into the skin (Lademann et al., 2011). This mechanism of skin carotenoid deposition was first proposed by Lademann et al. (2009) after detection of differential carotenoid content at different depths of the stratum corneum as measured by Raman spectroscopy. The authors proposed sebaceous transport of carotenoids to the surface of the skin

followed by penetration into the stratum corneum since such penetration characteristics had previously been observed with tocopherol (Thiele et al., 1999).

Serum carotenoid levels have also been inversely associated with NCDs (Namitha & Negi, 2010). A positive dose-response relationship has been noted between serum carotenoid concentrations and NCD prevention according to the Carotenoid Health Index, which was developed using 62 studies composed primarily of prospective cohort and case control studies examining the relation between plasma carotenoid content and health outcomes (Donaldson, 2011). Five cutoff points of serum carotenoid concentrations associated with varying levels of risk for NCDs were established (Donaldson, 2011). Raw-food dieters, consuming FV, nuts, and seeds as 95% of their diet, exhibit favorable plasma carotenoid concentrations with respect to such recommendations for NCD prevention (Garcia et al., 2008).

2.4.4 Benefits against NCDs

Primarily known for their antioxidant effects and provitamin A activity, carotenoids have been associated with disease prevention regarding cardiovascular disease (Iversen et al., 2014), cancer (World Cancer, 2007), diabetes mellitus (Mirmiran et al., 2016), metabolic syndrome (Holt et al., 2014), eye health (Mares, 2016), overall mortality (Zhao et al., 2016), and may even have anti-osteoporotic (Hayhoe et al., 2017) and cognitive (Kesse-Guyot et al., 2014) benefits (Donaldson, 2011). A study investigating epigenetic biomarkers of age related to mortality and chronic disease conditions such as NCDs found that blood carotenoid levels had the greatest significant association with extrinsic epigenetic age acceleration, a measure of intrinsic and extrinsic aging factors in 4,173 post-menopausal women of the Women's Health Initiative and 402 male and females from an Italian cohort study (Invecchiare nel Chianti) (Quach et al., 2016).

Carotenoid health benefits have largely been indicated from observational studies, which have suggested a strong synergistic effect between carotenoids and other FV constituents (Wang et al., 2013). Synergistic effects between carotenoids and other antioxidants have been noted at low concentrations in vitro, such as a combination of 2.5 μM zeaxanthin and 0.1 mM of α-tocopherol that effectively protected against singlet oxygen-mediated lipid photoperoxidation by almost two-fold more than 2.5 μM zeaxanthin alone, just above two-fold more than α-tocopherol alone, and greater than two-fold above the control (Wrona et al., 2003). A separate study exploring different combinations of four antioxidants (lycopene, β-carotene, and vitamins C and E) demonstrated that specific different concentrations and combinations of mixed antioxidants can be more effective than corresponding single antioxidants when assessed using a 2,2 diphenyl -1-picrylhydrazyl (DPPH) free radical scavenging capacity assay (Liu et al., 2008). One review concluded that physiological levels of carotenoids and mixtures of antioxidants obtained from an average FV intake are associated with positive health influence as opposed to doses of a single extracted or synthetic carotenoid (Darvin et al., 2006).

Some experimental studies using carotenoid supplementation in pill form (β-carotene extract), alone or accompanied with other antioxidants and micronutrients, have not demonstrated a considerable influence on various types of cancers (Druesne-Pecollo et al., 2010; Nagao et al., 2015; Wright et al., 2007), while some pro-oxidant effects of synthetic or extracted carotenoids have been observed, primarily with β-carotene (Galan et al., 2005; Omenn et al., 1996; The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994). Pro-oxidant effects are suggested to be caused by large doses of a single antioxidant as opposed to health benefit from physiologically appropriate antioxidant dosages obtained from FVs in the diet (Palozza et al., 2003). Pro-carcinogenic effects of β-carotene seem to be primarily evident at dosages far exceeding dietary intake levels at dosages of 20 mg (The Alpha-tocopherol, Beta-carotene Cancer

Prevention Study Group, 1994) or 30 mg (Omenn et al., 1996) in contrast to established typical intake of 2-6 mg/day for β-carotene based on six 24-hour dietary recalls over 18 months of a subsample (3128 subjects) from the SU.VI.MAX study (Galan et al., 2005).

2.4.3.1 Cardiovascular Disease (CVD)

There is accumulating evidence for FV-associated carotenoid intake and decreased risk of CVD and associated conditions. After adjustment for age, smoking, and other confounding factors, a significant inverse association between the highest intakes of α-carotene (1518 μg/day) and β-carotene (7639 μg/day), and the risk of coronary artery disease was demonstrated in a group of over 70,000 women, ages 30-55, over a 12 year follow up-period as a part of the Nurses' Health Study (Osganian et al., 2003). Another cohort study of 573 middle-aged individuals free of CVD symptoms at baseline and monitored over 18 months found a significantly inverse relation between atherosclerotic progression and lutein, zeaxanthin, β-cryptoxanthin, and α-carotene, after adjusting for age, sex, smoking, CRP, and other cardiac risk factors (Dwyer et al., 2004). A pureed FV drink providing ≥ 5mg of total carotenoids given daily for 6 weeks to 39 volunteers between 30 and 70 years of age significantly increased plasma α-carotene and β-carotene concentrations and was concurrently associated with endothelium-dependent dilation, a measure of vascular tone (George et al., 2012). An increase in plasma carotenoid concentrations was associated with activity of the HDL antioxidant modulating enzymes, paraoxonase-1 (PON-1) and lecithin cholesterol acyltransferase (LCAT) in the serum of 80 obese subjects with type 2 diabetes between the ages of 40 and 70 y following 8 weeks of ≥6 servings of FVs, which indicated carotenoid-mediated anti-atherogenic effects (Daniels et al., 2014).

Carotenoids have been implicated with anti-CVD and stroke properties (Raposo & Morais, 2015). CVD is characterized by oxidative stress that promotes endothelial dysfunction, lipid

peroxidation, and inflammation that can generate oxidation of low-density lipoprotein and foam cell formation that can lead to atherosclerosis (Wang et al., 2013). Lycopene is considered the strongest ROS quencher of the carotenoids due to its highly conjugated, long chain structure and has been associated with decreased LDL oxidation, oxidative stress at possible plaque sites, and lipoprotein sensitivity to oxidative damage (Hadley et al., 2003; Namitha & Negi, 2010). A group of participants from the Coronary Artery Risk Development in Young Adults study (4580, 18-30 year old African American and Caucasian participants) selected for an ancillary study called the Young Adult Longitudinal Trends in Antioxidants Study, which monitored for various serum markers of CVD over 15 years. This study established an inverse association with plasma carotenoids analyzed at years 0 and 7 with inflammatory, endothelial, and oxidative stress biomarkers including: C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (sICAM1), leukocytes, fibrinogen, and F2-isoprostanes; a positive association with serum superoxide dismutase was also observed (Hozawa et al., 2007).

Stroke and other related conditions have also demonstrated an inverse association with carotenoid intake. An enhanced dietary intervention plan consisting of energy, protein, and antioxidants (15 mg vitamin C, 9 mg vitamin E, and 300 μg carotenoids per 100 ml of oral sip feedings) was associated with a decreased mortality risk in 165 hospitalized acute stroke patients >65 years old following 5-7 years post-intervention (Ha et al., 2011; Iversen et al., 2014). The risk of ischemic heart disease and stroke increased with low dietary carotenoid status (< 0.23 μmol/l) and/or vitamin C (22.7 μmol/l) in a 12 year follow up of 2974 male patients of the Basel Prospective Study (Gey et al., 1993). In a comprehensive review on dietary antioxidants and stroke, most observational studies inversely associated stroke risk with β-carotene, α-carotene, and lycopene intake, while experimental studies failed to reduce risk of stroke through carotenoid supplementation (Cherubini et al., 2008). Such evidence supports the idea that supplementation of

individual carotenoids does not have the same protective influence as consumption of FVs, as there is a synergistic effect of antioxidants and bioactive phytochemicals when entire foods are consumed (Raposo & Morais, 2015).

2.4.3.2 Cancer

Cancer is an uncontrolled upregulation of cellular growth from mutations in genes that regulate cell cycle control (World Cancer, 2007). Cancerous cells are virtually deficient in cell-to-cell communication contributing to the uncontrolled growth in early development (Aasen et al., 2016; Bertram & Vine, 2005). Dietary intake of carotenoids may be protective against cancer development by cell proliferation inhibition via cell-to-cell communication modulation (Aasen et al., 2016; Bertram & Vine, 2005), or by affecting gap junctions (Bertram & Vine, 2005; Stahl et al., 1997) and estrogen receptors (Prakash et al., 2001), as well as inhibition of oxidative stress (Fiedor & Burdam 2014).

A possible pro-oxidative influence of carotenoids, eventually leading to increased risk of cancer, has been tested in RCTs for β-carotene. In a multicenter RCT, when 30 mg of β-carotene and 25,000 IU of retinol were administered daily to a randomly selected treatment group from 18,314 smokers, former smokers, and workers exposed to asbestos for a mean duration of 4 years, the relative risk for lung cancer (1.28) and overall mortality (1.17) was higher in the treatment group than the placebo group (Omenn et al., 1996). In another intervention RCT, 29,133 male smokers (ages 50-69) were randomly assigned to groups administered daily supplementation of 50 mg alpha-tocopherol, 20 mg β-carotene, both alpha-tocopherol and β-carotene, or a placebo, and those receiving only β-carotene exhibited a higher incidence of lung cancer than those who did not (The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994). These studies demonstrate the importance of physiologically appropriate dosages due to the pro-oxidative

capacity of large amounts of a single antioxidant, such as β-carotene, or few (i.e. alpha-tocopherol and β-carotene) antioxidants alone (Palozza et al., 2003), in contrast to the synergistic antioxidant protection provided by low dosages of antioxidants including carotenoids (Lademann et al., 2011).

An inverse association of breast cancer risk and consumption of α-carotene, β-carotene, β-crytoxanthin, and lutein and zeaxanthin, was demonstrated in a case-control study using a validated food frequency questionnaire (FFQ) on 561 cases and 561 respective controls of Chinese women (25-70 years old) with breast cancer (Wang et al., 2014). A similar inverse association was demonstrated between lung cancer and α-carotene, β-carotene, and total carotenoid levels in a meta-analysis based on 17 prospective studies (Abar et al., 2016). Greater intake of high β-crytoxanthin containing citrus fruits had an inverse association with lung cancer risk after a pooled analysis of seven cohort studies that estimated carotenoid intake from dietary questionnaires, even after adjusting for vitamin C content, folate, multivitamins, and other carotenoids (Männistö et al., 2004). Carotenoids that had been significantly associated with decreased lung cancer risk were no longer associated after adjusting for total FV intake in a case-control study with 587 primary cases and 624 controls, which suggests synergistic relationships of FV phytochemicals (Wright et al, 2003). Although evidence concerning putative carotenoid anti-cancer effects is still controversial, some authors have nonetheless suggested that there is probable evidence of benefits from carotenoid intake similar to that of intake of whole FVs (Boeing et al., 2012).

2.4.3.3 Diabetes

Type 2 diabetes involves the dysregulation of glycemic control and oxidative stress, often leading to vascular complications (Martini et al., 2010). Carotenoid supplementation alone is not recommended, as intervention trial evidence to date regarding carotenoid supplementation has not established strong associations between supplementation and type 2 diabetes (American Diabetes

Association, 2007). In an RCT with over 22,000 healthy physicians, aged 40-84 years from 1982-1995, supplementation with 50 mg of β-carotene on alternate days did not establish a reduction of diabetes risk (Liu et al., 1999).

On the other hand, observational trials examining carotenoid intake from FV consumption in relation to Type 2 diabetes have demonstrated more positive results. The Epidemiology of Vascular Aging study, studying 1,389 participants aged 59-71 years old over nine years, found a significantly decreased risk of dysglycemia in participants of the quartile with the highest plasma carotenoid levels, even after controlling for sociodemographic variables, lifestyle habits, CVD, BP, BMI, and lipid profile (Akbaraly et al., 2008). In the Tehran Glucose and Lipid Study, where 1106 men and women aged 19-74 years were monitored for fasting serum insulin and glucose with an FFQ at baseline and three years later, higher β-carotene and β-cryptoxanthin intake was associated with 58% and 49% decreased risk of IR, respectively (Mirmiran et al., 2016). A marginal inverse association was found between α-carotene and IR. Higher skin carotenoid levels measured by Raman spectroscopy have been associated with lower incidence of metabolic syndrome in 155 patients (mean age of 54 years) referred to a dietician for dietary assessment (Holt et al., 2014). More human trial studies are required to firmly establish the relationship between carotenoid intake and diabetes risk, but their contribution to FV associated decreases in diabetes risk has received validation (American Diabetes Association, 2007).

2.4.3.4 Inflammation and Oxidative Stress

Carotenoids have demonstrated immune system modulating effects, including suppression of pro-inflammatory events. Carotenoids can increase immune factors such as monocytes, white blood cells, T-helper cells, and lymphocytes associated with immune system responsiveness to infection and oxidative stress (Boileau et al., 1999; Hughes et al., 1997). CRP, a non-specific

indicator of the inflammatory response that is closely correlated with cardiovascular disease risk, demonstrated an inverse association with α-carotene and β-carotene intake in an RCT of 63 nonsmoking males subject to 4 weeks of low (2 servings/day) FV intake followed by randomization into a low, medium (5 servings/day) and high (8 servings/day) (Watzl et al., 2005). Participants consuming 8 FV servings/day for the following four weeks of the study showed significantly increased plasma carotenoids and decreased CRP concentrations as compared to 2 or 5 servings/day (Watzl et al., 2005). Preterm infants given diets with added lutein, lycopene, and β-carotene showed decreased CRP values together with maturation and protective effects on retina health (Rubin et al., 2012).

While some studies have demonstrated an inverse relationship between carotenoid intake and CRP and other inflammatory markers, there exists controversy. A study assessing the influence of a three-week carrot juice intervention (8 ounces/day) on 69 overweight breast cancer survivors found an increase in total plasma carotenoids and reduced oxidative stress but no influence was noted on inflammatory biomarkers including high-sensitivity C-Reactive Protein (hsCRP), thromboxane B2, and prostaglandin E2 (Butalla et al., 2012). In a three-dose (2, 5, and 10 daily FV servings) crossover feeding trail of 49 overweight or obese women with four week washout periods between three week intervention periods, increased FV intake led to increased plasma carotenoids, but the FV treatment did not exert a significant influence on inflammatory biomarkers or oxidative stress markers including hsCRP, 8-isoprostane F2α (8-iso-PGF2), and hexanoyl lysine (Crane et al., 2011). As the above studies have examined individuals with disease conditions over short periods of time (three weeks), their findings may not be of sufficient duration or necessarily be applicable to healthy individuals (Donaldson, 2011).

In general, stronger associations have been observed examining the antioxidant properties of FV-derived carotenoids in comparison to supplementation of synthetic carotenoids. A negative

correlation was established between baseline carotenoid levels and lymphocyte DNA damage in 40 healthy, non-smoking volunteers aged 25-45, but supplementation of 15 mg/day of carotenoids did not have a significant influence on endogenous oxidative damage (Collins et al., 1998). Conversely, in a study where only FV and eggs accounted for variations of carotenoid intake, determined by FFQs quantified by the USDA nutrient file, intake of lutein plus zeaxanthin, α-carotene, β-carotene, and total carotenoids exhibited a significant inverse association with oxidative stress markers, 8-iso-PGF2 and 8-hydroxy-2'-hydroxyguanosine (8-OHdG), determined by enzyme-linked immunosorbent assay on urine samples of 296 healthy men aged 40-59 years (Cocate et al., 2015). Such studies suggest an emphasis on the synergistic properties of carotenoids and FV components (Palazzo et al, 2003), and how they participate in an antioxidant protection chain (Darvin et al., 2011; Lademann et al., 2011).

2.4.3.5 Eye and Neurological Diseases

Some human trial evidence has suggested that lutein and zeaxanthin exert protective effects for eye health, which could be partly mediated through ROS quenching photo-protection (Namitha & Negi, 2010; Mares, 2016). When supplemented with 20 mg lutein or 10 mg lutein plus 10 mg zeaxanthin per day for 48 weeks, 107 subjects aged 50-79 exhibited a decreased risk of development of age-related macular degeneration (AMD) as assessed by increased macular pigment optical density (MPOD) and best-corrected visual acuity (BCVA) (Ma et al., 2012). MPOD has previously been inversely associated with AMD (Bone et al., 2001; Nolan et al., 2010). Addition of other antioxidants (15mg vitamin E, 150 mg vitamin C) and minerals (20 mg zinc and 0.4mg copper) to a supplementation of 12 mg lutein and 0.6 mg zeaxanthin resulted in a slower progression of AMD in the treatment group of 433 subjects aged ≥55, suggesting synergistic effects with other antioxidants (Beatty et al., 2013). When analyzing the ROS scavenging

mechanism of macular pigment in post-mortem human macula and retinal pigment epithelium, an equal ratio of lutein, zeaxanthin, and meso-zeaxanthin (a metabolite of zeaxanthin) acted synergistically to quench more singlet oxygen than any individual carotenoid (Li et al., 2010).

In addition, lutein and zeaxanthin have demonstrated neuroprotective and anti-inflammatory effects together with their well-established antioxidant effects regarding diabetic retinopathy in vivo (Neelam et al., 2017). Following a comparison of type 1 and type 2 diabetics, and normal healthy subjects (n=150, age 30-75), type 2 diabetes was associated with decreased MPOD with no difference in carotenoid intake suggesting carotenoids were not influential in this case (Scanlon et al., 2015). Cataract research has demonstrated a reduction in risk of the condition through carotenoid-rich FV intake, though the bioactive carotenoid components of FV such as lutein and zeaxanthin are difficult to isolate in such observational studies (Mares, 2016). Other conditions such as glaucoma and retinopathy have shown controversial results with respect to carotenoids, requiring further study (Mares, 2016).

The increase in carotenoids conferred by diets high in FVs, as well as increases in bioavailability from fat intake with meals (Brown et al., 2004), may account for the eye health protection associated with the Mediterranean diet, which was indicated after monitoring 4753 men and women aged ≥ 65 years for their Mediterranean diet score and neovascular AMD (Hogg et al., 2017). Additionally, supplementation of 10 mg lutein and/or 10 mg zeaxanthin in 34 men aged 18-40 y presented an increased MPOD and a potential to improve the contrast acuity threshold (CAT) for better visual acuity under low lighting conditions (Kvansakul et al., 2006). Following a review of the literature, there is accumulating evidence that suggests protective effects of carotenoid-rich FV, particularly those rich in lutein and zeaxanthin, on vision-related NCD (Mares, 2016). This suggestion is supported by an analysis of carotenoids in the serum of 175 men and 174 women

aged 25-45 y from five European countries, demonstrating that a high FV diet was associated with elevated lutein, zeaxanthin, β-cryptoxanthin, and xanthophyll levels (Olmedilla et al., 2001).

In summary, although carotenoids by themselves may not demonstrate a clear protection against some NCDs, they do serve as an indicator of FVs protective potential (Baldrick et al., 2011) in the 'network' of antioxidants and other anti-cancer, and anti-inflammatory FV components (Darvin et al., 2006). A change in diet and lifestyle may result in a lasting beneficial effect of increased antioxidants integrated in the diet through greater FV consumption (Takahashi et al., 2006), whereas supplementation of individual FV components alone may not be sustainable. Reduction in chronic disease risk and overall mortality resulting from antioxidant supplement intake (120 mg Vitamin C, 30 mg Vitamin E, 6 mg β-carotene, 100 μg selenium, and 20 mg zinc) in the SU.VI.MAX study disappeared 5 years post-intervention in 12,741 French adults (Hercberg et al., 2010). The unique photo-protective characteristics of carotenoids make them relatively easy to distinguish through various methods of assessment, allowing for more objective measures of FV intake (Mayne et al., 2013). Given the therapeutic potential of carotenoids against various NCD's, their association with FV intake and NCD prevention, and their role in acting as a biomarker for FV intake, a rapid and assessible method for carotenoid assessment would be of great value.

2.5 Common Methods of Carotenoid Assessment

There are various methods of assessment for human carotenoid status. The most common are the convenient and economical self-administered questionnaires for FV intake, which are used in most of the observational studies that establish associations between FVs and NCDs that are then compared to a nutrient database for carotenoid quantification (Woodside et al., 2017). The more specific and gold standard assessment of carotenoid status is the invasive, complex, and

expensive high-performance liquid chromatography (HPLC) method that measures carotenoid levels in tissue or blood samples (Zidichouski et al., 2009). Recent developments include non-invasive optical detection of skin carotenoids through analysis of the refraction/reflection of light from the unique carotenoid chemical structure (Whigham & Redelfs, 2015).

2.5.1 Self-administered Questionnaires

FFQs are one of the most convenient and cost-effective methods of dietary assessment, although they have shown a poor correlation with the more specific and time consuming 24-hour recalls and food diaries, but all questionnaires have inherent drawbacks (Forouzanfar et al., 2016; Willet, 2012). Food diaries and 24-hour recalls are based on food and amount consumed by an individual on one or several days while an FFQ involves questions that ask how often and how much of specific foods are consumed in a given period (Willet, 2012). Serving size demonstrations with images or models improve participant perception of general portion sizes for FFQs, but recall bias (i.e., the subject's ability to remember) and the tendency to over-report healthy foods, such as FVs, are innate weaknesses in these methods (Willet, 2012; Wrieden et al., 2003). While more practical in use, producing FFQs can take time as they should be developed for the specific study population and aims, considering differences in ethnicity, culture, social and economic status, etc. (Teufel, 1997).

Although FFQs are not very specific and are less accurate than food recalls and records, this quick cost-effective tool gives an overall measure of diet quality and has been validated for FV in various populations and for particular food components including carotenoids (Traynor et al., 2006; Zamora-Ros et al., 2012). Moreover, the FFQ serves as a comprehensive assessment for overall dietary components that may act as confounders in relation to study objectives. Thus, FFQ's can give a greater context to the more objective, accurate, complex, and expensive

biomarker assessments available (Shim et al., 2014; Zamora-Ros et al., 2012). More modern iterations of dietary assessment have evolved to include image-assisted (i.e., before and after meal pictures) and image-based automatic food identification, along with nutrient and energy estimations based on meal images in an attempt to reduce dietary assessment limitations (Boushey et al., 2016).

2.5.2 High Performance Liquid Chromatography (HPLC)

The current gold standard for carotenoid assessment is HPLC analysis, but it has its own drawbacks (Zidichouski et al., 2009). While this method is more accurate and objective than questionnaires, accounting for bioavailability and metabolism as it is directly measuring biomarkers, this method requires blood or tissue samples, expensive equipment, and the technical understanding to interpret results correctly (Zamora-Ros et al., 2012). After samples are processed to release biomarkers and various other food components with similar chemical properties from the sample, they are separated by HPLC based on intrinsic structural and chemical differences (Kupiec, 2004). The processed samples are flushed through a stationary phase within a mobile phase, typically together with varying solvent concentrations, which allow the sample molecules with different affinities for the stationary phase to separate at distinctive time points as the mobile phase slowly changes concentrations of its constituents (Scott, 1986). Different liquid phases or elution times can aid in the separation of the molecules over time, and then a detector (i.e., UV, fluorescence, etc.) responds to changes that are indicative of individual molecules (Jahns et al., 2014; Scott, 1986).

While both blood and skin samples can be used depending on the method of extraction (Zamora-Ros et al., 2012), a study monitoring 155 men and 109 women for their daily FV intake and plasma carotenoid concentrations found positive relationships between plasma β-

cryptoxanthin, lutein and zeaxanthin and daily FV servings, but found a negative association for α-carotene and lycopene (Couillard et al., 2016). Such studies have indicated that plasma carotenoid concentrations may not be the most robust biomarker of FV intake (Couillard et al., 2016). The authors suggest that α-carotene, β-carotene, and lycopene could simply be more sensitive to specific FV categories, while β-cryptoxanthin and lutein, two of the more yellow carotenoids (Chandra et al., 2012), may be better indicators of overall FV intake (Couillard et al., 2016). Measurement of blood plasma and serum carotenoids has been suggested as not sensitive enough as opposed to isotopic labeling, such as is used for retinoid measurements, that could provide more insight on carotenoid kinetics (Bohn et al., 2017). One method used to ensure greater sensitivity, in tandem with HPLC, is mass spectrometry, due to its high molecular specificity, detection sensitivity, and dynamic range (Dass, 2007).

2.5.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS)

LC-MS is a method that utilizes the physical separation capability of liquid chromatography (HPLC) with the greater sensitivity of mass analysis for a synergistically enhanced measurement capability (Dass, 2007). Following separation of a sample by liquid chromatography, the sample moves through the interface, which removes most of the mobile phase while transferring the maximum amount of the desired sample without degrading ionizing efficiency or influencing the vacuum system, and is transported to the mass spectrometer (Dass, 2007). Once entering the mass spectrometer, the sample goes through chemical or electron ionization and the mass analyzer separates the ions based on their mass to charge ratio before mass analysis and passing them onto the detector that measures and amplifies the ionic current (Dass, 2007). A computer processes, records, and stores the data while a vacuum system maintains a very low pressure to prevent the ions from colliding or interacting with other species (Dass, 2007).

LC-MS has been used to quantify vegetable (Mokhtar et al., 2016) as well as serum carotenoid content (Zhu et al., 2006). One challenge when using LC-MS for carotenoid analysis is that reagents necessary for extraction and good resolution can be incompatible with MS, but recent developments have demonstrated the use of LC-MS friendly conditions that can simplify and quicken the procedure (Abate-Paella et al., 2017). LC-MS methods have been successfully used to quantitatively profile four carotenoids along with six fat soluble vitamins in plant foods, demonstrating the high level of sensitivity provided by this method (Gentili & Caretti, 2010).

2.5.4 Resonance Raman Spectrophotometry

Resonance Raman Spectroscopy (RRS) has emerged as a valid, highly reproducible, and reliable noninvasive method of assessment for skin carotenoids (Mayne et al., 2013) when compared with HPLC skin sample analysis and for intraclass correlation (Mayne et al., 2010). Carotenoid uptake and depletion kinetics have also been measured successfully with RSS, demonstrating validity with changes in FV intake when compared to serum HPLC analysis (Jahns et al., 2014). The conjugated double bond structure of carotenoids makes them particularly well suited for RRS, as the double bonds (C=C), single bonds (C-C), and methyl group bonds ($C-CH_3$) exhibit specific spectral lines at discrete frequencies that are downshifted by the laser excitation and backscattered light is then detected by a spectrograph/CCD (charge coupled device – set of sensors that respond to red, green, and blue light) array combination (Ermakov & Gellerman, 2015; Whigham & Redelf, 2015). Raman scattered or inelastically reflected light, corresponds exactly to the vibrational and rotational energy transition the carotenoid molecules experience when excited by a laser (Hata et al., 2000; Whigham & Redelf, 2015). RRS exhibited less intra-individual variability than serum HPLC analysis for carotenoid concentrations in 372 healthy subjects that provided three blood samples (48 h between each) and RRS skin carotenoid measurements over 8

days (Zidichouski et al., 2009). While RRS is highly specific and sensitive, allowing for selective detection of carotenoids, it has a weak signal, can be attenuated by the strong absorbing and scattering melanin chromophore present in the skin, and requires expensive and complex instrumentation and external calibration (Ermakov et al., 2001; Ermakov & Gellerman, 2015; Whigham & Redelf, 2015).

2.5.5 Reflectance Spectrophotometry (RS)

A reflectance spectrophotometer uses a broad "white light" source as the standard illuminant along with a collection module that relates the absorption at a specific spectral location to the measured reflectivity (Ermakov & Gellerman, 2012; Ermakov & Gellerman, 2015; Konica Minolta, 2007). The machine determines the carotenoid absorbance and can calculate the difference between absorbance at 480 nm and 610 nm to disregard the background tissue absorbance (Ermakov & Gellerman, 2012; Ermakov & Gellerman, 2015). The drawbacks of the method are lower molecular specificity and overall sensitivity when compared to RRS (Ermakov & Gellerman, 2015; Konica Minolta Sensing Inc, 2007).

RS has been widely used to measure one of the tri-stimulus CIELab color space values (b*) that ranges from yellow (+) to blue (-), as an indicator of carotenoid levels (Ermakov & Gellerman, 2015; Foo et al., 2017; Li et al., 2013; Pathare et al., 2013; Pezdirc et al., 2015; Pezdirc et al., 2016; Stephen et al., 2011; Tan et al., 2015; Whigham & Redelf, 2015). CIElab color space values are derived from the device independent yet perceptually different XYZ color space, coming from the device dependent and perceptually different RGB color space, to produce a device independent and perceptually uniform color space (Mendoza et al., 2006; Pathare et al. 2013). The L* value is a measure of luminance ranging from 0 (black) to 100 (white); the a* value is a measure of green

(-120) to red (120) coloration; the b* value is a measure of blue (-120) to yellow (120) coloration (Mendoza et al., 2006).

The accurate representation of CIELab color space values from RS can be applied to various contexts. Quality of various fresh and processed foods has been verified through standardized illuminant photo imaging and CIELab color processing for carotenoid content (Li et al., 2013; Pathare et al., 2013). Differences between RS values of the back of the hand and the palm were determined in a 12-week supplementation trial (24 mg β-carotene/day from Betatene, an algal extract) as increasing by 17-fold and 2.2-fold, respectively; the palm RS measures correlated best with serum levels assessed by HPLC analysis ($r = 0.94$), proving spectrophotometry as an effective assessment for the FV biomarker (Stahl et al., 1998).

CIELab b* (yellow) values of the skin have become widely used for their association with increased carotenoid concentration attributed to the plant pigment's yellow-orange hue (Pathare et al., 2013; Pezdirc et al., 2015; Pezdirc et al., 2016; Stephen et al., 2011; Tan et al., 2015). Human skin b* values have been positively associated with the skin carotenoid increase seen following greater FV consumption (Pezdirc et al., 2015; Pezdirc et al., 2016; Stephen et al., 2011; Tan et al., 2015). A randomized crossover trial of 30 women found significantly greater skin yellowness and plasma carotenoid concentrations in women consuming a high carotenoid FV diet (~25 mg β-carotene/day) when compared to a low carotenoid FV diet (~0.25 mg β-carotene/day from mainly white vegetables and legumes) for four weeks (Pezdirc et al., 2016). Another RCT demonstrated a significant increase in yellow (b*) coloration of the skin of 81 Malaysian students following consumption of carotenoid-rich FV smoothies (~20-30 mg total carotenoids/day) for six weeks when compared to mineral water (Tan et al., 2015).

Spectrophotometry has demonstrated effective detection of carotenoid associated yellow coloration (b*) following supplementation as well. In an 8-week intervention trial, skin b* values

not only increased with greater FV intake measured by FFQ in 82 Caucasian participants, but also increased with 15 mg supplementation of β-carotene/day in 10 participants (Stephen et al., 2011). Another study found significant increases in b* values following capsulated β-carotene supplementation of 15 mg/day over 8 weeks in 10 black African women (Coetzee & Perrett, 2014). The validation of spectrophotometrically determined carotenoid status by b* values has led to the exploration of a less-expensive, possibly more convenient, optical method of skin carotenoid assessment.

2.5.6 Digital Image Analysis

Assuming conditions during measurement are standardized, CIELab color space will maintain an objective and consistent value that can be compared whether measured or displayed by a spectrophotometer or camera (Mendoza et al. 2006). In a recent double-blind RCT, Foo et al. (2017) demonstrated that photo processing with a calibrated camera can determine carotenoid associated color changes in the skin. First, facial images of the double-blinded RCT participants were taken with a color chart in frame and under standardized distance and illumination conditions. The images were then color calibrated according to the color charts. Finally, a region of interest (ROI) of standardized size was selected and the CIELab color space was calculated from the average RGB values within the ROI selected. Foo et al. (2017) showed significantly greater skin yellowness (b*) with capsulated β-carotene supplementation (~18 mg/day) over 12 weeks when compared to placebo (lactose capsules) in a double-blind RCT of 43 Caucasian men with a mean age of 21 years (Foo et al., 2017). The authors showed that an increase in skin b* values was not associated with improvement of indices of oxidative stress measured by urinary 8-OHdG and isoprostanes and semen DNA fragmentation, immune function assessed by salivary bacterial killing capacity and lysosomal activity, and semen quality measured by sperm concentration and

motility. These findings are in concert with other studies showing limited improvements in health-related biomarkers with carotenoid supplementation (Butalla et al., 2012; Collins et al., 1998; Crane et al., 2011; Druesne-Pecollo et al., 2010; Hercberg et al., 2010; Nagao et al., 2015; Omenn et al., 1996; The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994; Wright et al., 2007).

In contrast, studies have consistently shown that carotenoid-rich FV intake can positively influence various health biomarkers including: dysglycemia (Arkbalay et al., 2008); oxidative stress markers 8-iso-PGF2 and 8-OHdG (Cocate et al., 2015); HDL antioxidant modulating enzymes PON-1 and LCAT (Daniels et al., 2014), endothelium-dependent dilation (George et al., 2012); CRP (Rubin et al., 2012; Watzl et al., 2005), sICAM1, leukocytes, fibrinogen, F2-isoprostanes, and superoxide dismutase (Hozawa et al., 2007); and extrinsic epigenetic age acceleration determined by blood cell composition and DNA methylation (Quach et al., 2016). An interesting aspect that emerged from the Foo et al. (2017) study was that perceived health and attractiveness increased in concert with the skin carotenoid associated coloration. Such findings agree with the concept that carotenoids play a role in the synergistic antioxidative network in the skin that play a role in skin and overall health (Darvin et al., 2011; Lademann et al., 2011).

Smartphones have emerged as portable diagnostic tools with potential to decrease costs and improve healthcare monitoring capability (Oncescu et al., 2013). While many smartphone-based diagnostic tools tend to require a separate accessory system that works with the phone, such as for colorimetric detection of pH in sweat and saliva (Oncescu et al., 2013) or jaundice (Taylor et al., 2017) and pancreatic cancer diagnosis (Mariakakis et al., 2017), some applications use the smartphone's own sensors to address chronic wound and wound care identification (White et al., 2014), size and color detection of wounds (Poon & Friesen, 2015), or diagnosis of skin cancer (Abbott & Smith, 2018). This is all done through digital image capture and analysis.

The fact that the largest obstacles related to variations in lighting, zoom, and angle of image capture have already been surpassed via a large dataset and optimization of software (Abbott & Smith, 2018) suggest that there might be potential for reliable smartphone image applications for health assessment.

2.6 Perceived Health and Attractiveness

Increased perceived health and attractiveness resulting from greater skin carotenoid content is thought to have evolved through sexual selection (Whitehead et al., 2012c). In birds, amphibians, reptiles, and fish, bright colored ornaments signal the quality of the owner because carotenoids are being stored to serve as an 'honest' sexual signal rather than for self-maintenance (Alonso-Alvarez & Galvan, 2011; Simons et al., 2012). In a meta-analysis of 148 studies spanning 88 different species of birds, carotenoid levels and ornament color intensity were significantly related to greater swelling in response to phytohaemagglutinin, a lectin used to test immunocompetence and resistance to oxidative stress (Simons et al., 2012). Free radical exposure has been shown to induce a paler ornament in 77 red-legged partridges, similar to the notion that sickness is associated with pale skin, due to depletion of carotenoid reserves from the skin stores (Alonso-Alvarez & Galvan, 2011). A healthier individual, or organism of higher quality, is perceived as a more attractive mate when considering resistance to stressors and reproductive viability (Garcia-De et al., 2015). A healthier organism tends to be associated with more pro-vitamin A carotenoid activity, as well as the presence of anti-inflammatory, anti-oxidant, and anti-cancer carotenoids present in skin reserves, which would coincide with an increasing concentration of yellow pigmented molecules present in the skin (Garcia-De et al., 2015; Whitehead et al., 2012c; Stephen et al., 2011).

Increased perceived health and attractiveness has been associated with preference for lighter (L*) and yellower (b*) faces across different races in a perception trial involving 31 black

south Africans and 32 white Caucasians (Stephen et al., 2011). In another perception trial, when presented photos of a human face with the ability to change carotenoid or melanin skin coloration, participants chose to increase carotenoid coloration more than melanin coloration when instructed to choose the healthiest looking face (Stephen et al., 2011). Another study repeated the same experiment focusing on attractiveness, with three different groups of 60 participants (180 total, ~75% Caucasian and the rest other ethnicities) in choosing the most attractive face along a spectrum of melanin coloration alone, carotenoid coloration alone, and both melanin and carotenoid coloration combined (Lefevre & Perrett, 2015). This study demonstrated a greater perceived attractiveness with melanin or carotenoid coloration alone but found that carotenoid coloration was consistently preferred over the tanning associated melanin coloration (Lefevre & Perrett, 2015). Similar results of a preference for yellower (b*) skin, greater than the preference for melanin associated tanning, have also been demonstrated with Asian populations (Whitehead et al., 2012c). Interventions applying an appearance-based focus have been effective for motivating smoking (Semer et al., 2005) and tanning (Mahler et al., 2007) cessation, with the potential to drive improved FV intake (Whitehead et al., 2014).

2.7 Potential Motivational Nudge

While expert advice and/or nutritional counseling has demonstrated moderate (1-2 servings/day) increases in FV intake following a meta-analysis of 15 intervention trials with >3 month follow up (Maderuelo-Fernandez et al., 2015), potentially more effective methods of intervention are being studied. A review of 105 interventions to modify dietary behavior suggests interventions need to be frequent to reinforce the message, have a level of personalization, and have a duration of > 6 week (Racey et al., 2016). A prospective study using two self-reported surveys on 139 UK university students, one assessing demographics and FV intake predictor

variables at baseline, and another assessing average FV intake and perceived obstacles approximately two weeks later, demonstrated that motivation is an important determinant behind FV intake intervention (Evans et al., 2015).

Studies have demonstrated the influence of appearance in motivating healthy lifestyle change (Pezdirc et al., 2015). An RCT of 123 undergraduate students resulted in less skin darkening measured by skin spectrophotometric readings at 4- to 5- and 12-months following appearance-based interventions including exposure to photo-aged faces with extreme cases of wrinkles and sun spots at baseline and at 4- to 5-months following UV photographs depicting underlying skin damage (Mahler et al., 2007). A study using a facial overlay and digitally produced masks simulating facial disfigurements and wrinkling from tobacco use on participants' own facial photos demonstrated increased participation in smoking cessation programs (Semer et al., 2005).

Appearance also seems to have an influence on lifestyle change associated with a healthy diet. A survey of 250 UK adults found a strong association between importance attributed to appearance and personal health and dietary guideline conformity (Traill et al., 2012). When compared to a group of older individuals (35-50 y) from the National Weight Control Registry, younger participants (18-35 y) were more motivated by appearance and social factors and rated health concerns as less motivating (LaRose et al., 2013). This suggests that young adults would be more receptive to appearance-based motivational nudges, which could help form stronger healthy lifestyle habits. Moreover, the problem of low FV intake is most prevalent among adolescents (aged 11-17 years) (Minaker and Hammond, 2016), likely due to the age groups' poor self-regulation strategies (Taut et al., 2015).

While the appeal to vanity as a motivational nudge is still a novel concept, one study demonstrated a sustained improvement in self-reported FV intake over a 10 week follow up of 46 participants (aged 18-61 years) when they were shown the appearance of their own face on a

spectrum of carotenoid coloration representing ±10 servings of FV (Whitehead et al., 2014). One drawback from this study is the self-reported FV intake assessment, allowing for a major source of bias. There is a need for better FV intake interventions tailored toward motivation and extrinsic factors, such as attractiveness, along with improved monitoring of FV intake and NCD risk.

Chapter 3

Manuscript

Digital Photo Analysis for Tissue Carotenoid Status Assessment

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3.1 Introduction

Globally, human fruit and vegetable (FV) intake is not meeting dietary recommendations for health maintenance. In 2010, only 0.4% of a sample representing 87% of the world's adult population met United States Department of Agriculture (USDA) dietary guidelines of about 4 servings (2 cups) of fruit and 5 servings (2 ½ cups) of vegetables per day (Micha et al., 2015). Low FV consumption accounted for 41% of dietary risk associated deaths in 2015 and is one of the leading preventable causes for non-communicable diseases (NCDs) (Forouzanfar et al., 2016). While there are various FV components shown to prevent NCDs (Shashirekha et al., 2015), carotenoids also serve as an accurate biomarker for FV intake (National Academy of Sciences, 2000) beyond probable evidence for their chemopreventive properties against NCD risk (Donaldson, 2011).

There are various methods for carotenoid assessment. Food frequency questionnaires (FFQ) and food diaries are quick, simple measures but are prone to bias and lack accuracy of reporting (Willet, 2012; Wrieden et al., 2003). Biomarker measurement methods such as liquid chromatography, whether detected either optically or via mass spectroscopy, can accurately distinguish plasma carotenoids (Couillard et al., 2016; Gentili & Caretti, 2010). However, these methods require invasive sampling, are costly, time consuming, and require experience and expertise to analyze and interpret results (Zamora-Ros et al., 2012). Optical methods for carotenoid assessment using skin coloration based on storage of excess carotenoids in skin (Kiokias et al., 2016) are relatively accurate, quick, and non-invasive (Ermakov & Gellerman, 2015; Ermakov & Gellerman, 2015; Whigham & Redelf, 2015) but still lack accessibility.

Studies using spectrophotometric methods have demonstrated improvements in skin carotenoid coloration resulting from carotenoid-rich FV supplementation (Pezdirc et al., 2016), FV-rich smoothies (Tan et al., 2015), and FV or β-carotene alone (Stephen et al., 2011).

Reflectance spectrophotometry can assess skin coloration and convert color saturation values into a device independent and perceptually uniform L*a*b* color space (Pathare et al., 2013). The b* value represents blue (-120) to yellow (+120) coloration and is the value that corresponds to the carotenoid associated color changes in the skin. Recently, Foo et al. (2017) demonstrated that digital image analysis of skin regions for carotenoid associated coloration can accurately assess changes in carotenoid intake. Photos of the skin were taken before and after carotenoid supplementation and then analyzed for carotenoid associated coloration values (b*) but the analysis was not compared to another established method of carotenoid assessment. In addition, increases in carotenoid intake and skin carotenoid coloration were associated with significant enhancements in perceived health and facial attractiveness (Foo et al., 2017).

Carotenoid enhanced skin coloration has been referred to as an 'honest signal' for health and attractiveness in animals (Alonso-Alvarez & Galvan, 2011; Simons et al., 2012). Such increases in perceived health and attractiveness in association with high carotenoid skin coloration has been demonstrated across ethnicities (Stephen et al., 2011; Whitehead et al., 2012c), but it appears this increase is not the result of increased melanin skin content, which results from tanning. When compared to skin coloration changes associated with tanning or low F&V intake, skin coloration changes associated with high F&V intake were perceived as more attractive (Lefevre & Perrett, 2015). Behavioral interventions, such as a Mediterranean diet, aimed to increase FV intake have led to moderate improvements (Maderuelo-Fernandez et al., 2015), but more innovative approaches are necessary to prevent NCD development. In that regard, motivation (Evans et al., 2015) and appearance (LaRose et al., 2013) have been identified as important determinants that could drive improvements in FV intake and lifestyle change.

The development of a digital photo skin analysis for carotenoid status assessment can reduce the costs, invasiveness, time, and complexity that have limited widespread utilization of

current methods of carotenoid status assessment. To our knowledge, digital photo analysis for carotenoid status assessment has not been compared to spectrophotometric measurement, including the testing of various photo capture conditions or devices. Likewise, the impact of short-term carotenoid-rich supplementation on skin coloration and intra-individual attractiveness over a two-week period has not been studied. The present study used short-duration supplementation of a carotenoid-rich carrot juice to evaluate the utility of digital image analysis for skin carotenoid status under varying lighting conditions in comparison to previously established methods (FFQ, food diary, and spectrophotometer) and also determined possible changes in intra-individual perceived health and attractiveness.

3.2 Materials and Methods

3.2.1 Study Design

An open label carrot-based FV juice supplementation trial of two weeks was undertaken with a four-week follow-up period (Figure 3.1). Body weight, an FFQ validated for the local region (Traynor et al., 2006 skin spectrophotometric measures, and digital photos of the face and both sides of the right hand for skin coloration analysis were taken bi-weekly for a total of four measurement sessions. Photos for perceived health and attractiveness ratings were taken during all four measurement sessions as well. Baseline measurements for all the above parameters were taken along with height assessment prior to the start of supplementation. A food diary was completed at baseline, after supplementation, and after follow-up completion.

All participants were provided with the full amount of Buda Juice No. 03 (Buda Juice, Mississauga, ON) frozen, following their baseline measurements at the Mary Emily Clinical Nutrition Research Unit (Ste. Anne de Bellevue, QC). Participants were instructed to store the juice in the freezer and thaw the juice in the fridge according to their consumption of one cup (250

ml) of the daily for two weeks. They were also reminded to consume the carrot juice with daily email reminders for the first two days of the intervention trial. The instructions also advised the subjects to drink the juice with a fatty meal, as lipid consumption aids carotenoid bioavailability (Faulks & Southon, 2005). Compliance for juice consumption was monitored through return of empty juice bottles. Buda Juice No. 03 consisted of a cold pressed, certified organic, non-pasteurized, mixture of carrot, orange, apple and cucumber juices and was subsequently analyzed for β-carotene content.

3.2.2 Participants

Twenty-six McGill University Macdonald Campus students (9 male, 17 female) were recruited through fliers posted on the campus and a mass email of the flier was sent to the undergraduate and graduate student body. The sample size was calculated to detect a difference of about 3.0 in yellow (b*) skin coloration based on the results of Tan et al. (2015), assuming a standard deviation of 2.0 with an $\alpha = 0.05$ and 80% power. To account for 20% dropout, five participants were added.

Inclusion criteria were: 1) BMI between 18.5-24.9; and 2) 18-40 years of age (according to Statistics Canada, over 90% of university students were ≤40 years of age in 2006). Exclusion criteria were: 1) chronic or acute illness; 2) chronic or acute skin condition; 3) use of dietary supplements associated with the skin; 4) use of skin altering products; 5) smoking; 6) excessive alcohol use (>1 drink/day for females, >2 drinks/day for males); and 7) recent illness in the last month. If a participant became ill during the study (i.e., cold or flu), this event was recorded but data was still collected. The study was approved by the Faculty of Agricultural and Environmental Sciences Research Ethics Board at McGill University (Montreal, QC).

3.2.3 Weight and Height

Participants were asked to take their shoes off and align their heels to the wall using the NHANES (National Health and Nutrition Examination Survey) measurement protocol for height and weight (NHANES, 2007). Height was measured at baseline, and weight was measured at 0, 2, 4 and 6 weeks.

3.2.4 Dietary Assessment

Participants completed a short FFQ, which had been validated against a 24-hr recall for a similar region (Ontario, Canada), with verbal instructions provided during the beginning middle and end of the study (weeks 0, 3, and 6). Participants were also provided with instructions on how to complete a three-day food record for Weeks 0, 3, and 6. To analyze the nutrient content of the participants' diets from the food diaries, food quantities were entered into the Food Processor Nutrition and Fitness Software© ESHA Research 2011. The three-day food record provided greater detail of the overall diet, particularly the amount of fat consumption as this could affect carotenoid bioavailability (Faulks & Southon, 2005).

3.2.5 β-Carotene Quantification by LC-MS

Buda Juice No. 03 was subject to an extraction based on the method of Tan et al. (2015) that involved freeze drying five juice samples. From a sample weight of five ml, extraction was performed with five ml methanol/hexane (4:3, v/v) mixture for two cycles. The samples were sonicated for 15 min and centrifuged at 3500 rpm for 5 min following the addition of the methanol/hexane mixture.

Analyses were done on a triple quadrupole MS system (EVOQ Elite, Bruker, Billerica, MA) coupled with an ultrahigh-performance liquid chromatography pump (Advance, Bruker,

Billerica, MA) and equipped with a C18 reverse phase column YMC Carotenoid (4.6 × 250 mm, 3 µm, (p/n CT99S032546WT) (Waters, Milford, MN). Mobile aqueous phase A (0.1% formic acid in water) and mobile organic phase B was 100% acetone. Chromatographic runs were performed under isocratic conditions for 30 min using 95% solvent B. The column temperature was set at 40°C and the flow rate was 1 mL/min. The samples were injected in triplicate.

The operating parameters of the mass spectrometer were: APCI positive spray 99 uA, cone temperature 350°C, cone gas flow 20 psi, heated probe temperature 450°C, probe gas flow 40 psi, nebulizer gas flow 60 psi. The mass spectrometer was used in the multiple reaction monitoring mode (MRM). Two transitions were followed for β-carotene: 537.40→177.20 (CE 11 eV) and 537.40→137.2 (CE 20 eV).

Quantification of the different compounds, based on peak areas, was performed following an external calibration curve using a β-carotene standard (Sigma-Aldrich, Oakville, ON). The x-axis presented the concentration (ng/ml) and the y-axis displayed the peak area. Six points of calibration were used to produce a standard curve and the linearity was assessed by the correlation coefficient, R^2.

3.2.6 Spectrophotometer Skin Coloration Values

A CM-600d spectrophotometer (Konica Minolta, Tokyo, JPN) served as a source of validation for the digital imaging assessment since it yields color values in CIELab color space, thereby allowing direct comparison to values attained from digital image analysis. Participants were required to wash their hands with soap and clean their right cheek with an alcohol swab prior to the imaging measurements to ensure the absence of dirt or debris that may interfere with the spectrophotometric measurements. Seated participants extended their right hand out and the spectrophotometer was placed in the middle of the palm followed by the top of the hand for

readings. The spectrophotometer was then placed on the right cheek for readings. All measurements were performed in triplicate. The L*a*b* values were noted and the glass on the spectrophotometer was cleaned with a Kimtech Kimwipe following each measurement.

3.2.7 Digital Photo Capture and Processing

A table top light tent studio with two halogen bulbs was used to create standardized illumination conditions for image capture. Photos of the participant's right palm, top of the hand, and the right cheek were taken in Raw format with a Canon EOS Rebel T1i camera (Canon Inc., Tokyo, JPN) with ISO set to 400, F at 8.0, and 33 mm focal length. Additional photos were taken with an iPhone 4 smartphone (Apple Inc., Cupertino, CA) and Google Pixel 1 smartphone (Google, Menlo Park, CA). All photos were taken at a standard distance of 60 cm. To assess background color influence on the image coloration in standardized conditions, all photos were taken with different backgrounds: 1) black background to prevent residual reflection; and 2) white background to counter the possible excess contrast from the former. To evaluate uncontrolled lighting conditions and devices, additional photos were taken with the camera and both smartphone cameras in a room with lights off using the device's flash, as well as in a room using incandescent lighting conditions. Photos for the separate body regions taken in triplicate using the three devices under two standardized lighting conditions with two backgrounds (n = 54 images), as well as under the flash and general lighting conditions (n = 54 images), which provided a total to 108 images per timepoint per participant.

Participants were asked to put their hand in the light tent and place it in the middle of the photo frame. Each participant had a photo taken of their right palm as the primary outcome for carotenoid status assessment and the top of their right hand as sun-exposed comparison. A photo of the participants' face was taken to assess for perceived health and attractiveness ratings as well

as carotenoid-derived skin coloration. Each photo had a white piece of paper with the participants ID for the purpose of this study, as well as a color chart for use as internal standards of illumination for image processing and analysis. Canon camera images were only color calibrated, only white reference normalized, both color calibrated and white reference normalized, and kept without processing. All other device photos were initially processed by the device's standard proprietary filters and then white reference normalized and kept without further processing.

3.2.8 Perceived Health and Attractiveness Assessment

The perceived health and attractiveness assessment was conducted via an electronic survey that displayed randomized images at different timepoints of intervention of the participants based on a previously published methodology using a 10-point bipolar scale rankings from 1 (very unattractive) to 10 (very attractive) (Lydon et al., 2003). The assessment ratings of 11 trained evaluators who provided independent ratings on each subject were averaged. Regardless of culture of origin and ethnicity, agreement among trained raters has previously been noted to be high (Nestor et al., 2010). Inter-rater reliability was high after comparing ratings that the evaluators made individually on 20 practice headshots, which allowed measurement of subjective ratings due to the strong consensus among evaluators. The assistants were instructed to rate the pictures using an objective and holistic approach involving a bipolar scale of values ranging from very unattractive (1) to very attractive (10) and very unhealthy (1) to very healthy (10). The ratings were done for each timepoint in the dataset and this allowed us to compare changes in participant's perceived health and attractiveness over the time of the intervention (within person ratings).

3.2.9 Digital Photo Analysis

Once all the images were taken, organized, and labeled (for each participant, week, camera,

lighting condition, region of interest (ROI) and duplicate), an in-house developed Matlab program (MathWorks Natick, MA) was used to analyze the palm images for their yellow coloration through the CIELab color space. The images were processed using the Matlab program where a standardized square of 80 x 80 pixels was placed in the most central region of the right palm, right cheek, and top of the right hand of respective images to designate the region of interest (ROI). The program then converts the device-dependent and non-perceptually uniform RGB (Red, Green, Blue) values of this ROI and converts them to the perceptually uniform and device independent CIE (Commision Internationale de l'Eclairage) L*a*b* color space. The L* value is a measure of luminance ranging from 0 (black) to 100 (white). The a* value is a measure of green (-120) to red (120) coloration. For the purposes of this study, the b* values only were assessed, which measures blue to yellow coloration on a spectrum of -120 (blue) to 120 (yellow).

3.2.10 Statistical Analysis

Dietary data results are reported as means across participants ± SD. To ensure consistency across dietary data, separate repeated measures ANOVA were performed for each nutrient, using time as a factor with 3 levels (0, 3, and 6 weeks) and average nutrient content following food diary entries for each participant as the dependent variable.

Digital image analysis of yellow (b*) skin coloration values was estimated for each image with three different devices under four different lighting conditions and then compared to yellow (b*) skin coloration values measured with the spectrophotometer. All b* values are reported as means across participants ± SEM. To analyze the effect of carrot juice supplementation on carotenoid status using the different methods of assessment over the six-week period, separate one-way repeated measures ANOVA analyses were performed for the spectrophotometer readings for each of the three ROI. The above statistical analysis was also performed with the image analysis

data for different combinations of the three different ROI, across three different devices, with or without color calibration applied for the Canon camera images, with or without reference white normalization of the photos, and under each of four various lighting conditions to determine which permutation would be most sensitive. Time was used as a factor with 4 levels (0, 2, 4, and 6 weeks) and CIE L*a*b* color space b* values assessed by spectrophotometry and photo analysis were dependent variables. The above one-way repeated measures ANOVA analysis was also used to ensure consistency in the white reference used for the images. To relate carotenoid status measurement values of the various digital image devices and lighting conditions to the spectrophotometer values, two-tailed Pearson correlation matrices were computed for each ROI under the four different lighting conditions for each camera used. Correlations were Bonferroni corrected and a Pearson correlation coefficient of 0.7-0.9 was considered strong (Mukaka, 2012).

Perceived health and attractiveness ratings were reported as mean ratings for the participant from all the raters ± SEM per time point. Two, one-way repeated measures ANOVA were performed using time as a factor with 4 levels (0, 2, 4, and 6 weeks) and changes in perceived health and attractiveness ratings as the dependent variables. Values of $p < 0.05$ were considered significant. All statistical calculations were performed using JASP (JASP, Amsterdam, Netherlands).

3.3 Results

3.3.1 Dietary and physiological characteristics of participants

Nine males and sixteen female participants with an average BMI of 22.1 completed the study. One female dropped out after the fourth week for personal reasons. No significant differences were found for dietary nutrient intake (Table 3.1) or FV servings per day (Table 3.2) throughout the study. One participant did not submit their final food diary for the end of the study,

but when their data was removed, the data maintained no differences across time. Five participants developed a cold during the study, but their dietary data did not appear to be affected significantly and removal of these participants did not influence the results of the of the spectrophotometer data.

3.3.2 Carotenoid content of juice

The standard curve with six points of calibration at different concentrations of the β-carotene standard (0.049, 0.098, 0.198, 0.391, 0.781, and 1.563 μg/ml) and linearity was confirmed at a correlation coefficient R^2 of 0.9978. The mean range of AUC (area under the curve) for the five carrot-based FV juice samples was 2600 to 3500, corresponding to an average β-carotene concentration of about 99 ± 12 μg/ml of juice. The mean content of β-carotene of the carrot-based Buda Juice was therefore confirmed to be 25 ± 3.0 mg/250 ml. This value corresponds to previous findings using HPLC-UV analysis showing approximately 12 mg of β-carotene per 100 ml of carrot juice, which provides about 30 mg per cup of carrot juice (Törrönen, et al., 1996). Another study using HPLC with UV detection measured 8.25 and 10.65 mg β-carotene per 100 g of carrot puree and boiled-mashed carrot, respectively, that amounts to about 20-25 mg/250 ml (Edwards et al., 2002). Likewise, an average content of 12 mg/100 ml and 5 mg /100 ml depending on the blend was measured via TLC (thin layer chromatography) densitometry (Starek et al., 2015).

3.3.3 Change in skin color over time

There was a significant increase ($p < 0.05$) in skin yellow coloration of the palm following the first two weeks of supplementation as measured by the Minolta spectrophotometer b* values (Figure 3.2). There were no differences in b* values for the top of the hand, while the cheek values decreased significantly at Week 6 when compared to Weeks 2 and 4.

Color calibrated and white reference normalized images taken with the Canon camera yielded significant time dependent increases in b* values for palm images under conditions of: (a) black background under standardized lighting and (b) lights off using the device's flash as the only source of illumination (Figure 3.3). Only photos of the cheek taken in incandescent lighting conditions demonstrated a significant decrease in b* values over time (Table 3.9). Photos of the top of the hand taken in all conditions besides the black background demonstrated significant decreases over time (Table 3.9). Photos of the top of the hand taken with a black background demonstrated a significant increase from Week 2 to Week 4 and a significant decrease from Week 4 to Week 6 (Table 3.9). Interestingly, when white reference normalization of all images was removed, significant changes in b* values were demonstrated for palm images with a white background and standardized lighting, in addition to the black background and flash conditions (Table 3.9). All other ROI and lighting condition combinations maintained their significant differences except the top of the hand black background images, which demonstrated a significant increase from Week 2 to Weeks 4 and 6 (Table 3.9).

The white reference itself (Table 3.4) produced significant time dependent increases in b* values for palm images with a black background under standardized lighting conditions obtained via the Canon camera. All lighting conditions except the white background images resulted in significant time dependent changes in white reference b* value for the top of the hand. Black background conditions showed significant differences at Weeks 0 and 2 in comparison to Weeks 4 and 6, flash conditions showed week 0 with significantly greater values than the other timepoints, and incandescent conditions showed significant differences between Week 4 and Weeks 0 and 2 (Table 3.9). No differences in the white reference b* values were observed for the cheek images under any of the lighting conditions. Only the palm images showed increasing significant differences of the b* value over time when addressing the reference white normalized images

without color calibration (Table 3.4). Images taken with the Canon camera yielded significant differences for the black and flash conditions, while the white conditions demonstrated a significant increase from Week 2 to 4, but a significant decrease from Week 4 to 6 (Table 3.9).

Images taken with the iPhone 4 demonstrated significant increases in b* value for white and incandescent lighting conditions, while the Pixel 1 camera resulted in significant increases only in the flash condition (Table 3.10). For the cheek, Canon camera images did not show any differences, while the iPhone 4 images showed a decrease for only the flash condition, and the Pixel 1 images yielded decreasing significant differences for all conditions. Top hand images yielded decreasing significant difference for almost all combinations except no difference for iPhone 4 images taken in the incandescent condition.

Significant increases were only observed in palm images and top of the hand images from the Canon camera when addressing images without color calibration or white reference normalization (Table 3.11). The Canon camera palm images demonstrated increasing significant differences for all lighting conditions. Images taken with the iPhone 4 demonstrated increasing significant differences for white and incandescent conditions only, while the Pixel 1 images only demonstrated decreasing significant differences. The top hand images that demonstrated an increasing significant difference were in the black background condition. All other combinations resulted in either no difference or decreasing significant differences.

The b* values obtained from the images taken with the color calibrated camera under different lighting conditions were correlated to the Minolta spectrophotometric method. This is the first validation for color calibrated camera image analysis against an established method of carotenoid status assessment. The color calibrated images with reference white normalization (Table 3.5) of the palm correlated strongly ($r > 0.7 - 0.9$; $p < 0.001$) with the respective spectrophotometer b* values under the flash condition only ($r = 0.82$; $p < 0.001$), while the other

lighting conditions exhibited a moderate correlation. Images of the top of the hand showed strong significant correlations with spectrophotometer b* values under all lighting conditions except the black background condition. The color calibrated images without reference white normalization demonstrated similar results (Table 3.6). Overall, the strongest correlation occurred between spectrophotometer b* values and the b* values obtained from images of the palm in the flash condition, regardless of whether reference white normalization was used.

The b* values for images obtained from the non-color calibrated devices were correlated with respective b* Minolta spectrophotometer values with (Table 3.7) and without (Table 3.8) white-reference normalization. The threshold for statistical significance was increased to a p value < 0.001 to accommodate the number of correlations (12) ($p < 0.004$) generated for each set of conditions, via a Bonferroni correction (Curtin & Shulz, 1998). The white reference normalized images of the palm yielded strong correlations for all conditions apart from the black background for the Canon camera (highest: flash, $r = 0.850$) and the Pixel 1 camera (highest: flash (Figure 3.4), $r = 0.868$; $p < 0.001$). The iPhone 4 correlated strongly under all lighting conditions but the white background (highest: incandescent (Figure 3.5), $r = 0.861$; $p < 0.001$). The images of the top of the hand only correlated strongly when photos were taken with the Canon camera in all conditions but the black background condition (highest: flash, $r = 0.768$; $p < 0.001$). The images without white reference normalization showed very similar correlations to the white reference images, but with slightly smaller correlation coefficients and the iPhone images taken under flash conditions did not correlate (Tables 3.7 & 3.8). For the images of the top of the hand, the lack of white reference normalization reduced the number of imaging conditions that yielded strong correlations to only the flash condition and incandescent condition when taken with the Canon and iPhone camera, respectively (Table 3.4 & 3.9). The images of the cheek did not show strong correlations with the spectrophotometer values, whether normalized to the white reference or not.

Inter-individual responses to the changes in skin b* value coloration from the carrot-based FV juice supplementation were identified (Figure 3.6). Hence, we categorized our participants as responders, slow-responders and non-responders. Responder status was considered as an increase in b* value of 1.0 or more above baseline as significance following supplementation for the whole sample was established with less than 1.0 (increase of 0.82 b* value at Week 2). Twelve participants responded within the 2-week supplementation period, whereas seven participants showed a slow response with no skin coloration evident until 2- or 4-weeks post-supplementation (Weeks 4 and 6), and six participants were non-responders. According to one-way repeated measures ANOVA, the responders and slow responders demonstrated significant increases after supplementation (Week 2) and for the remainder of the study (Weeks 4 and 6), when compared to baseline (Week 0). Slow responders demonstrated significant increase ($p < 0.05$) in b* values between Week 2 and Week 6. In reality, the difference between b* values for Week 0 and Week 2 was about 1/3 of the difference that the responders experienced. The non-responders demonstrated no significant differences in b* values throughout the study period.

3.3.4 Change in perceived health and attractiveness over time

There were no significant differences in perceived health or perceived attractiveness over time within-individuals when all individuals were grouped together (Figure 3.7). The data demonstrated a slight tendency to increase after supplementation (Week 2) but there was a large variation among participants. Separation into responders (for spectrophotometer skin b* value increases above 1.0 unit in the palm and cheek), also showed no significant time dependent differences in ratings for perceived health or perceived attractiveness (Figure 3.8). Raters were relatively consistent as intra-class correlations on practice ratings demonstrated a consistency

agreement of 0.823 and 0.899 for perceived health and attractiveness, respectively, when using a two-way random model.

Seven participants demonstrated a rating increase of > 0.5 units for perceived health following the supplementation while four participants demonstrated a rating increase of > 0.5 units for attractiveness. Three participants exhibited changes in both perceived health and attractiveness, two of which were part of the slow responder group for the palm skin coloration change.

3.4 Discussion

The present study extends previous findings showing that increasing carotenoid intake by FV-based supplementation can cause skin yellowing in the palm (Pezdirc et al., 2016; Tan et al., 2015; Whitehead et al., 2012d), as findings demonstrated that a short two-week supplementation of a carrot-based FV juice was sufficient to show an associated increase in palm skin coloration that is linked with increased skin carotenoid content. The average change in palm skin coloration of approximately 1.0 b* value resulting from the supplementation is in concert with the 0.6 b* value increase of skin areas not exposed to the sun (i.e., palm, sole of foot, inner arm), seen following a four-week supplementation of high-carotenoid FVs (about 25 mg β-carotene/day) (Pezdirc et al., 2016) whereas an increase of b* value of 3.2 was observed in facial skin regions (forehead and cheeks) following a four-week supplementation of smoothies (about 20 mg β-carotene/day) (Tan et al., 2015). Another study demonstrated an increase in palm skin carotenoid status as measured by Raman spectroscopy following fresh orange juice supplementation for 25 days (Massenti et al., 2015). Study differences, which include factors such as dosage, duration of study, and sample size likely contributed to variations in palm b* values occurring from carotenoid supplementation.

There also appeared to be responders, slow responders, and non-responders to the supplementation to the carotenoid-rich carrot juice supplementation (Figure 3.6) regardless of similarity in dietary FV intake, health status and age range. There are a variety of factors that have indicated interindividual variations of carotenoid bioavailability including sex, age, lifestyle, genetics, and gut microbiota composition (Bohn et al., 2017). Recently, differences in gut microbiome profiles have been suggested to contribute a large part to the variations in carotenoid status among individuals (Djuric et al., 2018), especially since more than 50% of carotenoids from foods are not absorbed until the colon is reached (Bohn et al., 2017). Bioaccessibility gut model studies have reported that significant quantities of lycopene and β-carotene are released from plant foods following colonic fermentation (Goñi et al., 2009). Future studies using stool samples could evaluate the degree that gut microbiota can influence responsiveness of skin coloration to carotenoid supplementation.

While some studies have reported increases in skin yellow coloration for the face with increasing FV intake (Pezdirc et al., 2016; Tan et al., 2015), as well as other areas of the skin with varying levels of sun exposure (i.e., inner arm and palm, or outer arm and top of the hand) (Pezdirc et al., 2016; Stephen et al., 2011), the two-weeks of carrot-based FV juice supplementation was not associated with skin yellow coloration changes of the cheek or top of the hand (exposed). This latter finding may be due several factors, including a shorter supplementation period, diversion of carotenoids to other tissues via a triage effect (Donaldson, 2011), or differences in carotenoid bioavailability due to variations in microbiome profiles. A study limitation was the lack of skin assessment for the red component (a*) that is associated with α-carotene, which is found in significant quantities in carrot juice. An increase in a* skin values have been associated with FV smoothie intake (Tan et al., 2015) but have not necessarily been linked to a high-carotenoid FV diet per se (Pezdirc et al., 2016). Future studies need to assess red skin coloration (a*) in association

with α-carotene and lycopene supplementation as the predominantly yellow (α-carotene and β-carotene) and red (lycopene) carotenoids account for most of total skin carotenoid content (Ermakov & Gellermann, 2010).

In accordance with a lack of detectable changes in yellow coloration of the face, no observed changes in ratings of perceived health and attractiveness over the two-week supplementation period were found. Previous studies have related greater FV intake (Stephen et al., 2011; Whitehead et al., 2012d) or FV associated skin color changes (represented by greater b* values) (Lefevre & Perrett, 2015; Stephen et al., 2011) to greater ratings of perceived health (Stephen et al., 2011) and attractiveness (Lefevre & Perrett, 2015; Whitehead et al., 2012d). Two β-carotenoid supplementation studies of longer durations of 8 weeks (Stephen et al., 2011) and 12 weeks (Foo et al., 2017), have reported improvements in perceived health (Foo et al., 2017; Stephen et al., 2011) and attractiveness (Foo et al., 2017). The differences in perceived health and attractiveness ratings between previous studies and the present work is likely due to the shorter intervention time and method of perceptual analysis. The ratings may be less sensitive than simply choosing which face is healthier looking or attractive when comparing a baseline image to an endpoint image, as was done in other studies (Lefevre & Perrett, 2014; Foo et al., 2017). Other influencing factors could include confounding influences such as effects from red skin coloration (a*) (Stephen et al., 2012) and other dietary carotenoids and antioxidants in the diet (Darvin et al., 2006; Liu et al., 2008; Wang et al., 2013). Alternatively, or in addition, the methodology used to rate the images may not be sensitive enough to demonstrate intra-individual differences following the short intervention period despite the within rating consistency and the use of randomization and trained raters.

In addition to significant changes in yellow coloration of the palm skin following carotenoid supplementation, b* values of the palm and top of the hand obtained from the Canon

camera image analysis exhibited strong ($r > 0.7$) and medium ($r > 0.5$-0.7) correlations with the Minolta reflectance spectrophotometer b* values. Given that the black background condition did not yield consistent reference white b* values for the palm (likely due to too much contrast), and black, flash, and incandescent conditions were not consistent for images of the top of the hand, those conditions should be ruled out of any analysis for inconsistent color representation. The flash lighting condition showed the highest correlation between Canon camera image b* values of the palm with palm spectrophotometer b* values (Table 3.5), and so this appears to be the best imaging condition for assessment of palm skin for carotenoid status (Figure 3.3).

Discrepancies within the white reference color representation could have been due to reflections from clothing interfering with color representation, especially for the facial images (Whitehead et al., 2012d). Other confounding factors could have included too many features in frame; variations in contrast, exposure, focus, and the specific ROI selected (Pathare et al., 2013). The white reference used for normalization of the images was a piece of blank printer paper that was held up by participants at different angles, which likely produced shadows and variations in reflectance. When the white reference paper, however, was tested against a color chart standard white no significantly different coloration values were noted (data not shown). The close correlation coefficients between images taken in the same conditions and differing only by reference white normalization suggests that this processing did not influence the trend of the observed b*values. White reference normalization does, however, appear to reduce the impact of excess or improper illumination and narrow the range of b* values from image analysis. Overall, the Canon camera image analysis worked best with the images of the palm, taken in darkness with the flash of the device, with incandescent lighting and a neutral beige background, and standardized lighting conditions with a white background. In future studies, better control and standardization for capture conditions could be maintained such as covering the clothes of

participants with fabric that matches the color of the background or ensuring a stricter standard position and location of the objects in the image (i.e., reference white, hand, face) to prevent unpredictable shadows (Foo et al., 2017).

There were several limitations of the study including a small sample size with an uneven representation of sexes among the participants and a short intervention period. The compliance monitoring was a limitation and lighting conditions were not standardized sufficiently as the wavelength spectrum emission of the bulbs used for each lighting condition was not assessed.

In conclusion, a two-week supplementation of a carrot-based FV juice was sufficient to increase carotenoid associated coloration of the palm skin although slow or non-responders to the supplementation were noted. Across all tested devices, the use of the flash of the device as the only source of illumination appeared to be the best in comparison to the spectrophotometer as strong correlations were reached for the smartphones and the calibrated camera, with and without the reference white normalization. Further work needs to be focused on clear and reproducible image manipulation and capture settings (Cromey, 2013) to allow for measurement of skin coloration to work with different devices and under various lighting conditions. In addition, identification of a threshold for perceived health and attractiveness changes needs to be identified, along with an adequate carotenoid supplementation dosage and duration.

Table 3.1 Subject characteristics

BMI		Weight (kg)		Height (cm)		Age	
Female	Male	Female	Male	Female	Male	Female	Male
21.3 ± 2.1	23.5 ± 2.0	56.8 ± 5.8	71.0 ± 11.1	163.1 ± 4.4	175.6 ± 5.0	21.1 ± 2.2	24.3 ± 3.7

Mean subject characteristics including BMI, weight, weight, and age for males (n=9) and females (n=16) with SD

Table 3.2 Average nutrient profile for baseline, midpoint, and endpoints (Weeks 0, 3, and 6, respectively) from diet diaries

Diet Diary Nutrients	Week 0	Week 3	Week 6
Calories	1949.20 ± 515.4	1951.16 ± 480.9	1910.96 ± 594.3
Protein (g)	79.80 ± 31.3	86.28 ± 37.4	84.35 ± 36.7
Carbohydrate (g)	254.92 ± 91.9	255.76 ± 61.9	238.48 ± 79.2
Fiber (g)	28.28 ± 9.8	29.34 ± 12.2	26.91 ± 12.5
Fat (g)	70.00 ± 21.9	68.48 ± 24.1	70.87 ± 30.6
Saturated Fat (g)	19.88 ± 8.7	21.00 ± 10.3	21.22 ± 10.5
Cholesterol (mg)	280.36 ± 225.3	263.64 ± 204.2	259.87 ± 150.9
Vitamin A (IU)	8162.88 ± 7414.1	7173.96 ± 6392.8	6757.04 ± 6138.5
β-Carotene (mcg)	5581.44 ± 4189.9	5877.28 ± 5065.9	4962.35 ± 3903.6
F&V servings/d	5.00 ± 2.6	4.39 ± 2.1	4.44 ± 2.1

Table 3.3 Average daily fruit and vegetable (FV) servings ± SD for all participants at each timepoint determined by food frequency questionnaire (FFQ)

FFQ FV servings		
Week 0	**Week 3**	**Week 6**
5.0 ± 2.6	4.4 ± 2.1	4.4 ± 2.1

Table 3.4 Significances of the time-dependant changes in b* values for a reference white color obtained from the Canon camera under different lighting conditions

		White Reference Color Consistency			
		Lighting Condition			
		White	Black	Flash	Incandescent
ROI	Palm	p=0.54	p=<0.001**	p=0.19	p=0.22
	Cheek	p=0.20	p=0.07#	p=0.35	p=0.93
	Top Hand	p=0.464	p=<0.001**	p=0.005*	p=<0.001**

One-way repeated measures ANOVA p-values for color calibrated canon camera white reference b* values of different ROI across different lighting conditions over time

*Significant at the 0.005 level, **Significant at the 0.001 level, #Trend at p>0.05 & ≤ 0.07

Table 3.5 Canon camera color calibrated & reference white normalized image b* value correlation with respective spectrophotometer b* value

		Region of Interest		
Condition	**Pearson's Correlation**	**Palm**	**Cheek**	**TopHand**
White	r	0.674	0.487	0.723*
	p-value	< .001	< .001	< .001
Black	r	0.592	0.407	0.498
	p-value	< .001	< .001	< .001
Flash	r	0.821*	0.609	0.754*
	p-value	< .001	< .001	< .001
Incandescent	r	0.650	0.476	0.717*
	p-value	< .001	< .001	< .001

Pearson correlation coefficients for Canon camera color calibrated image b* values of all regions of interest with reference white normalization and under various lighting conditions (standardized lighting conditions with a white (white) and black (black) background, lights off using the device's flash (flash) and using common incandescent bulb lighting (incandescent)) with respective spectrophotometer b* values. *strong correlation

Table 3.6 Canon camera color calibrated image b* value correlations with respective spectrophotometer b* values

		Regions of Interest		
Condition	**Correlation**	**Palm**	**Cheek**	**TopHand**
White	r	0.692	0.424	0.743*
	p-value	< .001	< .001	< .001
Black	r	0.476	0.276	0.444
	p-value	< .001	0.005	< .001
Flash	r	0.810*	0.581	0.738*
	p-value	< .001	< .001	< .001
Incandescent	r	0.682	0.441	0.720*
	p-value	< .001	< .001	< .001

Pearson correlation coefficients for Canon camera color calibrated image b* values of all regions of interest (ROI) without reference white normalization and under various lighting conditions with spectrophotometer b* values of the same ROI. *strong correlation

Table 3.7 All camera image reference white normalized b* value correlations with respective spectrophotometer b* values

			Regions of Interest		
Camera	**Condition**	**Pearson's Correlation**	**Palm**	**Cheek**	**TopHand**
Canon	White	r	0.793*	0.568	0.723*
		p-value	< .001	< .001	< .001
	Black	r	0.567	0.467	0.471
		p-value	< .001	< .001	< .001
	Flash	r	0.850*	0.436	0.768*
		p-value	< .001	< .001	< .001
	Incandescent	r	0.798*	0.563	0.729*
		p-value	< .001	< .001	< .001
Pixel	White	r	0.711*	0.623	0.539
		p-value	< .001	< .001	< .001
	Black	r	0.687	0.630	0.556
		p-value	< .001	< .001	< .001
	Flash	r	0.868*	0.504	0.676
		p-value	< .001	< .001	< .001
	Incandescent	r	0.796*	0.440	0.487
		p-value	< .001	< .001	< .001
iPhone	White	r	0.666	0.241	0.566
		p-value	< .001	0.016	< .001
	Black	r	0.753*	0.407	0.684
		p-value	< .001	< .001	< .001
	Flash	r	0.793*	0.660	0.615
		p-value	< .001	< .001	< .001
	Incandescent	r	0.861*	0.676	0.678
		p-value	< .001	< .001	< .001

Pearson correlation coefficients for all devices' (Canon, Pixel 1, and iPhone 4) image b* values of all regions of interest (ROI) with reference white normalization and under various lighting conditions with spectrophotometer b* values of the same ROI. *strong correlation

Table 3.8 All camera image b* value correlations with respective spectrophotometer b* values

			Regions of Interest		
Camera	**Condition**	**Pearson's Correlation**	**Palm**	**Cheek**	**TopHand**
Canon	White	r	0.719*	0.447	0.680
		p-value	< .001	< .001	< .001
	Black	r	0.463	0.268	0.416
		p-value	< .001	0.007	< .001
	Flash	r	0.803*	0.291	0.758*
		p-value	< .001	0.003	< .001
	Incandescent	r	0.847*	0.629	0.683
		p-value	< .001	< .001	< .001
Pixel	White	r	0.706*	0.420	0.535
		p-value	< .001	< .001	< .001
	Black	r	0.641	0.418	0.592
		p-value	< .001	< .001	< .001
	Flash	r	0.850*	0.429	0.674
		p-value	< .001	< .001	< .001
	Incandescent	r	0.745*	0.341	0.637
		p-value	< .001	< .001	< .001
iPhone	White	r	0.364	0.070	0.285
		p-value	< .001	0.49	0.004
	Black	r	0.743*	0.248	0.683
		p-value	< .001	0.013	< .001
	Flash	r	0.609	0.531	0.463
		p-value	< .001	< .001	< .001
	Incandescent	r	0.860*	0.693	0.731*
		p-value	< .001	< .001	< .001

Pearson correlation coefficients for all devices' (Canon, Pixel 1, and iPhone 4) image b* values of all regions of interest (ROI) without reference white normalization and under various lighting conditions with spectrophotometer b* values of the same ROI. *strong correlation

Table 3.9 Significances of the time-dependant changes in b* values for color calibrated images obtained from the Canon camera under different lighting conditions

Separate One-Way repeated measures ANOVA for b*value over time									
CC & RW		**Condition**							
ROI	**Camera**	**White**	**White post-hoc**	**Black**	**Black post-hoc**	**Flash**	**Flash post-hoc**	**Ambient**	**Ambient post-hoc**
Palm	**Pro**	//////	wk2-4 p=0.071	p=0.009	wk2-wk4 p=0.040	p<0.001	wk0-2 p=0.026; wk0-4 p=0.004; wk0-6 p=0.009	//////	//////
Cheek	**Pro**	//////	//////	//////	//////	//////	//////	p=0.001	wk2-4 p<0.001; wk2-6 p=0.042
TopHand	**Pro**	p<0.001	wk0-6 p<0.001; wk2-6 p=0.032; wk4-6 p<0.001;	p=0.005	wk2-4 p=0.002; wk4-6 p=0.045	p<0.001	wk0-4 p=0.003; wk0-6 p<0.001; wk4-6 p=0.035	p<0.001	wk0-4 p=0.023; wk0-6 p<0.001; wk2-6 p=0.002;
CC, No RW		**Condition**							
ROI	**Camera**	**White**	**White post-hoc**	**Black**	**Black post-hoc**	**Flash**	**Flash post-hoc**	**Ambient**	**Ambient post-hoc**
Palm	**Pro**	p=0.030	wk2-4 p=0.027	p<0.001	wk0-4 p=0.011; wk0-6 p=0.006; wk2-4 p=0.005;	p=0.029	wk0-wk4 p=0.046	//////	//////
Cheek	**Pro**	//////	//////	//////	//////	//////	//////	p<0.001	wk2-6 p=0.002; wk4-6 p=0.005
TopHand	**Pro**	p<0.001	wk0-6 p=0.003; wk4-6 p<0.001	p<0.001	wk2-4 p<0.001; wk2-6 p<0.040	p<-.001	wk0-4 p<0.001; wk0-6 p<0.001; wk4-6 p=.005	p<0.001	wk0-6 p<0.001; wk2-6 p=0.002; wk4-6 p<0.001
RW_b*Value		**Condition**							
ROI	**Camera**	**White**	**White post-hoc**	**Black**	**Black post-hoc**	**Flash**	**Flash post-hoc**	**Ambient**	**Ambient post-hoc**
PalmRW	**Pro**	//////	//////	p<0.001	Jump in values for wk4 and wk6	//////	//////	//////	//////
CheekRW	**Pro**	//////	//////	//////	//////	//////	//////	//////	//////
TopHandRW	**Pro**	//////	//////	p<0.001	Jump in values for wk4 and wk6	p=0.003	wk0-2 p=0.002;	p<0.001	wk0-4 p=0.002; wk2-4 p=0.013

Canon Camera image b* value repeated measures ANOVA and Bonferroni post-hoc analysis for images under different lighting conditions for b* values of different ROI for color calibrated (CC) images with and without reference white (RW) normalization and for b* values of the white reference of these images. Green = increase, Grey = decrease, Light green = increase then decrease

Table 3.10 Significances of time-dependant changes in b* values for reference white normalized images obtained from all different devices, ROI, and lighting conditions.

RW, No CC		Condition							
ROI	**Camera**	**White**	**White post-hoc**	**Black**	**Black post-hoc**	**Flash**	**Flash post-hoc**	**Ambient**	**Ambient post-hoc**
Palm	**Pro**	p=0.01	wk2-4 p=0.048; wk4-6 p=0.031	p<0.001	wk0-4 p=0.001; wk0-6 p=0.042; wk2-4 p=0.009	p<0.001	wk0-wk4 p=0.034; wk0-6 p=0.002	//////	//////
Palm	**Pixel**	p=0.30 greenhouse geisser corrected	wk4-6 p=0.005	p<0.001 greenhouse geisser corrected	wk0-wk2 p=0.021; wk0-wk6 p<0.001	p=0.002	wk0-2 p=0.027; wk0-4 p=0.005	//////	//////
Palm	**iPhone**	p<0.001	wk0-4 p<0.001; wk2-4 p=0.002	//////	//////	//////	//////	p<0.001	wk0-4 p=0.001; wk0-6 p=0.025
Cheek	**Pro**	//////	//////	//////	//////	//////	//////	//////	//////
Cheek	**Pixel**	p<0.001	wk0-2 p=0.002; wk0-4 p<0.001; wk0-6 p<0.001	p<0.001	wk0-wk4 p=0.003; wk0-wk6 p<0.001	p=0.006 Greenhouse-geisser corrected	wk0-2 p<0.001,; wk0-wk4 p<0.001	p=0.015 Greenhouse-geisser corrected	wk0-2 p=0.003; wk0-wk4 p<0.001
Cheek	**iPhone**	//////	//////	//////	Minimal significane (p=0.049) for wk2-wk4	p<0.001	Wk0-wk6 p<0.001; wk2-wk4 p=0.018; wk2-wk6 p<0.001	//////	//////
TopHand	**Pro**	p<0.001	wk0-6 p=0.005; wk4-6 p=0.003	p=0.002	wk2-4 p=0.002; wk4-6 p<0.001	p<0.001	wk0-6 p<0.001; wk2-6 p<0.001; wk4-6 p=0.008	p<0.001	wk0-6 p<0.001; wk2-6 p<0.001; wk4-6 p<0.001
TopHand	**Pixel**	p<0.001	wk0-6 p=0.002; wk2-6 p<0.001; wk4-6 p<0.001	p<0.001	wk0-2 p=0.005; wk2-4 AND 6 p<0.001; wk2-6 p=0.002; wk4-6 p=0.041	p<0.001	wk0-4 p=0.003; wk0-6 p<0.001; wk2-6 p<0.001; Wk4-6 p=0.002	p<0.001	wk0-2 p=<0.001; wk0-6 p=0.014; wk2-4 AND 6 p<0.001; wk4-6 p<0.001
TopHand	**iPhone**	p=0.048	wk4-6 p=0.050	p<0.001	wk0-6 p=0.029; wk2-4 p=0.005; wk2-6 p<0.001	p=0.001	wk2-6 p=0.007	//////	//////

Repeated measures ANOVA and Bonferroni post-hoc analysis for b* values of images with reference white (RW) normalization and without color calibration (CC) that were taken with different cameras under different lighting conditions and at different regions of interest (ROI) over time. Green = increase, Grey = decrease, Light green = increase then decrease

Table 3.11 Significances of time-dependant changes in b* values for images obtained from all different devices, ROI, and lighting conditions.

No CC or RW		Condition							
ROI	Camera	White	White post-hoc	Black	Black post-hoc	Flash	Flash post-hoc	Ambient	Ambient post-hoc
Palm	Pro	p=0.017	wk2-wk4 p=0.013	p<0.001	wk0-4 p<0.001; wk0-6 p<0.001; wk2-wk4 p=0.001; wk2-6 p<0.001	p=0.005	wk0-6 p=0.028	p=0.006	wk0-4 p=0.007; wk0-6 p=0.039
Palm	Pixel	p=0.001	wk0-2 p=0.033; wk0-6 p=0.003	p<0.001	wk0-2 p<0.001; wk0-4 p=0.004; wk0-6 p<0.001	//////	//////	//////	//////
Palm	iPhone	p=0.003	wk0-4 p=0.005; wk2-4 p=0.014	//////	//////	//////	//////	p<0.001	wk0-2 p=0.023; wk0-4 p<0.001; wk0-6 p=0.011
Cheek	Pro	//////	//////	//////	//////	//////	//////	p=0.009	//////
Cheek	Pixel	p=0.001	wk0-2 p=.029; wk0-4 p=0.030; wk0-6 p=0.006	p=0.004	wk0-6 p=0.011; wk4-6 p=0.006	p=0.054	wk0-2 p<0.001; wk0-4 p=0.024	p=0.087	wk0-2 p<0.001; wk0-4 p<0.001
Cheek	iPhone	//////	//////	p=0.058	wk2-4 p=0.048	p<0.001	wk0-4 p=0.002; wk0-6 p<0.001; wk2-4 p=0.002; wk2-6 p<0.001	//////	//////
TopHand	Pro	p=0.001	wk0-6 p=0.040; wk4-6 p=0.012	p<0.001	wk0-4 p=0.016; wk2-4 p<0.001; wk2-6 p=0.043	p<0.001	wk0-4 p=0.009; wk0-6 p<0.001; wk2-6 p<0.001; wk4-6 p=0.019	p<0.001	wk0-6 p=0.004; wk2-6 p<0.001; wk4-6 p=0.001
TopHand	Pixel	p<0.001	wk0-4 p=0.029; wk0-6 p<0.001; wk2-6 p<0.001; Wk4-6 p<0.001	p<0.001	wk0-2 p<0.001; wk0-4 p<0.001; wk0-6 p<0.001; wk2-6 p=0.013	p<0.001	wk0-2 p=0.001; wk0-4 p<0.001; wk0-6 p<0.001; wk2-6 p<0.001; wk4-6 p=0.002	p<0.001	wk0-4p=0.027; wk0-6 p<0.001; wk2-6 p<0.001; wk4-6 p<0.001
TopHand	iPhone	//////	//////	p<0.001	wk0-4 p<0.001; wk0-6 p=0.020; wk2-4 p<0.001; wk2-6 p=0.003	p=0.009	wk0-6 p=0.006	//////	//////

Repeated measures ANOVA and Bonferroni post-hoc analysis for b* values of images without reference white (RW) normalization or color calibration (CC) that were taken with different cameras under different lighting conditions and at different ROI over time.

Figure 3.1 Study Design Timeline

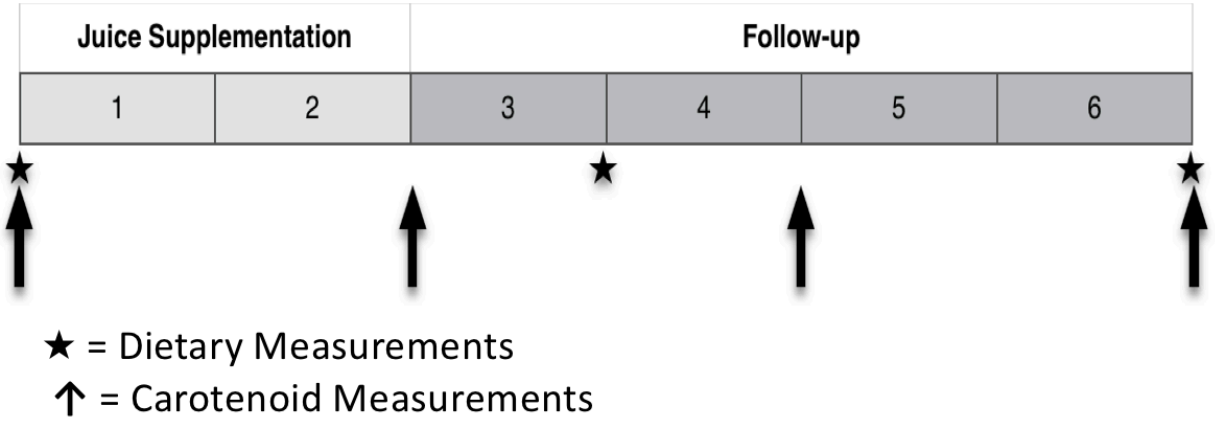


Figure 3.2 Average b* values following Minolta spectrophotometer measurement on different regions of the skin including top of the hand (grey), palm (blue), and cheek (orange) (error bars are SEM).

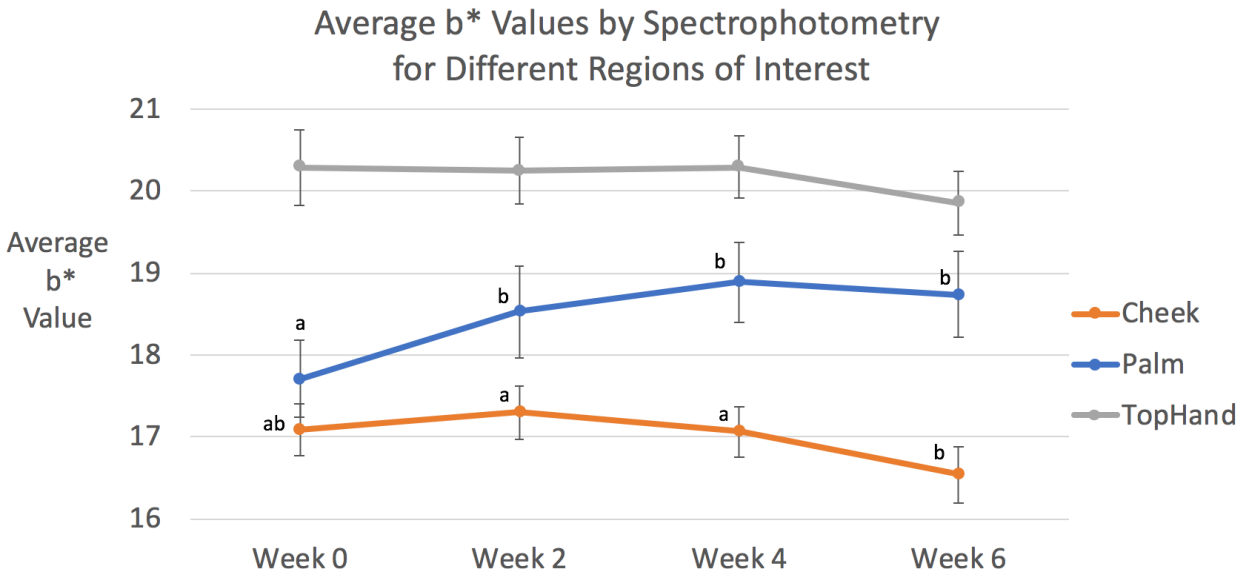


Figure 3.3 Average b* values of reference white normalized palm images taken with the Canon camera under the flash condition (error bars are SEM)

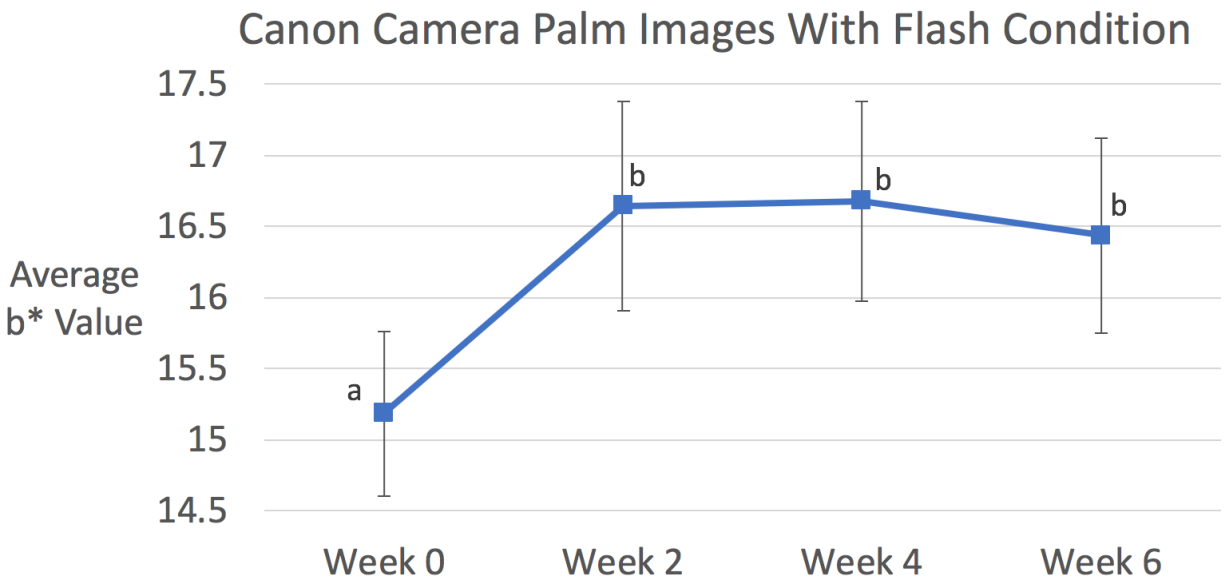


Figure 3.4 Average b* values of reference white normalized palm images taken with the Pixel 1 smartphone camera under the flash condition (error bars are SEM).

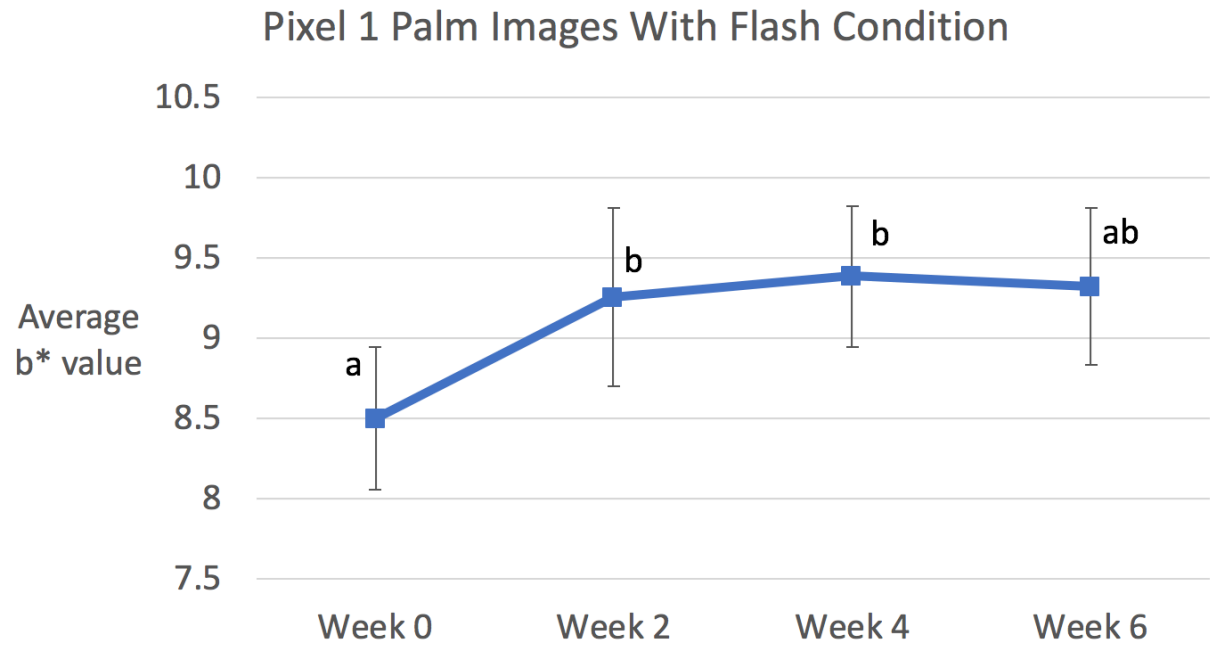


Figure 3.5 Average b* values of reference white normalized palm images taken with the iPhone 4 smartphone camera under the incandescent condition (error bars are SEM).

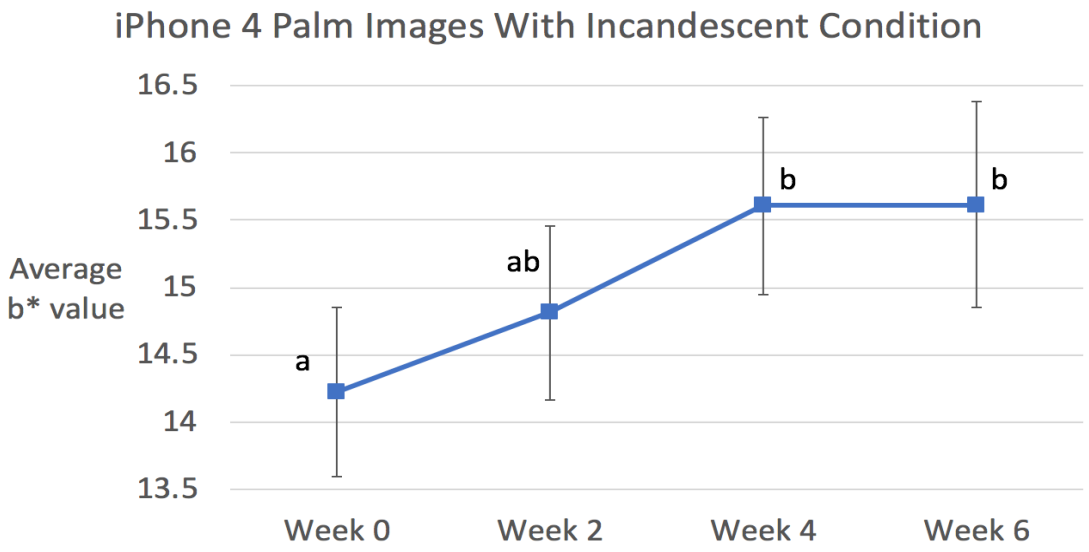
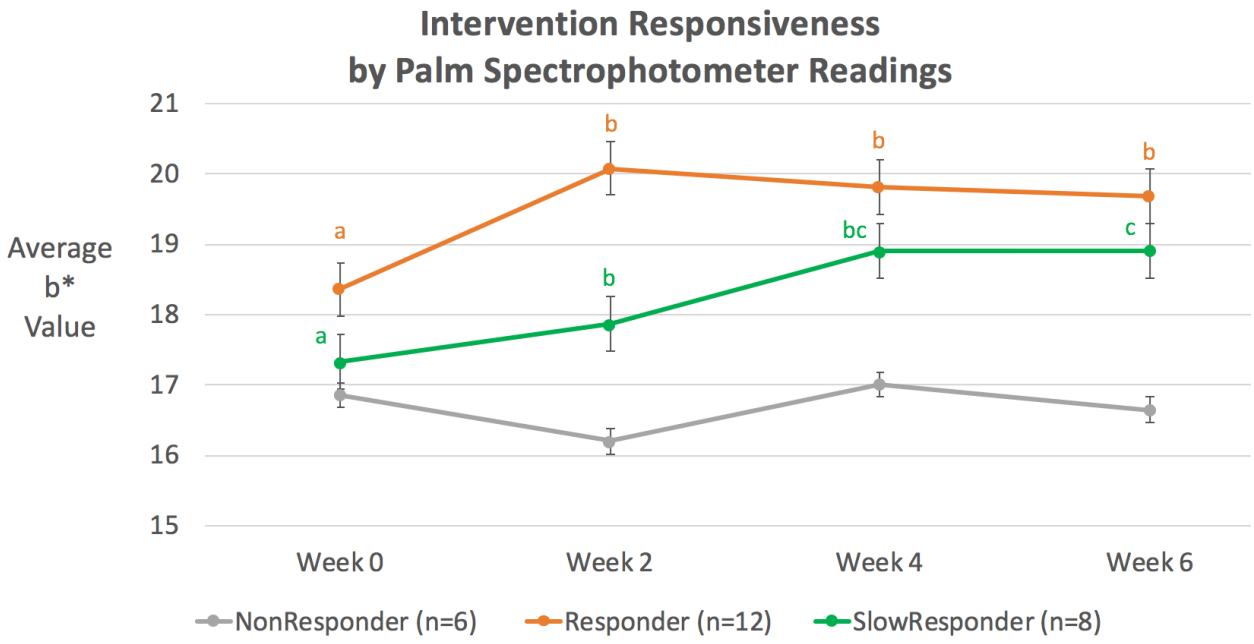


Figure 3.6 Intervention responsiveness by palm spectrophotometer readings



Intervention responsiveness demonstrated by separating participants into responders (increase ≥ 1.0 b* value following supplementation), slow responders (increase ≥ 1.0 b* value throughout the study), and non-responders (increases < 1.0 b* value) based on the spectrophotometer b* values of the palm (error bars are SEM).

Figure 3.7 Average ratings of perceived health and attractiveness for the 25 participants over time (error bars are SEM).

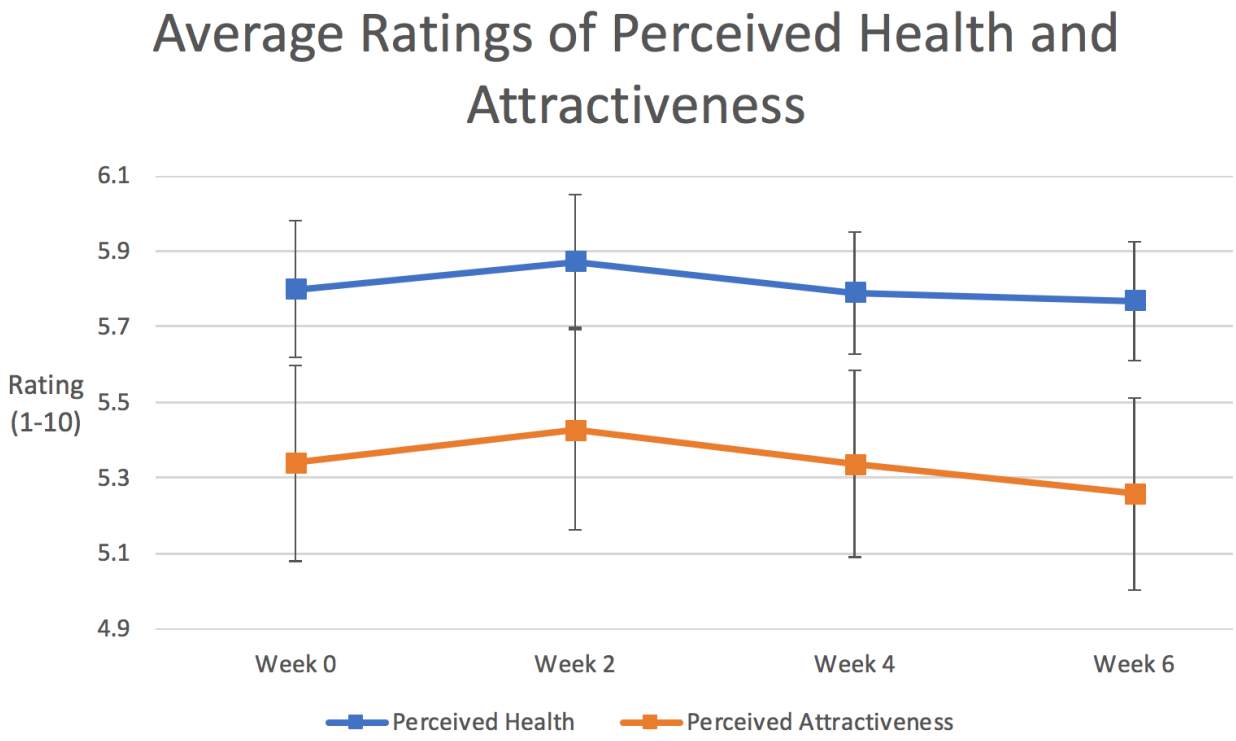
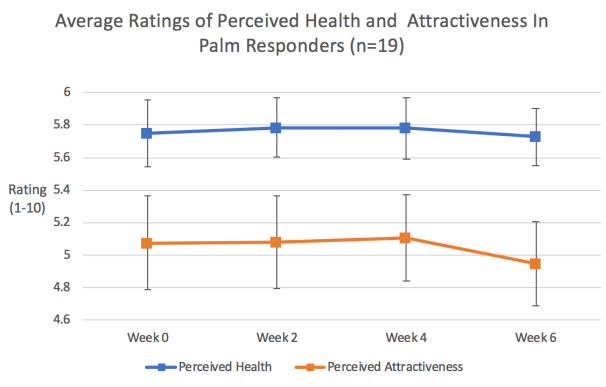
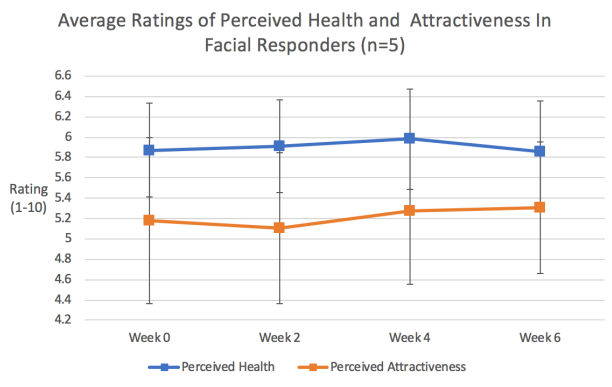


Figure 3.8 Average ratings of perceived health and attractiveness in responders to supplementation for palm and cheek b* values (> 1.0 increase) (error bars in SEM)





Chapter 4

Final Conclusions

4.1 General Summary and Conclusions

Most of the global population is not consuming enough FVs (Hall et al., 2009; Micha et al., 2015) to maintain health (Lim et al., 2012; WHO, 2017). Inadequate FV intake is one of the leading preventable risk factors for NCDs (Forouzanfar et al., 2016) and increased FV intake is associated with reduced risk of circulatory, respiratory, and digestive diseases (Leenders et al., 2014). In fact, a dose-dependent response of FV intake and decreased relative risk for cardiovascular diseases (CHD, CVD, and stroke), cancer, and all-cause mortality has been reported (Aune et al., 2017). In view of the above, there is the need for better monitoring of these relationships. The need for faster and more convenient monitoring is also evident as the healthcare system is increasingly becoming under pressure for quality and quantity of care because of rising NCD prevalence (Albahri et al, 2018).

Beyond high micronutrient and fiber composition, FVs carry several phytochemicals that have been attributed NCD-risk reduction potential (Shashirekha et al., 2015). Carotenoids are a class of yellow, orange, and red phytochemicals, primarily obtained from the diet (Namitha & Negi, 2010), that have shown antioxidant (Maiani et al., 2009), anti-inflammatory (Watzl et al., 2005), and anti-cancer properties (Gloria et al., 2014). Serum concentrations of the plant pigment have demonstrated a positive dose-response relationship with NCD prevention (Donaldson, 2011) and have been suggested as the best biomarker for FV intake (National Academy of Sciences, 2000).

Among all the methods of carotenoid status assessment, optical methods avoid the inherent biases of self-administered questionnaires (Willet, 2012; Wrieden et al., 2003) and exhibit a reliable level of sensitivity comparable to more invasive and complex methods such as HPLC sample analysis (Jahns et al., 2014; Stahl et al., 1998). Optical methods assess the carotenoid content of the skin as carotenoids are deposited there, as well as in the liver and adipose tissue, after meeting immediate needs throughout the systemic circulation (Kiokias et al., 2016). Reflectance spectrophotometry measures the yellow coloration of the skin associated with the plant pigment coloration (Ermakov & Gellerman, 2015), which has demonstrated a positive relationship with FV intake (Pezdirc et al., 2015; Stephen et al., 2011; Tan et al., 2015), carotenoid supplementation (Coetzee & Perrett, 2014; Stephen et al., 2011), and perceived health (Stephen et al., 2011) and attractiveness (Lefevre & Perrett, 2015; Stephen et al., 2011) irrespective of ethnicity (Stephen et al., 2011; Whitehead et al., 2012c) or skin melanin content from sun exposure (Lefevre & Perrett, 2015).

Digital image analysis of participant's skin for the carotenoid associated yellow coloration has demonstrated similar results following carotenoid supplementation as well as its influence on perceived health and attractiveness (Foo et al., 2017), but it has not been compared to existing assessment methods for carotenoid status. Since digital image analysis can detect carotenoid status and perceived health and attractiveness changes under standardized conditions, differences in capture conditions or image processing may allow for smartphone carotenoid status and perceived health and attractiveness assessment, potentially creating a highly accessible yet reliable method for FV intake assessment and intervention.

In the present study, detectable changes in carotenoid status were induced via supplementation of 250 ml of a cold-pressed carrot-based FV juice to 25 participants daily for an intervention period of two weeks. The juice was measured for its β-carotene content via LC-MS

analysis. Measurements consisting of a validated FFQ (Taylor et al., 2006), food record, and skin coloration values for three regions of interest (palm, top of the hand, and cheek) taken via spectrophotometer and digital image capture and analysis. Age, sex, and height and weight were measured to record changes in BMI. Dietary assessment data was recorded at the beginning, middle, and end of the study, while weight and skin carotenoid measurements were recorded bi-weekly following baseline measurement for a total of six weeks. Digital images were taken with Canon EOS Rebel T1i, Google Pixel 1 smartphone, and Apple iPhone 4 smartphone cameras under standardized lighting conditions within a light box with a full white and full black background, under the device's flash as the only source of illumination, and under general incandescent lighting. Different methods of digital image processing included color calibration, white reference normalization, both color calibration and white reference normalization, or no processing. The smartphones exhibited proprietary standard photo processing upon image capture and were only subject to white reference normalization or kept without further processing.

The study findings demonstrated that a two-week supplementation with a cold-pressed carrot-based FV juice appeared to increase skin carotenoid status in the palm when measured by spectrophotometry. Inclusively, a responder relationship was identified across participants. A lack of change for cheek and top of the hand measurements for skin coloration suggests that supplementation dosage or duration was insufficient for a substantial deposition of carotenoids in these areas. Accordingly, perceived health and attractiveness did not change. Although the literature suggests carotenoid intake improves perceived health and attractiveness, this thesis study suggests that changes in perceived health and attractiveness only occur after longer than two weeks of increased carotenoid intake. It may also have to do with the method of perception analysis used, having participants rate facial images from 1-10 rather than having participants choose the healthiest looking or most attractive photo between baseline and endpoint photos. The Canon and

smartphone camera image analysis was able to detect similar changes in skin carotenoid status of the palm and top of the hand under specific lighting conditions but did not show strong correlations for the cheek region analysis. White reference normalization of images helped to standardize color values across devices. There were no significant differences in nutrient profile, FV serving consumption, or other subject characteristics.

This thesis presents the first study to compare digital camera and smartphone camera image capture and analysis against spectrophotometric analysis for carotenoid status assessment. It is also the first study to demonstrate detectable changes following two-weeks of carotenoid supplementation. Some limitations include compliance monitoring, a small sample size, the perception assessment method used, and the short intervention period. More research is needed to determine: (a) the optimal dosage and timeframe to improve carotenoid deposition; (b) establish differences between responders and non-responders; (c) identify optimal image processing and capture conditions for any image capture device; (d) determine the threshold for changes in perceived health and attractiveness according to carotenoid associated skin coloration; (e) and determine how effective an intervention utilizing these tools could be for improving FV intake. Additionally, criteria to establish validation of digital imaging for carotenoid status assessment still needs to be studied by examining methodological issues such as precision, reproducibility, etc.

The use of smartphone cameras as a more convenient and practical way of image capture for skin carotenoid assessment appears viable; however, some major obstacles are the variations in coloration that each device demonstrates under different illumination conditions and the proprietary image processing and color calibration and/or white balance procedures that each smartphone exhibits. If smartphone images could be taken in a raw format that takes in all the visual information without enacting any processing, as was done with the Canon camera, then

proper standardized processing could be done in the same manner as with the Canon camera. Ideally, the process of raw image capture, standard processing, and color analysis would be completed within the same device for a cheaper and more accessible alternative to reflectance spectroscopy measures of carotenoid associated skin yellow coloration (b*).

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