# Plant Pigments for Human Health:

# Carotenoids and Anthocyanins in the Fight Against Chronic Disease

by

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As a lifelong competitive athlete who endured several medical challenges over the years, I became fascinated with the role of nutrition in human health and well-being. While during my time as a graduate student, the athletic portion of my life decreased immensely compared to

college, I still encountered medical challenges that posed as additional hurdles in getting my PhD. Yes, some of these medical issues pushed back some progress in my work here and there, or added stress on top of stress, I saw these as reminders as to what piqued my interest in nutritional science and research in the first place. While I obviously learned more about anthocyanins and carotenoids than anyone (outside of my lab) would care to hear about, I also learned a lot about myself. I grew as a student, scientist, researcher, mentor, and person overall, and am beyond excited to see where all that I learned will take me in the future. So, now that I am wrapping up my dissertation and time as a graduate student, when the question *"am I finishing this PhD, or is it finishing me?"* comes up, I can smile and be proud of the fact that I finished my PhD, thanks to the countless people who guided me along the way.

## ABSTRACT

**BACKGROUND:** Increasing bioactives, like anthocyanins and carotenoids, in fruits and vegetables may mitigate disease. Biofortified multicolored carrots, like purple-red, contain anthocyanins and carotenoids.

**OBJECTIVE**: We assessed purple-red carrots as functional foods through anthocyanin and carotenoid profiles, *in vitro* bioactivity potentials, and *in vivo* relative bioavailability.

**METHODS**: Fresh, freeze-dried, and juiced carrots were used to optimize HPLC analysis of anthocyanins. The *in vitro* antioxidant activity and carbohydrate digestive enzyme inhibition potential were evaluated in juice.

Two studies assessed relative bioavailability of β-carotene and lycopene from freeze-dried red and purple-red carrots in Mongolian gerbils. Treatments were equalized for provitamin A content. The first study varied in lycopene, the second had equalized lycopene with different anthocyanin content.

An acute clinical trial assessed the relative bioavailability of carotenoids from juices made with red, purple, and purple-red carrots in addition to investigating potential antidiabetic effects the pigments may exert.

**RESULTS**: Purple-red carrots contained the same five anthocyanins as purple, but at lower concentrations. *In vitro* analysis of red, purple, and purple-red carrot juices show the potential to provide antioxidant activity and inhibition of carbohydrate digestive enzymes.

In gerbils, lycopene content did not impact provitamin A carotenoid. Low starting weights influenced findings based on end liver reserves, which indicated vitamin A deficiency. Baseline vitamin A was maintained after consuming diets fortified with freeze-dried purple-red carrots. Varying anthocyanin content did not impact carotenoid bioavailability.

Data from humans indicated some evidence of competition between carotenoids for absorption, but there was no significant difference between treatments, no treatment effect or treatment X time effect on plasma carotenoid or postprandial plasma glucose or insulin concentrations. **CONCLUSIONS**: Currently, purple-red carrots have less anthocyanins than purple, but similar profiles. Carotenoids are bioavailable from red and purple-red carrots in gerbils, and co-ingestion of carotenoids and anthocyanins did not impact β-carotene bioavailability. Provitamin A carotenoids were bioavailable from juices made with purple, red, and purple-red carrots in humans with some evidence for absorption competition. This study provides evidence that purple-red carrots could be a viable functional food and the bioavailability of provitamin A carotenoids is similar to that of other single-colored carrot varieties.

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| ABBREVIATION | | *PAGE* |
|---|---|---|
| **aC** | alpha-Carotene | *3, 12, 78, 105* |
| **ABTS** | 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) | *28, 31, 33, 38, 44, 45, 47, 48, 57, 112, 113* |
| **ANSA** | 3-amino-5-nitrosalicylic acid | *45* |
| **AUC** | Area-Under-the-Curve | *77, 87, 103, 104* |
| **iAUC** | Adjusted Area-Under-the-Curve (Baseline subtracted) | *87, 89-91, 93, 95, 103, 104* |
| **BC** | Beta-Carotene | *3, 10, 12, 15, 77-80, 88-94, 105, 107-109, 113, 114* |
| **BCX** | Beta-Cryptoxanthin | *3, 78, 105* |
| **BMI** | Body Mass Index | *81, 101* |
| **CAT** | Catalase | *5, 23* |
| **CI** | Confidence Interval | *90, 104* |
| **CRU** | Clinical Research Unit | *82-84* |
| **CV** | Coefficient of Variance | *102, 103* |
| **Cy3X-** | | *42, 43, 59, 60* |
| **CGG** | Cyanidin-3-xylosyl(coumuroylglucosyl)galactoside | |
| **FGG** | Cyanidin-3-xylosyl(feruloylglucosyl)galactoside | |
| **G** | Cyanidin-3-xylogalactiside | |
| **GG** | Cyanidin-3-xylosylglucosyl-galactoside | |
| **SGG** | Cyanidin-3-xylosyl(sinapoylglucosyl)galactoside | |
| **DI $H_2O$** | Deionized water | *35, 36, 55, 87* |
| **DNSA** | 3,5-dinitrosalicylic acid | *45* |
| **DPPH** | 2,2'-diphenyl-1-picrylhydrazyl | *28, 31, 33, 36, 37, 43, 44, 47, 48, 57, 61, 112, 113* |
| **ELISA** | Enzyme-Linked Immunosorbent Assay | *38, 39, 45* |
| **FA** | Formic Acid | *13, 34-36, 40-42, 48, 55, 87* |
| **FRAP** | Ferric Reducing Ability of Plasma | *31* |
| **GIP** | Gastric Inhibitory Peptide | *83, 84, 94, 95,* |
| **GLUT** | Glucose Transporter(s) | *6, 26* |

| | | |
|---|---|---|
| **GPx** | Glutathione Peroxidase | *5, 25* |
| **HAT** | Hydrogen Atom Transfer | *31* |
| **HDL** | High Density Lipoprotein | *91* |
| **HPLC** | High Performance Liquid Chromatography | *14, 28, 31, 33, 35, 36, 40, 41, 47, 48, 55, 60, 86, 87, 93* |
| **IkB** | Inhibitor of kappa B | *4, 25* |
| **IL6** | Interleukin 6 | *5, 25* |
| **IL8** | Interleukin 8 | *5, 25* |
| **Keap1** | Kelch-like ECH-Associated Protein 1 | *4, 25* |
| **LDL** | Low Density Lipoprotein | *91* |
| **MAPK(KK)** | Mitogen Activated Protein Kinase | *4* |
| **MeOH** | Methanol | *34-36, 41, 42, 48, 55, 87* |
| **ND** | No Difference | *55* |
| **NFκB** | Nuclear Factor kappa B | *4, 25* |
| **Nfr2** | Nuclear factor erythroid 2-related factor 2 | *4, 25* |
| **NPAC** | Non-provitamin A Carotenoid(s) | *3, 14* |
| **ORAC** | Oxygen Radical Antioxidant Capacity | *31* |
| **PAC** | Provitamin A Carotenoid(s) | *3, 12, 24, 75, 105* |
| **PC** | Purple Carrot | *55* |
| **PI** | Pro-Inflammatory | *5, 25* |
| **PNPG** | 5 mM p-nitro-phenyl-α-D-glucopyranoside | *40* |
| **PPB** | Potassium Phosphate Buffer | *39* |
| **PRC** | Purple-Red Carrot | *55* |
| **PTFE** | Polytetrafluorethylene | *36, 40, 42, 55* |
| **ROS** | Reactive Oxygen Species | *4* |
| **SET** | Single Electron Transfer | *31* |
| **SGLT1** | Sodium-Glucose Transport Protein 1 | *5* |
| **SOD** | Superoxide Dismutase | *5, 25* |
| **SPE** | Solid Phase Extraction | *14, 36, 40, 42, 55* |
| **SR-B1** | Scavenger Receptor class B type 1 | *91, 92* |

| | | |
|---|---|---|
| **T2D** | Type 2 Diabetes | *4* |
| **TEAC** | Trolox Equivalent Antioxidant Capacity | *37* |
| **TFA** | Trifluoroacetic Acid | *84* |
| **TNFα** | Tumor Necrosis Factor alpha | *5, 25* |
| **TMB** | 3,3',5,5'-Tetramethylbenzidine | *86* |
| **VA** | Vitamin A | *3, 11, 12, 74, 75, 114* |
| **VAD** | Vitamin A Deficiency | *2, 12* |
| **VLDL** | Very Low Density Lipoprotein | *91* |

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***Supplemental Figure 1***. Final lung retinol concentrations per g tissue and total lung retinol for Study 1 (A, B) and Study 2 (C, D) in gerbils after consuming treatment feeds

# CHAPTER 1:

## INTRODUCTION

Mikayla S. Kaeppler

## Introduction

A handful of chronic health conditions and diseases that originate from poor diet and malnutrition are responsible for over 70% of all deaths globally, in addition to decreasing the overall quality of life (WHO, 2022). Chronic diseases are present for a long duration, are not contagious, and tend to be slow in progression. The most prevalent chronic diseases globally include cardiovascular disease, cancer, chronic respiratory diseases, and diabetes. Combined, they are responsible for over 80% of all deaths from noncommunicable diseases. In the United States alone, those over 50 with at least one chronic disease are expected to double between 2020 and 2050 (Ansah, 2022).

Another primary global health concern stemming from poor diet and malnutrition is vitamin A deficiency (**VAD**), which is one of the top three micronutrient deficiencies and the most common vitamin deficiency in the world (WHO, N.D.). The World Health Organization considers VAD to be a significant global form of malnutrition, affecting an estimated 210 million children and women (World Health Organization, 2009). Vitamin A is an essential nutrient crucial for multiple fundamental biological processes, including vision, immune function, formation of reproductive hormones, growth, and cell differentiation. Deficiency results in severe outcomes, including night blindness, infertility, and increased risk of morbidity and mortality (Tanumihardjo, 2016; Arscott, 2010b).

Preventing and treating chronic diseases and comorbidities associated with nutrient deficiencies includes increasing fruit and vegetable intakes, which is linked to decreased risk of malnutrition and adverse health outcomes, much of which is attributed to micronutrient and bioactive contents (Temple, 1994; Willett, 1994; Liu, 2002; Hung, 2004; Arscott, 2010b). Due to their proposed health benefits, plant-derived bioactives have become a popular area of research in

multiple fields, which has resulted in numerous studies that have started the exploration of all levels of potential, from molecular-level interactions to clinical studies and development of functional foods with the hope of improving human health. Specifically, the plant pigment groups of carotenoids and anthocyanins have been targeted. Both possess health-benefit activities, including provitamin activity, potent radical scavenging activity, anti-inflammatory and anticarcinogenic properties, and antidiabetic potential (Di Mascio, 1989; Sun, 2009; Arscott, 2010b).

## Bioactive Plant Pigments: Carotenoids & Anthocyanins

Carotenoids are a class of terpenoids that provide the orange, red, and yellow pigments in plants and can be classified as carotenes or xanthophylls based on the presence or lack of hydroxyl groups at the end of their isoprenoid units (Shmarakov, 2013). The six main carotenoids heavily present in the human diet include beta-carotene (**BC**), alpha-carotene (**aC**), beta-cryptoxanthin (**BCX**), lycopene, lutein, and zeaxanthin. Three of the six have provitamin A activity, meaning that when ingested, they are cleaved in half in the small intestine producing one or two molecules of vitamin A (**Figure 1**). All six have been linked to possessing other bioactivities such as antioxidant, anti-inflammatory, and anticarcinogenic properties/activities. The three main provitamin A carotenoids (**PAC**) are beta-carotene, alpha-carotene, and beta-cryptoxanthin, which are essential sources of vitamin A in large populations of the world, especially in those economically deprived. These three provitamin A carotenoids provide up to 80% of dietary vitamin A (**VA**) in many developing countries (Arscott, 2010b). The three other common dietary carotenoids, also known as the non-provitamin A carotenoids (**NPAC**), include lycopene, lutein,

and zeaxanthin, which, due to their structures are not a source of VA, but like their provitamin A counterparts, are still thought to exert other beneficial bioactivities.

Anthocyanins are another subgroup of plant-derived bioactive compounds originating from the flavonoid family (Welch, 2008; Arscott, 2010a). These compounds provide the purple, red, and blue hues in plants and, like their carotenoid counterparts, are profoundly prevalent in the human diet. The 6 common forms are cyanidin, pelargonidin, peonidin, delphinidin, petunidin and malvidin. All forms vary in number and spatial distribution of hydroxyl and methoxy groups, and the most common anthocyanins are those with the -3-glycoside structure. Both pigment groups, through their consumption, have been linked to reduced risk of chronic diseases including, but not limited to, Type 2 diabetes (**T2D**), cardiovascular disease, and various forms of cancers (Ghosh, 2007; Kaulmann, 2014; Khoo, 2017; Bhatt, 2020). Although anthocyanins do not have any provitamin activities, these compounds can exert other health-promoting bioactivities.

### *Health Benefits & Proposed Mechanisms of Action*

Two key health benefits specific to fighting chronic disease that carotenoids and anthocyanins may provide include their antioxidant and anti-inflammatory activities. Many chronic diseases stem from increased oxidative stress and inflammation, which are both present in obese people (Furukawa, 2004; Vendrame, 2015; Xie, 2017; Sathasivam, 2018; Yahfoufi, 2018). Reactive oxygen species (**ROS**) increase in parallel with fat accumulation, which leads to decreased expression of antioxidative enzymes and increased generation of pro-inflammatory cytokines. Both anthocyanins and carotenoids influence the inflammatory signaling pathways that lead to increased ROS and decreased proinflammatory cytokines, including the **NF-kB, MAPKK**, and **Keap1-Nfr2** signaling pathways. The exact mechanisms by which carotenoids and

anthocyanins influence these pathways are still under investigation. However, the main mechanisms theorized include: 1) inhibition of the degradation of the NF-kB-ikB complex, which subsequently prevents the movement of the NF-kB component into the nucleus, thus preventing upregulation of proinflammatory (**PI)** cytokines and other inflammatory mediators such as interleukin 6 (**IL6**), interleukin 8 (**IL8**), and tumor necrosis factor alpha (**TNFa**); 2) inhibition of the de-phosphorylation of MAPKKK to MAPK, thus also preventing upregulation of PI cytokines and other inflammatory mediators; and 3) activation of kinases that prevent the degradation of Nfr2, which allows it to enter the nucleus and upregulate antioxidative enzymes such as superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPx**) (**Figure 2)**. Although each compound separately influences these pathways, the synergistic aspects between carotenoids and anthocyanins have yet to be assessed in humans in this manner.

Type 2 diabetes is a significant chronic disease that is increasing in prevalence on a global scale. This disease is not directly caused by hyperglycemia alone but rather through the detrimental effects of chronically high blood glucose mediated by and complicated through oxidative stress (Sayahi, 2017). Due to the well-documented antioxidant and anti-inflammatory activities anthocyanins and carotenoids exert, there are multiple mechanisms postulated for the protective activity these compounds may have against insulin resistance and hyperglycemia (Ghosh, 2007). Studies have shown that dietary antioxidants improve insulin resistance, both in animal and human models, by increasing insulin sensitivity, protecting beta-cells from glucose-induced oxidative stress, and lowering plasma glucose (Ghosh, 2007; Kalt, 2020).

There is increasing evidence that various fruits and vegetables are significant sources of secondary metabolites, such as anthocyanins and carotenoids, that can exert antidiabetic activity by inhibiting key steps in carbohydrate digestion in the small intestine (Podsędek, 2014; Belwal,

2017; Papoutsis, 2021). More specifically, these compounds inhibit the enzymes alpha-amylase and alpha-glucosidase, which are responsible for breaking down starch to maltose and then further into glucose, or through inhibiting the critical transporters involved in glucose absorption, **SGLT1** and **GLUT** (**Figure 3**). Studies suggest anthocyanin glycosides are effective carbohydrate digestive enzyme inhibitors. Multicolored carrots contain both anthocyanins and carotenoids, specifically cyanidin-3-galactoside-based anthocyanins, which, in one study, was among the tested cyanidin analogs showing the highest activity recorded (Castro-Acosta, 2017). Despite this, many health benefits are often based on unestablished chemical properties or based on epidemiological studies, so more research is required to understand these potentials and what may influence them.

## Bioavailability

Plant-derived bioactive compounds, like anthocyanins and carotenoids, will not offer any benefits when consumed unless they are bioavailable, i.e., they must be released from the food matrix, digested, and absorbed. Numerous dietary and human factors influence and determine the bioavailability of any bioactive compound in food. The acronym SLAMENGHI, though first proposed in terms of carotenoid bioavailability, can be used to group and describe the main factors that seem to influence the bioavailability and bioefficacy of many nutrients (de Pee, 1996; Castenmiller, 1998; van het Hof, 1999). These factors include 1) Species of the nutrient/bioactive compound, 2) Linkages at the molecular level, 3) Amount consumed in a meal, 4) Matrix in which it is incorporated, 5) Effectors of absorption and bioconversion, 6) Nutrient status of the consumer, 7) Genetic factors, 8) Host-related factors, and 9) Interactions.

Both dietary carotenoids and anthocyanins are susceptible to degradation during processing, cooking, and storage. Although these methods have been improved in various manners over the

years to maximize retention and bioavailability, multiple factors impact the stability of the pigment molecules. Changes to and losses of both carotenoids and anthocyanins occur during any type of processing, from industrial-level processing to domestic preparation, which is attributed to physical removal, changes in isomerization, and enzymatic oxidation (Rodriguez-Amaya, 2004). Carotenoids are relatively stable molecules but are susceptible to degradation via light, oxygen, heat, and acidification (Palermo, 2013).

Most of the impact different processing and cooking methods may have on carotenoid content and bioaccessibility from various fruits and vegetables is dependent upon the fruit or vegetable. Carotenoids are generally well-protected in plants and can exist in the chloroplasts, where they are often associated with proteins, or in the chromoplasts, either as semi-crystalline forms or as oily droplets (Palermo, 2013). Regarding stability, different processing or cooking methods, and physical processing, such as chopping, peeling, and slicing, likely start to induce degradation but may increase overall bioaccessibility from various food matrices. Traditional domestic cooking methods, such as boiling, steaming, microwaving, and frying, may increase bioaccessible carotenoid content by softening the food matrix they are in and generally result in minor losses of overall content (<50%). Steaming and microwaving have been associated with higher retention than boiling and frying. However, results heavily depend on the fruit or vegetable and their varying food matrices, preparation method (size of slices or pieces), temperatures, and cooking durations.

Due to multiple characteristics, anthocyanins are much more susceptible to degradation and loss through processing and cooking than carotenoids. Unlike carotenoids, anthocyanins are water-soluble and often found in the outer layers of plants. In addition, their chemical structure and countless variations in side chains make them quite unstable, and highly sensitive to changes

in temperature and pH (Chen, 2022). Compared with carotenoids, physical and chemical processing methods lead to higher losses. Evidence shows that degradation or content loss from physical processing, like peeling, slicing, and grinding, ranges from minor to significant losses depending on the fruit or vegetable. High-pressure processing is one of the few methods that consistently shows higher anthocyanin retention and even some increases in concentration compared with other methods.

Regarding cooking methods, baking and air-frying lead to some loss and degradation, but less than that caused by microwaving and frying, and boiling and steaming lead to higher losses. Blanching for 30 seconds to 1 min is generally associated with minimal loss. While processing and cooking may make anthocyanins slightly more bioaccessible in the same manner as carotenoids, the overall retention of the total content is much more impacted. In terms of the impact of freezing and processing for storage, it varies between studies and is dependent on the fruit and vegetable analyzed and freeze-drying conditions. Generally, freeze-drying does not significantly reduce carotenoid or anthocyanin content, prolongs shelf life, and slows degradation (Oliviera, 2016; Shofian, 2011). Blanching followed by freezing leads to longer storage potential and slower degradation of carotenoids and anthocyanins compared with raw fruits and vegetables, but not as well as freeze-drying. Some associated losses still occur due to the blanching step (Chen, 2002).

## Biological and Chemical Interactions

In addition to the various ways that food matrix and processing and cooking methods can impact the bioaccessibility and bioavailability of anthocyanins and carotenoids, even the presence of other phytochemicals and nutrients, and the simultaneous presence of different carotenoids or

anthocyanins, have the potential to influence one another's bioavailability and bioactivities. Due to the presence of these bioactive compounds in many fruits and vegetables and the importance of PACs as sources of dietary VA, it is crucial to establish the impact of ingestion with other bioactive compounds on each pigment group's bioavailability. In addition to the impact on bioavailability, both carotenoids and anthocyanins provide health benefits that may be accentuated when ingested simultaneously. Both carotenoids and anthocyanins have independently been shown to be bioavailable in humans, but their chemical and biological relationship remains unclear (Phan, 2018c).

There is limited information on the interactions of bioactive compounds when ingested. Carotenoids and anthocyanins may impact one another at different points throughout their metabolism. Carotenoids and anthocyanins follow a similar metabolic pathway in that they both: a) begin to be released from the food matrix upon ingestion in the oral cavity and are further liberated in the stomach, b) are primarily absorbed into the enterocytes in the duodenum of the small intestine, c) are circulated in the blood to the liver for further metabolism and hepatic recirculation, and d) are distributed to other tissues or excreted. Despite the difference in polarity and solubility of the various forms, these bioactive compounds may impact one another's bioavailability and bioactivities.

One mechanism proposed is through interaction with lipid membranes during the metabolic path these compounds share when ingested and metabolized simultaneously (Tsuchiya, 2015). Many phytochemicals interact with lipid membranes, changing various aspects of the membranes' physicochemical properties, such as fluidity, permeability, and viscosity. Any changes in lipid membranes can impact the bioavailability and bioaccessibility of both lipophilic and hydrophilic bioactive compounds. Anthocyanins can alter lipid membranes either through electrostatic

interactions with the polar lipid heads or by integrating between the lipid molecules changing membrane fluidity (Bonarska-Kujawa, 2011). Although binding or integration of the anthocyanins may help protect membranes against oxidation, they may also impact the diffusion of lipophilic molecules, like carotenoids, altering their overall bioavailability and their respective antioxidant, anti-inflammatory, and antidiabetic effects (Tsuchiya, 2011).

Co-consumption of different bioactive compounds impacts one another's bioavailability when consumed from various food matrices and can have synergistic or antagonistic effects on each other's respective bioactivities. Few studies have examined the relationship between the simultaneous consumption of polyphenols and carotenoids, even fewer of which specifically examine anthocyanins and carotenoids. In Caco-2 cells, flavonoids, including anthocyanins, have been shown to impact carotenoid cellular uptake (Claudie, 2013; Phan, 2018a; Phan, 2018b; Phan, 2019a; Phan, 2019b). Phan et al. (2019c) looked specifically at the combination of anthocyanins from red cabbage and carotenoids from tomato, spinach, and carrot. They reported that the bioavailability of anthocyanins from red cabbage increased when combined with spinach and tomato. Still, both bioavailability of anthocyanins and carotenoids decreased when red cabbage was tested with carrot.

The primary focus of research in this field has been to assess the impact of bioactive pigment families on their respective antioxidant and anti-inflammatory activities. Most of these studies were conducted *in vitro* and *ex vivo* and reported mixed findings. Individually, anthocyanins and carotenoids from various fruits and vegetables reduce alpha-amylase and alpha-glucosidase activity (Papoutsis, 2021). Synergistic effects between polyphenols and BC on their alpha-amylase inhibition activity have been reported.

## Agriculture and Food Applications

There are many varieties of phytochemicals in fruit and vegetables consumed worldwide. As evidence increases for the link between the consumption of some of these phytochemicals, like carotenoids and anthocyanins, and improved health through their bioactivities, they have become compounds of interest in numerous research areas. One area is explicitly within the realms of agriculture, nutrition, and food chemistry in developing functional foods. Functional foods are defined as those that contain significant levels of biologically active components that, when consumed, provide additional physiological health benefits beyond those of essential nutrition (Villar, 2002; Doyon, 2008).

In theory, the increased phytochemical content could lead to increased health benefits. Over the years, plant breeders have bred crops to increase phytochemical content and variety to develop crops naturally dense in bioactive compounds. Specifically, various crops have been developed or are being developed to contain more bioactive pigments. Biofortification efforts to increase provitamin A carotenoid content of foods like maize, sweet potato, and rice have been implemented to provide a more nutritious version than white-colored phenotypes (Simon, 1997; Porter Dotsi, 2006; Arscott, 2010b). Plant breeders have also been working on biofortifying crops to contain more anthocyanins to increase health benefits. Most recently, some plant breeders honed explicitly in on developing even more nutrient-dense crops by creating multicolored versions that contain a variety of carotenoids in addition to anthocyanins.

### *Carrots as Functional Foods*

Carrots are a functional food because they provide essential VA and potentially reduce the risk of chronic disease through the other bioactive compounds they contain (Arscott, 2010a). Over

40 million tons of carrots and turnips were produced globally in 2022 (FAOSTAT). Carrots with various patterns of root pigment have been identified over time, including red, orange, white, yellow, and purple varieties (Arscott, 2010a). Wild carrot relatives have non-pigmented roots, and orange carrots were developed and propagated during the process of domestication and early breeding. Typical orange carrots are rich in carotenoids such as BC and aC, which are essential VA precursors, although there is variation in these traits among cultivars. Less common purple carrots are known for their anthocyanins, specifically, glycosides of cyanidin, that give them their dark, rich color. The extent to which purple carrots may provide more health benefits than orange carrots has yet to be thoroughly investigated. The role of anthocyanins as a functional food factor has yet to be fully understood, which is due mainly to the numerous factors that influence the molecules' color and stability, such as the type of anthocyanin pigment and co-pigments, light, high temperature, pH, metal ions, enzymes, oxygen, and antioxidants (Welch, 2008). Despite that, progress at the molecular level has been made.

Carrots are popular with consumers, acting as effective nutritional vehicles of these phytochemicals to help fight against VAD and other chronic diseases. In recent years, dual-color carrots, such as purple-red varieties, have been developed and may provide a more phytochemically-enriched alternative to their single-colored counterparts. In theory, the increased phytochemical content could lead to increased health benefits; therefore, it is essential to understand how these compounds interact to ensure the combination does not hinder the desired health benefits and decrease overall nutritional value. Previously, it has been shown that PACs are bioavailable in both gerbils and humans from red, orange, purple-orange, and purple carrots, but these compounds have yet to be shown to be bioavailable in humans from purple-red carrots (Horvitz, 2004; Molldrem, 2004; Mills, 2007; Mills, 2008; Charron, 2009; Tanumihardjo, 2009;

Arscott, 2010b; Arscott, 2010a). No studies have assessed the antioxidant and anti-inflammatory activities that the anthocyanins in purple-red carrots may provide.

Plant breeders have bred crops to contain multiple phytochemicals. For example, traditional orange carrots are rich in carotenoids, while other varieties of purple and red carrots are rich sources of both anthocyanins and carotenoids (Arscott, 2010a). Adding anthocyanins to carotenoid-rich carrots may enhance carotenoid bioavailability by reducing carotenoid oxidation and enhancing their antioxidant and antidiabetic effects, but this has never been investigated in humans (Phan, 2018c).

## Carotenoid and Anthocyanin Quantification Methods

### *Extraction*

No single gold standard method exists for analyzing and quantifying carotenoids or anthocyanins. There are general commonalities between extraction, purification, and analysis methods for carotenoids and anthocyanins. There are significant variations within the general methods, including specific solvents used, the amount of solvent or mixtures used to extract, additional purification steps, extraction duration, and more (Rodriguez-Amaya, 2004). Carotenoids are lipid-soluble compounds and extraction from biological samples (food, plant tissue, blood, body tissue, etc.) needs to include a polar organic solvent such as acetone, chloroform, methanol, or ethanol. The sample extract is then partitioned to separate the carotenoids from the water portion of the sample using nonpolar organic solvents such as hexane, petroleum ether, and dichloromethane. The resulting extract is transferred to glass, dried using nitrogen gas, and reconstituted.

Anthocyanins are the exact opposite of carotenoids in that they are water-soluble polar compounds. Hence, their extraction from biological samples includes polar organic solvents with the addition of an acidic agent to increase the stability of the anthocyanins in the sample (Singh, 2020). Generally, anthocyanins are extracted using methanol, water, or acetone acidified with formic acid (**FA**), hydrochloric acid, acetic acid, or phosphoric acid. The acid percentage of the solvents varies but generally ranges from 1-10%. The samples are analyzed immediately or subjected to further purification using solid phase extraction (**SPE**) to eliminate other compounds that may impact analysis, such as proteins, sugars, or other organic acids.

### *Analysis*

Both carotenoids and anthocyanins are generally analyzed and quantified using Reverse Phase High Performance Liquid Chromatography (**HPLC**) combined with UV-vis absorption detection. Both are commonly analyzed using C18 columns, which are popular due to their wide commercial availability, compatibility with an extensive range of polarity, and the larger surface area allows for increased interaction between the bonded phase and the sample (Rodriguez-Amaya, 2004). For carotenoids, the mobile phase is commonly acetonitrile or methanol combined with small amounts of other solvents (e.g. chloroform, dichloroethane, dichloromethane, water, isopropyl alcohol) and their spectra is recorded at 450 nm. Anthocyanins are analyzed using acidified aqueous mobile phases. Typically mobile phases include acetonitrile, methanol or water acidified with ranging amounts of formic, hydrochloric, acetic, or phosphoric acid (Singh, 2020).

## Summary & Conclusion

Through this dissertation, I will expand on the current information and understanding of the relationship between carotenoids and anthocyanins and their proposed health benefits. I will specifically assess this in terms of the impact of simultaneous consumption of PAC, NPAC, and anthocyanins from a single, natural food source on total relative bioavailability and other bioactivities through 3 specific aims and their associated studies:

1. Establish the anthocyanin profile of purple-red carrots by optimizing current extraction and analysis methods and explore the antioxidant and enzyme inhibition potential of multicolored carrots processed in different ways.
2. Determine the effect of co-consumption of non-provitamin A carotenoids and anthocyanins on the relative bioavailability of provitamin A carotenoids from multicolored carrots in Mongolian gerbils.
3. Determine the effect of anthocyanins on the acute relative bioavailability of provitamin A carotenoids and explore the antidiabetic potential of juice made with red, purple, and purple-red carrots in humans.

These aims will yield new information regarding the impact of simultaneous consumption of anthocyanins and carotenoids, specifically non-provitamin A lycopene and provitamin A BC and aC, from a single natural food source on each pigment group's respective bioavailabilities and bioactivities in humans. These studies will inform public health officials, consumers, and growers of the potential benefits of vegetables with greater concentrations of various phytochemicals.

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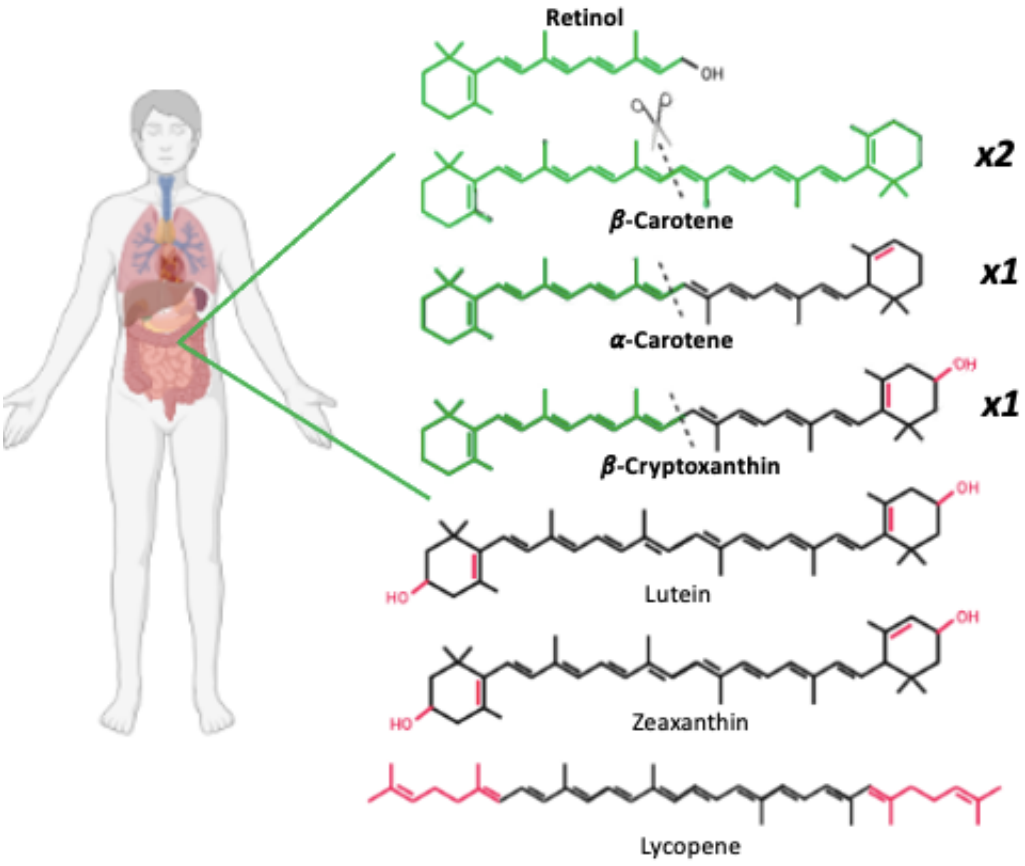


**Figure 1.** The six primary carotenoids that are part of the human diet. Three of them are provitamin A carotenoids (**PAC**), which are β-carotene, α-carotene, and β-cryptoxanthin. When ingested, these PAC get cleaved in the duodenum of the small intestine producing at least one molecule of retinol (vitamin A) (Green). Due to its structure, cleavage of β-carotene results in two molecules of vitamin A, while the cleavage of α-carotene and β-cryptoxanthin only produce one molecule of vitamin A. The other three common carotenoids are lutein, zeaxanthin, and lycopene, which cannot produce vitamin A due to their structures, (Red) but provide other health benefitting bioactivities such as antioxidant and anti-inflammatory activities.

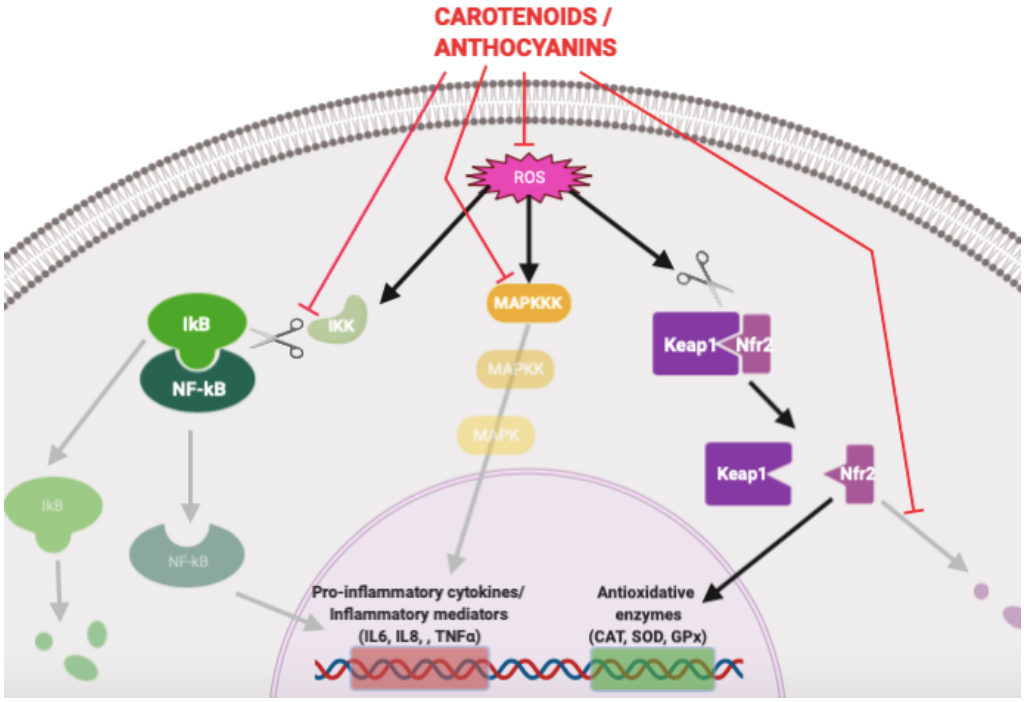


**Figure 2.** Schematic of antioxidant and anti-inflammatory mechanisms of carotenoids and anthocyanins. Both compounds are thought to decrease the transcription of pro-inflammatory cytokines and inflammatory mediators and increase transcription of antioxidative enzymes. Carotenoids and anthocyanins may influence the control of NF-kB, MAPKKK, and Keap-Nfr2 signaling pathways by inhibiting IKK from phosphorylating and splitting the NF-κB-IκB complex and through inhibition of the dephosphorylation of MAPKKK to MAPK. These inhibitions prevent NF-kB and MAPK from entering the nucleus and upregulating PI cytokines and inflammatory mediators such as IL6, IL8, iNOS and TNFα. The pigment groups may also activate the phosphorylation of Nfr2 after splitting from Keap-1 which prevents degradation of Nfr2 allowing it to enter the nucleus and upregulate antioxidative enzymes such as SOD, GPx, and CAT. **NF-κB**; Nuclear Factor kappa B, **MAPKKK**; Mitogen Activated Protein (3) Kinase, **Keap1**; Kelch-like ECH-associated protein 1, **Nfr2**; Nuclear factor erythroid 2-related factor 2; **IκB**; inhibitor of κB, **PI**; Pro-inflammatory, **IL6**; interleukin 6, **IL8**; interleukin 8, **TNFα**; Tumor necrosis factor alpha, **SOD**; superoxidedismutase, **GPx**; glutathione peroxidase, **CAT**; catalase.

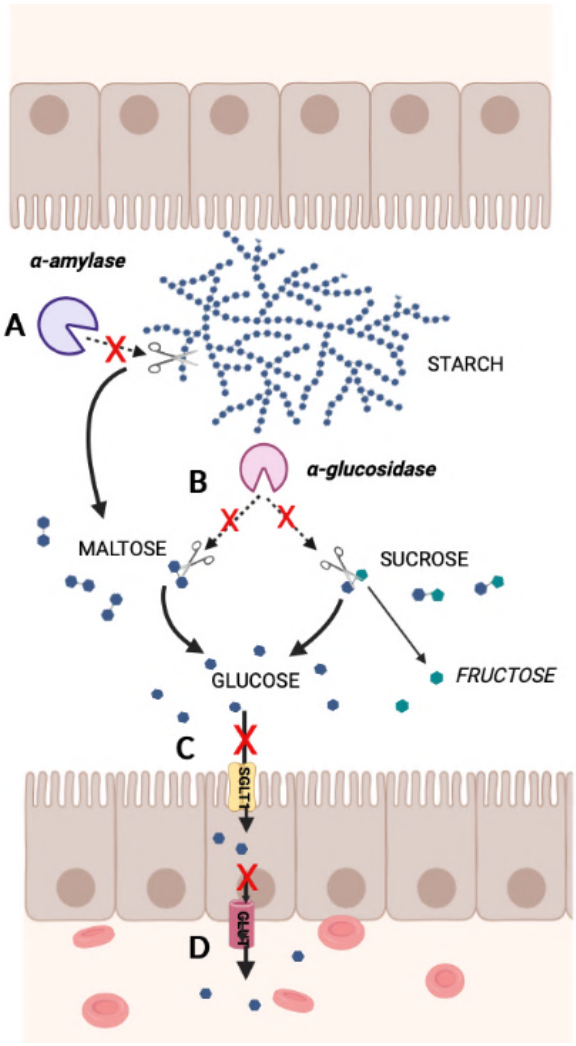


**Figure 3.** Postulated mechanisms of the role anthocyanins and carotenoids may play in postprandial glycemic activity via inhibition of enzymes and/or transporters of carbohydrate digestion including inhibition of starch digestion into maltose via alpha-amylase **(A)**, inhibition of disaccharide digestion via alpha-glucosidase **(B)**, or through the inhibition of glucose transporters SGLT1 **(C)** and GLUT**(D)**.

# CHAPTER 2:

## HPLC ANALYSIS OF ANTHOCYANINS AND *IN VITRO* ASSESSMENT OF ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITION POTENTIAL OF RED, PURPLE AND PURPLE-RED CARROTS (*DAUCUS CAROTA* L.)

Mikayla S. Kaeppler

**Abstract**

Methods for determining anthocyanin content of fresh, freeze-dried, and juice made from purple and purple-red carrots using HPLC were developed by testing and comparing different factors of extraction, purification, and analysis. Additionally, *in vitro* antioxidant activity and digestive enzyme inhibition potential of red, purple, and purple-red carrot juice was assessed using the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (**ABTS**) and 2,2'-diphenyl-1-picrylhydrazyl (**DPPH**) antioxidant assays, and alpha-amylase and alpha-glucosidase inhibition assays. Anthocyanin profiles were created for the purple and purple-red carrot samples. The data from these studies confirm the presence of anthocyanins in the purple-red carrots and showed evidence of some antioxidant and enzyme inhibitory activity. Both color varieties contained the same 5 main anthocyanins but varied in concentrations of each, and the purple varieties contained about 4-5x more total anthocyanins than the purple-red varieties. The results of the DPPH assay indicated antioxidant activity is highest in the purple carrot juice, followed by the purple-red, then red carrot juices. The results of the alpha-amylase inhibition assay indicated potential inhibition activity of all juice types, while the ABTS and alpha-glucosidase assays were inconclusive due to interference of the pigments with the colorimetric-based assays.

**Key words: Alpha-amylase; alpha-glucosidase; anthocyanins; carrot; HPLC**

## Introduction

Anthocyanins are a subgroup of plant-derived polyphenols originating from the flavonoid family responsible for the red, blue, and purple pigments in numerous fruits and vegetables consumed worldwide (Belwal, 2017). Anthocyanins aid in pollination by attracting pollinators. In humans, anthocyanin consumption has been linked to multiple health benefits due to their antioxidant, anti-inflammatory, antidiabetic, and anticancer activities. Additionally, anthocyanins may aid in preventing or treating various conditions and diseases, such as cardiovascular disease and obesity (Belwal, 2017; Mattioli, 2020; Perez, 2023). Research interest in these bioactive pigments has steadily grown over recent years because evidence increased for the potential health benefits.

Plant geneticists and food scientists have targeted specific fruits and vegetables, like purple carrot, as possible functional foods due to the bioactive compounds they contain (Alasalvar, 2001; Arscott, 2010b). Anthocyanins are responsible for the purple color in these carrots. Carotenoids, another major plant pigment group, are responsible for fruit and vegetables red, orange, and yellow colors. Some carotenoids are an essential source of vitamin A and, like anthocyanins, are also reported to exert other health-promoting activities (Ahmad, 2019). New carrot varieties have recently been developed to contain additional bioactive pigments to enhance their nutrient profile. One example is the novel purple-red carrot, which contains anthocyanins, provitamin A carotenoids, and the non-provitamin A carotenoid lycopene, a potent antioxidant.

Purple (or black) carrots are described as one of the first two colors of domesticated carrots (Simon, 2019). The origin of the domesticated purple carrots dates back to 900 AD in central Asia. However, even in the 21st century, they are relatively uncommon and unknown by consumers in the United States. Purple carrots have been extensively studied, and their anthocyanin and

carotenoid profiles are fairly well-established (Alasalvar, 2001; Kurlich, 2005; Charron, 2009; Garcia-Herera, 2016; Macura, 2019; Blando, 2021). On the other hand, the same cannot be said about the purple-red carrot because little literature describes the anthocyanin and carotenoid profiles of these varieties. Multiple studies show that purple carrot anthocyanins exert antioxidant and anti-inflammatory activity and even antidiabetic activity by inhibiting carbohydrate digestive enzymes (Sun, 2009 Arscott, 2010a; de Silva, 2014; Ahmad, 2019; Assefa, 2020; Blando, 2021; Yusuf, 2021). However, this has never been determined with purple-red carrots. It is essential to establish the pigment profiles of purple-red carrots and their potential bioactivities to understand and optimize this unique variety as a functional food before mass production and integration into the market.

Anthocyanins and their parent compounds (flavonoids) have been extensively studied. However, anthocyanins specifically are susceptible to degradation, and their stability is influenced by numerous factors making establishing consistent anthocyanin profiles and bioactivities complex **(Figure 1**) (Welch, 2008; Sing, 2009; Lao 2016; Constantin, 2022; Tena, 2022). There is no gold standard or well-established method for extracting and analyzing anthocyanins from fruits and vegetables. However, some general parameters and commonalities exist between many reported methods (Welch, 2008; Deineka, 2015; Lao, 2016; Singh, 2020; Chen, 2022; Tena, 2022). Due to their polar structures, anthocyanins are often extracted and analyzed using organic polar solvents with an acidic agent. For extraction, mainly acidified methanol is used, but acidified water, acetone, or ethanol are also common. The acidic agents include formic, hydrochloric, acetic, and phosphoric acids, which are added to help increase the stability of the anthocyanins being extracted (converted to flavylium ion)[especially from food samples]. After extraction, anthocyanins are often further purified using SPE, which helps eliminate sugars, organic acids, amino acids, or

proteins that can impact anthocyanin stability and recovery/accuracy. The analysis is often performed by reverse-phase HPLC equipped with a C18 column and PDA detector.

There are variations in *in vitro* methods used to assess the bioactivities that anthocyanins may exert. Four assays are commonly used to analyze antioxidant activity and total antioxidant capacity, which are the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid radical (**ABTS**+) scavenging, the Ferric Reducing Ability of Plasma (**FRAP**), the 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) radical-based, and the Oxygen Radical Antioxidant Capacity (**ORAC**) assays (Schlesier, 2002; Schaich, 2015; Ali, 2020). All four have been used in various forms of research, especially in the development of functional foods.

However, no universal assay is recommended, which complicates assessing and comparing antioxidant activity. Method variations and critical differences in the chemical reactions used to determine antioxidant capacity among assays complicate comparisons. For example, the ABTS and FRAP assays measure single electron transfer (**SET**), while the DPPH assay and the ORAC assay both measure antioxidant capacity based on hydrogen atom transfer (**HAT**) (Schaich, 2015). Due to the differences in antioxidant mechanisms measured by each assay, it is strongly recommended that two or more *in vitro* assays be used to assess antioxidant activity and total antioxidant capacity to understand the mechanism behind how antioxidants provide the highest activity. Despite the differences in the base chemical reaction in each type of assay, like with HPLC analysis, there is significant variation in the methods used. The results reported stem from the specific sample being analyzed and the methods used to extract the compounds of interest. The last thing to note is that most assays use parameters that do not apply to human physiology, and results cannot be interpreted regarding the potential antioxidant activity that could occur *in vivo*.

Sensitive assays are crucial for understanding antioxidants like anthocyanins and establishing a general idea of the potential protective activity of secondary plant metabolites in various foods (Schlesier, 2002; Tan, 2022). In addition to other limitations, these *in vitro* assays could better represent how antioxidants in functional foods impact human health. Commercial antioxidant assay kits with standardized protocols can be purchased, making results between studies comparable if the same kits are used. Some organizations are developing databases for antioxidants to help standardize methods and provide a compilation of the recorded activity of various foods. However, they are still in the development stage, so available information is limited and not always accessible (Deng, 2023; Taniguchi, 2023).

Lastly, there has been increasing evidence that secondary plant metabolites in common fruits and vegetables may be beneficial for controlling or mediating blood glucose levels (Li, 2015). Specifically, anthocyanins may exert antidiabetic activity by influencing key steps in carbohydrate digestion through the inhibition of two key enzymes found in the small intestine, alpha-amylase and alpha-glucosidase, which are responsible for the breakdown of polysaccharides and disaccharides into glucose (Castro-Acosta, 2016). Purple and purple-red carrots contain anthocyanins, specifically cyanidin-3-glucosides, demonstrating the highest inhibitory effect compared with other cyanidin analogs (Castro-Acosta, 2017). Assays that investigate digestive enzyme inhibition potential are based on colorimetric detection by UV-spectrophotometry and Enzyme-Linked Immunosorbent Assays. Assay kits are commercially available and vary by method and mode of detection. Unlike the case for antioxidants, multiple organizations and databases provide specific information on each enzyme and even standardized guidelines for analysis (Chang, 2009).

Due to building evidence linking the consumption of anthocyanins and carotenoids and improved health, interest in and popularity of foods containing these bioactive pigments, like purple carrots, has steadily increased in the past few decades. Purple carrot pigments' bioavailability and bioactivity potential have been repeatedly investigated, but there needs to be data on purple-red carrots (Kurlich, 2005; Porter Dosti, 2006; Sun, 2009; Arscott, 2010a). This paper discusses three sub-studies conducted to investigate three different parameters of novel purple-red carrots to further understand their potential as a functional food including: 1) developing a method to establish the first documented anthocyanin profile of purple-red carrots analyzed by HPLC, 2) assessing *in vitro* antioxidant activity of purple-red carrots using two different assays; ABTS and DPPH assays and 3) assessing *in vitro* alpha-amylase and alpha-glucosidase inhibition potential of purple-red carrots. The combination of the three projects will provide a basis for future clinical work looking at the bioavailability and potential bioactivities of pigments from purple-red carrots *in vivo*.

## Methods

***Carrot sample preparation.*** Red, purple, and purple-red carrots used in this work were developed and provided by Philipp Simon through the USDA carrot breeding and genetics program. Carrots were harvested at the University of California Desert Research and Extension Center, shipped to the University of Wisconsin-Madison at 2°C, and stored upon arrival at 4°C.

*Fresh/raw carrots.* All carrots were separated by genotype and processed. Processing included removing the carrot tops and visible damage, washing, and gently scrubbing in cold water to remove soil and other debris. The carrots were sliced into 2-3 inch pieces,

blanched for 1 min in boiling water, submerged in cold water, air dried, packed into freezer-safe bags, and stored at 4°C until analyzed.

*Freeze-dried carrots.* A portion of each processed genotype was lyophilized until dry and ground into a powder using an industrial blender. The freeze-dried samples were stored at -80°C until analysis.

*Juice.* Carrot juices were made by taking 200 g processed frozen carrots, microwaving for 1 min in two 30-second intervals, and blending in a Vitamix™ blender with 8 oz of water on high for 1 min. The resulting pulp was poured into cheesecloth and squeezed by hand until no more juice could be obtained. The juice was analyzed immediately or stored in air-tight glass test tubes at -20°C until analysis.

### *Extraction & Purification*

*Standards.* The anthocyanins in purple carrots are cyanidin-3-glucoside based with varying acylations and side chains (**Figure 2**). A standard cyanidin-3-glucoside powder was used as an initial test standard and as an external standard in each run. Malvadin-3-galactoside was used as an additional external standard. 5 mg of the standard powders were stored at -80°C until serial dilutions were made, with the most concentrated form using 4 mL 10% FA:MeOH. Dilutions of 1:20, 1:40, 1:100, and 1:200 were created to make initial standard curves. All methods tested are shown in **Table 1**.

*Fresh carrots.* Anthocyanins were extracted in triplicate from the frozen raw carrot samples by modifying a previously published method (Kurlich, 2005). Briefly, carrot macerate samples ranging from 1-5 g were weighed and placed into test tubes covered with

aluminum foil to keep out light and put on dry ice to keep cool during extraction. Two extraction methods were tested, which included one single extraction versus 4 separate extractions. The first included extracting the anthocyanins by adding 40 mL 10%FA: MeOH to the samples, which were mixed with a vortex, sonicated, and centrifuged; the supernatant was transferred into a separate tube. The second method used 10 mL 10% FA: MeOH four times, and the supernatants were combined. Following the initial extraction, for both methods, 1 mL of the supernatants was aliquoted into another amber vial and diluted with 3 mL cold DI $H_2O$; 200 mL of that mixture was placed in an HPLC vial for analysis and kept at -20°C until ready to run.

*Freeze-dried carrots.* Extraction of anthocyanins from the freeze-dried carrots was done in triplicate and utilized a previously published method with a few modifications (Kurlich, 2005). Briefly, 0.1-0.5 $\pm$ 0.05 g carrot powder was weighed and combined with 1 mL 10% FA:MeOH. The samples were mixed by vortex and centrifuged for 5 minutes. The top layers were transferred into separate tubes, kept on ice, and covered to keep the samples cold. An additional 1 mL 10% FA:MeOH was used to repeat the extraction, and the top layer was pooled with the initial supernatant. The pooled supernatants were mixed by vortex and centrifuged for five minutes to ensure no solids remained. Next, a 200 μL sample was aliquoted into a different tube and diluted with 600 μL DI $H_2O$. A final 200 μL was aliquoted into an HPLC vial and kept at -20°C until ready to run.

*Juice.* All carrot juice samples were extracted using modified versions of a previously published method and run in duplicate (Charron, 2009). Extraction methods of purple and purple-red carrot juices were tested using dilutions made with different ratios of juice:DI

$H_2O$ and extracted using a range of 10%FA:MeOH. Briefly, juice samples were mixed with DI $H_2O$, and aliquots were combined with 10%FA:MeOH and mixed again. Samples were either immediately centrifuged for 10 minutes or sonicated for 10 minutes first and then centrifuged for 10 minutes. The supernatant of each sample was aliquoted into separate tubes, and the remaining pellet was extracted in the same manner as the first time two more times. Extracts were either analyzed as is, dried down, and reconstituted with 10%FA:DI $H_2O$ and analyzed, filtered using Polytetrafluorethylene (**PTFE)**, 45 μm syringe filters and analyzed, or filtered using C18 SPE cartridges (Sigma) and then analyzed by HPLC.

***HPLC Analysis.*** Chromatographic analyses were conducted on a Waters HPLC system (Milford, MA, USA) equipped with a 1525 binary pump, a 717 autosampler, a 2996 photodiode array detector, and a reversed phase-C18 column (5 mm, 4.6 mm x 250 mm). The mobile phase consisted of two solvents: Solvent A was DI water acidified with formic acid (**FA**) (2%, 3%, 5%, and 10% tested), and solvent B was HPLC-grade methanol. The volume injected ranged from 20-80 μL. Although two gradients were tested, the primary gradient used had a profile of 100% A at 0 min, 85% A at 20 min, 80% at 38 min, 70% at 48 min, 45% at 51 min and 55 min, and then returned to initial conditions at 56 min. Chromatograms were obtained at 520 nm, and photodiode array spectra were used to confirm peaks associated with anthocyanins (**Figure 3**).

***Antioxidant activity.***

*DPPH Assay.* The antioxidant activity of red, purple-red, and red carrot juices was investigated using a commercially available assay kit (DoJindo, Japan) (Schlesier, 2021). All reagents and solutions used for the assays were prepared on the day of analysis

according to manufacturer's instructions. A Trolox standard curve was made using five dilutions. Samples were assessed using five different dilutions of each juice type. Three different blanks were used: control (Blank 1), sample (Blank 2), and solvent (Blank 3). All standards, blanks, and samples were assessed in triplicate.

In a 96-well microplate, 20 µL sample or standard was pipetted into designated wells, 20 µL ethanol into the wells for Blank 3, and 20 µL solvent into the wells for Blank 1 and Blank 2. Next, 80 µL assay buffer was added to all wells, 100 µL ethanol was added to the wells for Blank 2 and 3, and 100 µL DPPH working solution was added to the wells of the standards, samples, and Blank 1. The plate was gently shaken to mix the contents, incubated in the dark at 25°C for 30 minutes, and read on a Versamax™ Tunable Microplate Reader at 517 nm. The inhibition ratios of Trolox and the samples were calculated using the following equations:

$$\textit{Inhibition ratio of Trolox (\%)} = (A_C - A_R)/ A_C \times 100$$

$A_C$ = *Abs. of 0 µg/mL Trolox std - Blank 3* $A_R$ = *Abs of 40-80 µg/mL Trolox std - Blank 3*

$$\textit{Inhibition ratio of juice samples (\%)} = (A_{CS} - A_S)/ A_{CS} \times 100$$

$A_{CS}$ = *Blank 1 – Blank 2* $A_S$ = *Abs of samples - Blank 2*

A plot was made with the inhibition ratio of the samples (y) plotted against the sample concentration (x), and a regression line was drawn (**Figure 4**). Antioxidant capacity was expressed as Trolox Equivalent Antioxidant Capacity (**TEAC**), which was calculated using the following equation:

$$TEAC = IC_{50}\ (Trolox) / IC_{50}\ (Sample)$$

*ABTS Assay.* The antioxidant activity of red, purple-red, and red carrot juices was investigated using a commercially available assay kit (Sigma-Aldrich No. CS0790). All reagents and solutions used for the assays were prepared on the day of analysis according to the manufacturer's instructions. All samples and standards were assessed in triplicate. A Trolox standard curve was prepared using six different dilutions.

In the designated microplate wells, 10 µL Trolox standards or 10 µL juice samples were combined with 20 µL Myoglobin working solution. Next, 150 µL ABTS solution was added to each well and incubated at room temperature for 5 minutes. The reaction was stopped by adding 100 µL stop solution to each well, and the plate was read at 405 nm on a Versamax™ Tunable Microplate Reader. A Trolox standard curve was created by plotting the average absorbance of the Trolox standards versus the Trolox concentration. The antioxidant concentration of the juice samples was calculated using the standard curve using the following equation:

$$X\ (mM) = ((y(A_{405}) - Intercept) / Slope) \times Dilution\ Factor$$

*X (mM) = Antioxidant concentration [(mM) relative to the concentration of the Trolox std]*

### *Enzyme Inhibition Potential*

*Alpha-Amylase.* The inhibition of alpha-amylase of red, purple-red, and purple carrot juice samples was analyzed in triplicate by Enzyme-Linked Immunosorbent Assay (**ELISA**) using a standard protocol published by MilliporeSigma (Bernfeld, 1995). All reagents needed for the analysis were purchased from MilliporeSigma and Sigma Aldrich. Briefly, samples were prepared by combining 50 µL diluted juice samples and 50 µL 0.02 M

sodium phosphate buffer with alpha-amylase from the porcine pancreas. Controls and sample blanks had 100 μL buffer, the former including alpha-amylase and sample blanks without. All were incubated at 25°C for 10 minutes.

Next, 50 μL 1% starch solution was added to initiate the reaction, and samples were incubated again at 25°C for 10 minutes. The reaction was stopped by adding 100 μL color reagent solution made with 2 M sodium hydroxide solution, including 96 mM 3,5-dinitrosalicylic acid and 5.3 M potassium sodium tartrate tetrahydrate, and immediately incubated in a boiling water bath for 10 minutes. Samples were cooled to room temperature and diluted with 2 mL distilled water. For analysis, 200 μL standards, controls, samples, and sample blanks were pipetted into a 96-well microplate and read on a Versamax™ Tunable Microplate Reader at 540 nm. All standards, controls, and three samples of each juice color were calculated with the following equation:

$$\%inhibition = [(Abs_{Ctrl}) - (Abs_{Sample} - Abs_{SampleBlank})/ (Abs_{Ctrl})] \times 100$$

*Alpha-Glucosidase.* The inhibition of alpha-glucosidase by juices made from red, purple-red, and purple carrots was analyzed in triplicate by ELISA using a standard protocol published in the Worthington Enzyme Manual (Worthington, 2011). All reagents needed for the analysis were purchased from MilliporeSigma and Sigma Aldrich. Briefly, a standard curve was created using ranging concentrations of maltose standard. Samples were prepared by pipetting 50 μL sample and 50 μL 0.1M potassium phosphate buffer (**PPB**) (pH 6.9 at 25°C), or 100 μL PPB for controls, into 96-well microplates. Next, 100 μL 2.0 U/mL enzyme solution made with lyophilized *Sacch. cerevisiae* was pipetted into

each well except for the controls, which had another 100 μL PPB added. The plate was incubated at 25°C for 10 minutes, followed by the addition of 50 μL 5 mM p-nitro-phenyl-α-D-glucopyranoside (**PNPG**) solution into each well to initiate the enzymatic reaction, and incubated once more at 25°C for 5 minutes. To stop the reaction, 100 μL 0.1 M sodium carbonate was added to the wells, and the absorbance was recorded at 405 nm on a Versamax™ Tunable Microplate Reader. Inhibitory activity was expressed as a percentage and was calculated using the following equation:

$$\%inhibition = [(Abs_{Ctrl}) - (Abs_{Sample} - Abs_{SampleBlank})/ (Abs_{Ctrl})] \times 10$$

**Results & Discussion**

***HPLC Analysis.*** Methods for analyzing the anthocyanins in fresh, freeze-dried, and juiced purple and purple-red carrots were optimized by adjusting various factors of the extraction and purification steps and the HPLC. Purple carrots were more concentrated in anthocyanins than purple-red carrots, meaning any previously published methods on purple carrot anthocyanin analysis had to be adjusted. While optimizing an analysis method specific for purple-red carrots, different analysis parameters were adjusted and compared. External standards were used to compare run efficacy and efficiency.

The parameters that most impacted the analysis were the sample weight/volume, centrifugation, acid concentration in the mobile phase, and the HPLC gradient. The larger sample weights generally had more apparent peaks in fresh and freeze-dried samples. Due to anthocyanin concentration, the purple carrot samples had better results using 3 g fresh or 0.3g freeze-dried carrots. The purple-red carrots required 5 g fresh and 0.5 g freeze-dried carrots to produce clear and distinct peaks. Including sonication and centrifugation steps, no matter

the duration, improved extraction efficiency. Omitting the centrifugation step, despite the inclusion of sonication or not, resulted in hazier samples and, therefore, messier chromatograms.

Filtering the fresh and freeze-dried sample extracts did not benefit the analysis and generally resulted in less peak resolution and missing peaks (due to retention loss). In the purple carrot samples, injecting more than 20 μL was not beneficial. It was only beneficial when smaller amounts of sample were used, so the concentration of anthocyanins was lower overall. In the purple-red carrot samples, 40-50 μL injection volumes generally showed more apparent peaks, while anything above 50 μL caused co-elution.

The analysis heavily depended on the type of juice being analyzed in the samples. The purple carrot juice samples consistently showed peaks, but lower sample volumes and higher dilutions resulted in the best chromatograms. In the purple-red carrots, there needed to be 2-3x more juice used than the purple carrot juice samples or a lower dilution volume to see consistent peaks. There was no impact on the number of extraction intervals (whether extraction with the 10%FA:MeOH was done in one step or multiple). Sonication did not impact the extraction, while centrifugation was necessary for clarity, especially in the purple-red carrots. Due to the relatively dilute extracts from the purple-red juice samples, an additional step was tested, including drying down the extract and reconstituting in either 10%FA:MeOH or 10%FA:$H_2O$. Although drying and reconstituting resulted in what looked like a more concentrated sample, despite the solvent the samples were reconstituted in, the chromatograms were noisy and inconsistent between runs.

All filtration steps tested in the juice analysis resulted in either no difference or a detrimental effect on the anthocyanin content. The filter paper and SPE Pak resulted in

coelution, low peak resolution, or missing peaks. In contrast, the PTFE syringe filter resulted in either no differences or losses of peaks depending on the sample volume and dilution. All sample types had the clearest chromatograms when the mobile phase (Solvent A) had 5% or 10% FA concentration. When the samples were run using 10% concentration, the peaks were generally similar in size and resolution to 5%, but the analytes eluted 10 minutes earlier.

Purple carrots have been analyzed numerous times for anthocyanin content, and five cyanidin derivatives are commonly reported (Alasalvar, 2001; Kurlich, 2005; Charron, 2009; Algarra, 2014; Garcia-Herera, 2016; Macura, 2019; Blando, 2021). The same five anthocyanins were seen in the analysis of the fresh, freeze-dried, and juice from the purple carrots in this work but ranged in total concentration between sample types (**Table 2**). The anthocyanins eluted in the following order: 1. Cyanidin 3-xylosylglucosyl-galactoside (**Cy3XGG**), 2. Cyanidin 3-xylogalactiside (**Cy3XG**), 3. Cyanidin 3-xylosyl(sinapoyl-glucosyl)galactoside (**Cy3XSGG**), 4. Cyanidin 3-xylosyl(feruloylglucosyl)galactoside (**Cy3XFGG**) and 5. Cyanidin 3-xylosyl-(coumuroylglucosyl)galactoside (**Cy3XCGG**). The highest concentration observed was Cy3XFGG, followed by either Cy3XG or Cy3XSGG, with the smallest concentrations being Cy3XGG and Cy3XCGG. The purple-red carrots had the same profile, except the Cy3XG and Cy3XSGG peaks were a much smaller proportion of the total anthocyanin content than the purple carrots' total anthocyanin content. The total anthocyanin content of the fresh purple carrots was about 3-4x that of the fresh purple-red carrots, the freeze-dried purple about 3x that of the freeze-dried purple-red, and the purple carrot juice about 4-5x that of the purple-red juice.

***Antioxidant Activity.*** Other studies have assessed the antioxidant activity of purple carrots using various assays, which, unfortunately, are hard to compare between studies (Arnao, 2000;

Olszowy, 2018 ). Multiple factors can influence the results observed using these assays, including differences in the food sample being assessed, variation between assay types and suppliers, methods used between assays, and human error.

*DPPH Assay.* The antioxidant activity of each of the three juice types was analyzed using a commercially available kit. The results were expressed as $IC_{50}$ values, representing the juice concentration needed to scavenge 50% of the DPPH radicals present (**Table 3**). The purple carrot juice had the lowest $IC_{50}$, which was 0.54, followed by purple-red, which was 0.62, and the red carrot juice was 1.66. The purple carrot juice was expected to have the highest antioxidant activity based on the anthocyanin concentration and known potent antioxidant activity that anthocyanins can exert. Any substance analyzed using colorimetric assays with absorbances within the range of that used in the assay itself may compromise observed results. The absorption region of anthocyanins is between 480-600 nm, with maximal absorption recorded around 520 nm. This is very similar to the wavelength used to assess the change in color in the assay, which was 517 nm. Although the pigments in the juices did not appear to affect the analysis, it is possible that the absorbance changes observed could have been influenced by the presence of anthocyanins or other compounds in the juice.

*ABTS Assay.* Analysis of the antioxidant activity of red, purple, and purple-red carrot juices was attempted using a commercially available assay kit. Multiple rounds were conducted, but the results were inconclusive when compared with the standards. It is possible that due to the presence of pigments in the juice, affected the color changes and resulted in abnormal absorbance readings and calculations leading to negative numbers. As the ABTS radical is

neutralized, it becomes colorless, unlike the color reaction in the DPPH assay, which goes from yellow to purple when antioxidants are present. The use of the Trolox standards in the assay allowed for the confirmation that the assay was conducted correctly, but despite consistent standard curves, only some assay runs resulted in normal and usable numbers. This was still the case when sample blanks were included, or samples were diluted to the point that it was unable to be sure if pigments were still present to be tested.

One of the most significant factors that led to the unsuccessful analysis of the antioxidant activity of the carrot juices using the ABTS assay was the specific wavelength used, per the manufacturer's instructions. In the kit used, all absorbances were to be read at 405 nm, less common than other methods and kits that use a wavelength between 730-750 nm for analysis (Arnao, 2000; Schlesier, 2002; Olszowy, 2018). The specific ABTS kit used in this study was chosen due to cost, simplicity, and availability. The kit was standard from a large distributor, and the different wavelength the plates would be read at was not considered a problem, as other ABTS assays use similar wavelengths. Results of assays used to assess antioxidant activity in samples with substances that may be absorbed in the same range of wavelengths used in the assay can lead to inaccurate or misleading results (Olszowy, 2018). These compounds can influence the recorded changes of absorbance, resulting in an erroneous estimation of the actual antioxidant activity of the samples or compounds of interest.

### *Enzyme Inhibition*

*Alpha-Amylase.* Alpha-amylase inhibition potential of the bioactive pigments in carrot juices made with red, purple-red, and purple carrots was assessed using a commercially available kit and analyzed by ELISA. Due to the possible interference of the anthocyanin

pigmentation of the samples, a sample blank was used and was subtracted from the sample absorbances (Lankatillake, 2021). In this assay, alpha-amylase breaks down starch into maltose, measured by colorimetry using 3,5-dinitrosalicylic acid (**DNSA**). As maltose is produced, it reacts with the DNSA molecules reducing it to 3-amino-5-nitrosalicylic acid (**ANSA**), which causes the color to change from yellow to orange/red. The more maltose produced represents more alpha-amylase activity resulting in a darker color. Inhibition was assessed by looking at how adding the carrot juices hinders the alpha-amylase from breaking down the starch, leading to less reduction of the DNSA to ANSA and, therefore, less color change.

A maltose standard curve was created using eight dilutions. After the assay was complete, the observed antioxidant activity of the control (+enzyme, +starch, -juice) was compared to the samples (+enzyme, +starch, +juice) after correction by subtracting the blanks (-enzyme, +starch, +juice). The calculated %Inhibition and mg maltose produced are shown in (**Table 3**). The red juice inhibited the alpha-amylase by about 70%, and the purple and purple-red inhibited the enzyme activity by about 75%. The assay was conducted three separate times, each time assessing the samples, blanks, and control in triplicate. The red juice had much more consistent results between the three assays performed. It ranged from 66-72% inhibition, while the purple-and purple-red carrots had an extensive range between assays, with purple-red %inhibition ranging from 58-96% and purple from 55-91%.

Due to the dilution of the juices and the small amount of sample used, despite thorough mixing of the juice samples prior to use, there may have been a range in the anthocyanin content within each sample pipetted into the wells. It could also have been due

to human error, including pipetting accuracy of the sample, or that some of the wells in one assay may have been mixed better than others. Like the case with the antioxidant assays, the purple and purple-red pigmentation of the juices, no matter how dilute, may have impacted the results despite adjusting with the sample blank.

*Alpha-Glucosidase.* Multiple assays were performed because the concentration and presence of the pigments in the carrot juices impacted the colorimetric analysis. For example, five dilutions of each juice were assessed, and even at the lowest concentration, the wells with the purple and purple-red juices had grayish tints, both in the sample (including the alpha-glucosidase) verse the sample blank (no alpha-glucosidase). Efforts were made to dilute the juice samples to prevent pigment interference. The dilutions needed to not impact the assay were so low that the activity of the juices could not be determined.

The assay used to assess alpha-glucosidase inhibition was unsuccessful, which used the same wavelength as the ABTS assay. The alpha-amylase and the DPPH assays were not affected similarly. The pigments in the juice may have impacted the change in absorbances recorded due to the wavelengths used being similar to that of other compounds. However, the two assays that were successful in this study, the alpha-amylase inhibition assay (514 nm) and the DPPH antioxidant assay (540 nm), used wavelengths even closer to the wavelength that anthocyanin absorbances are read at (520 nm).

Carotenoid absorption spectra are read at 450 nm, so the carotenoids in the juice could have influenced the assays instead of the anthocyanins. This is unlikely due to the concentration of carotenoids compared to the anthocyanins, and the red carrot juice generally had more consistent results in all assays. It could also have been due to the different principles of the assays and chemical reactions utilized or that there are other

compounds present in the purple and purple-red juices that may be absorbed in similar wavelengths like other flavonoids, specifically flavanols like quercetin or kaempferol, or metabolites of anthocyanins, such as ferulic acid, all of which can be present in carrots (da Silva Dias, 2014).

## Conclusion

This paper aimed to investigate the anthocyanin profile, antioxidant, and digestive enzyme inhibition potential of novel purple-red carrots. These analyses are the first to report the anthocyanin profile of fresh, freeze-dried, and juice from purple-red carrots and the *in vitro* antioxidant potential and digestive enzyme inhibition potential of purple-red carrot juice compared to juice made from purple or red carrots. Various components of anthocyanin analysis methods were investigated. The following are summarized points of what conditions were optimal or not beneficial for analyzing anthocyanins in purple and purple-red carrots by HPLC: Extraction methods including larger sample weights or volumes, and inclusion of sonication and centrifugation steps in either a single extraction step, or multiple was the most beneficial. Purification by filtering or drying down and reconstituting the samples was not beneficial. The HPLC conditions that gave the best results included using a mobile phase solvent that is made up of 5-10% acid and not any lower than 3%, with the use of 10%FA:$H_2O$ and MeOH as solvent A and solvent B, the longer run time allowed for more separation of the five peaks, and the smaller the injection volume, the better, if working with concentrated extractions.

The pigments, or potentially other compounds in the juices, made colorimetric assessments of *in vitro* antioxidant and enzyme inhibition activity complex. While antioxidant activity observed by the DPPH assay indicated that the purple and purple-red carrot juices exerted more antioxidant

activity than the red carrot juice, the ABTS assay was inconclusive. The same was observed in the enzyme inhibition analysis; purple and purple-red juices showed better alpha-amylase inhibition compared to the red juice, but the recorded inhibition of the purple and purple-red juices varied more between runs than the red carrot, indicating that this assay may have been influenced by the presence of the anthocyanins in the juice.

The alpha-glucosidase analysis was inconclusive, and the complications of the assay were attributed to the interference of the pigments in addition to the lower wavelength (405 nm) that absorbance was measured, versus higher wavelengths (730-740 nm) used more commonly in other ABTS assays, which is further from the wavelengths that the anthocyanins and carotenoids in the juice are absorbed (520 nm, 450 nm). Future studies need to assess purple-red carrots' antioxidant activity and enzyme inhibition potential using other kits or protocols. They should also evaluate the difference between fresh, freeze-dried, and juice from the carrots. Despite the limitations, this paper provides insight into method development and optimization for the analysis of anthocyanins in purple and purple-red carrots, the anthocyanin profile of purple-red carrots compared to purple carrots, and a first look into the antioxidant and alpha-amylase inhibition potential of red, purple, and purple-red carrot juices.

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**Table 1.** Methods tested in anthocyanin analysis

| | Fresh | | Freeze-Dried | | Juice | |
|---|---|---|---|---|---|---|
| | Tested | Best | Tested | Best | Tested | Best |
| **Extraction** | | | | | | |
| Sample wt./vol. | 1, 3, 5g | PC: 3g, PRC: 5g | 0.1, 0.3, 0.5g | PC/PRC: 0.5g | 1, 2, 3 mL[a] | PC: 1:3:3[b] PRC: 2:2:2 |
| Dilution vol. (mL) | --- | --- | --- | --- | 1, 2, 3 | PC: 1:3:3[b] PRC: 2:2:2 |
| Extraction vol. (mL) | --- | --- | --- | --- | 1, 2, 3 | PC: 1:3:3[b] PRC: 2:2:2 |
| Extraction intervals | 1, 4 | ND[c] | --- | --- | 1, 2, 4 | ND[c] |
| Sonication (min) | 0, 5, 10 m | 5-10 m | --- | --- | 0, 5, 10 m | ND[d] |
| Centrifugation (min) | 0, 5, 10 m | 5-10 m | --- | --- | 0, 5, 10 m | 5-10 m |
| **Purification** | | | | | | |
| #1 Whatman™ Paper | W / WO | WO | W / WO | WO | --- | --- |
| PTFE Syringe Filter | W / WO | WO | --- | --- | W / WO | WO |
| C18 SPE Pak | --- | --- | --- | --- | W / WO | WO |
| Dry / Reconstitute | --- | --- | --- | --- | 10%FA:H2O, 10%FA:MeOH | WO |
| **HPLC Conditions** | | | | | | |
| %Acid mobile phase | 2, 3, 5, 10% | 5-10% | 2, 3, 5, 10% | 5% | 2, 3, 5, 10% | 5-10% |
| Run time/Gradient | 46[e], 66[f] m | 66 m | --- | --- | 46, 66 m | 66 m |
| Injection vol. (μL) | 20, 40, 50, 80 | PC: 20 PRC: 40-50 | 20, 40, 50, 80 | PC: 20 PRC: 40-50 | 20, 40, 50, 80 | PC: 20 PRC: 40-50 |

PC = Purple carrot, PRC = Purple-red carrot, ND = No difference, W = With, WO = Without

[a] Juice samples were analyzed using different ratios of the juice samples, water used for dilution, and 10%FA:MeOH for extraction ranging from 1-4 mL.

[b] Ratio of Juice:Water:10%FA:MeOH

[c] No difference observed between samples extracted in one step versus multiple steps.

[d] When analyzing the juice samples, there was no observed effect of including a sonication step.

[e] Gradient used for 46 min run time: 0-17 min- 85% A, 18 min- 80% A, 28 min- 70% A, 31 min- 45% A, 35 min- 45% A, 36 min- 85% A, 46 min- 85% A. Solvent A = DI water with formic acid, Solvent B = MeOH.

[f] Gradient used for 66 min run time: 0-19 min- 100% A, 20 min- 85% A, 38 min- 80% A, 48 min- 70% A, 51 min- 45% A, 55 min- 45% A, 56 min- 100% A, 66 min- 100% A. Solvent A = DI water with formic acid, Solvent B = MeOH

**Table 2.** Anthocyanin content of fresh, freeze-dried, and juiced purple and purple-red carrots.

| Sample | | Cy3XGG[a] | Cy3XG[b] | Cy3XSGG[c] | Cy3XFGG[d] | Cy3XCGG[e] | Total |
|---|---|---|---|---|---|---|---|
| **Purple Carrot** | Fresh *(μg/g)* | 32.0 ± 27.7 | 28.5 ± 41.4 | 210 ± 202 | 930 ± 866 | 21.9 ± 23.9 | **1220 ± 1020** |
| | Freeze-Dried *(μg/g)* | 116 ± 96.0 | 140 ± 230 | 905 ± 1020 | 3900 ± 3300 | 106 ± 120 | **5160 ± 3910** |
| | Juice (*μg/100mL)* | 439 ± 112 | 4140 ± 684 | 947 ± 122 | 3250 ± 425 | 1520 ± 1090 | **10300 ± 318** |
| **Purple-Red Carrot** | Fresh *(μg/g)* | 20.8 ± 11.9 | 36.7 ± 10.8 | 10.6 ± 15.6 | 245 ± 82.0 | 23.6 ± 11.7 | **336 ± 118** |
| | Freeze-Dried *(μg/g)* | 38.6 ± 33.2 | 46.8 ± 79.5 | 302 ± 350 | 1300 ± 1140 | 35.4 ± 41.6 | **1720 ± 1350** |
| | Juice (*μg/100mL)* | 112 ± 41.9 | 179 ± 90.7 | 287 ± 134 | 1690 ± 779 | 78.4 ± 44.4 | **2340 ± 1090** |

[a] Cyanidin 3-xylosylglucosyl-galactoside (**Cy3XGG**)

[b] Cyanidin 3-xylogalactiside (**Cy3XG**)

[c] Cyanidin 3-xylosyl (sinapoylglucosyl)galactoside (**Cy3XSGG**)

[c] Cyanidin 3-xylosyl(feruloylglucosyl)galactoside (**Cy3XFGG**)

[e] Cyanidin 3-xylosyl(coumuroylglucosyl)galactoside (**Cy3XCGG**).

**Table 3.** Comparison of *in vitro* antioxidant activity and enzyme inhibition potential of juices made from purple, purple-red, and red carrots.

| | **DPPH**[a] (514nm) | **ABTS**$^{+}$ (405nm) | **α-Amylase** (540nm) | | **α-Glucosidase** (405nm) | **Total Carotenoids**[d] | **Total Anthocyanins**[d] |
|---|---|---|---|---|---|---|---|
| | | | %Inhibition | Maltose[c] | | | |
| **Red** | 1.66 | N/A[b] | 69% ± 3% | 0.24 | N/A[b] | 10.4 ± 6.60 | --- |
| **Purple-Red** | 0.62 | N/A[b] | 75% ± 16% | 0.19 | N/A[b] | 33.5 ± 10.9 | 2680 ± 1250 |
| **Purple** | 0.54 | N/A[b] | 75% ± 20% | 0.19 | N/A[b] | 31.9 ± 11.7 | 13980 ± 736.6 |

[a] Antioxidant activity expressed as $IC_{50}$

[b] Not available; assay did not produce usable results

[c] Maltose concentration produced from starch, expressed as mg

[d] Average concentration expressed as μmol/L of juice

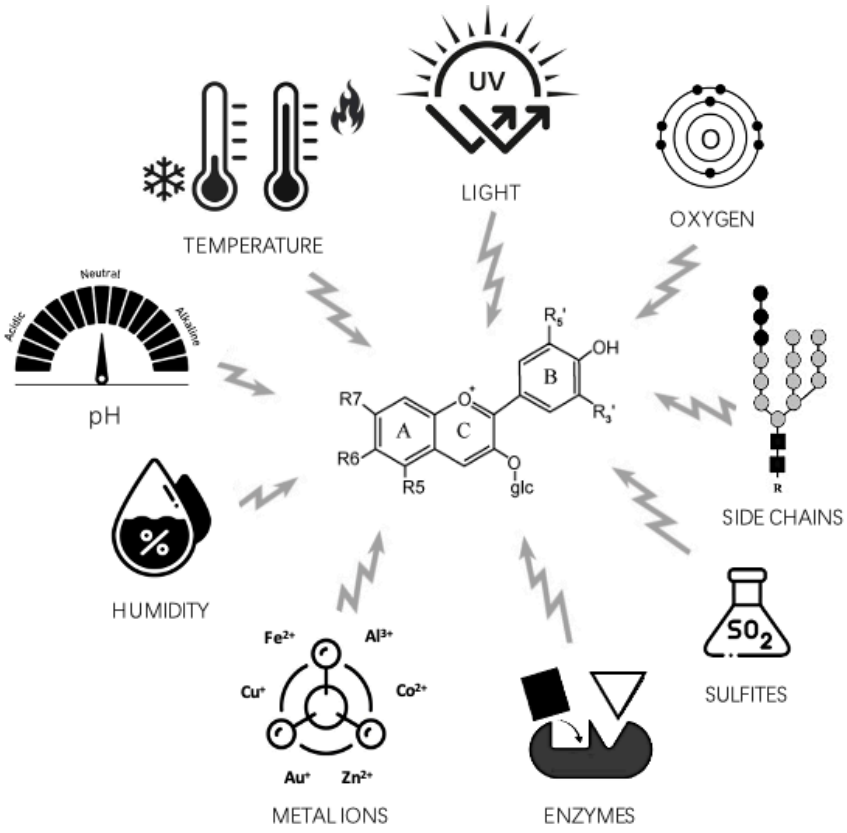


**Figure 1.** Factors that impact anthocyanin stability and analysis.

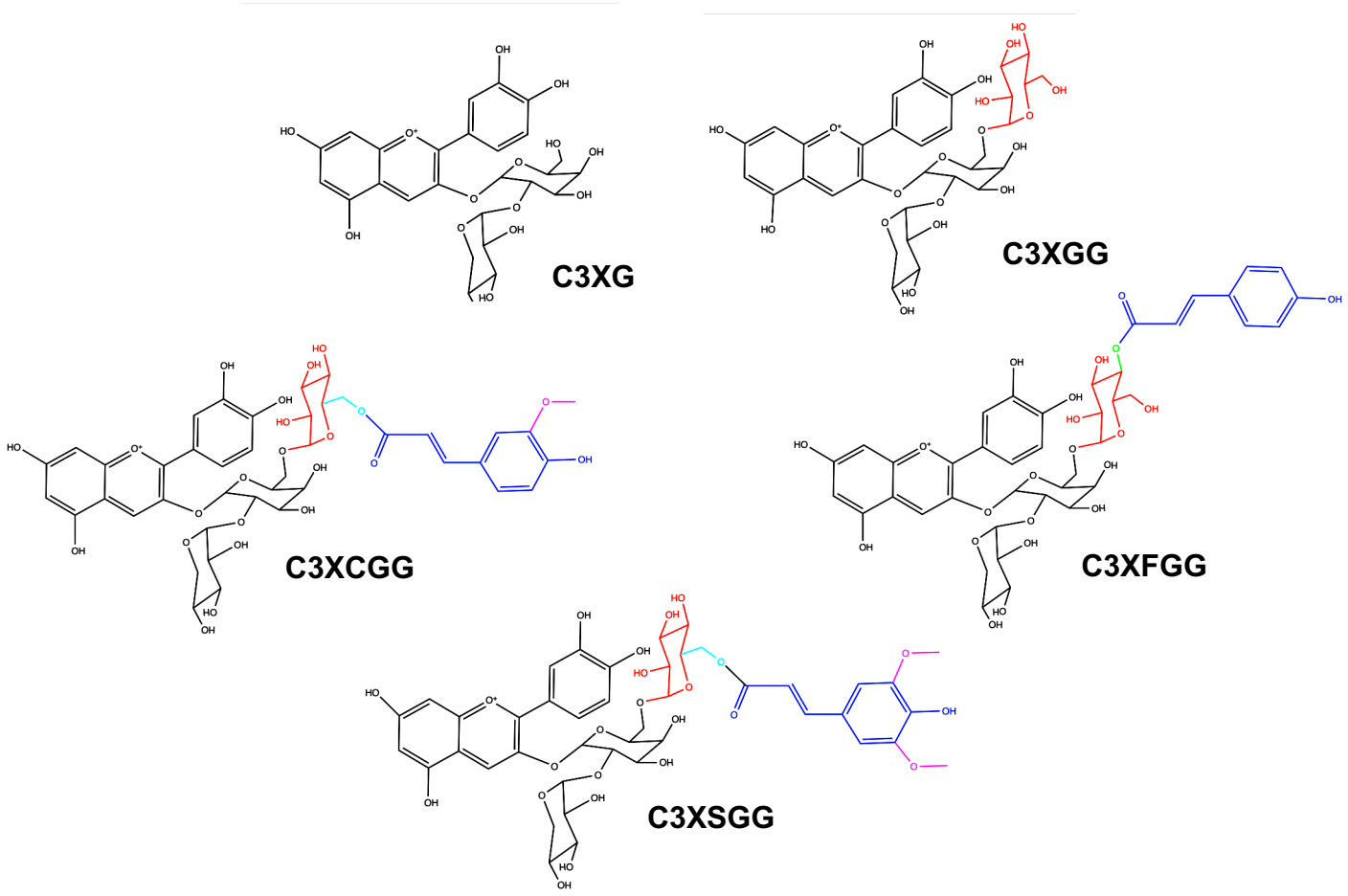


**Figure 2.** Structures of anthocyanins found in purple and purple-red carrots, **Cy3XGG**: Cyanidin 3-xylosylglucosyl-galactoside, **Cy3XG**: Cyanidin 3-xylogalactiside, **Cy3XSGG**: Cyanidin 3-xylosyl(sinapoylglucosyl)galactoside, **Cy3XFGG**: Cyanidin 3-xylosyl (feruloylglucosyl) galactoside, **Cy3XCGG**: Cyanidin 3-xylosyl-(coumuroylglucosyl)galactoside.

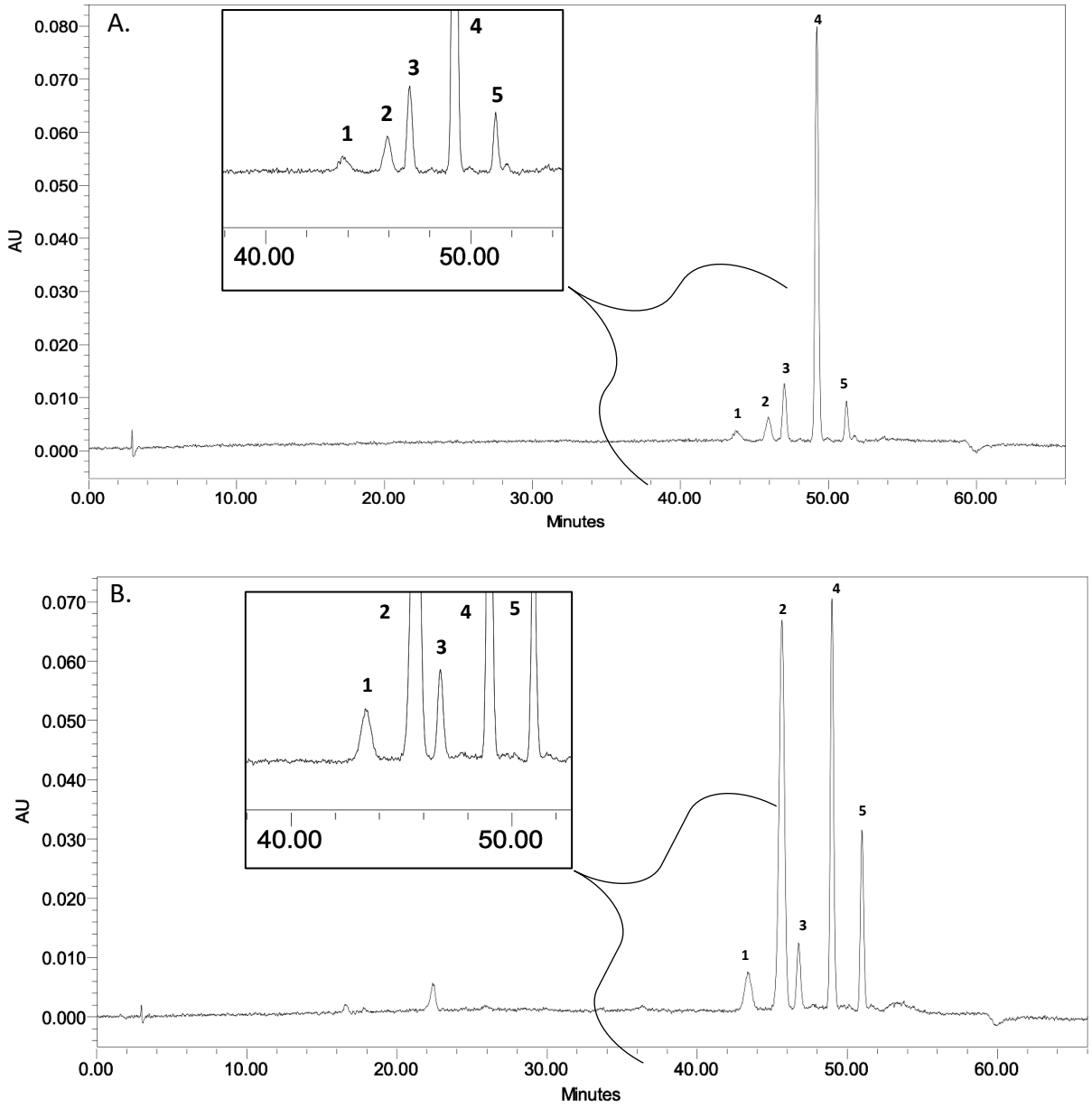


**Figure 3.** Chromatographic profile of anthocyanins in extracts at 520 nm of **a)** purple and **b)** purple-red carrots analyzed by HPLC at 520 nm. 1. Cyanidin 3-xylosylglucosyl-galactoside (**Cy3XGG**), 2. Cyanidin 3-xylogalactiside (**Cy3XG**), 3. Cyanidin 3-xylosyl (sinapoylglucosyl)galactoside (**Cy3XSGG**), 4. Cyanidin 3-xylosyl(feruloylglucosyl)galactoside (**Cy3XFGG**) and 5. Cyanidin 3-xylosyl-(coumuroylglucosyl)galactoside (**Cy3XCGG**).

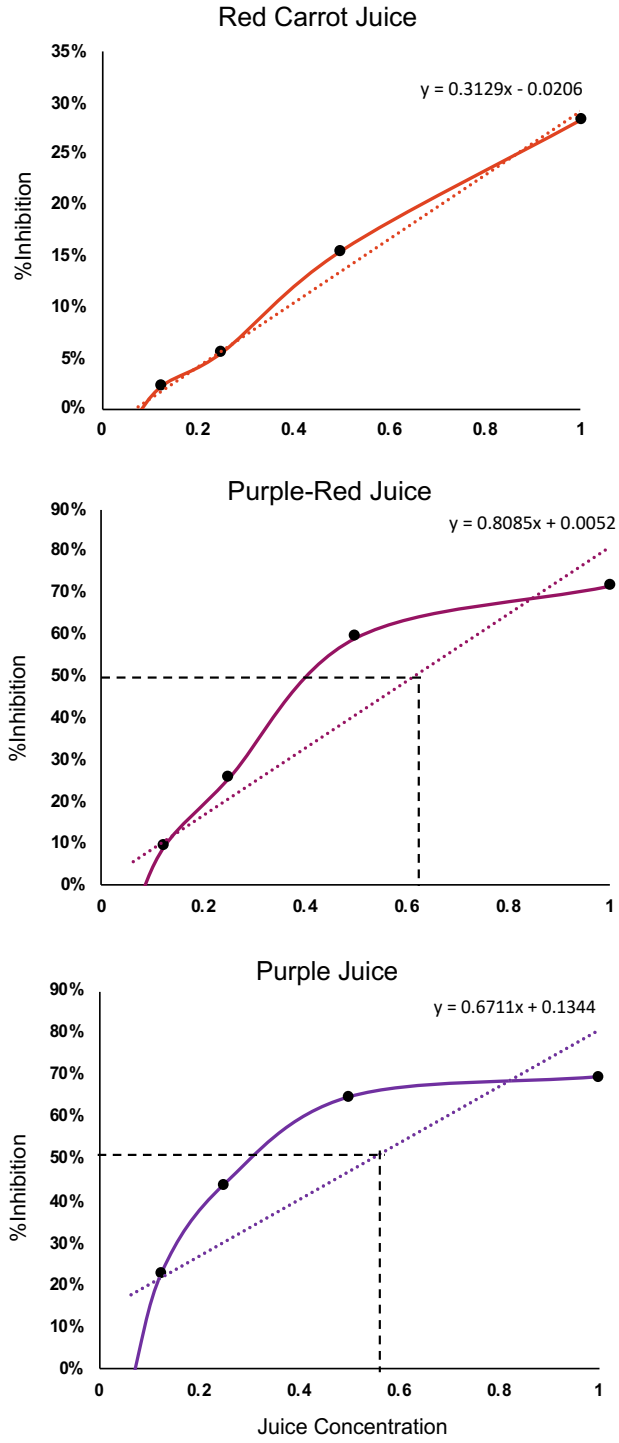


**Figure 4.** Plots of the inhibition ratio vs. sample concentration and coinciding regression lines that were used to calculate the $IC_{50}$ value of purple, purple-red, and red carrot juices assessed using DPPH (517 nm).

# CHAPTER 3:

## ANTHOCYANIN AND LYCOPENE CONTENTS DO NOT AFFECT BETA-CAROTENE BIOEFFICACY FROM MULTICOLORED CARROTS (*DAUCUS CAROTA* L.) IN MALE MONGOLIAN GERBILS

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Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

# Anthocyanin and Lycopene Contents Do Not Affect β-Carotene Bioefficacy from Multicolored Carrots (*Daucus carota* L.) in Male Mongolian Gerbils

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A B S T R A C T

**Background:** Anthocyanins and carotenoids are phytochemicals that may benefit health through provitamin A carotenoid (PAC), antioxidant, and anti-inflammatory activities. These bioactives may mitigate chronic diseases. Consumption of multiple phytochemicals may impact bioactivity in synergistic or antagonistic manners.

**Objectives:** Two studies in weanling male Mongolian gerbils assessed the relative bioefficacy of β-carotene equivalents (BCEs) to vitamin A (VA) with simultaneous consumption of the non-PAC lycopene or anthocyanins from multicolored carrots.

**Methods:** After 3-wk VA depletion, 5–6 gerbils were killed as baseline groups. The remaining gerbils were divided into 4 carrot treatment groups; the positive control group received retinyl acetate and the negative control group was given vehicle soybean oil ($n = 10$/group; $n = 60$/study). In the lycopene study, gerbils consumed feed varying in lycopene sourced from red carrots. In the anthocyanin study, gerbils consumed feed varying in anthocyanin content sourced from purple-red carrots, and positive controls received lycopene. Treatment feeds had equalized BCEs: 5.59 ± 0.96 μg/g (lycopene study) and 7.02 ± 0.39 μg/g (anthocyanin study). Controls consumed feeds without pigments. Serum, liver, and lung samples were analyzed for retinol and carotenoid concentrations using HPLC. Data were analyzed by ANOVA and Tukey's studentized range test.

**Results:** In the lycopene study, liver VA did not differ between groups (0.11 ± 0.07 μmol/g) indicating no effect of varying lycopene content. In the anthocyanin study, liver VA concentrations in the medium-to-high (0.22 ± 0.14 μmol/g) and medium-to-low anthocyanin (0.25 ± 0.07 μmol/g) groups were higher than the negative control (0.11 ± 0.07 μmol/g) ($P < 0.05$). All treatment groups maintained baseline VA concentrations (0.23 ± 0.06 μmol/g). Combining studies, serum retinol had 12% sensitivity to predict VA deficiency, defined as 0.7 μmol/L.

**Conclusions:** These gerbil studies suggested that simultaneous consumption of carotenoids and anthocyanins does not impact relative BCE bioefficacy. Breeding carrots for enhanced pigments to improve dietary intake should continue.

*Keywords*: anthocyanins, β-carotene, bioefficacy, carotenoids, carrot (*Daucus carota*), lycopene, vitamin A

## Introduction

Fruit and vegetables provide essential nutrients and complex assortments of bioactive phytochemicals, especially in multicolored carrots [1,2], which may be crucial for optimal health and protection against chronic diseases [3]. Common phytochemicals include anthocyanins and carotenoids, which likely play a significant role in purported health benefits due to antioxidant, anti-inflammatory, and anticancer properties [3,4]. Carotenoids are responsible for the red, orange, and yellow pigmentation of plants. The most prominently studied, that is, β-carotene (BC) and lycopene, are potent free radical scavengers [5]. More importantly, in the human diet, provitamin A carotenoids (PACs) (e.g., BC, α-carotene, β-cryptoxanthin) can be

*Abbreviations used:* ANC, anthocyanin; B, baseline; BC, β-carotene; BCE, β-carotene equivalents; H, high lycopene; HA, high anthocyanin; L, low lycopene; LA, low anthocyanin; Lyc, lycopene; MH, medium-to-high lycopene; MHA, medium-to-high anthocyanin; ML, medium-to-low lycopene; MLA, medium-to-low anthocyanin; PAC, provitamin A carotenoid; VA, vitamin A; VAD, vitamin A deficiency.

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cleaved during digestion resulting in 1 or 2 molecules of vitamin A (VA) [6]. VA is needed for vision, epithelial cell regeneration, immune competence, reproduction, and retinoid-target genes [7]. WHO considers VA deficiency (VAD) a paramount problem affecting an estimated 210 million children and women [8]. VAD leads to xerophthalmia, infertility, and increased morbidity and mortality. One way to combat VAD is to consume plant foods that contain PACs, which provide up to 80% of dietary VA in low- and middle-income countries [1,9]. It is essential to better understand PAC's relationship with other bioactives to inform biofortification strategies and dietary patterns to maximize health benefits.

Anthocyanins, the most abundant flavonoids in fruits and vegetables, are water-soluble phytochemicals that confer red, blue, and purple pigmentation to plants [10,11]. Anthocyanins and carotenoids released from the food matrix follow a similar digestion pathway, providing opportunities for interaction during metabolism with the majority of absorption occurring in the small intestine. Limited studies have investigated the interactions between carotenoids, especially lycopene and BC, or between anthocyanins and carotenoids. To exert health benefits, they must be bioavailable, that is, released from the food matrix, absorbed, and available to be utilized or stored. BC is bioavailable from multicolored carrots, and lycopene is bioavailable from red carrots, but the extent of interactions among anthocyanins, BC, and lycopene from different varieties of orange, red, and purple-red carrots is undetermined [1,12–16). Some evidence suggests that anthocyanins increase BC absorption and that lycopene can either increase or decrease BC absorption, but the impact that carrot-derived lycopene and anthocyanins have on BC bioaccessibility and bioavailability has not been thoroughly investigated [17–20).

Many chronic diseases stem from a combination of oxidative stress and inflammation [21]. Due to antioxidant and anti-inflammatory activities, anthocyanins have increased in dietary popularity for consumers with heightened dietary awareness because many studies link their consumption to decreased risk of chronic disease. Efforts to breed carrots focus on color through increases in anthocyanins and carotenoids. Carrots are a popular vegetable, widely available, and a great source of bioactives. Approximately 41 million tons of carrots and turnips were produced globally in 2020 [22]. Historically, multicolored carrots, such as purple-yellow and purple-white, were cultivated. Over time, a few single-colored carrots were domesticated through breeding and cultivation, for example, orange in Europe and the United States and red in India and China. In recent years, dual-colored carrots, such as purple-red varieties, have been selected to provide a more phytochemically enriched alternative to their single-colored counterparts [1,2,12,23]. In theory, the increased phytochemical content could lead to increased health benefits.

The complex array of bioactives in fruits and vegetables, when ingested simultaneously, may interact during absorption and metabolism, and it is essential to understand these interactions. The mechanisms by which gerbils absorb, convert, and store BC are similar to that of humans [12,24]. The combination of lycopene and BC from red carrots and the combination of anthocyanins and carotenoids from purple carrots were studied in Mongolian gerbils (*Meriones unguiculatus*) to determine the potential influence of lycopene on BC bioefficacy in the first study, and the influence of anthocyanins on BC bioefficacy in the second. The primary outcomes included evaluation of the serum, liver, and lung carotenoid and retinol concentrations. Secondary outcomes included the evaluation of serum, liver, and lung retinol concentrations based on initial body weights. We hypothesized that higher concentrations of lycopene would negatively impact BC absorption and the anthocyanins would protect the carotenoids during digestion and perhaps improve bioavailability.

## Methods

### Animals and study design

Mongolian gerbils ($n = 66$/study) were obtained from Charles River Laboratories. Gerbils were 28–35 and 35–42 days old in the lycopene and anthocyanin studies, respectively. Upon arrival, gerbils were weighed and pair housed in plastic cages. Initial body weights were lower in the lycopene study group (12.3–42.3; 26.3 ± 7.3 g) than in the anthocyanin study group (25.9–45.4; 33.8 ± 4.0 g). Gerbils consumed ad libitum an anthocyanin-, carotenoid-, and preformed VA-free feed with or without added carrot powder for the study duration. Gerbils were weighed daily and monitored for health until killed by exsanguination under isofluorane anesthesia. One gerbil in the lycopene study died before allocation to treatment. Blood samples were centrifuged 3000 x *g* for 15 min to isolate serum in BD Vacutainer Gel and Clot Activator tubes. The liver and lungs were excised, placed in bags, and immediately put on dry ice or submerged in liquid nitrogen. Liver samples from the anthocyanin study were fixed in formalin for histological evaluation. Prepared slides were stained with hematoxylin and eosin or Masson's trichrome to evaluate fibrosis or cirrhosis. Slides were evaluated by a trained pathologist not involved in the study, and a report was generated. Tissues were stored at −80°C until analysis. All procedures were approved by and performed in compliance with the guidelines of the Animal Care and Use Committee of the University of Wisconsin-Madison College of Agricultural and Life Sciences.

Basal-purified feeds used carotenoid-free ingredients and were VA-deficient (Teklad Custom Diet TD.06632) with carrots added to the treatment feeds (Table 1), which used a published mineral mix [36]. For the VA-depletion phases, the basal feed was consumed for 3 wk. Feed was stored at −20°C and mixed each week to maintain equal concentrations. After depletion, a baseline group was killed ($n = 5$ or 6), and tissue and serum VA concentrations were established before the commencement of treatments. In the lycopene study, gerbils with extremely high and low weights were included in the baseline group. In the anthocyanin study, the baseline group was random because the gerbils did not have extreme weights. The remaining gerbils were sorted into 6 weight-matched treatment groups ($n = 10$/group) and placed on their respective treatments for 4 wk (Table 2). For the treatment phase, freeze-dried carrot powder was added to the feed at 3.5% in the lycopene study and 1.4% in the anthocyanin study. In the lycopene study, gerbils were assigned to 1 of 4 treatments and fed high (H), medium-to-high (MH), medium-to-low (ML), or low (L) lycopene content feed with equalized BC content based on BC equivalents (BCEs), which was 5.59 ± 0.96 μg/g. In the anthocyanin study, anthocyanin content was varied between 4 groups (i.e., HA, high anthocyanin; MHA, medium-to-high anthocyanin; MLA,

**TABLE 1**
Composition of vitamin A-deficient feed used to determine the impact of lycopene and anthocyanin content on the bioefficacy of provitamin A carotenoids (α- and β-carotene) from multicolored carrots in male Mongolian gerbils[1]

| Feed component | Feed (g/kg) |
|---|---|
| Casein (vitamin-free) | 200.0 |
| L-cystine | 3.0 |
| Sucrose | 360.5 |
| Maltodextrin | 120.0 |
| Corn starch | 150.0 |
| Cottonseed oil | 60.0 |
| Cellulose | 60.0 |
| Mineral mix[2] | 35.0 |
| Magnesium oxide | 1.75 |
| Calcium phosphate, dibasic | 2.0 |
| Vitamin mix (without choline, A, D, E)[3] | 5.0 |
| Choline bitartrate | 2.5 |
| Vitamin $D_3$, cholecalciferol (500,000 IU/g) | 0.0044 |
| Vitamin E, DL-α-tocopheryl acetate (500 IU/g) | 0.242 |
| Dehydrated carrot[4,5] | Varied |

[1] Provided by Harlan Teklad, Madison, WI, USA.
[2] AIN-93M-MX (94049) mineral mix [36].
[3] The vitamin mix (83171) provided the following (feed [g/kg]): biotin, 0.08; calcium pantothenate, 13.22; folic acid, 0.4; inositol, 22.02; menadione, 9.92; niacin, 19.82; *p*-aminobenzoic acid, 22.02; pyridoxine–HCl, 4.4; riboflavin, 4.4; thiamin (81%), 4.4; vitamin $B_{12}$ (0.1% in mannitol), 5.94; ascorbic acid (97.5 %), 203.32.
[4] In the lycopene study, dehydrated carrot powder from red carrots was added as fortificant (3.5%) to the feeds fed to the 4 treatment groups, which provided 5.59 μg BCE/g feed and the mean lycopene content (nmol/g) of each treatment group's feed were as follows: high lycopene, 25.7; medium-high lycopene, 19.6; medium-to-low lycopene, 15.3; and low lycopene 9.42.
[5] In the anthocyanin study, dehydrated carrot powder from purple-red carrots was added as a fortificant (1.4%) to the feeds fed to the 4 treatment groups, which provided 7.02 μg BCE/g and 15.9 nmol lycopene/g of feed and the mean anthocyanin contents (μmol/g) of each treatment group's feed were as follows: high anthocyanin, 0.098; medium-to-high anthocyanin, 0.083; medium-to-low anthocyanin, 0.056; and low anthocyanin, 0.038.

medium-low anthocyanin; LA, low anthocyanin). In the anthocyanin study, BCE (7.02 ± 0.39 μg/g) and lycopene (15.9 ± 0.95 nmol/g) contents were equalized among the 4 treatment feeds. In both studies, when needed to maintain the amount of carrot, the white carrot was used to equalize concentrations among the feeds.

## Carrot preparation and analysis

Carrots were shipped from the University of California Desert Research and Extension Center at 2°C and refrigerated upon arrival. Each genotype was prepared separately by washing and scrubbing to remove soil, but peels were retained. In the lycopene study, carrots were cut into 1-in discs and blanched for 1.5 min in boiling water, submerged in cold water, and allowed to air dry. In the anthocyanin study, the carrot discs were blanched for 1 min to limit the amount of exposure of the anthocyanins to high temperatures but long enough to inactivate enzymes. All carrots were freeze-dried, ground to a powder, and stored at −80°C.

Fresh and freeze-dried carrots were analyzed in triplicate for carotenoid content using modified published methods [12] [Table 3). Briefly, 0.03 g powder or 0.1 g fresh carrot macerate was weighed into 50 mL glass tubes and combined with 50:50 acetone:dichloromethane and β-*apo*-8′-carotenal as an internal standard. The samples were mixed with a vortex, sonicated, and centrifuged (3000 x *g*). The supernatants were filtered into 25-mL volumetric flasks, and the remaining sample was extracted 3 more times with 50:50 acetone:dichloromethane. From the collected supernatants, 2 mL aliquots were dried under nitrogen and reconstituted in 50:50 methanol:dichloromethane. Each sample was analyzed on HPLC. HPLC-purified standards and absorption spectra were used for identification. The lycopene from the carrots is approximately 98% in the all-*trans* configuration.

Anthocyanins were assessed in triplicate in fresh and freeze-dried samples using a modified method [25] (Table 2). Briefly, 1 g fresh carrot macerate was placed in a 50-mL test tube wrapped in foil; 10 mL 90:10 methanol:formic acid was added. The tubes were capped, mixed for 1 min, sonicated for 10 min, and centrifuged for 10 min at 3000 x *g*. The supernatant was collected into 40-mL brown glass vials. Two more extractions were conducted with 15 mL 90:10 methanol:formic acid. From the combined supernatants, 1 mL aliquots were poured into a 10-mL brown glass vial and mixed with 3 mL water. A 500 μL aliquot of the diluted sample was used for analysis.

Freeze-dried carrot powder (0.1 g) was weighed into 5-mL brown glass vials, and 1 mL 90:10 methanol:formic acid was added. The samples were mixed for 2 min and centrifuged for 5 min at 3000 x *g*. The supernatant was pipetted into a 5-mL brown glass vial, the extraction was repeated, and supernatants were combined. A 200 μL aliquot was taken in a 10-mL brown glass vial, diluted with 600 μL water, and mixed by vortex for 30 sec.

Aliquots of the diluted samples were injected and analyzed on a Waters HPLC with a Zorbax SB-C18 column (5 μm, 250 mm × 4.6 mm), and guard cartridge (Agilent Technologies). The solvent system consisted of 10% aqueous formic acid (solvent A) and methanol (solvent B). The gradient was linear from 5% to 55% solvent B over 20 min, then 100% solvent B for 5 min to flush the column, and back to 5% solvent B for 10 min for equilibration. The flow rate was 1 mL/min, and the injection volume was 50 μL.

## Feed analysis

Feeds were analyzed for carotenoid concentrations using a modified method in triplicate [12]. Briefly, ~0.5 g was weighed, combined with ethanol (0.1% butylated hydroxytoluene (BHT) as an antioxidant), mixed by vortex, and placed into a hot water bath (85°C) for 5 min. Samples were removed and 500 μL 80% KOH:$H_2O$ was added. After mixing, the samples were placed into the bath, mixed, and heated for 5 min. At completion, pure water and β-*apo*-8′-carotenal as an internal standard were added. Samples were mixed and extracted 3 times with hexanes. Extracts were combined, dried under nitrogen, reconstituted in 500 μL 50:50 dichloroethane:methanol, and 50 μL was injected into an HPLC system (Waters Corporation). HPLC-purified standards were used for quantification.

For anthocyanin analysis, 1.5 g feed was placed into 15 mL glass test tubes. Each sample was extracted 3 times with 4 mL, 3 mL, and 3 mL methanol:formic acid (90:10, v:v), mixed for 1 min, and centrifuged for 8 min at 3000 x *g*. The supernatants were transferred into 10 mL brown glass vials and kept on ice. A 700 μL aliquot was dried under nitrogen and reconstituted in 100

**TABLE 2**
Treatment feeds consumed for 28 d by male Mongolian gerbils in 2 studies. Gerbils were fed a provitamin A carotenoid (PAC), lycopene, and anthocyanin-free feed for 21 d before treatments and baseline measurements [1]

| **Lycopene study** | Treatments | | | Controls | | |
|---|---|---|---|---|---|---|
| Group *(n)* | 1 (10) | 2 (10) | 3 (10) | 4 (10) | Positive (10) | Negative (10) |
| Treatment feed[2] | | | | | | |
| PAC[3] | + | + | + | + | — | — |
| Lycopene | H | MH | ML | L | — | — |
| (nmol/g) | 25.7 ± 5.88 | 19.6 ± 2.78 | 15.3 ± 4.11 | 9.42 ± 2.67 | — | — |
| Oil dose | Vehicle | Vehicle | Vehicle | Vehicle | VA[4] + | Vehicle |
| **Anthocyanin study** | | | | | | |
| Group *(n)* | 1 (10) | 2 (10) | 3 (10) | 4 (10) | Positive (10) | Negative (10) |
| Treatment feed[2] | | | | | | |
| PAC[3] | + | + | + | + | — | — |
| Lycopene[4] | + | + | + | + | — | — |
| Anthocyanins | HA | MHA | MLA | LA | — | — |
| (μmol/g) | 0.098 ± 0.18 | 0.083 ± 0.19 | 0.056 ± 0.15 | 0.038 ± 0.005 | — | — |
| Oil dose | Vehicle | Vehicle | Vehicle | Vehicle | VA[5] +/Lyc[6] + | Vehicle |

H, high lycopene; HA, high anthocyanin; L, low lycopene; LA, low anthocyanin; Lyc, lycopene; MH, medium-to-high lycopene; MHA, medium-to-high anthocyanin; ML, medium-to-low lycopene; MLA, medium-to-low anthocyanin; PAC, provitamin A carotenoid; VA, vitamin A

[1] Baseline group measurements were taken in $n = 5$ and 6 for the lycopene and anthocyanin studies, respectively.

[2] Treatment feeds fortified with freeze-dried red (the lycopene study) and purple-red carrot (the anthocyanin study).

[3] Equalized as BCE = β-carotene equivalents, 1 μg = 1 μg β-carotene or 2 μg α-carotene. BCE content of the 4 treatment feeds was 5.59 ± 0.96 μg/g in the lycopene study and 7.02 ± 0.39 μg/g in the anthocyanin study.

[4] Lycopene content was 15.9 ± 0.95 nmol/g.

[5] Provided as retinyl acetate in vehicle soybean oil, equalized to theoretical VA intake the prior day. The retinyl acetate concentrations were 0.874 and 2.03 nmol/μL for the lycopene and anthocyanin studies, respectively.

[6] Provided as purified lycopene extracted from commercial supplements and dissolved in vehicle soybean oil with a final concentration of 2.19 nmol/μL.

**TABLE 3**
Concentrations of α-carotene, β-carotene, lycopene, and anthocyanins in fresh and lyophilized carrots that were incorporated into Mongolian gerbil feed

| Carrot | α-Carotene | β-Carotene | Lycopene | Anthocyanins |
|---|---|---|---|---|
| Fresh red, nmol/g | 12.6 ± 8.61 | 76.8 ± 29.5 | 186 ± 91.2 | — |
| Lyophilized red, nmol/g | 49.3 ± 20.2 | 362 ± 135 | 570. ± 261 | — |
| Fresh purple, nmol/g | 17.8 ± 8.68 | 50.2 ± 19.5 | 69.2 ± 38.2 | 61.3 ± 56.1 |
| Lyophilized purple, nmol/g | 104 ± 70.9 | 332 ± 89.8 | 533 ± 166 | 7480 ± 5950 |

Values are mean ± SD, $n = 3$

μL of 1:3 methanolic formic acid:water. Samples were injected onto the HPLC using the same conditions as for carrots.

## Oil doses

In the lycopene study, the positive control dose was solely retinyl acetate for comparison with the BC intake from the red carrots. For the anthocyanin study, a mixed supplement was prepared that contained retinyl acetate (Sigma–Aldrich) and lycopene dissolved in soybean oil, which were first quantified separately by spectrophotometry before mixing. Five 20 mg lycopene capsules (Whole Foods tomato sourced gelatin capsules) were dissolved in dichloromethane and concentrated by rotary evaporation. The lycopene was purified on an open column with 2% water-deactivated alumina and eluted with hexanes. To ensure that the resulting supplement was predominantly in the all-*trans* configuration (>95%), the lycopene fractions that had the all-*trans* spectra were combined and evaporated, soybean oil was added, and the remaining hexanes were removed under vacuum. The working solutions were sonicated to dissolve all crystals and prepared to administer approximately 35 nmol VA and 54 nmol lycopene each day by mixing 2.87 mL lycopene and 1.72 mL VA oils. The supplements were kept at −20°C. The positive control group represented the intake of the treatment groups by calculating how much was consumed by the 4 carrot treatment feeds the previous day. All other gerbils were administered a dose of soybean oil in the same amount as the supplemental oil to eliminate dietary influences. The gerbils were dosed daily with 29–43 μL/d in the lycopene study and 46–58 μL/d in the anthocyanin study.

## Serum and tissues

Modified procedures were used for VA and carotenoid analyses of serum and tissues [12,13]. For all samples, C-23 β-*apo*-carotenol was added as an internal standard to determine extraction efficiency. Briefly, serum (500 μL) was denatured with ethanol (0.1% BHT) and extracted with hexane 4 times (2 mL and 3 × 1 mL). Supernatants were pooled, dried under nitrogen, and reconstituted in 200 μL 75:25 methanol:dichloroethane. For tissues, 0.5

g liver and 0.1 g lung were weighed, ground with anhydrous sodium sulfate, and extracted with dichloromethane into 50-mL and 25-mL volumetric flasks, respectively. Liver extract (5 mL) was dried under nitrogen and mixed with ethanol (0.1% BHT) and 50:50 KOH:$H_2O$ for saponification at 45°C for 1 h; 1 mL water was added and 2 mL hexanes (3×) was used for extraction. The extract was dried and reconstituted in 150 μL 75:25 methanol:dichloroethane. For the lung, 1 mL aliquots were dried under nitrogen and reconstituted in 100 μL 75:25 methanol:dichloroethane. Serum and tissue extracts were run on a Waters UPLC system.

## Statistical analysis and calculations

Data are presented as means (± SD) and analyzed using Statistical Analysis System Studio software (SAS Institute Inc., 3.7 Enterprise Edition). ANOVA using the PROC GLM command and Tukey's studentized range test were used at $\alpha < 0.05$ to evaluate all outcomes of interest in all groups including the baseline concentrations. Effect sizes were determined using the $Eta^2$ test where values > 0.14 are considered large. Data were considered significant at $P < 0.05$. Pearson correlation was used to compare 2 variables of interest. Grubb's test was used to evaluate outliers. Specificity and sensitivity of serum retinol concentrations were determined relative to liver VA concentrations, using the following equations:

$$\% \text{ Specificity} = [\text{True Negative} / (\text{False Positive} + \text{True Negative})] \times 100$$

$$\% \text{ Sensitivity} = [\text{True Positive} / (\text{False Negative} + \text{True Positive})] \times 100$$

The serum retinol concentration to define VA deficiency was 0.7 μmol/L as recommended by WHO [26]. A liver VA concentration of 0.10 μmol/g was used as the reference test for VA deficiency. *t*-Tests were applied when testing combined data above and below reference values. This value was suggested by an expert panel [7] and recently reviewed against published biological evidence [27].

# Results

## Gerbil weights and intakes

In both studies, final body weights did not differ between groups, that is, 73.8 ± 8.5 and 73.9 ± 5.8 in the lycopene and anthocyanin studies, respectively. The starting body weights in the lycopene study were low (26.1 ± 7.4 g); therefore, gerbils with a range of weights at baseline kill were selected (56.6 ± 20.3 g). In the anthocyanin study, the initial weights were similar to previous studies (33.8 ± 4.0 g) and the baseline group's body weight was 62.7 ± 4.1 g. Daily feed intake was not different between groups. In the lycopene study, intake was 4.9 ± 0.5 g/d per gerbil in treatment groups and 5.1 ± 0.5 g/d in controls. In the anthocyanin study, intake was 4.7 ± 0.4 g/d in treatment groups and 4.3 ± 0.4 g/d in controls.

Over the 28-day treatment period in the lycopene study, each gerbil consumed 817 ± 35.0 μg BCE. During the anthocyanin study, each gerbil consumed 924 ± 51.5 μg BCE and 2090 ± 125 nmol lycopene. The carrot-treated groups, from HA to LA, consumed 12.9 ± 2.4, 10.9 ± 2.6, 7.4 ± 1.8, and 5.0 ± 0.6 μmol anthocyanins.

## Liver and lung lycopene, β-carotene, and retinol concentrations

Total liver lycopene and lycopene/g liver were significantly different between the treatment groups and negative controls in both studies. In the lycopene study, all 4 treatment group's liver lycopene concentrations significantly differed from the baseline, positive control, and negative control groups ($P \leq 0.05$) (Figure 1A). The effect size for lycopene concentration and total lycopene were ≥0.70. Total liver VA content and liver retinol concentrations did not differ between groups (Figure 1B and **C**). All carrot-fed groups did not differ from the positive control. Large ranges were observed in hepatic VA, and almost two-thirds of the gerbils were VA deficient (≤0.1 μmol/g) at the end of the study between groups. The unanticipated range in starting weights in the lycopene study was significantly correlated to liver VA concentrations (Figure 2A). The gerbils received were smaller than those in prior studies using the same age range.

In the lycopene study, lung retinol concentrations did not differ from the positive control but all treatment groups were higher than the negative control group ($P < 0.05$) (Supplemental Figure 1). The retinol concentration of the lycopene treatment groups was higher than the baseline ($P < 0.05$), and the effect size was 0.35. Total lung retinol was maintained from baseline in all treatment groups ($P = 0.011$). Lycopene was not detected in the lung tissue. BC was not detected in the liver or the lungs, likely due to bioconversion to retinol to meet requirements. Like the liver, lung retinol also significantly correlated with starting weight in the lycopene study (Figure 2B). Unlike the liver and the lungs, serum retinol concentrations were not correlated with starting body weight in either study (Figure 2C). Interestingly, lung retinol (0.53 ± 0.14 μmol/g) and liver lycopene concentrations (40.7 ± 19.1 μmol/g) were significantly correlated ($r = 0.282$; $P = 0.023$), but the correlation only trended to significance when adjusted for total tissue concentration of retinol (0.18 ± 0.06 μmol) and lycopene (116 ± 58.1 μmol) ($r = 0.217$; $P = 0.082$).

In the anthocyanin study, all purple-red carrot groups and the positive control group had significantly higher liver lycopene concentrations than baseline and negative control groups ($P < 0.05$) (Figure 3A), and the effect size was 0.60. Total liver retinol content and concentrations in the purple-red carrot groups did not differ from the positive control (Figure 3B and **C**). However, total liver retinol was significantly higher in the HA and MLA groups than in the negative control group, which resulted in an effect size of 0.30.

In the anthocyanin study, no differences in lung retinol concentration or total retinol were noted between groups, but concentrations were generally higher than in the lycopene study. In the anthocyanin study, total lung retinol concentration (0.23 ± 0.08 μmol) correlated with total liver lycopene (28.2 ± 12.7 μmol) ($r = 0.296$; $P = 0.016$) but was not correlated by retinol concentration (0.66 ± 0.20 μmol/g lung) or liver (10.1 ± 3.72 μmol/g) ($r = 0.186$; $P = 0.13$). In both studies, lung retinol concentration was correlated to liver retinol concentration ($P < 0.0001$ in the lycopene study and $P = 0.048$ in the anthocyanin study) (Figure 4).

In the histological report from the evaluation of the livers ($n = 4$/group) fixed in formalin, liver retinol concentrations were significantly lower (0.14 ± 0.07 μmol/g; $P = 0.027$) in gerbils that were experiencing degeneration consistent with cirrhosis than in those that were not (0.22 ± 0.13 μmol/g). However, none of the livers had moderate or severe increases in fibrosis as noted in the pathologist's report.

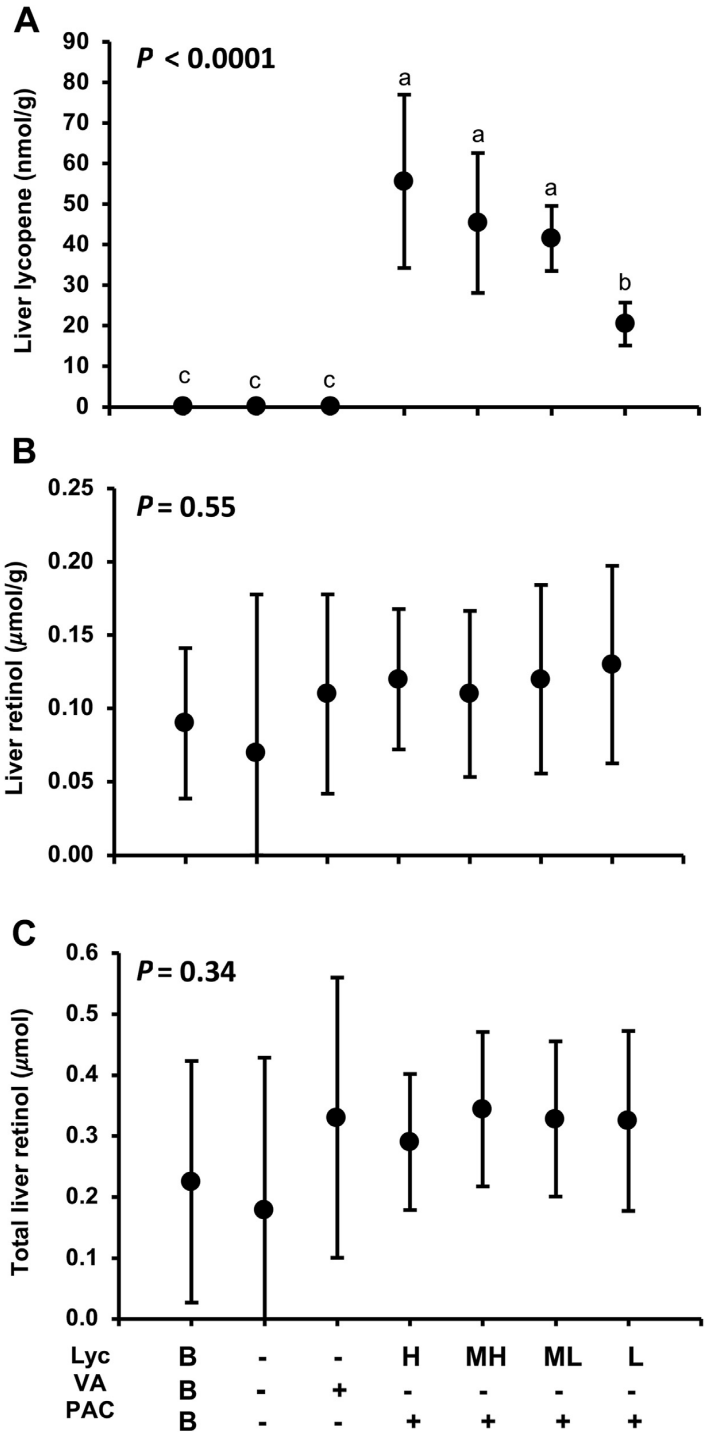


**FIGURE 1.** Final liver lycopene (A), liver retinol concentrations per gram of the tissue (B), and total liver content (C) for the lycopene study in male Mongolian gerbils that consumed treatment feeds consisting of H, MH, ML, and L concentrations of lycopene that were equalized in PACs, all sourced from red carrots (Lycopene study). The positive control was dosed with preformed vitamin A in soybean oil. All values are group means ± SD ($n$ = 10/group; baseline $n$ = 5) and groups labeled with different letters were significantly different ($P < 0.05$). H, high lycopene; L, low lycopene; Lyc, lycopene; MH, medium-high lycopene; ML, medium-low lycopene; PAC, provitamin A carotenoids; VA, vitamin A.

## Serum concentrations

In the lycopene study, there was an outlier in the L group with an extreme serum retinol concentration (7.7 μmol/L) that was detected by Grubb's test ($P < 0.0001$), which had a significant influence on the model. With the outlier included, there was no difference in serum retinol concentration between groups. However, when the outlier was removed, there was a significant difference between the negative control group and the MH lycopene group, and the positive control group, whereas the H and ML lycopene groups trended toward being different. In the

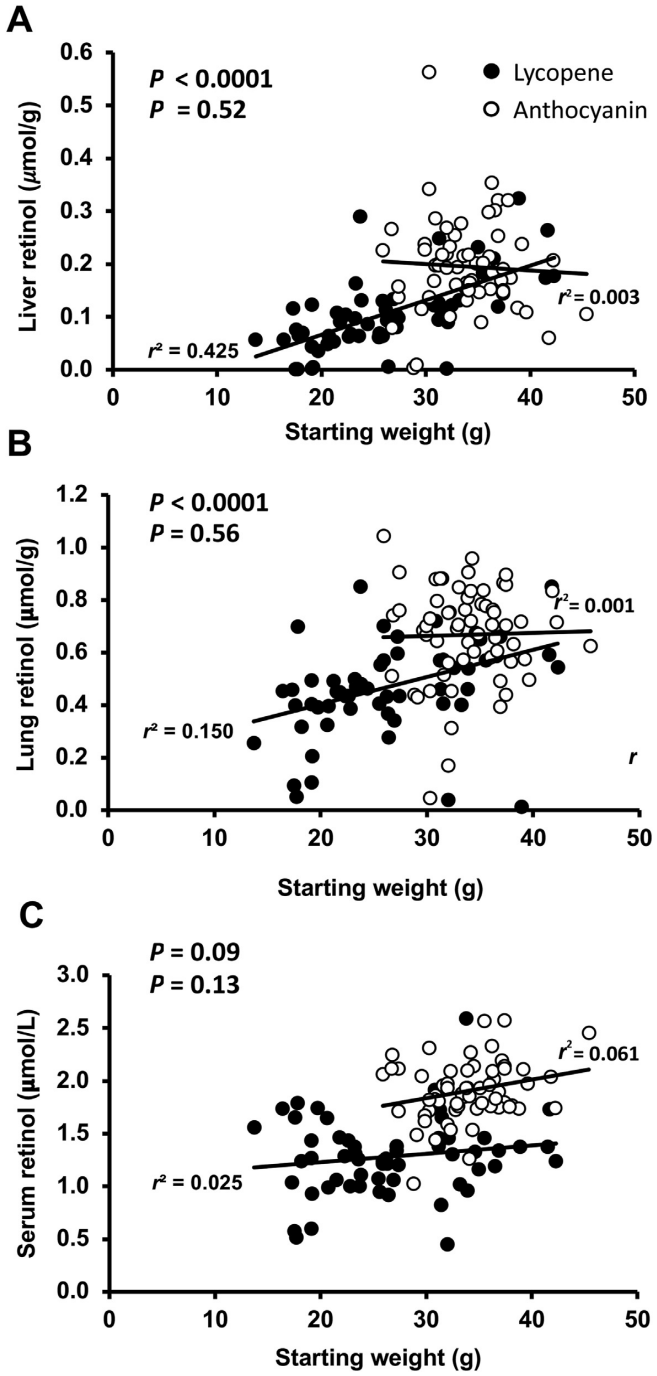


**FIGURE 2.** Association between liver retinol concentrations (µmol/g) (A) and lung retinol concentrations (µmol/g) (B) versus the starting weight of male Mongolian gerbils (g) in the lycopene study ($P < 0.05$) and the anthocyanin study (NS). Serum retinol concentrations did not correlate with starting body weights in either study (C).

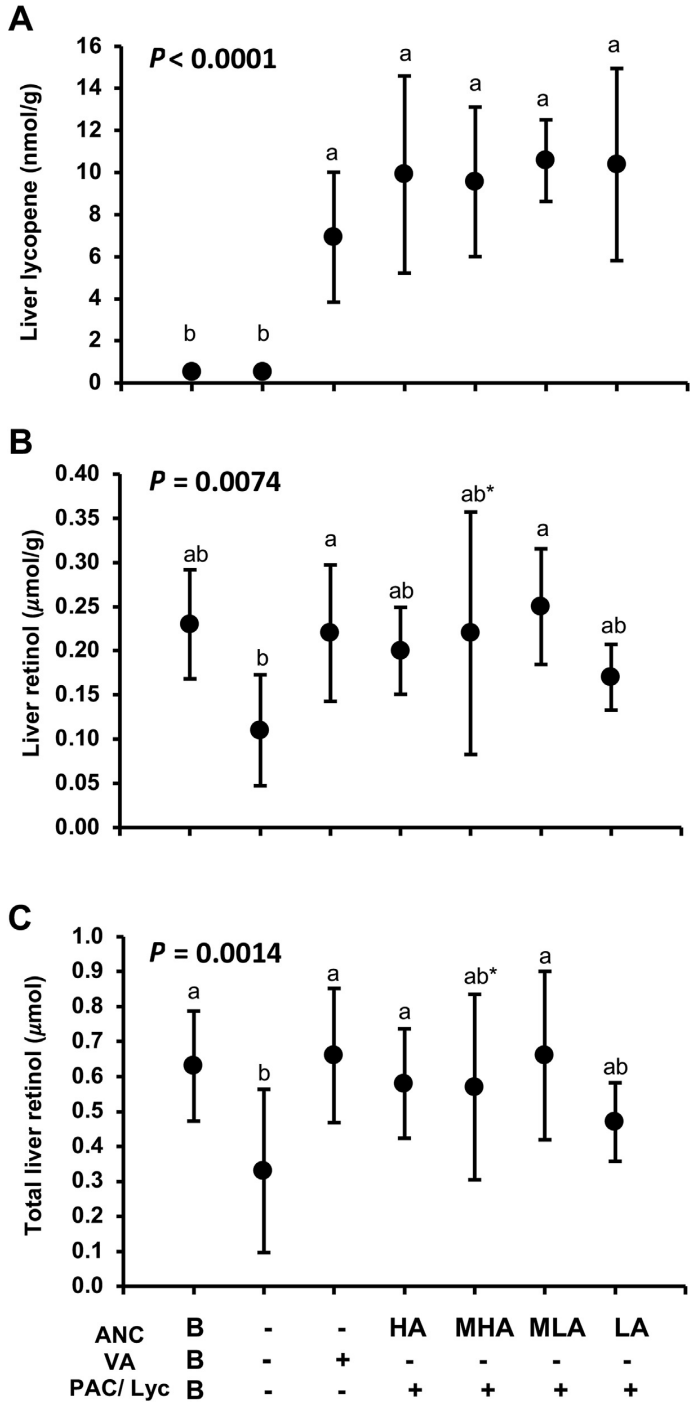


**FIGURE 3.** Final liver lycopene (A), liver retinol concentrations per gram of the tissue (B), and total liver retinol content (C) for the anthocyanin study in male Mongolian gerbils that consumed treatment feeds consisting of HA, MHA, MLA, and LA concentrations of anthocyanins. Feeds were equalized in PACs and lycopene, all sourced from purple-red carrots. The positive control was dosed with preformed vitamin A in soybean oil. The * denotes $P = 0.057$ and 0.053 for liver retinol concentration and total retinol being higher than the negative control group. All values are group means ± SD and groups labeled with different letters were significantly different ($P < 0.05$). ANC, anthocyanin; HA, high anthocyanin; LA, low anthocyanin; Lyc, lycopene; MHA, medium-high anthocyanin; MLA, medium-low anthocyanin; PAC, provitamin A carotenoids; VA, vitamin A.

anthocyanin study, serum retinol concentrations did not differ between groups. Serum carotenoids were not detected in either study.

Liver VA reserves are the reference standard for assessing VA status, but for ethical reasons, serum retinol and retinol-binding protein concentrations are commonly accessible biomarkers. In the lycopene study, nearly half of the gerbils had deficient liver VA stores (≤0.10 μmol/g), but only 4 gerbils had serum retinol concentrations indicating VA deficiency (<0.70 μmol/L). These 4 gerbils had severely low liver reserves of 0.0020 ± 0.00092

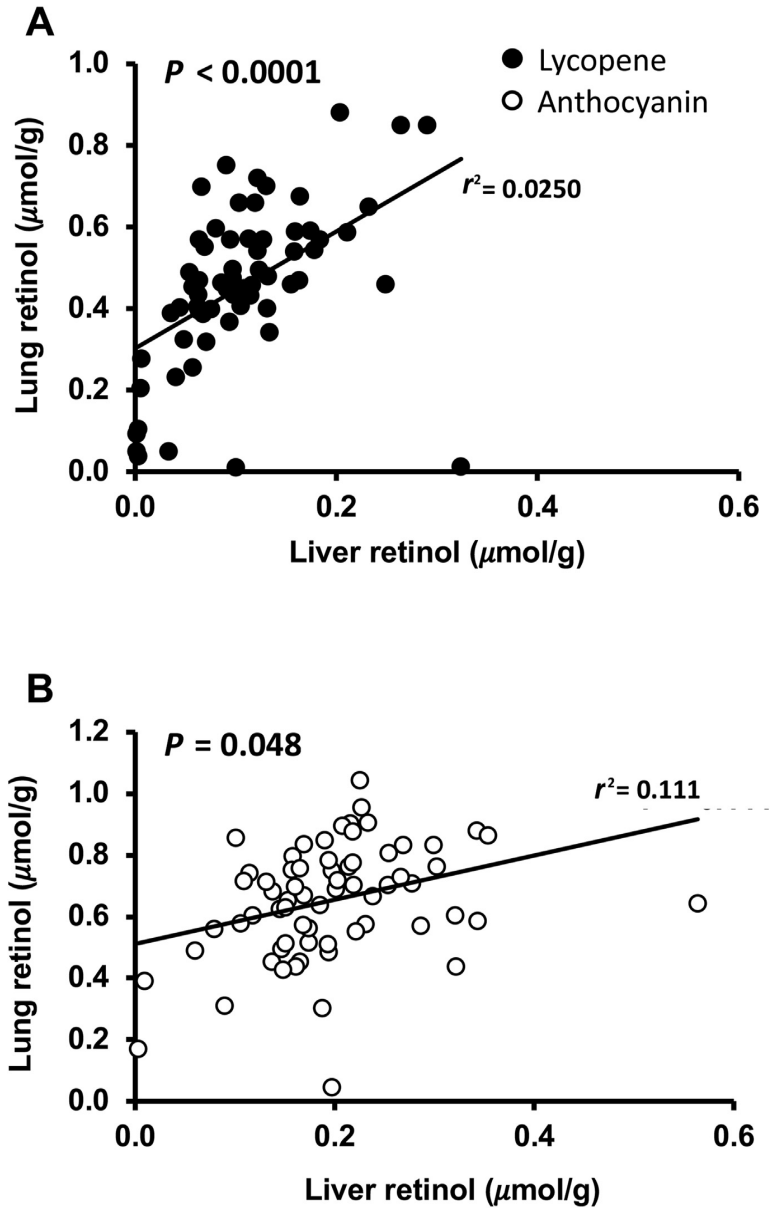


**FIGURE 4.** Lung retinol concentrations significantly correlated to liver retinol concentrations in the lycopene study (A) where treatments to male Mongolian gerbils include a variety of red carrots and the anthocyanin study (B) where treatments to male Mongolian gerbils included a variety of purple-red carrots. Liver retinol concentrations were generally lower in the lycopene study.

μmol/g (Figure 5). In the anthocyanin study, 6 gerbils were VA deficient by liver reserves but none of them had deficient serum retinol concentrations (Figure 5).

Of the paired serum and liver samples from both studies ($n$ = 129), there were 29 false negative, 97 true negative, 4 true positive, and 0 false positive cases. The sensitivity of serum retinol to predict VAD was calculated to be 12% [4/(29 ± 4)], indicating that out of the samples in which the liver VA indicated VAD, 12% of the samples had serum retinol concentrations correctly indicating VAD (true positive), whereas 88% had serum retinol concentrations that did not detect VAD (false negative). The specificity of serum retinol was 100% [97/(0 ± 97)], meaning that 100% of the gerbils that had liver reserves above the VAD cutoff (0.1 μmol/g) also had serum retinol concentrations above the VAD cutoff (0.7 μmol/L) (true negatives). In the VA-adequate gerbils from both studies, that is, >0.1 μmol retinol/g liver, serum retinol concentrations were significantly lower in gerbils from the lycopene study ($n$ = 28, 1.33 ± 0.35 μmol/L) than the anthocyanin study ($n$ = 60, 1.94 ± 0.62 μmol/L) ($P$ < 0.0001).

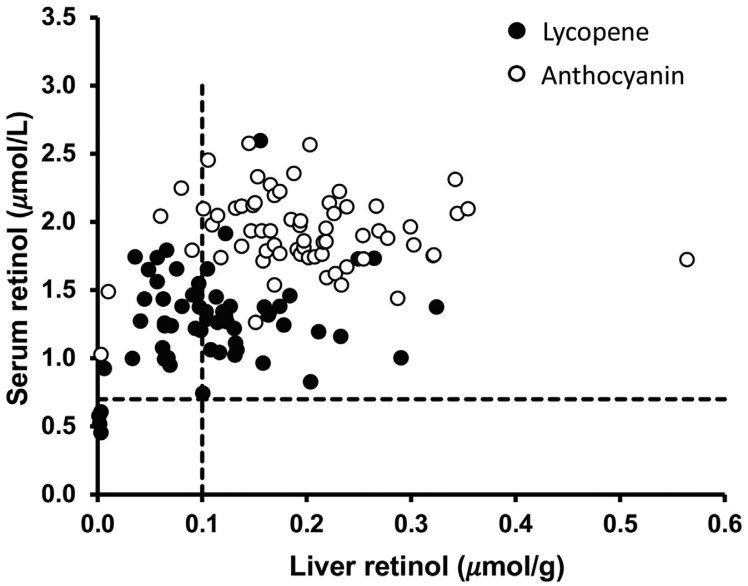


**FIGURE 5.** Comparison of vitamin A deficiency (VAD) based on serum and liver retinol concentrations in 129 male Mongolian gerbils fed a variety of feeds and supplements. The vertical dashed line represents the cutoff for VAD based on 0.10 μmol/g liver. The horizontal line represents the cutoff for VAD based on a serum retinol concentration of 0.70 μmol/L. Data in the top right quadrant are considered to be adequate VA status by both serum and liver retinol concentrations (96 true negative), whereas the ones in the upper left quadrant are considered adequate by serum retinol but deficient by liver retinol concentrations (30 false negative). In the lower left quadrant, the data points are considered deficient by both serum and liver concentrations (4 true positive), whereas those in the lower right quadrant are considered deficient by serum retinol but not by liver retinol concentrations (0 false negative). VAD, vitamin A deficiency.

## Discussion

Consistent evidence suggests that the consumption of fruits and vegetables decreases the risk of developing chronic diseases, some of which are associated with plant phytochemicals [28–30]. These studies created feeds with multicolored carrots with differing amounts of either lycopene or anthocyanins to assess the impact of simultaneous consumption of lycopene or anthocyanins with PACs on BCE bioefficacy. All groups maintained VA status from a basal state by consuming feeds with carrots as the sole source of VA indicating that BC is bioavailable from red and purple-red carrots with minimal effects of lycopene. A prior study demonstrated reduced lycopene bioavailability from red carrot compared with red tomato paste, which did not have a high BC concentration [13].

In the lycopene study, liver VA concentrations did not differ between groups, but there were large ranges in hepatic VA, and almost two-thirds of the gerbils were deficient (≤0.10 μmol/g). Some gerbils were deficient in every group, including the positive control. This may have been due to the unanticipated range in starting weights, which were significantly correlated to liver VA concentrations at the end of the study. In the anthocyanin study, all groups maintained baseline VA concentrations and total liver content, which suggests that anthocyanins do not have a negative impact on BCE bioavailability and bioefficacy nor a positive impact at high concentrations as hypothesized. These findings are similar to studies conducted by Mills et al. [14] and Arscott et al. [1]. Mills et al. measured the antioxidant potential and VA bioefficacy in Mongolian gerbils of 4 biofortified carrot varieties, including prototype purple-red carrots [14]. Neither the presence of lycopene nor anthocyanins from multicolored carrots influenced the bioconversion of PACs to VA. Arscott et al. found that in women who were fed smoothies containing anthocyanins and carotenoids from purple-orange carrots [1], anthocyanins did not affect the relative bioavailability of BC following an acute feeding. At the time of that study, purple-red carrots were still undergoing breeding to enhance lycopene content. Phan et al. reported an increase in cellular BC absorption when co-present with anthocyanins in isolated Caco-2 cells [19], which may not directly translate to humans.

In the lycopene study, liver lycopene concentrations of all 4 red carrot-treated groups differed from baseline and control groups, which did not receive lycopene. Interestingly, as dietary lycopene increased, the liver lycopene increased reflecting relative intakes, which confirms the differences between the feeds. In the anthocyanin study, lycopene concentrations did not differ between the carrot-fed groups, which confirmed the equalized feed content. These data indicate that lycopene absorption was likely not impacted by the presence of BC in the lycopene study or anthocyanins in the anthocyanin study. Other studies have reported similar findings in that lycopene content did not impact BC bioavailability and bioefficacy. Mills et al. reported in 2 different studies that co-consumption of lycopene and BC in gerbils had no impact on BC bioavailability [13,14] but may have led to a decrease in lycopene bioavailability. In a double-blind study evaluated by area-under-response curve, Johnson et al. reported no significant impact of lycopene on BC but saw an increase in lycopene bioavailability in 10 adult males when 60 mg doses were consumed individually or combined [20]. On the other hand, no BC was detected in the tissues in either of the current studies, indicating that all absorbed BC was metabolized to meet VA requirements.

In both studies, lung VA was correlated with liver VA. The lung contains VA-storing cells similar to hepatic stellate cells [31], and VA is delivered to the lungs by chylomicra [32]. Vitamin A is essential for development both pre- and postnatally [33]. In humans, lung differentiation occurs mainly during the final trimester of pregnancy and continues throughout the first year of life, whereas in rats, this phase does not begin until postpartum, and alveolar structures slowly develop through life [31]. Gerbils likely develop similarly. Comparable to humans, dams pass retinol to rat fetuses in the last third of pregnancy [34]. Although VA

accumulates in the liver in the final period before birth, physiological levels of lung and liver retinol are low at birth; however, plasma is not necessarily low in offspring of VA-adequate mothers [33]. It could be that the mothers in the lycopene study were not VA-adequate causing extremely low liver reserves at birth.

Human neonates postpartum have little tissue VA stores to draw from, suggesting that to build reserves, they are highly dependent on getting VA from their mother's milk, supplements, or food [33]. In rats, retinol stores start to accumulate in the liver after the postnatal 21-day weaning period. This may explain why the small gerbils in the lycopene study had low liver VA stores at baseline. It is possible that they may not have been properly weaned or had not reached the stage of VA accumulation. This may also explain why starting weight was significantly correlated with lung VA, especially in those that started less than 25 g upon arrival. Postweaning, a steady input of VA from the diet is necessary to accumulate and maintain lung retinol. The significant correlation between liver and lung VA in both studies indicates the importance of VA intake postweaning. It is likely that the small gerbils that may have been in their weaning period were consistently using dietary VA for growth and development and did not accumulate tissue stores.

Serum retinol concentrations are under homeostatic control and the current cutoff for VAD is 0.7 μmol/L [7,26]. Although serum retinol is a common method for assessing VA status in humans, it is not a good measure of total liver VA until liver stores are extremely low. Even during VAD, if the animals or humans are without infection and remain otherwise healthy, adequate serum retinol concentration is maintained through increased enterohepatic recycling [27]. This was likely the case in the lycopene study. There were no clinical signs that these gerbils were VA deficient, such as reduced body weight at kill. If serum retinol concentration was the only biomarker assessed, almost all of the gerbils appeared as though they had sufficient VA status when in fact many had depleted liver stores. Poor sensitivity of serum retinol indicates that VA-deficient individuals may be misdiagnosed, supporting previous claims that serum retinol has limited utility as a predictor of VA status. The difference in serum retinol concentrations of the VA-adequate gerbils from both studies indicates that the gerbils in the lycopene study may have had a lower serum retinol set-point due to the initial VA deficiency.

Although a limitation, the unanticipated wide range of starting weights in the lycopene study likely influenced serum and tissue retinol. At the time the gerbils arrived, according to their growth curve, they should have weighed 28–35 g [35], but almost half of them were <28 g, with the smallest weighing 12.6 g. A strong correlation was observed between the starting body weights and final liver retinol concentrations. In the anthocyanin study, all gerbils had typical starting weights, and there was no correlation between serum retinol and liver retinol concentrations, or between starting weight and liver retinol concentration. Both studies add more evidence that serum retinol concentrations have limited utility as predictors of VA status.

Red and purple-red carrots may be considered novel functional foods that provide combinations of different bioactive compounds, such as anthocyanins and carotenoids, which are linked to optimal human health. Future studies should address the relationship of lycopene on BC relative bioavailability in humans from purple-red carrots as well as evaluate the reverse relationship to get a more concise understanding of the impact BC has on lycopene bioavailability. To our knowledge, the anthocyanin study is the first to assess the impact of anthocyanins on the bioavailability of BC from a single food source, that is, purple-red carrots, in an in vivo model. The results from the second study suggest that simultaneous consumption of carotenoids and anthocyanins does not negatively impact the bioefficacy of BC.

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## Author disclosures

The authors report no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2022.10.010.

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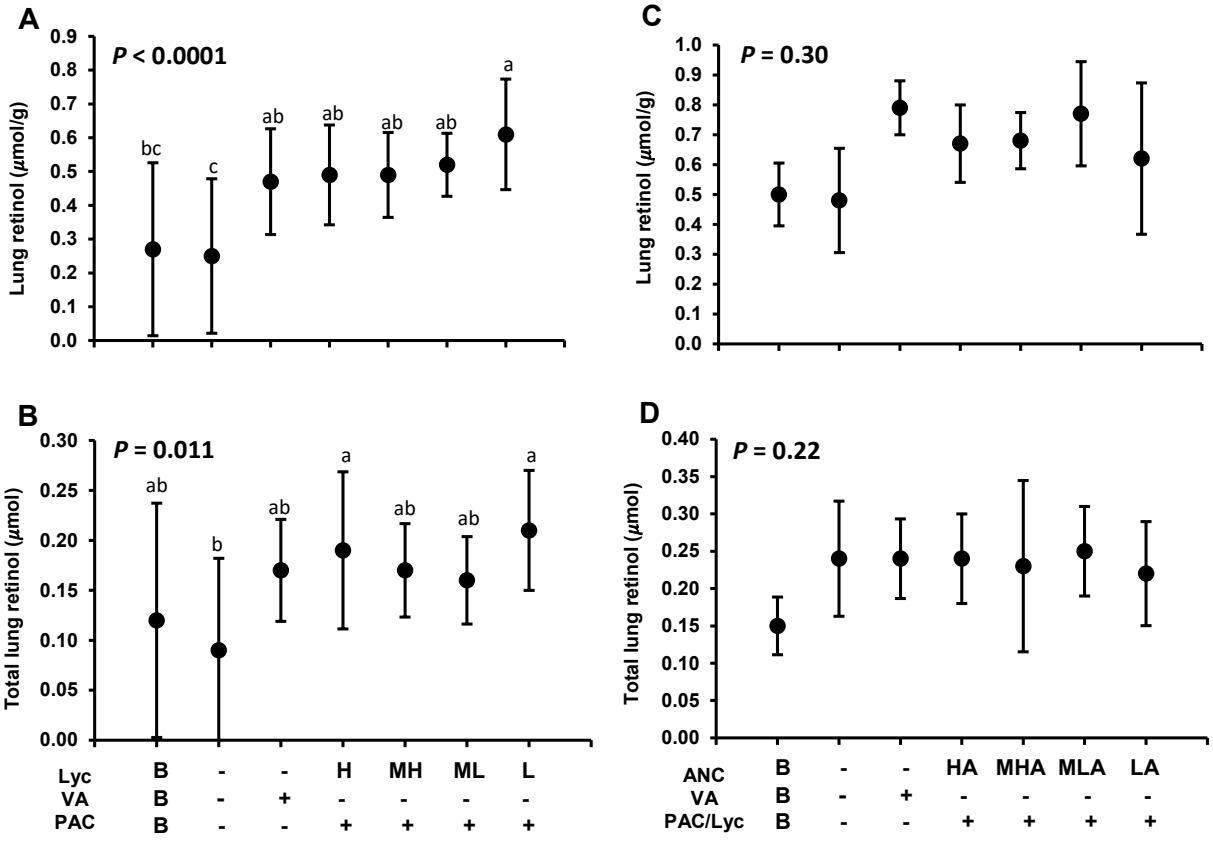


**Supplemental Figure 1.** Final lung retinol concentrations per g tissue and total lung retinol for Study 1 (A, B) and Study 2 (C, D) in gerbils after consuming treatment feeds consisting of H, MH, ML, and L concentrations of lycopene, or anthocyanins. Treatment diets were equalized in provitamin A carotenoids (PAC) in both studies, and lycopene was also equalized in the second study. During the treatment period, the positive control group in both studies received doses of preformed vitamin A (VA) in soybean oil. All values are group means + SD and groups labeled with different letters were significantly different ($P < 0.05$). B, baseline; H, high lycopene; HA, high anthocyanin; L, low lycopene; LA, low anthocyanin; Lyc, lycopene; MH, medium-high lycopene; MHA, medium-high anthocyanin; ML, medium-low lycopene; MLA, medium-low anthocyanin; PAC, provitamin A carotenoids; VA, vitamin A.

# CHAPTER 4:

# *β*-CAROTENE AND LYCOPENE ARE BIOAVAILABLE IN HUMANS FROM JUICE MADE FROM PURPLE-RED MULTICOLORED CARROTS (*DAUCUS CAROTA* L.) COMPARABLE TO THAT OF JUICE MADE FROM RED AND PURPLE SINGLE-COLORED VARIETIES

Mikayla S. Kaeppler

## Abstract

**BACKGROUND:** Purple-red carrots contain the provitamin A carotenoids α-carotene (**aC**) and β-carotene (**BC**) and the non-provitamin A carotenoid lycopene, in addition to anthocyanins. The combination of the plant pigments in this multicolored variety may provide additional health benefits compared to the single-colored red and purple varieties. In contrast, the combination of pigments may impact the bioavailability of the carotenoids and influence their respective bioactivities.

**METHODS**: An acute feeding trial was conducted that investigated the potential role of purple-red carrots as a functional food compared to that of red carrots and purple carrots. Carrot juice made with each of the three carrot varieties was served to healthy males and females (n = 11) following a week-long washout period on a low-carotenoid and low-anthocyanin diet. Plasma carotenoids were analyzed by HPLC to assess bioavailability following each treatment. Additionally, plasma glucose and insulin response were assessed to explore the potential of the pigments in each juice to inhibit alpha-amylase and alpha-glucosidase.

**RESULTS:** There were some indications of competition between lycopene and BC absorption, but there were no significant differences between peak plasma concentrations or area-under-the-curve (**AUC**) of response to the treatment juices. Postprandial glucose response did not differ among treatments.

**CONCLUSION:** The data of this study indicate that carotenoids are bioavailable from juice made with purple-red carrots, similar to that of juices made with red or purple carrots. The combination of lycopene, BC, and anthocyanins in the purple-red carrots does not appear to impact bioavailability.


**Key words: Anthocyanin; β-carotene; bioavailability; carrot; lycopene**

## Introduction

Carrots are a commonly consumed vegetable considered to be a prime example of an affordable, well-liked, accessible, functional food due to the bioactive compounds they contain in addition to micronutrients (Simon, 1997; Arscott, 2010b; Ahmad, 2019). Carotenoids and anthocyanins are groups of plant pigments found in fruits and vegetables that provide the red, orange, and yellow, and the purple, blue, and red colors, respectively. β-Carotene (**BC**), α-carotene (**aC**), and β-cryptoxanthin (**BCX**) are three common provitamin A carotenoids, meaning that when ingested, they can be metabolized into at least one molecule of the essential nutrient, vitamin A. These three carotenoids provide over 80% of dietary vitamin A in numerous countries (FAO, 2001). Other commonly studied non-provitamin A carotenoids include lycopene, lutein, and zeaxanthin. Like these non-provitamin A carotenoids, anthocyanins do not offer provitamin activity, but consumption of carotenoids and anthocyanins, is associated with improved health due to other bioactivities including antioxidant, anticancer, antidiabetic, anti-inflammatory activity and more (Hung, 2004; Khoo, 2017).

Carrots are important dietary sources of BC, especially the orange varieties. Other colored varieties include purple, red, and yellow, which all contain some BC, but differ in amount as well as the other pigments. Purple carrots contain BC, lutein, and anthocyanins; red carrots contain BC and lycopene; and yellow carrots contain BC and lutein. Due to the beneficial association between these pigments and human health, multicolored carrot varieties have been produced. The novel purple-red variety has a red core containing BC and lycopene, and a purple outer layer due to anthocyanins. The goal of developing and producing fruits and vegetables to include an assortment of bioactive phytochemicals, specifically carotenoids and anthocyanins, is to create more phytochemically enriched varieties with health benefits.

The development of purple-red carrots combines multiple bioactive pigments to provide an array of health-benefiting bioactivities. Specifically, purple-red carrots, like red carrots, contain BC, a provitamin A carotenoid and an antioxidant, and lycopene, an antioxidant, and consumption is associated with decreased risk of cancer. Additionally, the purple-red variety contains anthocyanins, which are beneficial to human health due to their antioxidant and anti-inflammatory activities and, most recently, have been associated with antidiabetic activity through inhibition of parameters of glucose metabolism.

Vitamin A deficiency is a prominent global health issue in developing regions, and dietary provitamin A carotenoids are a safe, affordable strategy to combat it (Tanumihardjo, 2008). Despite the promising health benefits linked to the consumption of lycopene and anthocyanins, the most critical aspect of fruits and vegetables that contain provitamin A carotenoids is to ensure that the addition of other biochemical compounds does not negatively impact the amount of vitamin A that they provide. As purple-red carrots may contain more bioactive pigments, it is critical to make sure that the provitamin A carotenoids, specifically BC, are bioavailable, meaning they can be released from the food matrix, absorbed, metabolized into vitamin A, and utilized in physiologic functions. BC is bioavailable from purple carrots and has been shown in animal and human models to be comparable to typical orange carrots (Kurlich, 2005; Charron, 2009; Arscott, 2010a). Carotenoids in purple-red carrots are bioavailable in Mongolian gerbils, but this has never been tested in humans (Kaeppler, 2023).

To assess this, an acute randomized cross-over study was designed to test the bioavailability of BC from juice made with purple-red carrots and compare that to juice made with its purple and red single-colored counterparts. The food matrix greatly influences the bioavailability of bioactive compounds like carotenoids and anthocyanins, and strained juice

eliminates the impact that carrot fiber content may have. This study hypothesized that lycopene's presence might negatively impact BC's bioavailability from purple-red carrots. However, anthocyanins may protect BC during digestion and absorption, positively impacting bioavailability. Additionally, the antioxidant activity and capacity of purple carrots and red carrots have been investigated, but not purple-red carrots and the antidiabetic potential of the anthocyanins in purple or purple-red carrots has not been tested in humans (Kurlich, 2005; Porter Dosti, 2006; Charron, 2009; Sun, 2009; Singh, 2018). As a secondary outcome, this study hypothesized that the juices made with purple and purple-red carrots might improve postprandial blood glucose compared to the juice made with red carrots. The overall objective of this study was to determine the bioavailability of BC and lycopene and investigate the antidiabetic potential of juice made with purple-red carrots in humans using plasma concentrations of carotenoids and biochemical markers of glucose metabolism following acute feeding in healthy individuals.

## Methods

***Participants.*** The Health Sciences Institutional Review Board at the University of Wisconsin-Madison approved the study protocol (2021-1544), which was registered at ClinicalTrials.gov (NCT05319548). Subjects were recruited through flyers posted around the University of Wisconsin-Madison campus and via ClinicalTrials.gov from April 2022-February 2023. Anyone interested in participating was given a packet of information. If they were still interested and met baseline criteria, they met with the lead researcher to discuss the study, ask questions, and complete screening with an initial medical history questionnaire.

Inclusion criteria included healthy, non-smoking males or females (18-40 y) with a body mass index (**BMI**) between 18.5 and 30 $kg/m^2$, who could understand the study information, were

willing to comply and have their blood drawn, and could give written consent. Participants were excluded from the study if they did not meet the weight requirement, had significant comorbidity (cardiovascular disease, diabetes, cancer, kidney disease, liver disease, bowel disease), a history of malabsorptive or other gastrointestinal disorders, abnormal diet or specific dietary restrictions, are pregnant or trying to become pregnant, have any food intolerances, allergies, or hypersensitivities, have a history of substance abuse or alcoholism, or were unwilling to restrict their consumption of specific foods before the study.

Potential subjects who completed questionnaires, met all inclusion criteria, and were still interested, signed informed consent and were enrolled. They were given a personalized folder that included a more in-depth study information packet, a calendar and day-by-day schedule of the study designed for each participant, a packet of food and meal options, and the food diary they would use during each of the study arms. Once enrolled, participants could contact the lead researcher at any time by text, email, or phone to ask any questions or get more information.

***Study Design.*** This study was an acute randomized, crossover study with treatment groups that received 250 mL juice made from red, purple-red, and purple carrots, a slice of white bread, followed by blood draws. The study was conducted in the Clinical Research Unit (**CRU**) at the University Hospital in Madison. All researchers, participants, and hospital staff involved in the study were blinded to the treatment juice, except for the lead researcher who made the juice. Participants took part in three arms (test periods), in which they received a different carrot juice each time. Each arm of the study was 11 days, including a 7-day pre-treatment washout period and a 4-day treatment period. After the treatment period, subjects had a 10-day break during which they could return to their regular diet until the next test period began.

During the washout period, participants were instructed to avoid foods containing carotenoids 7 d before each treatment period and foods containing anthocyanins 3 d before each treatment and to record their food and drink intake in their provided journal. Participants were given lists of foods allowed, recipe ideas, and sources of information if they needed clarification on whether a food was acceptable. Participants were prohibited from consuming vitamins and other nutraceutical supplements throughout the study. They completed food diaries during each of the three arms, which they brought with them at the beginning of each treatment period to assess compliance. Additionally, participants were instructed to contact the lead researcher with questions about food options at any time.

During the 4-day treatment period, the participants continued the low-carotenoid and low-anthocyanin diet and logged their intake, and were instructed not to participate in strenuous exercise or consume alcohol or caffeine one day prior to and during the treatment period. The treatment period was broken down into four "treatment days", and participants were required to come to the CRU on day 1 for treatment, and 8 post-consumption draws, on treatment day 2 for a 24-hour post-consumption blood draw, and on treatment day 4 for a 72-hour blood draw.

On the first day of each treatment period (treatment day 1), participants arrived at the CRU in the morning after at least a 10-hour overnight fast. Upon arrival, CRU staff recorded the participants' heart rate, respiration rate, blood pressure, weight, and height. A venous cannula was inserted into an antecubital fossa or forearm vein, followed by baseline blood draws to analyze carotenoids, anthocyanins, glucose, insulin, C-peptide, and gastric inhibitory peptide (**GIP**). Participants were then instructed to consume the treatment juice and a slice of white bread. Blood was drawn at 15 m, 30 m, 45 m, 1 h, 2.5 h, 4 h, 6 h, 9 h, 24 h, and 72 h post-consumption of the juice and bread. All draw times were recorded.

During the first treatment day of each arm, participants were provided a carotenoid/anthocyanin-free lunch after their 2.5 h draw. They were provided snacks and drinks they could consume any time after that excluding 30 minutes before the next draw. On treatment days 2 and 4, participants returned to the CRU after a 10-hour fast, at the same time that they consumed their treatment juice on day 1 for a 24 h and 72 h post-consumption blood draw. Once their 72 h draw was complete, participants could begin their 10-d break and return to their regular diet.

***Treatments.*** Juices were made the morning of each treatment using 200 g of previously processed and frozen red, purple-red, or purple carrots that were microwaved for two 30-second intervals, and blended in a Vitamix™ blender with 8 oz water on high for 1 min. Pulp was scraped down from the walls of the blender, and the mixture was mixed once more for 30 seconds. The resulting pulp was poured into cheesecloth and squeezed by hand until no more juice could be obtained. Each batch produced ~350 mL juice, and 250 mL was aliquoted back into a clean blender with 1 tsp of vegetable oil and mixed on medium for 30 seconds. The remaining 100 mL juice was kept in air-tight glass tubes for analysis and held at -20°C.

The 250 mL treatment juice was poured into opaque black plastic cups with opaque black plastic sipper lids. The treatment juice and a slice of white bread were put into a secured bag, transported immediately to the clinic, and kept at 20°C until treatment. All participants consumed the same amount and brand of white bread purchased from the same convenience store throughout the study. After baseline blood draws, participants were instructed to drink the juice and eat the slice of white bread in 5 minutes. Cups were checked by the CRU nurse to ensure it was all

consumed, and the start and stop times of when the participant began consuming the treatment and finished were recorded.

***Sample Collection and Analysis.*** Blood samples were collected in BD Vacutainer $K_2$EDTA Hemogard tubes for serum anthocyanin, carotenoid, and GIP analysis, into BD Vacutainer SST Hemogard serum tubes for insulin and C-peptide analysis, and BD Vacutainer fluoroxide/oxalate Hemogard tubes for glucose analysis. All samples were inverted 8-10x and processed within 20 minutes. Anthocyanin and carotenoid samples were centrifuged at 2200 x g for 20 min at 4°C. The glucose, GIP, insulin, and C-peptide samples were centrifuged at 1300 x g for 15 min at 4°C. All serum or plasma for each sample was aliquoted into cryovials and kept at -80°C. The cryovials that the anthocyanin plasma was aliquoted into contained 0.44 M aqueous Trifluoroacetic Acid (**TFA**) to help preserve the anthocyanins in the sample.

*Carotenoids*. All samples were assessed in duplicate using a modified previously published method (Porter Dosti, 2006). Briefly, 250 µL plasma was combined with 100 µL of C-23 β-apo-carotenol as an internal standard, and the sample was denatured with ethanol (0.1% BHT) and extracted with hexane three times (2 mL, 1 mL, 1 mL). Supernatants for each sample were pooled, dried under nitrogen, reconstituted with 200 µL 75:25 methanol:dichloroethane, and 5 µL was injected into a Waters UPLC system.

*Glucose*. All blood samples were analyzed for glucose content in duplicate using a commercially available kit (FujiFilm #997-03001). Reagents, solutions, and standards were brought to room temperature. 20 µL standard or sample was mixed with 3 mL buffer

containing the color reagent and incubated in a water bath (37°C) for 5 minutes. Standards and samples were assessed by spectrophotometry at 505 nm against buffer. Glucose concentrations were calculated using the following equation:

$$Glucose\ (mg/dL) = A_S / A_{Std} \times C_{Std}$$

$A_S$ = *Absorbance of the sample*, $A_{Std}$ = *Absorbance of standard*, $C_{Std}$ = *Concentration of standard in mg/dL*

*Insulin*. All blood samples were analyzed for insulin content in triplicate using a commercially available kit (ALPCO, Salem, NH). Reagents, solutions, standards, controls, and plasma samples were prepared following manufacturer's instructions. A standard curve was made using provided insulin dilutions, including a blank, and two different concentrations of provided glucose standards were used as controls. The analysis included pipetting 25 µL standard, control, and sample into a 96-well microplate. In each well, 100 µL detection antibody was added, and the plate was covered and incubated for 1 h at room temperature on a plate shaker. All contents were decanted from the wells, and the plate was washed six times with wash buffer. Once the plate was washed and free of bubbles, 100 µL 3,3',5,5'-Tetramethylbenzidine (**TMB**) substrate was added to all wells, and the plate was covered and incubated for 15 min.

To stop the reaction, 100 µL stop solution was added to each well, the plate was gently shaken to mix the content, and the plate was read immediately on a Versamax™ Tunable Microplate Reader at 540 nm. Insulin concentration was calculated based on the standard curve (x = insulin concentration (uIU/mL), y = recorded absorbance) created by

subtracting the standard blank from the average value of each standard. Sample concentration was calculated by subtracting the standard blank from the recorded absorbances of the samples. That value was applied to the regression equation from the standard curve to get the final sample insulin concentration.

***Analysis of Treatment Juices.***

*Carotenoids*. All treatment juices were analyzed in duplicate for carotenoid content using an adjusted method from serum carotenoids (Charron, 2009). Briefly, 500 μL juice was combined with 1 mL ethanol, and 100 μL of C-23 β-apo-carotenol as an internal standard. Carotenoids were extracted with 4 mL hexane (2 mL, 1 mL, 1 mL), dried under nitrogen, reconstituted with 200 μL 75:25 methanol:dichloroethane, and 20 μL was injected onto a Waters HPLC system (Milford, MA, USA) equipped with a 1525 binary pump, a 717 autosampler, a 2996 photodiode array detector, and a reverse phase-C18 (5 mm, 4.6 mm x 250 mm).

*Anthocyanins*. Treatment juices were analyzed for anthocyanin content using a modified published method, which included taking 1 mL purple carrot juice and diluting with 3 mL DI $H_2O$, or 2 mL purple-red carrot juice and diluting with 3 mL DI $H_2O$, followed by addition of 2 mL 10% FA:MeOH to each sample. After the samples were mixed by vortex and centrifuged, the supernatant was put into separate tubes, and the remaining pellet was subjected to two more extractions with 1 mL 10% FA:MeOH. A 1 mL aliquot was taken from the 4 mL extracted supernatant and centrifuged to ensure no more solids remained.

20 μL purple carrot juice extract and 50 μL purple-red carrot juice extracts were injected into the above Waters HPLC System.

***Statistical Analysis.*** Using G*power, the sample size calculated to have a high probability for detecting a difference in plasma carotenoid concentrations using area-under-the-curve (**AUC**) for this study was 8 subjects ($\alpha = 0.05$) (Faul, 2007). We aimed to recruit 12 subjects to allow for dropouts or missed blood draws. The plasma concentrations for carotenoids, glucose, and insulin from the subjects who completed all three arms were adjusted for baseline and plotted against time. The baseline-adjusted AUC (**iAUC**) was calculated for each using trapezoidal approximation. Carotenoid iAUCs were created for 0-72 h ($iAUC_{0\text{-}72h}$) and 0-2.5 h for glucose and insulin ($iAUC_{0\text{-}2.5h}$). All statistical analyses were conducted using Statistical Analysis System (SAS) Studio software (SAS Institute Inc., 3.7 Enterprise Edition). PROC MIXED and Tukey's Studentized Range test were used to evaluate and compare all outcomes of interest. T-tests were applied to analyze baseline characteristics. Results with $P < 0.05$ were considered significant, and all results were discussed with a statistical consultant.

**Results & Discussion**

***Carrots and Treatments.*** Juices were made the day of treatment and kept cold in sealed, opaque cups to minimize degradation of pigments. The carotenoid and anthocyanin concentrations of the juices varied by carrot color but were relatively similar between juices made with the same color carrot (**Figure 1**). Purple carrot juice had about 1/3 of the carotenoids as red and purple-red juice. Out of the total carotenoid content in each juice, about 60%, 78%, and 84% of carotenoid content in the purple, purple-red, and red juices were provitamin A carotenoids, respectively, with about

90% of the total provitamin A carotenoid content from BC. Purple juice had the highest anthocyanin concentration, ~5x more than the purple-red juice, and as expected, no anthocyanins were detected in the red juice.

***Participant characteristics and compliance.*** A total of 11 subjects participated in this study **(Figure 2).** Nine subjects completed all three arms, and two subjects completed two arms. One subject had schedule conflicts, and the other tested positive for COVID before the third period. Participant and baseline characteristics are described in **Table 1**. There were no differences between subjects or between males and females for any of the baseline parameters.

Blood was drawn for carotenoid and anthocyanin analysis 11 times during each of the three treatment periods. Sample collection for glucose and insulin analysis was at the first six draws (baseline-2.5 h post-treatment). Only two timepoints were missed; one 72-h draw for carotenoids and anthocyanins for one subject and the 2.5 h time point draw for glucose and insulin for another subject.

Food journals were checked during the first treatment day of each arm and at the end of the study; based on the entries, all subjects were compliant with the diets. This was further confirmed by baseline plasma carotenoid concentrations **(Table 1)**. Subjects reported that they avoided certain foods they were unsure of or contacted the lead researcher to check before consuming. Other than one subject briefly feeling lightheaded upon arrival, no adverse events were noted, and no adverse effects were reported as a result of the treatments.

***Serum and Plasma Results.*** The mean peak concentration changes for individual subjects and iAUCs of BC, lycopene, total carotenoids, glucose, and insulin are depicted in **Table 2 & Table**

**3**. The mean iAUC for BC was highest after the purple-red carrot juice treatment, followed by the red carrot and purple carrot juices. Interestingly, the mean lycopene iAUC was highest after the purple carrot juice treatment, despite having almost no lycopene content. The next highest was in the purple-red carrot juice, and the red carrot juice, which had the highest lycopene content, had the lowest average iAUC. The iAUCs of total carotenoids followed the same pattern.

The purple juice treatment resulted in the lowest iAUCs for glucose, but the largest for insulin, while the iAUCs of the purple-red and red had similar glucose concentrations, and the red juice treatment had the lowest insulin concentration. There were no differences in iAUCs between treatments. Variation in peak concentrations and iAUCs was high, meaning there were large differences in the data between subjects, as shown by the coefficient of variation (**CV**) values calculated for each. Almost all parameters in the table have CVs >100%, indicating the standard deviations are larger than the means, which may have been influenced by small and negative data points, data were assessed by LSMeans, and the standard error (**SE**) and corresponding confidence intervals (**CI**), shown in **Table 4**, showed a similar pattern in that the data points varied between and within subjects for different treatments.

iAUCs for 0-72 h for carotenoids and 0-2.5 h for carotenoids, glucose, and insulin were plotted (**Figure 3A, 3B**, **3C**). Both the red and purple-red juice treatments showed similar initial peaks at 6-h for lycopene, BC, and total carotenoids and then dropped at the 9-h mark. The purple-red juice lycopene, BC, and total carotenoid concentrations increased again, while the red juice carotenoids continued to decrease. By the 72-h draw, the mean lycopene, BC, and total carotenoid concentrations after the purple-red treatment decreased. At this point, the purple-red carrot juice and red carrot juice treatments resulted in similar concentrations. In contrast, the lycopene and total carotenoid concentrations after the purple carrot juice treatment remained higher. The purple

carrot juice treatment had fewer extreme peaks than the other two juices. It was the lowest iAUC for BC, which was expected because it had lower BC concentration. It also had the highest iAUC for total carotenoids, which was attributed to lutein/zeaxanthin (not pictured). The purple juice had 4x more lutein and zeaxanthin content than the other two juices.

Based on mean lycopene concentrations of the purple, purple-red, and red juice amounts, which were 6.25 nmol/250 mL, 1450 nmol/250 mL, and 1000 nmol/250 mL, respectively, one would expect that the red and purple-red treatment juices would result in higher peaks and iAUC compared to the purple treatment juice which had minimal lycopene. Overall, the lycopene concentrations at each time point are above baseline, meaning that the participants likely did get some lycopene from the juice. The fact that it was maintained through 72 h after the purple juice treatment, unlike the purple-red and red juices, could suggest that something in the purple carrot juice protects lycopene or prevents it from being excreted.

Upon ingestion, carotenoids are released from the food matrix and incorporated into micelles, which transport the carotenoids in the intestinal lumen. The absorption of carotenoids into the enterocyte is facilitated mainly by scavenger receptor class B type 1 (**SR-BI**) (Shamrakov, 2013). It is possible that the high carotenoid concentration in the juices played a role in bioavailability (Liu, 2003). BC and lycopene compete for absorption, which may have been why the lycopene concentrations were lower for the red carrot juice compared with the purple and purple-red (Prince, 1991; White, 1993; Gaziano, 1995; O'Neill, 1998; van den Berg, 1998; Mills, 2007; Phan, 2018a).

Additionally, BC is a provitamin A carotenoid, so the plasma concentrations following the treatments partially depend on each individual's vitamin A status. Cholesterol levels or associated lipoproteins were not analyzed in this study, but they may have influenced carotenoid response.

BC circulation is associated with low-density lipoproteins (**LDL**) and very low-density lipoproteins (**VLDL**), lycopene is mainly associated with LDL but can also be found in VLDL, and lutein and zeaxanthin are equally distributed between LDL and high-density lipoproteins (**HDL**) (Shamrakov, 2013).

The average lycopene iAUC after the purple-red juice was higher than that of the red, suggesting anthocyanin involvement. Purple-red juice had lower anthocyanins than the purple juice. Plasma lycopene after the purple-red juice treatment was similar to the red carrot juice by 72 h. Even at high concentrations, anthocyanins have relatively low bioavailability and are quickly metabolized and eliminated from the body (Gonçalves, 2021). The potential impact they may have on lycopene absorption would likely not be in effect at later times. It could be that one or some of the metabolites from the anthocyanins or other polyphenols present in the purple juice could have played a role, because they have been shown in *in vitro* studies to increase lycopene absorption, cellular uptake, and SR-B1 expression in different cell types (Chen, 2021). Additionally, it may have simply been the total carotenoid concentration of the juices that had the most impact. Although the purple carrot juice had the lowest concentration, the resulting plasma carotenoid concentrations were not significantly different from the other juices. The lycopene concentrations of the juices may have been too low to observe a response.

Lastly, the pre-treatment washout period may not have been long enough to get carotenoid baseline levels depleted to see a response. The week-long washout period included in each arm of the study was similar to that used in other studies (Thürmann, 2002; Horvitz, 2004; Tanumihardjo, 2009; Arscott, 2010a). According to Burri et al., carotenoid depletion follows first-order kinetics, and the average depletion of plasma BC is 32-42 d, lycopene is 24-30 d, and lutein is approximately 76 d (Burri, 2001). Even though baseline levels were similar to other studies that had week-long

washout periods, in subjects that regularly consume fruits and vegetables, one week may not have been enough time to get their plasma levels down to see a response following juice consumption.

Strengths of this study include it being the first clinical study that investigated the bioavailability and potential interactions between BC, lycopene, and anthocyanin following consumption of juice made from multicolored carrots. This study assessed the bioavailability of BC from the novel purple-red carrot, which was the single food source of carotenoids and anthocyanins utilized in this study. All treatments and blood draws took place at the Clinical Research Unit at the University Hospital in Madison, WI, which included staff explicitly trained in clinical research. Nine subjects completed all three arms of the study, which was predicted to have statistical power to compare differences in carotenoid concentrations after the treatments ($\alpha = 0.05$).

The design of the study was acute consumption of carrot juice for each treatment period. The acute design and single treatment may not have been enough to induce an observable response or truly assess the effect of ingestion of lycopene, BC, and anthocyanins on bioavailability. Additionally, even though the subjects were compliant with the pre-treatment diet restrictions, the washout period may have needed to be longer to get blood concentrations low enough to see a significant response after drinking the juices. There were large variations in the mean peak concentrations and iAUCs among subjects. Only a small amount of plasma was collected for the anthocyanin analysis, which ultimately resulted in the inability to observe plasma anthocyanins. During the period of sample analyses, logistical and mechanical problems occurred, which restricted further analysis of the plasma anthocyanins and prevented the analysis of the GIP and C-peptide samples. These parameters may have provided more clarity or insight into the data successfully collected and discussed.

**Conclusion**

This is the first study to investigate the impact of co-consumption of anthocyanins and carotenoids from juice made with red, purple, and purple-red carrots on BC and lycopene bioavailability and potential antidiabetic effects. Although there were no effects or differences between treatment juices, there was some evidence of possible synergism between anthocyanin concentration and lycopene bioavailability in the purple and purple-red carrot juices. There also may have been potential competition between BC and lycopene, as evidenced by the lowest lycopene concentrations observed following consumption of the red carrot juice. Although the concentration of bioactive pigments in the juice may not have been enough to observe a notable response following ingestion, this is a realistic and physiologically relevant amount that the general public would consume.

There were no differences between carotenoid, glucose, and insulin concentrations between treatments. Due to the lack of effect of the treatments on glucose or insulin responses and the small sample size, there would likely be no differences in the GIP or C-peptide iAUCs. In future studies, these analyses would allow for a further understanding of the potential mechanism of action behind the antidiabetic effect that these pigments may have if differences were observed in the glucose and insulin responses after treatment.

Additional studies may include a larger sample size, repeated consumption of juice over a longer duration of time, and additional collection and analysis of other biological samples, including cholesterol and lipoproteins, triglycerides, and other parameters of glucose metabolism like GIP, GLP-1, and C-peptide. This study provides additional evidence that supports carrots as functional foods and is the first study in humans to show that carotenoids are bioavailable from

purple-red carrots and that the bioavailability of carotenoids from this carrot type is similar to that of red and purple carrots.

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**Table 1.** Participant baseline characteristics[a]

| *Characteristic* | *Total* | *Male* | *Female* |
|---|---|---|---|
| Participants, *n* | 11 | 6 | 5 |
| Age (yr) | 23.1 ± 6.3 | 22.9 ± 7.36 | 23.4 ± 5.13 |
| Weight (kg) | 71.9 ± 10.3 | 77.0 ± 5.3 | 64.8 ± 11.9 |
| BMI (kg/m$^2$) | 24.0 ± 2.4 | 23.5 ± 2.1 | 24.2 ± 3.1 |
| Total carotenoids (μmol/L) | 0.98 ± 0.55 | 1.07 ± 0.68 | 0.83 ± 0.24 |
| Lycopene (μmol/L) | 0.22 ± 0.13 | 0.27 ± 0.13 | 0.17 ± 0.06 |
| β-carotene (μmol/L) | 0.35 ± 0.28 | 0.32± 0.35 | 0.35 ± 0.15 |
| Glucose (mmol/L) | 5.59 ± 0.45 | 5.02 ± 0.34 | 5.56 ± 0.09 |
| Insulin (uIU/mL) | 7.32 ± 4.60 | 6.70 ± 3.49 | 7.63 ± 3.99 |
| HOMA-IR[b] | 1.76 ± 1.16 | 1.68 ± 0.91 | 1.73 ± 1.02 |

[a] Expressed as mean ± SD; Average baseline levels of all subjects from each arm

[b] Homeostatic Model Assessment for Insulin Resistance; fasting glucose (mmol/L) x fasting insulin (uIU/mL) / 22.5

**Table 2.** Mean plasma peak concentration of carotenoids, glucose and insulin following carrot juice treatments

| Treatment Juice | Peak concentration change[a] *(CV)*[b] | | | | |
|---|---|---|---|---|---|
| | β-Carotene | Lycopene | Total | Glucose[c] | Insulin[d] |
| Purple | 0.032 ± 0.014 *(42.5)* | 0.012 ± 0.002 *(17.2)* | 0.063 ± 0.030 *(97.3)* | 0.55 ± 0.88 *(161.9)* | 24.2 ± 16.5 *(68.0)* |
| Purple-Red | 0.043 ± 0.037 *(85.4)* | 0.003 ± 0.015 *(525.3)* | 0.020± 0.057 *(280.2)* | 0.42 ± 0.64 *(151.2)* | 16.0 ± 13.7 *(85.4)* |
| Red | 0.047 ± 0.031 *(67.0)* | -0.004 ± 0.013 *(-297.1)* | -0.003 ± 0.0.46 *(-1420)* | 0.58 ± 0.66 *(113.7)* | 17.9 ± 11.8 *(66.0)* |

[a] Mean (±SD) peak concentration change from baseline expressed as μmol/L

[c] Coefficient of variation

[d] Expressed as mmol/L

[e] Expressed as uIU/mL

**Table 3.** Mean adjusted Area-Under-the-Curve (AUC) for plasma carotenoids, glucose, and insulin after treatment

| Treatment Juice | iAUC[a] *(CV)*[b] | | | | |
|---|---|---|---|---|---|
| | β-Carotene | Lycopene | Total | Glucose[df] | Insulin[ef] |
| Purple | 3.10 ± 4.69 *(151.)* | 0.97 ± 2.18 *(189.)* | 6.72 ± 7.37 *(110.)* | 238 ± 900. *(378.)* | 2350 ± 1970 *(83.7)* |
| Purple-Red | 5.49 ± 4.29 *(78.2)* | 0.97 ± 2.34 *(241.)* | 4.89 ± 8.69 *(178.)* | 369 ± 1320 *(357.)* | 1280 ± 1040 *(81.6)* |
| Red | 4.74 ± 8.68 *(183.)* | -0.39 ± 2.41 *(-673.)* | 1.29 ± 6.01 *(465.)* | 361 ± 1710 *(474.)* | 1720 ± 1250 *(72.5)* |

[a] Average area under the curve after subtracting baseline over 72 h treatment period ($iAUC_{0\text{-}72h}$)

[b] Coefficient of variation

[d] Expressed as mmol/L

[e] Expressed as uIU/mL

[f] Values are baseline through 2.5 h ($iAUC_{0-2.5h}$)

**Table 4.** LSMEANS estimates and confidence intervals for adjusted Area Under the Curve (iAUC) values

| Treatment Juice | AUC LSMEANS[a] *(CI)*[b] | | | | |
|---|---|---|---|---|---|
| | β-Carotene | Lycopene | Total | Glucose[ce] | Insulin[de] |
| Purple | 2.41 *(6.72, -1.90)* | 1.25 *(2.90, -0.40)* | 5.83 *(11.6, 0.09)* | 294 *(1220, -629)* | 2270 *(3240, 1300)* |
| Purple-Red | 5.49 *(9.41, 1.57)* | 0.68 *(2.13, -0.77)* | 4.97 *(10.1, -0.07)* | 254 *(1090, -579)* | 1410 *(2320, 497)* |
| Red | 4.18 *(8.55, -0.19)* | -0.17 *(1.48, -1.82)* | 1.74 *(7.48, -4.00)* | 543 *(1480, -392)* | 1530 *(2520, 544)* |

[a] LSMEANs estimates based on average area under the curve after subtracting baseline over 72 h treatment period ($iAUC_{0\text{-}72h}$)

[b] Confidence Interval

[c] Expressed as mmol/L

[d] Expressed as uIU/mL

[e] Values are baseline through 2.5 h ($iAUC_{0-2.5h}$)

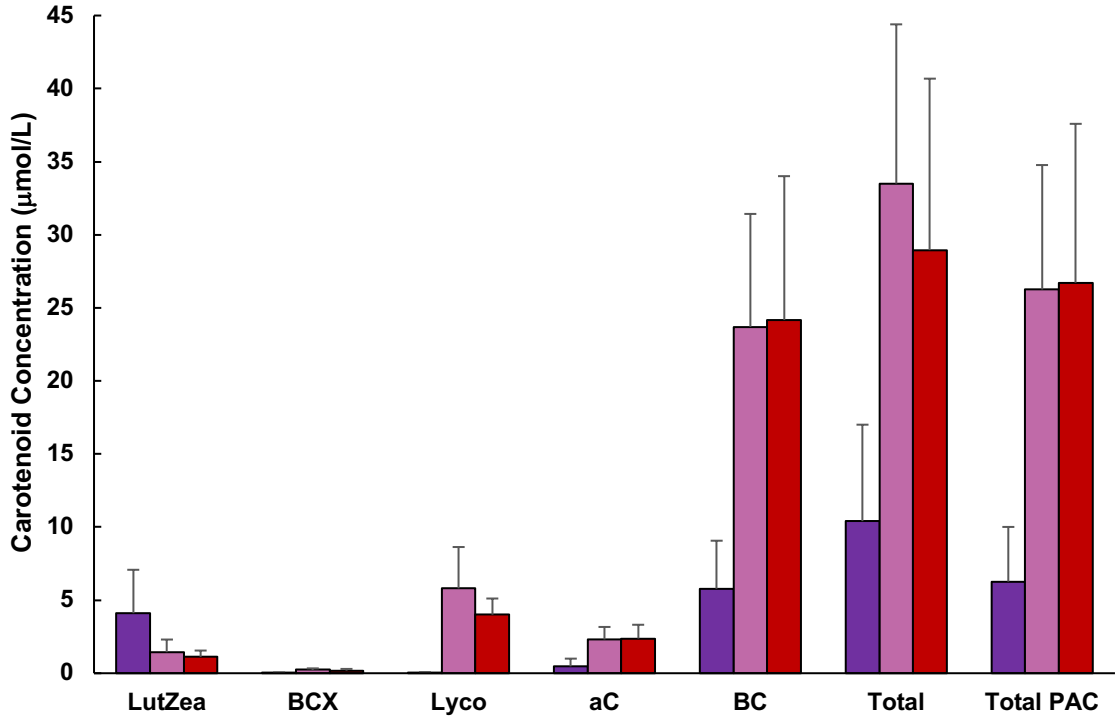


**Figure 1.** Average carotenoid content in the treatment carrot juices made with red (gray bars), purple (black bars) and purple-red (dotted bars) carrots; Lutein + Zeaxanthin **(LutZea);** Beta-Cryptoxanthin **(BCX);** Lycopene **(Lyco);** alpha-Carotene **(aC);** Beta-Carotene **(BC);** Total sum of carotenoids **(Total)**; Total sum of provitamin A carotenoids- BCX+aC+BC **(Total PAC).**

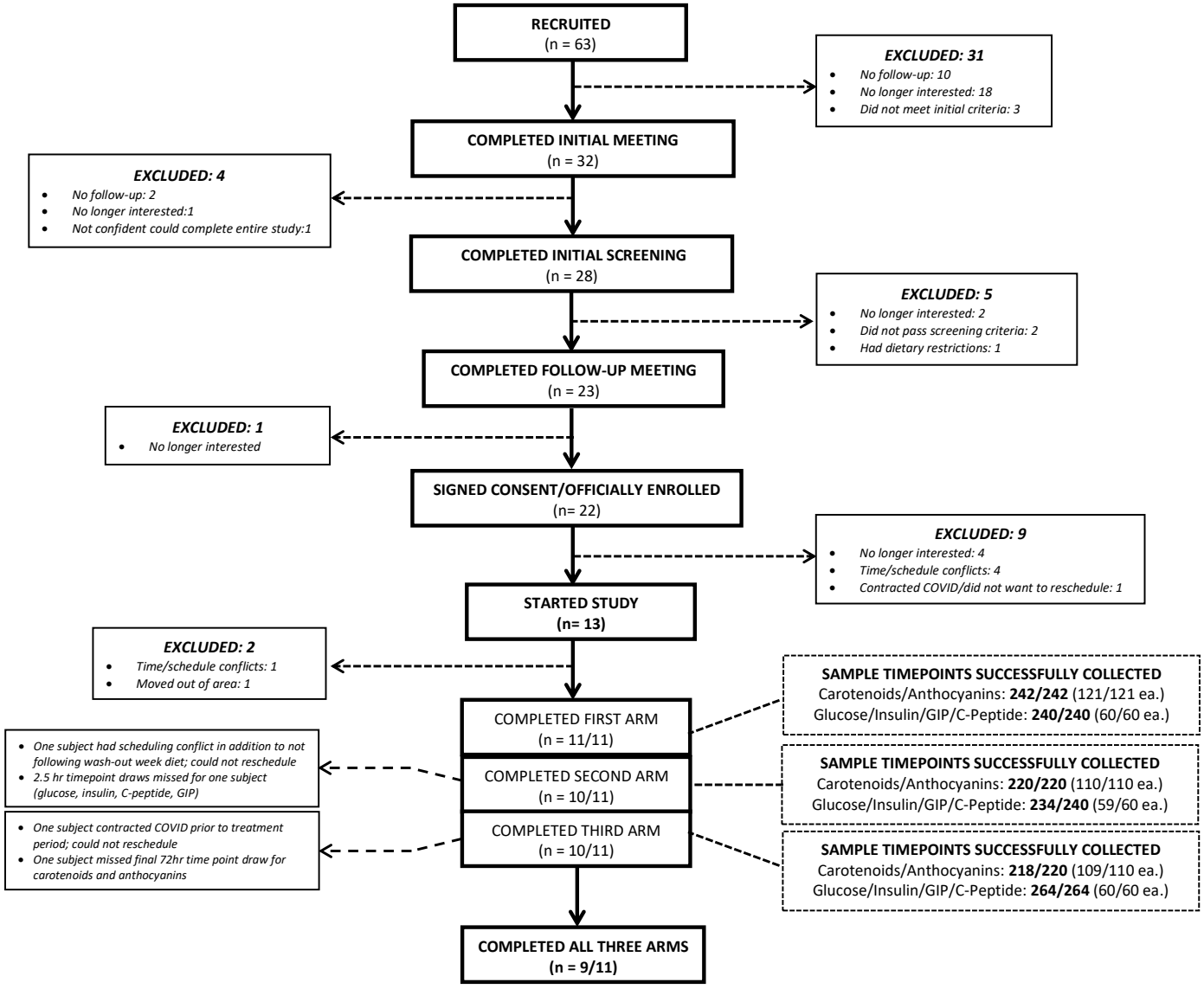


**Figure 2.** CONSORT flow diagram of study participant recruitment procedures and reasons for exclusions

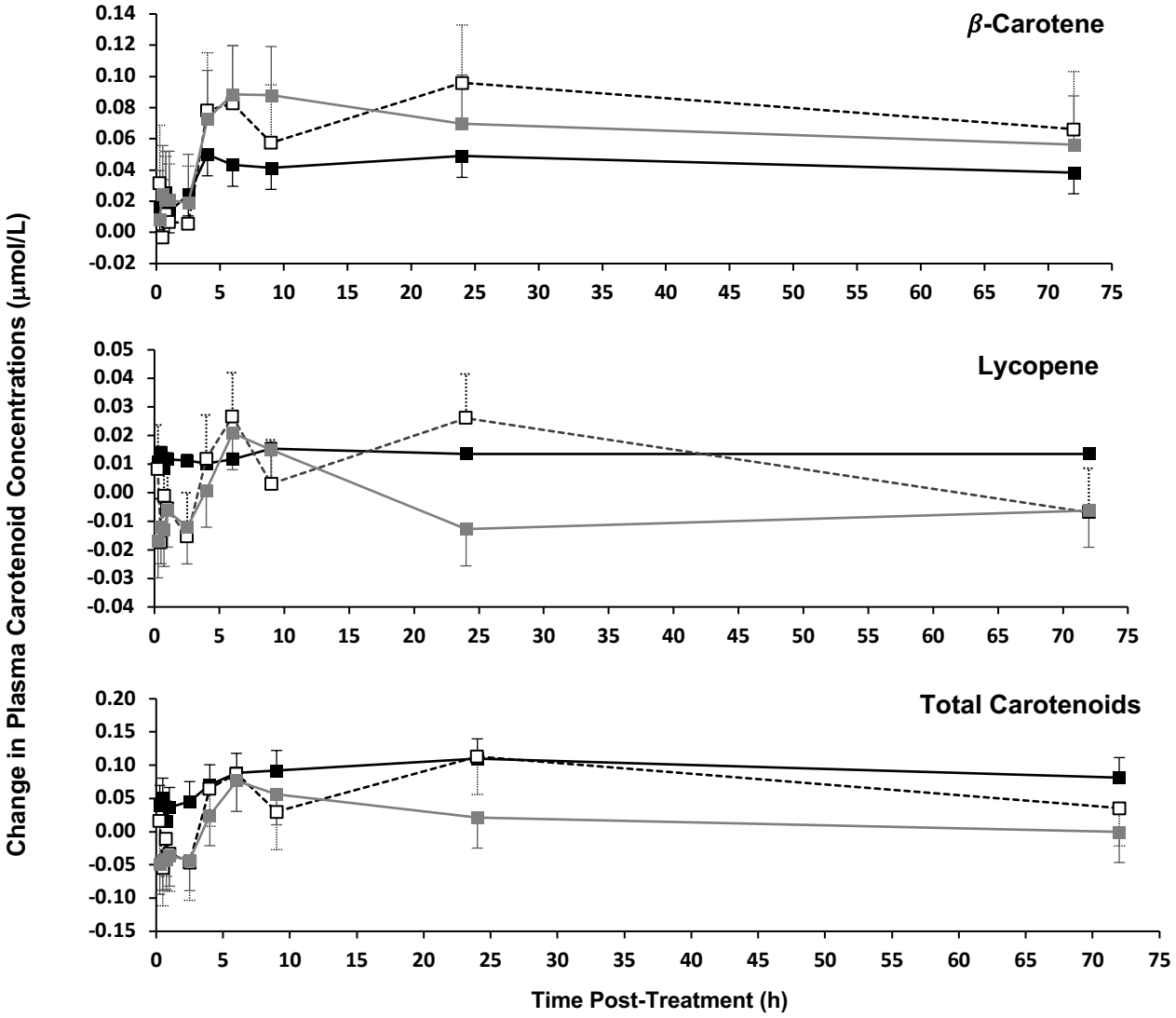


**Figure 3A.** Mean plasma concentrations (± SD) after subtracting baseline BC, lycopene and total carotenoids of all subjects at each timepoint over 72 h following consumption of each treatment juice. *Purple carrot juice = Solid black line, Red carrot juice = Solid gray line, Purple-red carrot juice = Dotted line*.

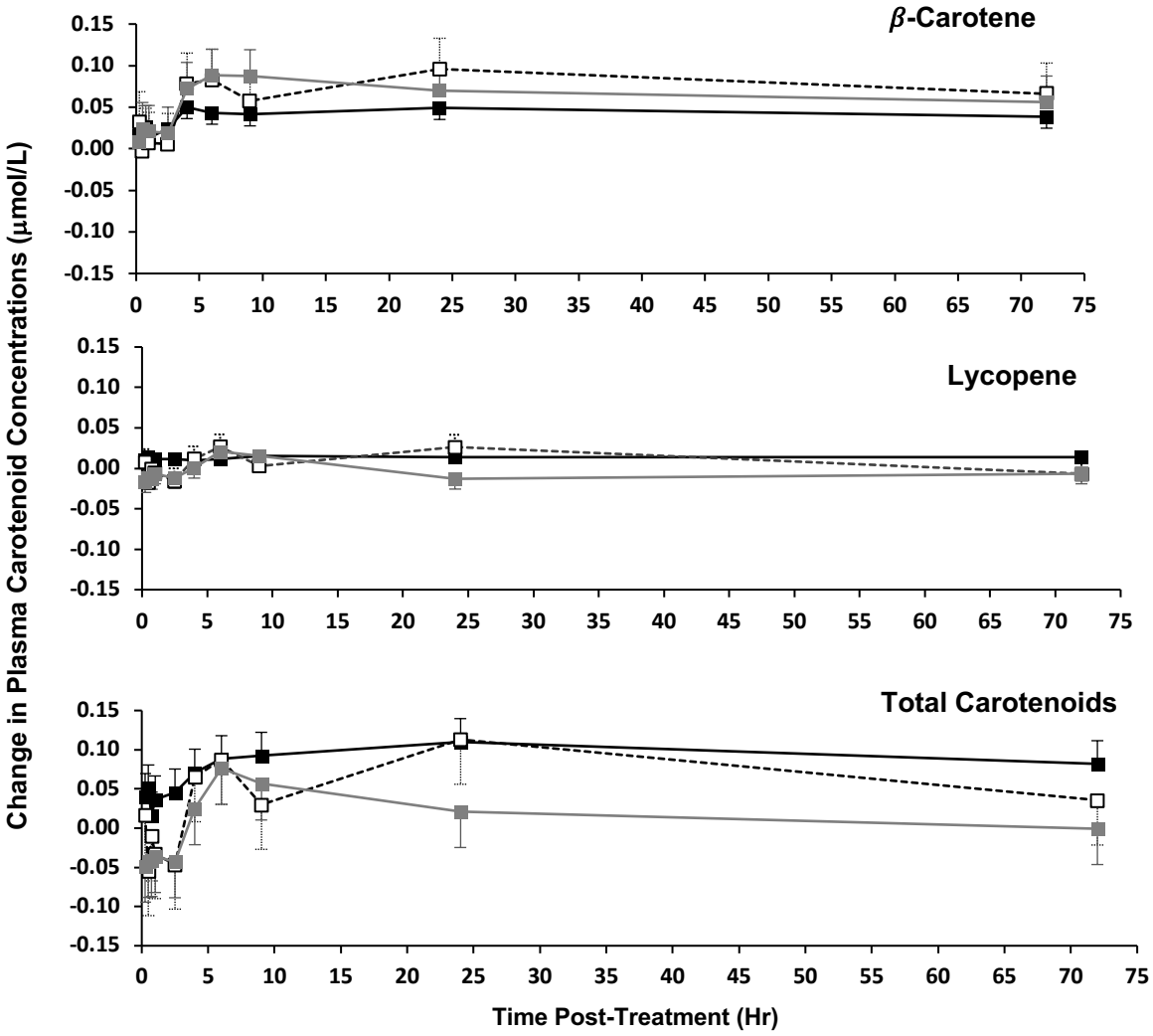


**Figure 3B.** Mean plasma concentrations (± SD), after subtracting baseline, of BC, lycopene and total carotenoids of all subjects at each timepoint over 72 h following consumption of each treatment juice. Scales adjusted to be the same to show relative differences in concentrations. *Purple carrot juice = Solid black line, Red carrot juice = Solid gray line, Purple-red carrot juice = Dotted line*.

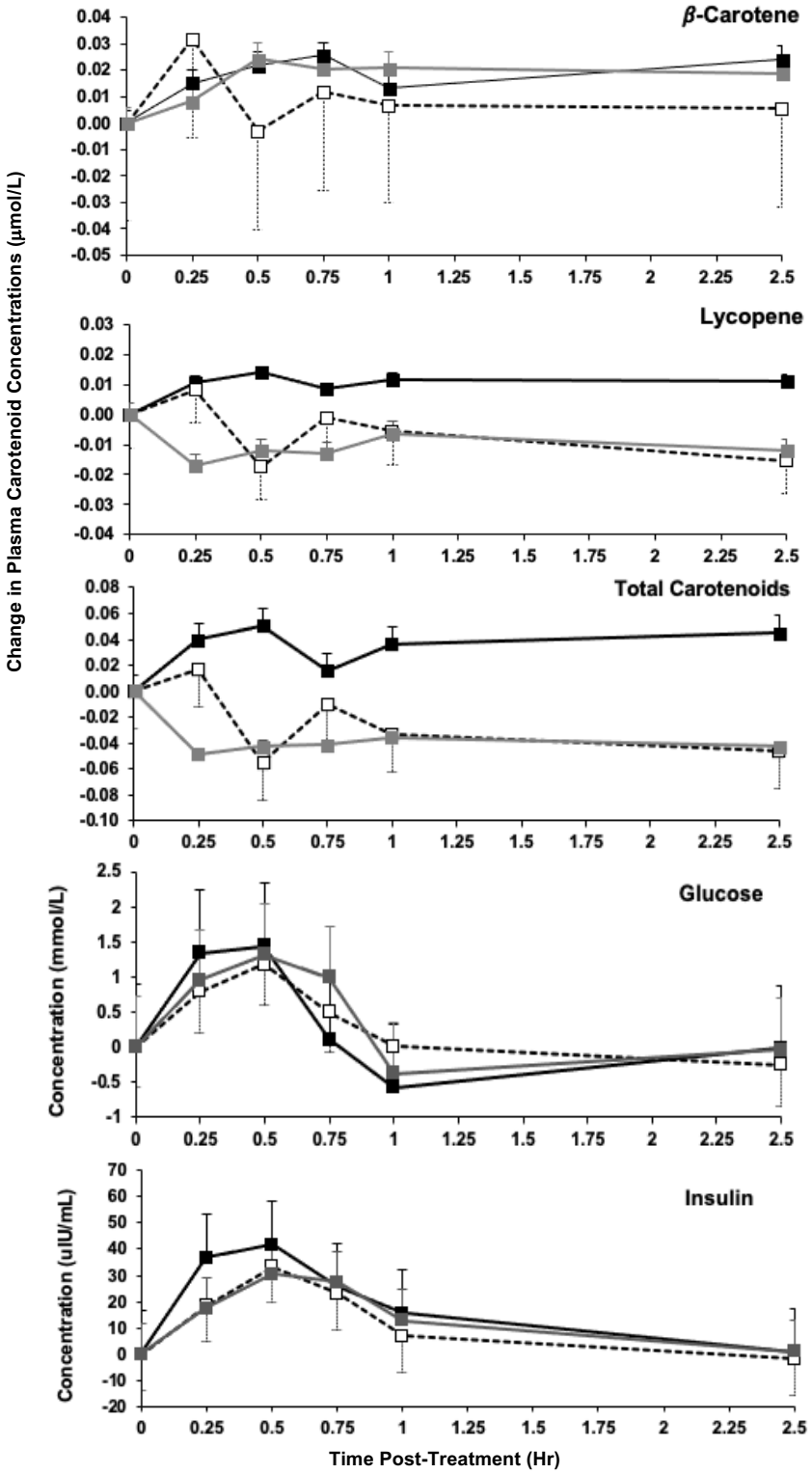


**FIGURE 3C.** Mean plasma concentrations (± SD) of BC, lycopene. total carotenoids, glucose and insulin of all subjects at each timepoint over 2.5 hours following consumption of each treatment juice. *Purple carrot juice = Solid black line, Red carrot juice = Solid gray line, Purple-red carrot juice = Dotted line*.

# CHAPTER 5:

## CONCLUSIONS & FUTURE DIRECTIONS

The studies presented in this dissertation emphasize the importance of investigating biofortified functional foods and the array of challenges and complexity associated with the health-benefiting properties and potentials of the bioactive phytochemicals that they contain. To better understand the interactions, three studies were implemented to investigate the bioavailability and bioactivities of carotenoids and anthocyanins sourced from carrots. All studies used multicolored carrots (red, purple-red, and purple) as the source of carotenoids and anthocyanins. Methods were developed to analyze the pigment profiles of purple-red carrots, and *in vitro* assays were used to investigate other potential bioactivities, like antioxidant activity and inhibition of alpha-amylase and alpha-glucosidase (**Chapter 2**). The bioavailability of carotenoids and anthocyanins was investigated *in vivo* using Mongolian gerbils (**Chapter 3**), and an acute feeding clinical trial in humans (**Chapter 4**).

Carrots are nutrient-dense vegetables that are a good source of vitamins, minerals, fiber, and bioactive phytochemicals like carotenoids and anthocyanins. Single-colored red carrots, which contain lycopene and provitamin A carotenoids, and purple carrots, which contain provitamin A carotenoids and anthocyanins, were assessed alongside the novel purple-red carrot, which contains lycopene, provitamin A carotenoids, and anthocyanins. Carrots can be prepared in multiple ways, and processing of any kind is known to influence the bioavailability of carotenoids and anthocyanins. In the gerbil studies, treatment diets were fortified using freeze-dried red and purple-red carrots, while in the clinical trial, carrot juices made with red, purple, and purple-red carrots were used to minimize variabilities that may come with the food matrix and use a form of carrot that is relevant.

Chapter 2 presented the results of three sub-studies—the first focused on developing and optimizing extraction and analysis methods of anthocyanins. This work highlights the challenges

associated with optimizing the extraction and analysis of anthocyanins, especially sourced from food samples. Multiple published methods were studied, and parameters that were similar between methods were compared. Methods for anthocyanin extraction of fresh, freeze-dried, and juice made from purple-red carrots were developed for analyzing the anthocyanin profile of purple-red carrots. A primary lesson learned from developing these methods was that even small, subtle changes in factors of extraction and analysis (like increasing solvent acid content by only 1%) could significantly impact the results. In contrast, other factors (such as duration of sonication or centrifugation) did not have as much of an impact. Optimal extraction and analysis methods may vary drastically in the same lab or other labs adopting these methods. There is considerable variation in anthocyanin content between carrots from the same cultivar and even more so between different cultivars. The successful development of methods to analyze anthocyanins in fresh, freeze-dried, and juice made with purple-red carrots and initial anthocyanin profiles will be useful for future bioavailability studies or biofortification strategies.

In the second and third sub-study, the antioxidant activity and enzyme inhibition potential of juice made with red, purple, and purple-red carrots was assessed by two different antioxidant assays (DPPH, ABTS) and two different enzyme assays (alpha-amylase, alpha-glucosidase). The DPPH antioxidant and the alpha-amylase inhibition assays were successful. Due to the challenges and differences between the assays used, methods used, and food samples analyzed, comparing the data complicated because purple-red carrots have not been assessed before. The complexity of analysis is due to multiple parameters such as differences in sample preparation methods, food samples used, differences in assays by manufacturers, and the difference in mechanism of the assay used (i.e., DPPH vs. ABTS- both analyze antioxidant activity, but are based on different mechanisms).

The most significant impact on the success of the assays was the presence of pigments, especially anthocyanins. All four assays tested were based on colorimetry, and the purple color of the anthocyanins in the juice influenced the assay results by interfering with the changes in absorption that the assay calculations are based on. The results of these sub-studies will be beneficial for future work as they provide initial evidence of antioxidant activity and alpha-amylase inhibition potential that red, purple, and purple-red carrot juice may exert and are an example of how analysis of bioactivities like the ones tested are challenging. Future work should investigate alternative assay options or kits to find better options for these analyses that utilize colorimetry and change in absorbance to estimate *in vitro* bioactivity potential.

In the third chapter, two animal studies were conducted using Mongolian gerbils to assess the bioavailability of provitamin A carotenoids, specifically BC, in the presence of lycopene in varying concentrations in the first study and the presence of an equalized amount of lycopene, but varying concentrations of anthocyanins in the second study. The results of both studies show that provitamin A carotenoids are bioavailable from red and purple-red carrots, and the anthocyanins in the purple-red carrots do not have a negative impact on bioavailability. Additionally, the correlations between lung and liver VA concentration indicate that there are likely stellate-like storage cells in the lung, which are crucial for developing the respiratory system. One limitation of the first study was the small starting weight of the gerbils upon arrival. While it may have impacted the final results on how lycopene influenced BC bioavailability, other important findings were observed that may not have been seen otherwise. The correlation between starting weight and VA stores at the end of the study further emphasizes the importance of dietary VA intake post-birth and proper weaning because low VA status post-weaning

significantly impacts the ability to build up vitamin A stores because dietary VA is used immediately.

Lastly, sensitivity and specificity analysis of serum retinol vs. liver retinol as indicators of vitamin A deficiency added more evidence to the argument that serum retinol values are not a good indicator of vitamin A status. These data further emphasize the need for the development of better tests and biomarkers or increased implementation of superior tests (such as the Modified Relative Dose Response) for accurate assessment of vitamin A deficiency. Future studies should include similar designs but use higher carrot concentrations to see the impact that higher carotenoid and anthocyanin contents may have on bioavailability. Only male gerbils were used in these studies to decrease variability, but it is crucial to test this in female gerbils because there may be sex differences in bioavailability. Lastly, future studies should incorporate isotope dilution methods to understand the metabolism, bioavailability, and potential interactions between carotenoids and anthocyanins.

Chapter 4 presented data from an acute clinical randomized crossover trial that assessed the difference in carotenoid bioavailability among three treatment juices made from red, purple, and purple-red carrots in humans. While there were no significant differences between the treatment juices in any plasma sample concentrations, the data indicate that carotenoids in the treatment juices are bioavailable in healthy human subjects. There there was considerable variation among subjects and within subjects between treatments. One lesson learned from this study includes the numerous factors and complexities involved with successfully planning and executing a clinical trial. In addition to the challenges associated with assessing the bioavailability and potential bioactivities of carotenoids and anthocyanins discussed in the previous chapters, human subjects bring in a new assortment of variables, intricacies, and

complications. (Not to mention the extra protocols that come with the occurrence of a global pandemic during a clinical trial).

At the same time, clinical studies are necessary to understand how best to optimize the nutritional aspects of fruits and vegetables to maximize the health benefits they provide and observe how various factors impact those benefits. Future clinical studies should assess similar parameters using a chronic design that includes larger sample sizes and multiple rounds of drinking the treatment juices over a more extended period to see if bioavailability is impacted differently. Additionally, the chronic design would allow for investigating other bioactivities that these pigments can provide, such as antioxidant and anti-inflammatory activity. These could not be assessed in an acute feeding trial. These activities are commonly described as reasons that plant pigments can influence human health, but they must be further investigated in human subjects.

Consumption of fruit and vegetables is consistently associated with better health outcomes. Yet, if asked, most of the general population would struggle to describe what specifically about fruits and vegetables make them healthy and what may make some healthier. The same goes for the common saying, “Eat the rainbow”; many people know the saying but associate it solely with eating various fruits and vegetables. This may have been the initial origin of the slogan, but hopefully, in the near future, it can be associated with the specific plant pigments in the fruits and vegetables, and just by looking at different colored varieties, consumers will be able to have an idea of how the chemicals in those fruits and vegetables that give them their colors are beneficial for health.

Through research projects like the ones discussed in this dissertation, evidence will compile, ultimately resulting in a better understanding of exactly how these pigments function in

the human body and how to maximize their benefits. Increasing consumption of provitamin A carotenoids through biofortifying popular crops to include more provitamin A carotenoids has proven to be an effective and safe way to combat vitamin A deficiency, and prevalence, although still high, has been decreasing on a global scale. Knowing that carotenoids, both provitamin A and non-provitamin A forms, and anthocyanins have high potential to provide additional health-promoting activities, are already present in a majority of commonly consumed fruits and vegetables, and are relatively easy for consumers to decipher which foods contain them, and which ones do not, all emphasize the importance of future research on the bioavailability and bioactivities of plant pigments to decrease the prevalence of common chronic diseases and associated malnutrition through their consumption.