The dose-response effects of the amount of oil in salad dressing on the bioavailability of carotenoids and fat-soluble vitamins in salad vegetables

by

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CHAPTER 1

INTRODUCTION

1.1 Overview

High intakes of fruits and vegetables have been recommended to prevent the development of chronic diseases. The beneficial effects of fruit and vegetable intake on human health have been closely linked to substances in plant foods, such as fiber, micronutrients and phytonutrients. Numerous studies have shown an association between vegetable intake and plasma level of fat-soluble nutrients (Campbell et al., 1994; Chopra et al., 2000; Drewnowski & Popkin, 1997; Olson, 1984; Su & Arab, 2006). Among the most frequently consumed plant foods, salad vegetables significantly contribute to vitamin and other micronutrient intakes in the U.S. population. In the Third National Health and Nutrition Examination Survey (NHANES) salad consumption was reported by one third of US adults and was associated with higher serum concentrations of micronutrients, including vitamins A and E, as well as provitamin A carotenoids (α- and β-carotene) (Su & Arab, 2006). Salad vegetables are among the major sources of vitamin E in the U.S. diet (Maras et al., 2004; Murphy et al., 1990). However, the amounts of micronutrients absorbed from raw salad vegetables may not be adequate to meet requirements, especially for the fat-soluble nutrients, due to the low bioavailability of fat-soluble nutrients in raw vegetables. The bioavailability of carotenoids and phylloquinone was reported to be significantly lower from raw vegetables compared with processed vegetables (Gartner et al., 1997; Rock et al., 1998). Many factors influence the bioavailability of fat-soluble nutrients from vegetables, including dietary factors, physicochemistry of fat-soluble nutrients and host-related factors. Dietary factors such as the plant matrix, nutrient species, amount of co-ingested fat and other compounds can be modified in order to enhance the absorption of fat-soluble nutrients from vegetables. Improving the absorption of fat-soluble nutrients in salad vegetables may help in meeting the recommended dietary intake of these nutrients. One way to improve the bioavailability of the

fat-soluble nutrients is to incorporate fat during processing and/or ingestion of vegetables. Absorption of fat-soluble nutrients from vegetables was improved by increasing the fat content of a meal (Gijsbers et al., 1996). An in vitro digestion model showed a high bioaccessibility of α-tocopherol, but not γ-tocopherol, in romaine lettuce homogenized with fat (Reboul et al., 2006b). In our previous study, the absorption of carotenoids from salad vegetables was higher when ingested with full fat compared with reduced and fat-free salad dressings (Brown et al., 2004). The necessity to increase the amount of co-ingested fat raises a concern considering the association between high fat intakes and the obesity epidemic (Corella et al., 2007; Snell-Bergeon et al., 2009). It is necessary to identify the minimal amount of fat needed to improve the bioavailability of carotenoids or other fat-soluble micronutrients in unprocessed vegetables. Although oil is known to be essential for the absorption of fat-soluble micronutrients, little or no information is available on the effect of low levels of oil on the relative bioavailability of a wide range of fat-soluble micronutrients from salad vagetables. In addition, no detailed information is available on the relationship between the overall amount of oil and the effect on the relative bioaccessibility of salad vegetables.

1.3 Study objectives

The overall objective of this study was to improve the bioavailability of dietary fat-soluble nutrients and bioactives to lower the risk of chronic disease and enhance overall health. The specific goals of this study were therefore to: 1) obtain the dose-response relation in terms of the influence of the amount of added fat (oil) on the intestinal absorption of carotenoids, phylloquinone and tocopherols in salad vegetables; 2) investigate whether low levels of oils, i.e. 2 g and 4 g, can result in a statistically significant increase in the absorption of the salad bioactives compared with 0 g of oil.

CHAPTER 2

LITERATURE REVIEW

2.1 Association between vegetable intake and micronutrient status

The 2005 Dietary Guidelines for Americans (USDA, 2005) recommend 3 or more servings of vegetables per day. That dietary guideline has not been met by the majority of the population. Kimmons et al. reported less than 1 in 10 Americans consume the recommended servings of fruits or vegetables (Kimmons et al., 2009). Concurrently, inadequate intakes of various micronutrients have been reported in the U.S. population. Data from the 1994 to 1996 Continuing Survey of Food Intakes by Individuals (CSFII) with added values for α-tocopherol (vitamin E) from the US Department of Agriculture National Nutrient Database for Standard Reference Release 15 showed that only a small percentage of the U.S. population (8.0 % of men and 2.4 % of women) met the estimated average requirements (EARs) for vitamin E intake from foods alone (Maras et al., 2004). Daily vitamin A and vitamin K intakes were lower than the recommended amounts as reported in the National Health and Nutrition Examination Survey (NHANES) IV (Center for Disease Control, 2004). These findings support the recommendation to increase vegetable consumption in order to provide adequate intakes of these nutrients. Vegetables are among the primary contributors of fat-soluble nutrients, such as carotenoids, tocopherols (vitamin E) and phylloquinone (vitamin K_1). Some vegetable groups serve as the major source of micronutrients due to their micronutrient contents and the frequency of consumption in many populations (Maras et al., 2004). According to 1990 Food and Drug Administration (FDA) Total Diet Study, the top five sources of phylloquinone in American diets are salad vegetables, such as spinach, collards, broccoli, iceberg lettuce, and coleslaw with dressing (Booth et al., 1996). Green leafy vegetables consumption significantly contributes to carotenoid (Takyi, 1999), tocopherol (Murphy et al., 1990) and phylloquinone (Booth et al.,

1996) intakes. Salad vegetables are among the major sources of vitamin E in the U.S. diet (Maras et al., 2004, Murphy et al., 1990).

The association between vegetable consumption and fat-soluble nutrient intakes prompts the question as to whether vegetable intake affects plasma levels of these nutrients. Total plasma carotenoid concentrations were shown to be proportional to total intakes of fruits and vegetables (Campbell et al., 1994). Salad consumption was associated with higher serum concentrations of micronutrients, including vitamins A and E, as well as provitamin A carotenoids (α and β carotene) (Su & Arab, 2006). Plasma α-carotene, β-cryptoxanthin and lutein concentrations, were found to be indicative of fruit and vegetable intakes (Campbell et al., 1994). Serum β-carotene concentration was reported to be a useful biomarker for fruit and vegetable consumption in French adults (Drenowski & Popkin, 1997). Increased plasma lycopene was observed after supplementation with tomato-based products in both smokers and non-smokers (Chopra et al., 2000). In breastfeeding Indonesian women, significant improvements in vitamin A status were observed after supplementation with pure β-carotene in a simpler matrix, but not after consumption of an additional daily serving of dark green leafy vegetables (de Pee et al., 1995).

2.2 Benefits of vegetable intake related to micronutrient status

Dietary patterns characterized by a variety of plant foods, including vegetables, have been linked to a wide range of health benefits. These health benefits include protection against various chronic diseases and increased longevity. Reduction in the risk of chronic diseases has been found in populations with high vegetable intakes. For example, a prospective study in Chinese women (Villegas et al., 2007) showed inverse correlations between the amount of vegetable consumption and the risk of type 2 diabetes. A review of 22 case-control studies and four prospective cohort studies in the 1999 World Cancer Research Fund report showed that vegetable but not fruit consumption, was associated with reduced risk of colorectal cancer (Park et al., 2007). Moreover, vegetable intake was also reported to have implications on bone health due primarily to the nutrient content in vegetables. New et al. (New et al., 2000) found that high intakes of nutrients in plant foods such as zinc, magnesium, potassium, fiber and vitamin C were associated with higher bone mass in

premenopausal women. Further study by the same group reported dietary effects on bone mass and bone metabolism that may link plant foods, including vegetable consumption, to higher bone mass and decreased bone resorption (New et al., 2000). Moreover, a diet consisting of olive oil and raw vegetables was shown to improve longevity in Italian elders (Masala et al., 2007).

Specific groups of vegetables have been reported to promote health. Consumption of dark green leafy vegetables was associated with reduced risk of colorectal cancer in men (O'Neill et al., 1998). An association between reduced risk of type 2 diabetes and consumption of green vegetables, but not yellow/red vegetables, was observed in a middle-aged Finnish population (Montonen et al., 2005), whereas the same association was found with green and dark yellow vegetable intakes in a female Chinese population (Villegas et al., 2007). Salad and raw vegetable intakes were inversely associated with type 2 diabetes (Williams et al., 1999), cardiovascular disease (Cox et al., 2000) and various types of cancer (Park et al., 2007; Cox et al., 2000; Sant et al., 2007). The World Cancer Research Fund reported strong evidence of inverse relationships between cancer at different locations and the consumption of plant foods (American Institute for Cancer Research, 1997).

The nutrient and bioactive components in vegetables are reported to link vegetable consumption to various health benefits. High levels of vitamins, minerals, phytochemicals and polyphenols in vegetables provide antioxidant effects against oxidative stress and inflammation, the initial biomarkers of the development of chronic diseases. Dietary fiber in vegetables may account for the association between vegetable intake and gastrointestinal health, as well as metabolic parameters. Vegetable consumption, but not fruit consumption, was inversely correlated with glucose intolerance (Williams et al., 1999). Frequent vegetable consumption was reported to prevent weight gain (He et al., 2004) and to be indicative of overall healthy lifestyles (e.g., not smoking, moderate alcohol consumption, infrequent consumption of many high-fat foods) (Wichelow & Prevost, 1996). The effects of vegetable consumption both from the nutritional standpoint and through overall lifestyle may serve as protection against the development of cancer. Some of the biological mechanisms linking vegetable intake and cancer prevention include the decline in oxidative disturbance to lipids and DNA, activation of phase I and II enzymes, and initiation of DNA repair and apoptosis

(Park et al., 2007). Considering the effects of vegetable consumption on micronutrient status and consequently on overall health, the bioavailability of fat-soluble nutrients in raw vegetables needs to be optimized.

2.3 Fat-soluble nutrients in salad vegetables

2.3.1 Chemistry of carotenoids, phylloquinone and tocopherols

Carotenoids are the yellow, orange and red pigments that give coloration in fruits and vegetables. They are C40 isoprenoid polyene compounds characterized by conjugated double-bonds with cyclic or acyclic structures at the ends of the chain. There are 600 identified carotenoids in nature, approximately 10% of them can be converted into vitamin A in mammals (Yeum & Russell, 2002), and about 3% are detected in human plasma (Thompson et al., 1985). Carotenoids are produced by photosynthetic plants, algae, fungi and bacteria. Animals do not produce carotenoids, but obtain these compounds from their diets. Two general classes of carotenoids are carotenes and xanthophylls. The main property distinguishing the two is the presence of oxygen atoms on xanthophylls, whereas carotenes lack oxygen atoms (Clinton, 1998). Most abundant and commonly found carotenes in plant foods are β-carotene, α-carotene and lycopene. Lycopene lacks cyclic end groups and is the precursor for synthesis of α-carotene and β-carotene through generation of cyclic ends by cyclases in plants. Xanthophylls are also called oxy-carotenoids and are more hydrophilic than carotenes because of the oxygen containing functional groups, including hydroxyl, methoxyl, carboxyl, oxo, epoxy and keto groups. The most abundant xanthophylls in salad vegetables include β-cryptoxanthin, zeaxanthin and lutein. Most carotenoids are present as all-*trans* isomers in vegetables and may undergo chemical-, thermal- and photo-isomerization (Koyama & Fujii, 1999) affecting the proportion of *cis* and *trans* isomers in body fluids and tissues (Rodriguez-Amaya, 2003).

Phylloquinone is one of the family of compounds comprising vitamin K. These coumpounds share a common parent structure, 2-methyl-l,4-naphthoquinone (menadione) with differing isoprenoid side chains at the 3 position. Phylloquinone (vitamin K_1) and menaquinones (vitamin K_2) are naturally occurring forms of vitamin K produced by plants and bacteria, respectively. The chemical structure of phylloquinone is 2-methyl-3-phytyl-

1,4-naphthoquinone having the same phytyl side chain as chlorophyll. Menaquinones, on the other hand, are named according to the number of prenyl groups (up to 13) in the unsaturated side-chain (Lambert & de Leenheer, 1986) and have the chemical structure of 2-methyl-3-$(prenyl)_n$-1,4-naphthoquinone.

Tocopherols are commonly known as vitamin E, which refers to eight structurally related chemical structures consisting of a chromanol ring and 16-carbon isoprenoid side chain. Tocopherols and tocotrienols are the two groups of vitamin E with different substitution pattern of methyl groups at positions 5, 7 and 8 of the head group (α-, β-, δ- and γ-). Tocopherols are characterized by a saturated side chain, while tocotrienols are indentified by an unsaturated side chain with double bonds in the positions 3', 7' and 11'. Among naturally occuring vitamin E species, α-tocopherol possesses the most potent antioxidant activity. Other vitamin E homologs, β-, δ- and γ-tocopherols and the tocotrienols are absorbed but are not included toward meeting the dietary requirement because they can neither be converted into α-tocopherol nor effectively transported from the liver (Schneider, 2005).

2.3.2 Biosynthesis of carotenoids, tocopherols and phylloquinone in plants

Carotenoids, tocopherols and phylloquinone are isoprenoid compounds produced in the plastids and are involved in the photosynthetic processes in higher plants (Fraser & Bramley, 2004; Gaudille`re et al., 1984; Maeda et al., 2008). Carotenoids can be synthesized from acetyl-CoA (mevalonic acid pathway) or from glycolytic intermediates (methylerythritol phosphate pathway) (Kopsell & Kopsell, 2006). The initial step is the isomerization of isopentenyl diphosphate (IPP), a precursor of all isoprenoid compounds, into dimethylallyl diphosphate (DMAPP). Geranylgeranyl diphosphate (GGPP) synthase catalyzes the condensation of IPP and DMAPP to form GGPP (Bramley, 2002). GGPP can be reduced to phytyl-diphosphate (PDP) used for phylloquinone, chlorophyll and tocopherol synthesis and is a key intermediate in the synthesis of phytoene, chlorophylls and tocotrienols (DellaPenna & Pogson, 2006). The first step unique to carotenoid biosynthesis is the condensation of two molecules of GGPP to form the first C40 carotenoid, phytoene (Gross, 1991). Phytoene desaturase and β-carotene desaturase catalyze dehydrogenation reactions of

phytoene converting it into lycopene (Cunningham & Gantt, 1998). Lycopene β -cyclase or lycopene ε –cyclase generate the β - ring or ε - ring, respectively, leading to the branching of carotenoid biosynthesis at lycopene. Two β-rings lead to carotenoids with β,β branch (e.g., β-carotene, zeaxanthin, violaxanthin, antheraxanthin and neoxanthin), whereas one β and one ε - ring leads to carotenoids with the β, ε branch (e.g., α-carotene, zeinoxanthin and lutein). The biosynthesis pathway continues with the additions of oxygen moieties, which convert the hydrocarbon α- and β-carotenes into xanthophylls (Kopsell & Kopsell, 2006). Tocopherols are biosynthesized from two converging pathways, which are tyrosine catabolism and isoprenoid biosynthesis via the non-mevalonate pathway (Schneider, 2005). Phylloquinone biosynthesis occurs in the chloroplast and begins with chorismate, an important biochemical intermediate in plants and microorganisms.

2.4 Factors affecting the amount of fat-soluble nutrients in vegetables

The concentrations of carotenoids, tocopherols and phylloquinone in vegetables generally (i) are determined by the growing conditions (weather, growing season, intensity of sunlight and soil state), (ii) vary with plant types (species, variety), (iii) increase as the plant matures, and (iv) are higher in dark-green leaves than in pale-green tissues. Most carotenoids are present as all-*trans* isomers in vegetables and may undergo chemical-, thermal- and photo-isomerization (Koyama & Fujii, 1999), which may affect their bioavailability and biological activities. Lycopene can occur in several forms in fresh plant foods, including carotenoid-protein complexes in chloroplasts or in crystalline form inside chromoplasts (Parada & Aguilera, 2007).

Carotenoid content increases as vegetable plants mature (de Azevedo & Rodriguez-Amaya, 2005) but decreases during senescence (Gross, 1991). Factors affecting the accumulation of carotenoids in plants include changes in the growing air temperature, irradiance level, irradiance photoperiod and nutritional fertility (Kopsell & Kopsell, 2006).

The vitamin E content (e.g., RRR-α-tocopherols) in plant foods is generally low to moderate (Eitenmiller & Lee, 1999). The most abundant tocopherol in green leafy tissues is α-tocopherol with relatively low concentrations of total tocopherols. Seeds on the other hand

are often more concentrated in total tocopherols (McLaughlin and Weihrauch, 1979). The amounts of tocopherols in fruits and vegetables is affected by species, variety, maturity, growing conditions (weather, growing season, intensity of sunlight, and soil state), and uneven distribution of tocopherols, as well as time and manner of harvesting (5). Post harvest variation in tocopherol concentrations is caused by many factors including processing procedures, storage time and conditions, sample preparation and variation in analytical methods (Booth and Bradford, 1963a; Booth, 1963; McLaughlin and Weihrauch, 1979). Tocopherol increases as plants reach maturity and continues to increase during senescence. Booth and Bradford (Booth and Bradford, 1963b) listed some rules in estimating tocopherol concentrations in plant products; tocopherol levels are high in dark-green tissues, moderate in fast-growing leaves, light green tissues and colored fruits, and low in roots, etiolated tissues and colorless fruits. For instance, lettuce grows very fast and has the lowest tocopherol content among leafy vegetables (Booth and Bradford, 1963b).

Vitamin K_1 or phylloquinone content increases during maturation for most vegetables. The amount of phylloquinone in vegetables differs in different locations due to variability in the climate, soil and growing conditions (Ferland and Sadowski, 1992). Phylloquinone content is greater in vegetables grown in a location with less sun exposure, lower temperature and soil with a great cation exchange capacity (high P, Ca, Mg and K levels) (Ferland and Sadowski, 1992). Less amount of daylight may provide protection against photodecomposition of vitamin K and may influence the chlorophyll levels affecting vitamin K_1 synthesis in the chloroplast (Ferland and Sadowski, 1992). However, summer months were reported to yield more phylloquinone in vegetables than winter months (Koivu et al., 1997). The amount of chlorophyll and carotenoids determine the coloration of leaves, which can be used to qualitatively assess the vitamin contents in vegetables. Plasma phylloquinone concentrations have been reported to be higher in fall compared with the winter months (McKeown et al., 2002; Sadowski et al., 1989), which may be due to the different phylloquinone contents in vegetables during those two seasons.

2.5 Carotenoid, tocopherol and phylloquinone requirements, intakes and dietary sources

Salad vegetables are among the top contributors of carotenoids, tocopherols and phylloquinone even though the bioavailability of these nutrients in salad vegetables is low. This may be explained by the frequent salad consumption, which is reported by more than one-third of U.S. adults (Su & Arab, 2006). There is no Recommended Daily Allowance (RDA) for carotenoids, except the RDA for vitamin A that can be extrapolated for pro-vitamin carotenoids (e.g., α-carotene, β-carotene and β-cryptoxanthin). Data from 1999-2000 NHANES show average adult intakes for vitamin A to be about 1000 µg RAE per day, while the RDA for vitamin A is 900 µg RAE suggesting that most Americans get enough vitamin A (Center for Disease Control, 2004). The amount of vitamin A is presented as retinol activity equivalence (RAE), which equals 1 µg of retinol. Two micrograms of β-carotene in oil contributes the same vitamin A activity as 1 µg of retinol (1 µg RAE), whereas 12 µg of -carotene in dietary fruits and vegetables are estimated to provide the same vitamin A activity due to lower bioavailability. α-Carotene and β-cryptoxanthin provide 50% as much retinol as dietary β-carotene, because only half of the molecule is convertible to usable vitamin A. Therefore, 24 µg of α-carotene or β-cryptoxanthin equals to 1 µg RAE. The non-provitamin A carotenoids, such as lutein and lycopene, are categorized as bioactive food components, which are thought to have health protective effects. In the US, the average daily lycopene intake ranges from 6.6–10.5 mg/day for men and from 5.7–10.4 mg/day for women (Porrini & Riso, 2005) and is more than 80% obtained from tomato processed products, such as ketchup, tomato juice, spaghetti sauce, pizza sauce and tomato juice (Clinton, 1998). All-*trans*-lycopene is the primary isomer (> 90%) present in processed tomato products (Boileau et al., 2002).

The major form of vitamin E in human tissues and supplements, as well as in vegetables is α-tocopherol, whereas fortified foods and oils contain primarily γ-tocopherol. γ-Tocopherol represents ~70% of the vitamin E consumed in the typical U.S. diet (McLaughlin & Weihrauch, 1979). According to the 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII), only a small portion of the US population, 8.0% of men and 2.4% of women, meets the EAR for α-tocopherol from foods alone (Maras et al., 2004). The primary contributors of α-tocopherol, such as ready-to-eat cereal, sweet baked products, white bread, beef, oils and salad dressing, are not particularly high in α-tocopherol, but are consumed

frequently (Maras et al., 2004). The RDA for vitamin E is 15 mg per day as α-tocopherol (RRR- and 2R-stereoisomeric forms of α-tocopherol) for both adult males and females. *RRR*-α-tocopherol occurs naturally in foods, while the *2R*-stereoisomeric forms of α-tocopherol (*RRR*-, *RSR*-, *RRS*-,and *RSS*-α-tocopherol) and the *2S*-stereoisomeric forms of α-tocopherol (*SRR*-, *SSR*-, *SRS*, and *SSS*-α-tocopherol) are found in fortified foods and supplements. Data from the 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII) reported that the mean α-tocopherol intake ranges from 6.1-7.1 mg/d in men and 4.4-4.8 mg/d in women (Maras et al., 2004).

Phylloquinone or vitamin K_1 is the predominant form of vitamin K in foods. Naturally occurring phylloquinones only exist in the *trans* form; all-*trans* menaquinones are also the most commonly found in nature. The *cis-trans* isomers, which are formed during UV light exposure or synthetic production of vitamin K, are considered to have low bioactivity (Parrish, 1980). There is no RDA set for phylloquinone but rather an Adequate Intake (AI) of 90 µg/day for women and 120 g/day for men (Institute of Medicine, 2001) due to the contribution of gut bacteria in producing significant amounts of functional vitamin K_1. Dietary surveys and observational studies (Booth et al., 1996; Feskanich et al., 1999) as well as HPLC analysis have shown that green, leafy vegetables are the primary dietary source of phylloquinone. According to data from 11 studies of vitamin K intake, young adults consumed lower dietary phylloquinone (~80 µg/d) than older adults (~120 µg/d) (Booth et al., 1996).

Table 1. Daily carotenoids, tocopherol and phylloquinone requirements, intakes and dietary sources in U.S. population

Nutrient	Life stage group	Dietary Reference Intakes (DRIs)[1] RDA[a]/AI[b]* (µg/day)	UL[c] (µg/day)	Dietary intake[2] (µg RAE/day)	Dietary sources[1]
Vitamin A Includes provitamin A carotenoids (e.g. α-carotene, β-carotene, β-cryptoxanthin)	Children: Adults (>20 y.o.):	♂ and ♀: 400 ♂: 900 ♀: 700	♂ and ♀: 900 ♂ and ♀: 3000	♂: 703 ♀: 579 ♂: 649 ♀: 580	Liver, dairy products, fish, darkly colored fruits and leafy vegetables
		(µg/day)	(µg/day)	(µg/day)	
Vitamin K	Children: Adults (>20 y.o.):	♂ and ♀: 55* ♂: 120* ♀: 90*	ND[d] ND[d]	♂: 61.2 ♀: 54.5 ♂: 101.0 ♀: 96.8	Green vegetables (collards, spinach, salad greens, broccoli, brussel sprouts, cabbage, plant oils, margarine
		(mg/day)	(mg/day)	(mg/day)	
Vitamin E	Children: Adults (>20 y.o.):	♂ and ♀: 7 ♂ and ♀: 15	♂ and ♀: 300 ♂ and ♀: 1000	♂: 5.8 ♀: 5.5 ♂: 8.6 ♀: 7.1	Vegetable oils, unprocessed, cereal, grains nuts, fruits, vegetables, meats

Sources: [1]Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids (2000); and Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc (2001). [a]RDA= Recommended Daily Allowance, [b]AI= Adequate Intake, [c]UL = The maximum level of daily nutrient intake that is likely to pose no risk of adverse effects. Unless otherwise specified, the UL represents total intake from food, water, and supplements. Due to lack of suitable data, ULs could not be established for vitamin K, thiamin, riboflavin, vitamin B12, pantothenic acid, biotin, or carotenoids. In the absence of ULs, extra caution may be warranted in consuming levels above recommended intakes; [d]ND = Not determinable due to lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake. [2]U.S. Department of Agriculture, Agricultural Research Service. 2008. Nutrient Intakes from Food: Mean Amounts Consumed per Individual, One Day, 2005-2006. www.ars.usda.gov/ba/bhnrc/fsrg. [2]What We Eat in America, NHANES, 2005-2006, individuals 2 years and over (excluding breast-fed children), Day 1 dietary intake data, weighted.

Table 2. Carotenoid concentrations in commonly consumed salad vegetables

Salad Vegetable	Nutrient content (µg/g of edible portion)			
	α-Carotene	β-Carotene	Lutein + zeaxanthin	Lycopene
Broccoli	0.25 ± 0.03	3.61 ± 0.07	14.03 ± 0.40	1.02 ± 0.04
Carrot	34.77 ± 5.32	82.85 ± 10.82	2.56 ± 0.63	0.01 ± 0.01
Cauliflower	0.00 ±0.00	0.00 ±0.00	0.01 ± 0.00	0.00 ± 0.00
Celery	0.00 ± 0.00	2.70 ± 0.36	2.83± 0.37	0.00 ± 0.00
Cherry tomato	1.01 ± 1.01	4.49 ± 0.02	1.23 ± 0.04	25.73 ± 0.54
Cucumber	0.11 ± ND[1]	0.45 ± ND	0.23 ± ND	0.00 ± ND
Romaine lettuce	0.00 ± 0.00	52.26 ± 5.10	23.12 ± 3.31	0.00 ± 0.00
Spinach	0.00 ± 0.00	56.26 ± 7.67	121.98 ± 19.31	0.00 ± 0.00

Source: USDA National Nutrient Database for Standard Reference, Release 22 (2009)

[1]ND: No Data

Table 3. Tocopherol and phylloquinone concentrations in commonly consumed salad vegetables

Salad Vegetable	Nutrient content (μg/g of edible portion)			
	α-Tocopherol	δ-Tocopherol	γ-Tocopherol	Phylloquinone
Broccoli	7.80 ± 0.99	0.00 ± 0.00	1.70 ± 0.44	1.02 ± 0.04
Carrot	6.60 ± 2.69	0.00 ± 0.00	0.00 ± 0.00	0.13 ± ND
Cauliflower	0.80 ± ND	0.00 ± ND	2.00 ± ND	0.16 ± 2.56
Celery	2.70 ± 0.72	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.03
Cherry tomato	5.40 ± 0.7	0.00 ± 0.02	1.20 ± 0.16	0.08 ± 0.05
Cucumber	0.30 ± 0.01	0.00 ± 0.04	0.30 ± 0.05	0.16 ± ND
Romaine lettuce	1.30 ± 0.27	0.10 ± 0.00	3.60 ± 0.52	1.03 ± 0.07
Spinach	20.30 ± 0.15	0.00 ± 0.00	1.80 ± 0.36	4.83 ± ND

Source: USDA National Nutrient Database for Standard Reference, Release 22 (2009)

[1]ND: No Data

pathways (Ziouzenkova & Plutzky, 2008).

The presence of conjugated double bonds in carotenoid molecules accounts for their antioxidant properties. These double bonds receive energy transfer from singlet oxygen of reactive oxygen species to generate ground state oxygen and a triplet or excited carotenoid molecule. The greater the number of conjugated double bonds, the lower the triplet energy level. Thus, lutein, α-carotene, β-carotene, zeaxanthin and *β*-cryptoxanthin (CTX) are all highly active quenchers of singlet oxygen. Lycopene, the open ring carotene, is the most efficient quencher of singlet oxygen (Stahl & Sies, 2003). Many of biological functions of carotenoids are derived from the antioxidant properties of carotenoids. For example, carotenoids function in the light-harvesting process, photo-protection, singlet oxygen scavenging and dissipation of excess energy from excited chlorophylls in plants (Frank & Cogdell, 1996). Lutein and zeaxanthin accumulate in the human macula and lens where they are believed to protect the eyes from phototoxic damage (Krinsky et al., 1958). Carotenoids also may protect human skin against UV light-induced photo-oxidative stress, preventing erythema formation, premature aging and cancer as well as other diseases associated with oxidative stress in this organ (Lee et al., 2000).

Phylloquinone is a cofactor for an enzyme involved in the post translational modification of specific γ-carboxyglutamyl residues in several proteins (Suttie, 1993) including 1) bone gamma-carboxyglutamic acid (Gla) protein (osteocalcin) and matrix Gla protein (Vermeer, 1990) plasma procoagulants and anticoagulants, as well as 3) cellular growth factor (Ferland, 1998). Phylloquinone involvement in modification of these proteins accounts for its role in bone health, blood clotting, prevention of cadiovascular disease, energy metabolism and inflammation. The role of phylloquinone in bone health is closely linked to γ-carboxylation of 3 glutamic acid residues of osteocalcin. Osteocalcin serves as a regulator of bone mineral maturation through its calcium binding ability, the development of which is dependent on phylloquinone (Cairns & Price, 1994). Phylloquinone intake has been associated with lower risk of bone loss and hip fracture, also inconsistently with greater bone mass density (Booth et al., 2004; Booth et al., 2000). Vitamin K deficiency as defined by either low dietary phylloquinone intake or low plasma phylloquinone was associated with a high serum percentage of undercarboxylated osteocalcin and a high risk of hip fracture

(Shearer, 2007).

Phylloquinone involvement in cardiovascular prevention is related to its role in the formation of matrix Gla protein. Matrix Gla protein represses vascular calcification through a variety of mechanisms, including the binding of calcium ions and crystals, and extracellular matrix (Proudfoot & Shanahan, 2006). The calcium-regulation activity of matrix Gla protein depends upon the phylloquinone-dependent γ-carboxylation of specific Glu residues. As a result, phylloquinone plays a role in the prevention of vascular calcification by ensuring proper action of matrix Gla protein.

Moreover, high phylloquinone intake is associated with lower triglycerol concentrations (Braam et al., 2004), as well as greater insulin sensitivity and glycemic status (Yoshida et al., 2008). The mechanism underlying high phylloquinone intake and greater insulin sensitivity is related to carboxylation of osteocalcin. Osteocalcin has been reported to influence β-cell function, insulin sensitivity, adiponectin production, energy expenditure and adiposity (Lee et al., 2007). These beneficial effects of phylloquinone in glucose homeostasis suggest potential use of phylloquinone in the prevention and/or treatment of diabetes. In addition, both vitamin A and vitamin E are agonists of vitamin K activities. Studies in rats have shown that higher than normal intake of dietary vitamin A and vitamin E result in depressed prothrombin levels (Matschiner & Doisy, 1962) and thus increased vitamin K requirement.

Tocopherols help lower the risk of certain chronic diseases such as heart disease, type 2 diabetes, hypertension and cancer and may combat some of the negative effects associated with aging, including cognitive decline and Alzheimer's disease. Such health benefits of tocopherols may be due to one or more of their biological activities, such as antioxidant activities, regulation of gene transcription, inhibition of enzymatic activities and cell cycle. Among other tocopherols, α-tocopherols is a highly efficient antioxidant because (i) it reacts with peroxyl radical extremely fast; (ii) it removes the radical character from the oxidizing fatty acid and prevents it from further radical reactions; (iii) it is converted into a fairly stable radical in antioxidant reaction (Schneider, 2005). Antioxidant activities of tocopherols may result in the reduction of oxidative stress and inflammation, which are closely linked to the development of many diseases. In plants, α-tocopherol protects the photosynthetic complex

(Schultz-Siebert, 1987), as well as seeds during storage, germination and early development, from lipid peroxidation (Sattler et al., 2004). The protective role of α-tocopherol in atherosclerosis has been linked to its antioxidant properties, as well as nonantioxidant functions of α-tocopherol, including inhibition of monocyte-endothelial cell adhesion and inhibition of platelet adhesion and aggregation (Williams et al., 1997). In addition, γ-tocopherol also contributes in atherosclerosis prevention via inhibition of inflammatory mediators (e.g. prostaglandin E2, leukotriene B4) formation by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) (Jiang et al., 2000). A role of vitamin E in the regulation of gene transcription has been shown in human and rat smooth muscle cells. α-Tocopherol can induce the gene expression of connective tissue growth factor (Villacorta et al., 2003) and α-tropomyosin (Aratri et al., 1999), which link the role of vitamin E in vascular health.

The anti-cancer effects of tocopherols are related to their molecular and enzymatic actions on the cell cycle and cellular signalling. α-Tocopherol can increase protein phosphatase 2A activity inhibiting the action of protein kinase C (PKC) in vascular smooth muscle cells, which leads to growth arrest (Boscoboinik et al., 1991) and induces apoptosis. α-Tocopherol has been shown to induce apoptosis through various pathways in different human cancer cell lines (Gyzin et al., 2002).

2.7 Absorption and metabolism of carotenoids, phylloquinone and tocopherols

Carotenoids, tocopherols and phylloquinone, as well as other fat-soluble nutrients from plant foods share similar digestive and absorptive pathways with some modifications unique to each nutrient. Digestion begins in the oral cavity where plant matrices are broken down by chewing and lubricated with saliva. Salivary α-amylase breaks down starch in the plant matrices releasing carotenoids, tocopherols and phylloquinone. As chyme enters the gastric lumen, pepsin, HCl and gastric lipases further degrade food matrix to release fat-soluble nutrients for dissolution in lipid droplets within the chyme. Gastric lipases hydrolyze dietary triacylglycerol (TG) to diacylglycerol (DG) and free fatty acids, which facilitate emulsification for continued digestion of lipid droplets. Polar carotenoids tend to be distributed at the surface of the oil droplets, whereas nonpolar carotenoids are located in the

core (Borel et al., 1996). Lypolysis continues in the intestinal lumen followed by formation of dispersed aggregates containing lipolytic products, phospholipids, cholesterol and lipophilic nutrients in the bile salt-rich aqueous duodenum (Carey et al., 1983). These aggregates are solubilized within micelles to mediate transport of fat-soluble substances across unstirred water layers on the apical surface of small intestine. Different ester forms of vitamin E (i.e., α-tocopheryl acetate) require additional steps, such as hydrolysis by pancreatic bile acid-dependent lipase (Zu & Ip, 2003) and digestion by intestinal mucosa esterase (Livny et al., 2003) prior to uptake into enterocytes. Intestinal uptake of carotenoids, tocopherols and phylloquinone has long been known to occur via passive diffusion. However, recent evidences showed the involvement of the scavenger receptor class B type 1 (SR-B1) and other intestinal membrane transporters, such as Niemann-Pick C1-like 1 (Davis et al., 2004) and cluster determinant 36 (Traber & Sies, 1996) in the absorption of these fat-soluble micronutrients. In vitro studies using CaCo2 cells demonstrated a rapid, saturable and temperature dependent tocopherol absorption (Anwar et al., 2006). The free alcohol forms of tocopherol are non-selectively absorbed in the human GI tract (Swanson et al., 1999). Moreover, the newly absorbed fat-soluble nutrients are secreted by the intestine into lipoproteins, primarily chylomicrons, as the carriers to target tissues, where they are utilized, processed and/or stored.

The transport mechanism of carotenoids, tocopherols and phylloquinone from enterocytes to target tissues varies for each nutrient and may be influenced by dietary and host-related factors. Absorbed carotenoids are carried almost exclusively by chylomicron to the systemic circulation where the chylomicron is rapidly degraded by lipoprotein lipase. Chylomicron remnants are cleared by the liver where carotenoid modification, bioconversion and storage take place (Wang et al., 1992). Very low density lipoproteins (VLDL) and low density lipoprotein (LDL) become the next major carotenoids carriers, consecutively (O'Neill & Thurnham, 1998). The VLDL, LDL and HDL carry 14%, 55%, and 31% of the total carotenoids in the plasma of fasting subjects, respectively (Clevidence & Bieri, 1993). Hydrocarbon carotenoids, such as α- and β-carotene and lycopene, are mainly found in the LDL, whereas the xanthophylls, such as lutein and zeaxanthin, are primarily detected in HDL (Clevidence & Bieri, 1993).

Intracellular vesicular trafficking directs vitamin E for incorporation into chylomicron while protein-mediated traffic may be involved in transport of vitamin E into HDL (Anwar et al., 2006). A study using the CaCo-2 cell model showed that the proportion of vitamin E absorbed as HDL versus chylomicrons seems to be regulated by fatty acid availability (et al., 2006). The majority of plasma tocopherols is transported by LDL and HDL; only a small portion (< 20%) is carried by VLDL (Perugini et al., 2000). In the liver, α-tocopherol transport protein (α-TTP) discriminates between R,R,R-α-tocopherol and S,R,R-α-tocopherol for transport to VLDL, explaining the different biological activities of vitamin E isomers. Vitamin E homeostasis is closely related to lipoprotein metabolism (Rigotti, 2007).

More than half of the absorbed phylloquinone is carried by chylomicrons, whereas progressively more phylloquinone is found in LDLs and HDLs (Lamon-Fava et al., 1998). The progressive increase of phylloquinone in LDLs and HDLs may be due to the fact that LDLs are products of VLDL metabolism (Havel & Kane, 1995), as well as a result of phylloquinone exchange between lipoproteins (Lamon-Fava et al., 1998). Plasma fat-soluble vitamin concentrations usually peak at a later time than triglyceride (Krasinski et al., 1990; Meydani et al., 1989; van Vliet et al., 1995) possibly because 1) phylloquinone remains bound to the particle remnants when triacylglycerols are shed from chylomicron during lipolysis; 2) newly formed VLDLs containing phylloquinone are secreted as more chylomicron remnants are taken up by the liver and 3) selective intestinal absorption may result in delayed phylloquinone absorption compared with fatty acid absorption (Lamon-Fava et al., 1998).

Moreover, proteins involved in lipoprotein metabolism have been related to lipid status and thus play significant roles in the absorption, intracellular trafficking and plasma transport of vitamin E, carotenoids (Borel et al., 2007) and perhaps other fat-soluble micronutrients. The association between plasma concentrations of vitamin E and carotenoids with genetic polymorphisms in genes involved in lipid metabolism (Borel et al., 2007) may support the statement above.

2.8 Factors influencing the bioavailability of carotenoids, tocopherols and phylloquinone in salad vegetables

The bioavailability of fat-soluble nutrients is influenced by interfering factors in the digestion and absorption steps of plant foods, such as food matrix, processing, physicochemical properties, interactions with other dietary components and host-related factors.

2.8.1. Food matrix and processing

Micronutrients have to be released from the food matrix in order to be absorbed. The location of these nutrients within the plant matrix and the type and the particle sizes of plant foods influence micronutrient bioavailability. Carotenoids are located in the chromoplasts and the chloroplasts of fruits and leafy vegetables, respectively. It has been suggested that chromoplasts are more easily disrupted than chloroplasts in the intestine, hence the greater bioavailability of these nutrients from fruits than green leafy vegetables (de Pee et al., 1998). Purified or supplemental forms of these micronutrients are more bioavailable compared with fresh sources (Garber et al., 1999; To¨ rro¨ nen et al., 1996). Absorption of phylloquinone is higher from a supplement tablet than from food sources, while the choice of food source does not have a major influence on phylloquinone bioavailibility (Garber et al., 1999). Moreover, food processing methods alter the food matrix by fragmenting the food plants into smaller pieces (i.e., chopping, grinding or liquefying) and thus increase the release of carotenoids from plant matrices (Kopsell & Kopsell, 2006). Cooking has been shown to improve the absorption of β-carotene, lycopene (Gartner et al., 1997; Rock et al., 1998) and phylloquinone (Damon et al., 2005) possibly due to increased extractibility from the vegetable matrix. Heating leads to degradation of carotenoids in some food plants (Kopsell & Kopsell, 2006), whereas freezing preserves the carotenoid content by reducing potential enzymatic oxidation (Kopas-Lane & Warthesen, 1995; Rodriguez-Amaya, 2003).

2.8.2 Chemical properties and isomeric composition of nutrients

Species of carotenoids, linkages at molecular level and the amount of carotenoid are among the determining factors of carotenoid bioavailability (Castenmiller & West, 1998). Moreover, different degrees of lipophilicity account for different bioavailability between carotenes and xanthophylls. Lutein in vegetables has been shown to be more bioavailable than β-carotene (Castenmiller & West, 1998; van het Hof et al., 1999). Due to lower lipophilicity, xanthophylls are released in a greater amount in the aqueous environment

(Castenmiller & West, 1998) and are located at the surface of oil droplets with ready access to mixed micelles and more readily partitioned into micelles than carotenes (Ribaya-Mercado et al., 2007; Rich et al., 2003). In vivo studies demonstrate that the *cis*-isomers of lycopene appear to be more bioavailable than the all-*trans* isomer (Stahl & Sies, 2003, Unlu et al., 2005), which is at least partially due to more efficient micellarization and uptake by the enterocyte relative to all-*trans* lycopene (Failla et al., 2007). Animal studies have shown a lower bioavailability of γ-tocopherol compared with α-tocopherol due to (i) preferential binding of α-tocopherol to α-TTP spell out in the liver for incorporation into VLDLs (Traber et al., 1992) and (ii) degradation of γ-tocopherol to γ-CEHC followed by excretion in the urine (Swanson et al., 1999).

2.8.3. Interactions with co-ingested dietary components

During digestion and absorption, carotenoids, tocopherols, phylloquinone and co-ingested dietary components interact at various steps, such as micellar incorporation, intestinal uptake and lymphatic transport, which results in competition for absorption (van Het Hof et al., 2000). These dietary components include fiber, phytosterols, protein, other fat-soluble nutrients and dietary lipids. Dietary fiber has been reported to impair carotenoid absorption from plant foods by interfering with micelle formation due to partition of bile salts and fat in the gel phase of dietary fiber (van Het Hof et al., 2000), and by entrapping carotenoids and increasing fecal excretion of fat-soluble substances (Riedl et al., 1999; Rock & Swendseid, 1992; Yeum & Russell, 2002). The effect of dietary fibers on the absorption of fat-soluble nutrients is not conclusive because of the difficulty in comparing studies with various sources of dietary fiber (Unlu et al., 2005). Sucrose polyester and plant sterols also reduce bioavailability through interaction with hydrophobic compounds to form hydrophilic cores, which are not subject to lipase degradation and thus decrease micellarization (Westrate & van het Hof, 1995). High protein diets lower carotenoid and tocopherol bioavailability (Sundaresan et al., 2005). The effects of co-ingested dietary lipids on the bioavailibility of carotenoids, tocopherols and phylloquinone will be discussed in section 2.9.

2.8.4. Host-related factors

The absorption of carotenoids, tocopherols and phylloquinone is modulated by the health status of individuals, which includes gut health, nutritional status, physiological status,

genotype and lifestyle. Disorders in the production and activity of digestive enzymes in the gut lumen, abnormalities in the metabolism of lipids as well as pathological changes in morphological and functional integrity of the absorptive epithelium may affect the efficiency of absorption. For example, increased gastric pH decreases β-carotene absorption (Takyi, 1999). Some diseases (cholestasis, biliary cirrhosis, cystic fibrosis) that cause fat malabsorption also inhibit the absorption of fat-soluble nutrients (Bennett & Medwadowski, 1967; Zaripheh & Erdman, 2002). The expression and activity of intestinal membrane transporters (e.g., SR-B1 & ABCA1) also determine the absorption of these nutrients. Moreover, diarrhea and intestinal parasitism impair fat absorption and in turn the uptake of carotenoids and fat-soluble vitamins (Rosenberg et al., 1977; Swanson et al., 1999).

Nutritional status is another important factor affecting absorption of carotenoids, tocopherols and phylloquinone. Once per day consumption of cooked spinach with 0 g and 5 g of added fat led to similar improvement of serum retinol level in children with poor vitamin A status; whereas no improvement in serum retinol level with 0 g added fat in children with better vitamin A status (Jayarajan et al., 1980) indicating that vitamin A status may affect the absorption and/or conversion of pro-vitamin A carotenoids from the diet.

Eating disorders are among the diseases that not only alter nutritional status, but may also lead to physiological disturbances, such as acid-base and electrolyte disturbances and abnormalities of lipid metabolism. Women with eating disorders, especially the amenorrheic ones, tend to be hypercholesteremic (Mira et al., 1989) and have elevated concentrations of the fat-soluble vitamins A and E (Mira et al., 1988). In this study, the SCOFF questionaire was used to screen applicants for eating disorders (Morgan et al., 1999). In addition, severe vitamin A deficiency impairs the integrity of intestinal epithelium, which induces malabsorption of pro-vitamin A carotenoids and possibly other fat-soluble nutrients.

Individual lifestyles, such as smoking and frequent alcohol consumption, may affect the absorption of these nutrients. Smoking has been reported to influence energy and nutrient intake as well as intestinal absorption. Smokers were found to have lower dietary and plasma levels of vitamin C, β-carotene and vitamin E compared with non-smokers (Chopra et al., 2000). Smoking induces the stimulation of sympathetic nervous system, which in turn increases fasting plasma cortisol (Chiolero et al., 2008). Cortisol concentration influences the

formation of visceral adipose tissue that may affect lipid metabolism, which may explain how smoking influences absorption of fat-soluble nutrients. Frequent and excessive alcohol consumption may promote alterations of the mucosal lining in the gastrointestinal tract due to chemical damage, which subsequently lead to malabsorption of nutrients (Rajendram & Preedy, 2005). Alcohol metabolism generates reactive oxygen species both in the mitchondrial electron transport chain and by mitogenic oxidase (MOX) inducing oxidative stress, which is attributed to alcoholic liver disease (1). The use of hormonal contraceptives has been reported to affect lipid and vitamin metabolism. Estrogen-containing oral contraceptives tend to raise plasma lipids and lipoproteins (Wallace et al., 1979). Oral contraceptives also affect the mobilization and redistribution of tissue vitamin A stores resulting in increased vitamin A requirement. Animal studies showed that oral contraceptive agents increase the activities of intestinal enzymes (Prasad et al., 1975). Taken all together, oral contraceptive agents may very likely affect the absorption of fat-soluble nutrients.

2.9 Effects of dietary lipids on the bioavailability of carotenoids, tocopherols and phylloquinone

Dietary lipids influence the bioavailability of fat-soluble nutrients through their involvement in micelle and chylomicron formation. The type and amount of dietary lipids, as well as the digestibility of fat-soluble components have been shown to affect carotenoid, tocopherol and phylloquinone absorption. Increased fat content of a meal has been shown to enhance the bioavailability of these fat-soluble nutrients. For instance, salad vegetables ingested with full fat salad dressing (28 g canola oil/serving) resulted in higher levels of carotenoids in the postprandial chylomicron fraction compared with reduced fat (6 g canola oil/serving) and fat free salad dressings (Brown et al., 2004). Results of a study involving ingestion of ^{2}H-labelled RRR α-tocopheryl acetate capsule with a test meal of toast with butter (17·5 g fat), cereal with full-fat milk (17·5 g fat), cereal with semi-skimmed milk (2·7 g fat) and water (0 g fat) showed that both the amount of fat and the food matrix influence absorption (Jeanes et al., 2004). In addition, phylloquinone absorption from spinach was improved by addition of butter (Gijsbers et al., 1996). An in vitro digestion model showed a high biacessibility of α-tocopherol, but not γ-tocopherol, in romaine lettuce when

homogenized with fat (Reboul et al., 2006b). The presence of fat in the intestinal lumen induced the secretion of vitamin E with intermediate density lipoproteins. Available lipids and oleic acids facilitated enterocytes to secrete vitamin E with chylomicrons (Rigotti, 2007).

The amount of co-ingested fat needed for optimal bioavailability of carotenoid and other fat-soluble nutrients is however still controversial (van het Hof et al., 2000; Yeum & Russell, 2002; Zaripheh & Erdman, 2002). Optimal carotenoid bioavailability was achieved with only a small amount of dietary fat (2 g-5 g/test meal) from cooked vegetables and supplements (Erdman et al., 1993; Ribaya-Mercado et al., 2007; Roodenburg et al., 2000). Dubois et al. (Dubois et al., 1994) reported that 42 g is the threshold amount of fat to promote significant postprandial changes in lipoprotein particles. Moreover, a lower amount of co-ingested fat may be needed for optimal carotenoid bioavailability in cooked compared with raw vegetables. Specific carotenoids may require different amounts of co-ingested fat for optimal absorption (Jayarajan et al., 1980; Ribaya-Mercado et al., 2007).

Different types of lipid in the diet have different absorbability (van Het Hof et al., 2000), which affects the incorporation of fat-soluble nutrients into chylomicrons. Hu et al (Hu et al., 2000) reported that ingestion of β-carotene with a meal rich in polyunsaturated fatty acid (PUFA) resulted in lower appearance of β-carotene and greater appearance of triacylglycerol in triacylglycerol-rich lipoproteins compared with a meal rich in saturated fatty acids. Long-chain triglycerides facilitate a greater formation of chylomicrons and consequently a greater carotenoid absorption compared with medium-chain triglycerides (Borel et al., 1998). The fat composition of the second meal was shown to play a role in carotene packaging into chylomicrons. A medium-chain fatty acid-containing second meal did not result in the secretion of chylomicrons (Borel et al., 1998). In contrast, unsaturated fatty acids were shown to be efficiently incorporated into lipoprotein (van Greevenbroek et al., 1996), thus contributing to chylomicron formation. The efficient incorporation of unsaturated fatty acids into lipoprotein may explain the observation of enhanced carotenoid absorption from a diet containing unsaturated fatty acids, especially oleic acid (Unlu et al., 2005). In addition, the presence of a non-absorbable fat replacer, such as sucrose polyesters, in the diet significantly decreased the plasma carotenoids concentrations (Koonvitsky et al., 1997; Schlagheck et al., 1997; Westrate & van het Hof, 1995) due to reduced bioavailability

of carotenoids.

Furthermore, a fat-containing previous meal has been reported to cause an early post-prandial peak in chylomicron and plasma triacylglycerol concentrations (Evans et al., 1998). Griffiths et al. (Griffiths et al., 1994) reported that an early plasma triacylglycerol peak after an overnight fast was observed following a low-fat meal suggesting that chylomicrons are pre-formed rather than newly formed before they are released into the circulation (Evans et al., 1998). The proposed explanation for this phenomenon is as follows; the release rate of pre-formed chylomicron into the plasma is accelerated by an increase in intestinal lymph flow (Evans et al., 1998), which may be mediated by an increase in small intestine blood flow or intestinal muscle contractions (Schmid-Schonbein et al., 1990) as results of environmental (i.e. smell, sight of foods) or physiological (i.e., presence of bolus) cues.

2.10 Carotenoid, tocopherol and phylloquinone extraction and analysis

2.10.1 Extraction from plant matrices and biological fluids

Routine and standard methods to simultaneously extract and analyze carotenoids, tocopherol and phylloquinone have not been established. Several factors contribute to the difficulty in developing a single extraction method and detection system to analyze all of these analytes. Some of these factors are (i) the mixture of different isomeric forms in nature, (ii) the different stability among fat-soluble nutrients, (iii) the presence of metabolites and synthetic forms in the sample, and (iv) the different detection parameters for each nutrient. The focus of this review is the multi-analyte extraction and quantification methods of these compounds from vegetetables and human serum.

Conventional extraction methods for fat-soluble nutrients include saponification, direct solvent extraction (DSE) and solid phase extraction (SPE) (Eitenmiller & Landen, 1999b). Saponification or alkaline hydrolysis requires these following steps: (i) addition of ethanol-based basic solution (e.g., KOH, NaOH) containing antioxidant (e.g., pyrogallol, ascorbic acid), (ii) thorough mixing to ensure the basic solution can get access to sample matrix, (iii) flushing the saponification medium with inert gas (e.g. nitrogen (N_2)), (iv)

refluxing using air condenser, (v) cooling of the digest, (vi) addition of 1% NaCl or water, (vii) phase separation of the digest with the suitable solvent mixtures, (viii) collection of the organic phase, (ix) washing of the organic solvent to remove fatty acid soaps, and (x) concentration of the nonsaponifiable fraction. Fat-soluble nutrients are released from the food matrix through alkaline hydrolysis of ester linkages and phospholipids, as well as separation of nutrients from proteins, lipid and carbohydrate complexes. Saponification also facilitates the removal of pigments (e.g. chlorophylls) and other substances that may interfere with the chromatography. The effectiveness of saponification in penetrating complex matrices makes it ideal for extraction from tissues, vegetables, fruits, prepared foods (Eitenmiller & Landen, 1999a). However, it may not be suitable for multi-analyte analysis because some fat-soluble nutrients are sensitive towards oxygen, light, extreme pH and heat. For example, extreme alkaline environment leads to complete degradation of vitamin K and destruction of vitamin E homologs. Additionally, high temperature decreases xanthophyll recovery (de Quiros & Costa, 2006). Additional steps such as flushing with nitrogen, protection from light and addition of antioxidant (e.g. ascorbic acid, pyrogallol) are included to minimize these destructive effects. Another disadvantage of saponification is the formation of artifacts in the extract, which can reduce the accuracy of peak identification, the estimation of vitamin A activity, and the chromatographic resolution. Some of the commonly used solvents in conventional extraction methods are tetrahydrofuran, hexane, acetone, methanol, ethanol and petroleum ether (Eitenmiller & Landen, 1999a). Extraction of tocopherol and tocotrienol homologs from the saponification medium is affected by the composition of the solvent mixture and the amount of lipid and ethanol in the sample medium (Ueda & Igarashi, 1985).

Direct solvent extraction utilizes a mixture of organic solvents to extract fat-soluble nutrients directly from the sample matrix. DSE is preferred over saponification for extraction from simpler sample matrices, such as physiological fluids, because of time, lower expenses for solvents and reduced risk of artifacts formation in the extract. Steps in the direct solvent extraction are (i) proteins denaturation with ethanol, methanol or acetonitrile, (ii) addition of buffer to enhance the extraction efficacy, (iii) addition of organic solvent to extract the analytes, (iv) centrifugation to promote phase separation and (v) removal of organic solvent

via evaporation. The solvent mixtures used for each class of fat-soluble nutrients vary. Some of the commonly used solvent mixtures for carotenoids analysis are acetone:methanol (50:50), tetrahydrofuran:methanol (50:50), hexane:ethyl acetate (85:15) and ethanol:hexane (4:3) (de Quiros & Costa, 2006), while for tocopherols, the Folch extraction with choroform:methanol (2:1), acetone, diethyl ether, and Soxhlet extraction are used (Lambert et al., 1986). Phylloquinone in serum can be extracted through protein denaturation with water-soluble organic solvents (e.g., ethanol, isopropanol and acetonitrile) followed by partitioning with nonpolar solvents (e.g., hexane); whereas phylloquinone in more complex matrices requires elimination of lipid components before chromatographic analysis. Lipase hydrolysis is performed to eliminate interferences in the chromatogram. Interfering lipids may also be removed by using SPE with silica cartridges (Eitenmiller & Landen, 1999c). SPE is also used in extraction of phylloquinone from fruits and vegetables after treatment with hexane (Perez-Ruiz et al., 2006). Similar to solvent extraction, SPE requires large amounts of organic solvents (Turner et al., 2001).

More recent extraction methods utilize supercritical fluids, such as carbon dioxide, nitrous oxide, ethane, propane, n-pentane, ammonia, fluoroform, sulphur hexafluoride and water (Turner et al., 2001). A supercritical fluid is any substance at a temperature and pressure above its critical point allowing them to diffuse through solids like gas and to dissolve materials like liquid. In principle, supercritical fluid extraction (SFE) system consists of extraction chamber, oven, high pressure pumps, restriction device and collection device (Turner et al., 2001). The mechanism of SFE is presented by Turner et al, as follow (i) weighed sample is placed into extraction cell, where the heating and pressurization take place, (ii) supercritical fluid is then pumped through the extraction cell to extract the target analytes from the sample matrix, (iii) extract enters the restriction device in which the pressure is reduced to ambient pressure and (iv) reduction in the pressure turns supercritical fluid into its gaseous phase leaving target analytes trapped in the collection device. Two types of collection mode include solvent collection and solid-phase trapping. Turner et al. (Turner et al., 2001) suggested that SFE offers some benefits, such as shorter extraction time, higher selectivity, minimal use of organic solvents, exclusion of oxygen, reduction of heat and increased sample throughput compared with conventional extraction techniques.

2.10.2 Analysis and detection methods

Liquid chromatography (LC) is the gold standard in fat-soluble nutrient analysis. Some of the strengths of LC compared with previous approaches are the ability to separate cis-isomers from all-*trans*-retinol and all-*trans*-carotenoids and to isolate pro-vitamin A carotenoids from carotenoid mixtures. One disadvantage of liquid chromatography is the use of large amounts of organic solvents (Turner et al., 2001). Normal-phase and reversed-phase liquid chromatography are widely used for fat-soluble nutrient analysis. Normal phase liquid chromatography uses more polar stationary phase than the mobile phase, while reversed-phase liquid chromatography uses more polar mobile phase than the stationary phase according to the IUPAC definition (Eitenmiller & Landen, 1999a). Most LC methods use reversed-phase C18 or C30 systems. Compared with normal-phase, reversed-phase chromatography provides some benefits, such as (i) lower tendency for retention time shift due to the inclusion of water, (ii) greater stability against minor changes in mobile phase content, (iii) less complicated removal of contaminants (iv) more flexible toward changes in mobile phase composition and (v) ability to resolve substances varying in polarities (Eitenmiller & Landen, 1999a). However, normal phase chromatography is suitable for separation of *cis*- from *trans* isomers of retinoids and carotenoids. Years of studies have shown the superiority of different chromatographic columns for specific analytes. For example, monomeric C18 provides a great resolution for some xantophylls, while plymeric C18 allows a greater shape selectivity toward geometric isomers and is routinely used in *cis*- and *trans*-carotenoids studies. The recent C30 column gives the greatest resolution, effectiveness to resolve *cis*- from *trans*-carotenoids, as well as multi-analyte analysis. Liquid chromatographic systems are equipped with a detection system for identification and quantification purposes. Some of the common detection systems used for fat-soluble nutrients include ultraviolet-visible (UV-VIS), mass spectrometry (MS), fluorescence and multiwavelength. Less commonly used detectors include atmospheric pressure chemical ionization (APCI) and electrochemical detectors (ECD). UV detection is considered to be the standard approach in carotenoid analysis. The quantification limit for carotenoids is less than 1 ng/mL. Fluorescence detection reduces the presence of chromatographic interferences in

tocopherol analysis compared with UV detection (Eitenmiller & Lee, 1999).Van Niekerk's work (Eitenmiller & Landen, 1999c) showed that fluorescence detection is ideal, sensitive and specific for vitamin E analysis.

Electrochemical detection (ECD) is less commonly used but is highly advised when the amount of sample is limited. Coulometric array ECD provides great sensitivity and very low detection limits that are three times lower compared with UV detection (Hagen et al., 1996). The basis of electrochemical detection is represented by Faraday's law; the detector measures the voltage generated based on "the number of moles converted to product in the cell and the number of electrons involved in the reaction" (Kissinger, 1977). The coulometric detector has 100% conversion efficiency, whereas the amperometric detector has much lower conversion efficiency (1-10%). In order to maximize the conversion efficiency, the coulometric detector consists of more surface electrode area downstream, which leads to less participation of each segment of surface electrode in converting analytes into products proportionately to the total conversion, but relatively equal to background current due to solvent breakdown (Kissinger, 1977). Unlike other detection methods, ECD can not be coupled with normal phase separations because of incompatibility between non-polar mobile phase and electrochemical reactions (Kissinger, 1977) leaving reversed-phase and ion-exchange stationary phase as the options. Affecting factors in ECD analysis are pH, electrochemical reactivity of the solvent and electrolytes, ionic strength and presence of contaminants (oxygen, hallides, trace metals). The selectivity of ECD is inversely related to oxidation or reduction potentials chosen (Kissinger, 1977). ECD with amperometric detection was used for the simultaneous analysis of fat-soluble vitamins in milk and milk powder with a glassy carbon electrode at +1050 mV giving low ng detection limits (28-30). In this study, amperometric detectiont is used to simultaneously analyze 9 analytes in serum samples at very low detection limits, including α-carotene, β-carotene, lutein, 13-*cis*-lycopene, 9-*cis*-lycopene, α-tocopherol, δ-tocopherol, γ-tocopherol and phylloquinone demonstrating the sensitivity, versatility and economy of ECD.

Coupling of LC and mass spectrometry (MS) contributes to significant advantages in fat-soluble nutrients analysis. The application of LC/MS has been extended through coupling with other detection methods. For example, coupling LC/MS and APCI eliminated the need

for purification and derivatization steps in retinyl palmitate and β-carotene analysis in human serum (Wang et al., 2000). Gas chromatography (GC) with MS and flame ionization detection (FID) has also been used to analyze fat-soluble nutrients. GC/MS is ideal for phylloquinone analysis in biological samples. The sample preparation method includes reductive acylation step to prevent phylloquinone degradation (Fauler et al., 2000). The detection limit and the quantification limit were 1.0 pg and 2.0 pg/mL, respectively. GC can not be used for analysis of carotenoids and other thermolabile compounds because it imposes a high risk of thermal degradation even after derivatization process (Eitenmiller & Landen, 1999a). Several developed methods for simultaneous serum carotenoids, tocopherols and phylloquinone analysis utilize various detection mechanisms, including multiwavelength (Wang et al., 2000), fluorescence after phylloquinone reduction (Biere et al., 1979), combination between UV-VIS and fluorescence (Jakob & Elmadfa, 1995), and atmospheric pressure chemical ionization (APCI) positive ion mode with SIM (Heudi et al., 2004).

2.10.3 Lipoprotein separation and extraction

The different hydrated densities, particle sizes, floatation rates at a specific density and composition of lipoprotein fractions in human serum are the basis of their separation methods. Several lipoprotein separation methods have been developed since 1950s, which include ultracentrifugal floatation, Cohn fractionation and zone electrophoresis (Havel & Kane, 1995). Isolated lipoprotein fractions can be identified and quantified from the lipid content of the fractions or using a number of methods, including refractometric methods (Havel & Kane, 1995), gel electrophoresis, HPLC, electron microscopy and spectroscopy; while the recovery and purity of lipoprotein fractions can be measured using enzymatic measurements (e.g. western blot), immunoprecipitation, radioimmunoassays and ^{125}I-labeled lipoproteins (Chung et al., 1980). Analytical ultracentrifugation is one of the most applied techniques in lipoprotein separation methods. Although, these methods vary in the instruments used, sample volume required, duration, and separation ability, they apply the same basic principle. Lipoproteins float at rates dependent on their densities, sizes and shapes at solvent densities higher than the lipoprotein density. Different lipoprotein fractions will float accordingly by layering blood serum with solvents at different densities after

ultracentrifugation. The basic procedures in ultracentrifugal lipoproteins extraction include (i) density adjustment using salt solutions (e.g. NaCl, KBr) (ii) ultracentrifugation and (iii) collection of different fractions. In general, there are three commonly used lipoproteins separation and extraction methods in post-absorptive fat-soluble nutrients studies done in humans. They are sequential floatation ultracentrifugation, density gradient centrifugation and cumulative rate ultracentrifugation. Sequential floatation ultracentrifugation involves more than one ultracentrifugation after gradual addition of solvent with higher density (Havel & Kane, 1995). This method separates lipoproteins on the basis of their hydrated densities. Density gradient ultracentrifugation isolates lipoprotein fractions according to particle sizes and facilitates extraction of highly purified fractions with only a single spin ultracentrifugation. The density range of the gradient allows for the variations in lipoprotein distributions, which often occur in disease states. Cumulative rate ultracentrifugation is the combination of the two methods described previously. It requires multiple centrifugations at different speeds and density gradients, which allows separation on the basis of both density and particles sizes. Although, it is less commonly used, cumulative rate ultracentrifugation results in high purity and high recovery lipoprotein fractions, as indicated by the absence of apolipoprotein B-48 in the higher density lipoproteins (Paetau et al., 1997). In order to isolate lipoprotein fractions and analyze their contents (e.g., fat-soluble nutrients), the ultracentrifugal extraction is followed by chemical analysis of the separated fractions.

CHAPTER 3

The dose-response effects of the amount of oil in salad dressing on the bioavailability of carotenoids and fat-soluble vitamins in salad vegetables

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3.1 Abstract

The objectives of the study were to define the dose-response relation of the amount of added oil and: 1) the absorption of carotenoids, phylloquinone and tocopherols in salad vegetables and 2) the absorption of retinyl palmitate formed from the ingested provitamin A carotenoids, α- and β-carotene. Women (n = 12) each consumed 5 salads containing equivalent amounts of carrot, cherry tomato, romaine lettuce and spinach. The salads with salad dressings containing 0, 2, 4, 8 or 32 g tocopherol-stripped soybean oil were ingested in random order separated by ≥ 2 weeks. Blood samples were collected at baseline and 2, 3.5, 5, 7, and 9.5 h postprandially. Chylomicron fractions were extracted and analyzed by HPLC with coulometric array electrochemical detection. When the salads were ingested with 0 g oil, there was negligible absorption of α- and β-carotenes, lutein, lycopene, phylloquinone, retinyl palmitate, α- and γ-tocopherols. For α- and β-carotenes, lycopene, retinyl palmitate, and α- and γ-tocopherols, absorption was increased with each amount of oil compared with 0 g oil (P <0.05). Starting from 4 g oil, all analytes (α-carotene, β-carotene, lutein, *trans*-lycopene, vitamin A, α-tocopherol, γ-tocopherol, and vitamin K_1) showed significant

increases in absorption compared with 0 g oil salad dressing. The absorption of each carotenoid and fat-soluble vitamin was highest with 32 g ingested oil ($P < 0.002$).

3.2 Introduction

In the Third National Health and Nutrition Examination Survey (NHANES) salad consumption was reported by one third of U.S.adults. Salad consumption was associated with higher serum concentrations of micronutrients, including vitamins A and E, as well as provitamin A carotenoids (α and β carotene) (Su & Arab, 2006). Salad vegetables are among the major sources of vitamin E in the U.S. diet (Maras et al., 2004; Murphy et al., 1990). However, food consumption data may be misleading because the amounts of micronutrients absorbed from raw salad vegetables may be low, especially for the fat-soluble nutrients. The bioavailability of carotenoids and phylloquinone was reported to be significantly lower from raw vegetables compared with processed vegetables (Garber et al., 1999; Rock et al., 1998). One approach to improve the bioavailability of fat-soluble nutrients is to incorporate fat during processing and/or ingestion of the vegetables. Absorption of fat-soluble nutrients from vegetables was improved by increasing the fat content of a meal (Gijsbers et al., 1996; Stahl & Sies, 2003). By using an *in vitro* digestion model, a high bioaccessibility of α-tocopherol, but not γ-tocopherol, in romaine lettuce was shown when the lettuce was homogenized with fat (Reboul et al., 2006b). In our previous study, the absorption of carotenoids from salad vegetables was higher with full fat compared with reduced-fat and fat-free salad dressings (Brown et al., 2004). However, increasing the amount of co-ingested fat raises a concern considering the association between high fat intakes and the obesity epidemic (Corella et al., 2007; Snell-Bergeon et al., 2009). It is necessary to identify the minimal amount of fat needed to improve the bioavailability of carotenoids and other fat-soluble micronutrients in unprocessed vegetables.

The overall objective of this study was to improve the bioavailability of dietary fat-soluble nutrients and bioactives to lower the risk of chronic disease and enhance overall health. The specific goals were to quantitatively determine the effects of the amount of added fat on the absorption of carotenoids, phylloquinone and tocopherols in salad vegetables and

to investigate whether low fat levels can significantly increase the uptake of these compounds compared with zero fat level.

3.3 Subjects and Methods

3.3.1 Subject recruitment

The screening of subjects consisted of two steps including an interview and a blood screening. The initial screening procedures consisted of a standardized interview addressing health and lifestyle factors and a written SCOFF questionnaire (Morgan et al., 1999). The interview was followed by measurements of height and weight. Subjects that passed the initial screening were invited to complete blood screening consisting of a cell blood count, blood biochemistry profile, and plasma lipid panel. The exclusion criteria were designed to eliminate factors known to interfere with lipid metabolism, as well as the absorption and metabolism of target analytes. The inclusion criteria included females 18-39 years of age and excellent health as indicated by health history, complete blood count, and blood biochemistry profile, and normolipidemia as indicated by the plasma lipid panel. The exclusion criteria included cigarette smoking (previous 12 months), frequent alcoholic beverages consumption (> 1 drink/day), current or recent (previous 1 mo) use of dietary supplements, plant sterols and/or medications known to affect lipid metabolism, use of hormonal contraceptive (previous 6 months), pregnant or planning a pregnancy, history of eating disorder, BMI $\geq$ 30, food allergies and modified diet (e.g., vegetarian). Also excluded were those with a history of restrictive eating - which was assessed by the SCOFF questionnaire (Morgan et al., 1999). Subjects with 2 or more "yes" responses were excluded. All documents and procedures involving human subjects were approved by the Iowa State University Institutional Review Board.

3.3.2 Experimental diets

Subjects were given a list of foods and beverages that are high in carotenoids, vitamins A, E, and K (Appendix A) and were asked to avoid the listed foods and beverages for days 1-3 of each of the five study periods. On day 4, subjects consumed a weighed,

standardized diet low in carotenoids, and vitamins A, E, and K (Appendix B). The nutrient contents of the diet were analyzed by Nutritionist Pro™ nutrient analysis software, version 4.4 (Axxya Systems, Stafford, TX). The daily diet provided an estimated 9.49 MJ, 73.9 g protein (12.8% of energy), 52.5 g fat (20.4% of energy), and 386.4 g carbohydrate (66.8% of energy). The calculated carotenoid, tocopherol, vitamin A, and vitamin K_1 contents of the experimental diet were 0.0 µg α-carotene, 10.8 µg β-carotene, 4.3 µg β-cryptoxanthin, 97.6 µg lutein (plus zeaxanthin), 0.0 µg lycopene, 0.7 mg α-tocopherol, 54.7 µg RAE vitamin A, and 20.0 µg vitamin K_1. On day 5, subjects consumed a reduced fat (2 g fat) snack and a low fat (3 g fat) lunch following the consumption of the test salad. These foods contained low amounts of carotenoids, tocopherols, vitamin A and vitamin K. All foods were consumed under supervision at the Iowa State University Nutrition and Wellness and Research Center (NWRC) except for the lunch andafternoon snack, which were carried out.

3.3.3 Test Salads

The test salad consisted of 48 g spinach (Spinach; Dole Food Company, Thousand Oak, CA), 48 g romaine lettuce (Hearts of Romaine, Fresh Express, Salinas, CA), 66 g raw shredded carrots (Shredded Carrots; Dole Food Company), and 85 g raw cherry tomatoes (NatureSweet, San Antonio, TX). The romaine lettuce and spinach were sorted to include only uniformly green leaves to minimize variation in chlorophyll and, by extrapolation, the carotenoid contents of the salads. All salad vegetables were washed with cold water and dried using paper towels followed by overnight storage at 4 °C. The day before the intervention day, salad vegetables were weighed in a glass bowl,covered and stored at 4 °C.

The salad dressings were supplied by Unilever and approved by the Unilever Safety and Environmental Assurance Centre. The salad dressings were of a two-phase oil-in-water type prepared by combining different amounts of soybean oil with complementary aqueous salad dressing base formulations (**Table 3**) to create 60 g of salad dressing with different oil contents (0, 2, 4, 8, or 32 g/60-g serving) (**Table 4**). The phylloquinone and tocopherol were stripped from the oil by a standard Short Path Distillation procedure to better study the absorption of these micronutrients from the salad. Five salad dressing base formulations were used for the corresponding treatments (0, 2, 4, 8, or 32 g of soybean oil) to ensure uniform

amounts of sugar, salt, and vinegar in the salad dressings. On the day of the intervention, soybean oil was added onto the test salad using a glass pipette. The appropriate weighed amount of the aqueous base formulation was then poured onto the salad. The salad was tossed carefully using plastic tongs to ensure even distribution of the salad dressing.

The total weight of the test salad (247 g) and the serving of salad dressing （60 g） were chosen as the 90th percentile of the quantity of salad (lettuce and other vegetables) consumed by adults per one occasion and the quantity of salad dressings reported by adults eating salad components at an eating occasion, respectively, in the 1994–1996 Continuing Survey of Food Intakes by Individuals (CSFII) (USDA, Agricultural Research Service, 1996). These amounts were chosen to simulate the typical intakes of salad dressing and salad vegetables for a person eating a salad as all or most of a meal.

Table 4. Salad dressing (SD) base formulations

Component	SD Base 1	SD Base 2	SD Base 3	SD Base 4	SD Base 5
Ultrapure RO water	89.7125	86.3825	83.0425	76.3825	36.3825
Sucrose	6.0000	6.0000	6.0000	6.0000	6.000
Salt, refined	0.6000	0.6000	0.6000	0.6000	0.6000
Spirit vinegar 12%	3.5000	3.5000	3.5000	3.5000	3.5000
Citric acid monohydrate	0.1800	0.1800	0.1800	0.1800	0.1800
EDTA Dissolvine	0.0075	0.0075	0.0075	0.0075	0.0075
Soybean oil (%)	0.0000	3.3300	6.6700	13.3300	53.3300
Total	100	100	100	100	100

Table 5. Amount of soybean oil and salad dressing (SD) base formulation on the salads

Treatment	Soybean oil (g)	SD base (g)	Total (g)
1	0.00	60.00 g SD base 1	60.00
2	2.00	58.00 g SD base 2	60.00
3	4.00	56.00 g SD base 3	60.00
4	8.00	52.00 g SD base 4	60.00
5	32.00	28.00 g SD base 5	60.00

3.3.4 Experimental design

Each subject consumed five test salads that were identical in vegetable composition with salad dressings that had different oil contents (0 g, 2 g, 4 g, 8 g, or 32 g of soybean oil). The order of treatments was randomly assigned to each subject in order to avoid carryover effects and to ensure the validity of the treatment effect. The 5 test salads were consumed in 10 treatment orders given by the Williams Design (Chinchilli & Esinhart, 1996), which were randomly assigned to ten of the twelve subjects. One of the treatment orders and its inverse were randomly assigned to the remaining two subjects. Each of the test salads was ingested separated by a washout period of ≥ 2 weeks.

The subjects ingested 240 mL of bottled water and waited 15 minutes before consuming the test salads. All subjects consumed the test salad along with 240 mL of water within 30 minutes.

Blood samples were collected from a forearm vein via a butterfly needle blood collection set (BD Safety-Lok™ Blood Collection Set, Becton, Dickinson, and Co, Franklin Lakes, NJ). Additional blood samples were collected at t = 0 (after a 12-h overnight fast and before salad consumption) and at 2, 3.5, 5, 7 and 9.5 hours after salad consumption.The blood samples were then transferred to 10-mL plastic vacutainer tubes containing spray-dried K2 EDTA as anticoagulant (BD Vacutainer®, Beckton, Dickinson, and Co). Blood samples were immediately placed on ice with minimum light exposure and centrifuged (700 × g, 4 ˚C, 30

min) to separate plasma.

3.3.5 Chylomicron isolation

Chylomicrons as the carriers of newly absorbed carotenoids and other fat-soluble dietary components (Paetau et al., 1997) were isolated from plasma. The amounts in the chylomicron fraction reflect the carotenoids, phylloquinone and tocopherols absorbed from the test salad without the contribution from endogenous stores of these nutrients. Chylomicron fractions were isolated from plasma samples using cumulative rate ultracentrifugation (Lindgren, 1975) with high purity and high recovery (Paetau et al., 1997). The densities were validated by using a digital density meter (Anton-Paar USA, DMA-48, Ashland, VA). Four milliliters of plasma were transferred to a centrifuge tube (Ultra Clear; Beckman Instruments, Inc, Spinco Division, Palo Alto, CA), and the density was adjusted to 1.10 kg/L by adding 0.14 g potassium bromide/mL plasma. The density gradient is formed by overlaying the 4 mL plasma with the prepared salt solutions in order of most to least dense to form a density gradient as follows: 3.0 mL of 1.065 g/ mL salt solution; 3.0 mL of 1.020 g/ mL salt solution; and 2.0 mL of 1.006 g/ mL salt solution.

Plasma samples were centrifuged in an ultracentrifuge (L8-70 M; Beckman Instruments Inc) with the use of a swinging bucket rotor (SW 40 Ti) at 28.3K for 43 min. The chylomicron fraction was removed from the centrifuge tube and stored at -70 °C until analyzed. The procedures were performed under dim light.

3.3.6 Extraction and HPLC-ECD analysis of carotenoids, phylloquinone and tocopherols

Analysis of analytes in the chylomicron fractions

Carotenoids, phylloquinone and tocopherols were extracted from the plasma chylomicron fractions by deproteination with methanol followed by three extractions with hexane containing 1.0 g butylated hydroxytoluene (BHT)/L (Brown et al., 2004). The combined hexane layers were evaporated to dryness and then redissolved in 60 µL methyl alcohol (MeOH): methyl-*tert*-butylether (MTBE) (1:1, by vol). A 25 µL aliquot was injected into the

HPLC-ECD system. The analytes were separated using a 5 µm C_{30} Carotenoid Column (Waters, Milford, MA, USA), as previously described (Brown et al., 2004). The column was eluted by a gradient consisting of different portions of MeOH, MTBE, and 1.0 M aqueous ammonium acetate buffer, pH 4.6. The proportions of MeOH: MTBE:ammonium acetate in Solvent A and Solvent B were 95:3:2 and 25:73:2 (by vol), respectively. The flow rate was 1.0 mL/min. An ESA CoulArray system (Chelmsford, MA, USA) consisting of a 16-channel 5600 CoulArray electrochemical detector, a 542 autosampler set at 4˚C, two 582 solvent delivery modules, and a CoulArray thermal organizer was operated using CoulArray version 3.10 software. The cells potentials were set at 100 mV, 300 mV, 400 mV, 450 mV, 550 mV, 600 mV, 750 mV, 800 mV, -1000 mV, and 200 mV. The dominant channels were 300 mV, 450 mV, and 750 mV for tocopherols, carotenoids and retinyl palmitate, respectively. A reductive potential (-1000 mV) followed by an oxidative potential (200 mV) on an upstream channel was applied for analysis of phylloquinone. To monitor inter-assay precision, an aliquot of a plasma triacylglycerol-rich lipoprotein pool was analyzed daily by HPLC-ECD. External standard calibration curves were generated for each analyte using commercially available standards.

Analysis of analytes in the salad vegetables

Representative samples of the salad vegetables from each of the five study periods (10 g to 30 g of each vegetable) were stored at -70 ˚C. Samples were analyzed in duplicate. A modification of the method of Granado et al. (2001) was used to extract and analyze carotenoids and tocopherols in the homogenized samples. Lipoxygenase activity in the vegetables was prevented by adding MeOH (EMD, Gibbstown, NJ) (0.01% BHT) (2:1, MeOH:sample weight) to each bottle immediately after opening. Frozen vegetable samples were thawed at room temperature and under yellow light before being processed in a food processor (Handy Chopper Plus HC3000; Black&Decker Corp, Towson, MD). The weight of the empty glass bottle was subtracted from the total weight of the vegetable sample (bottle plus sample plus MeOH) after thawing to obtain the sample weight post-freezing. An equivalence of 1 g of the original vegetable weight was obtained by dividing the post-freezing weight by the weight of fresh vegetable sample to correct for the losses during freezing and storage. The analytes were extracted twice from the vegetable puree by adding 6

mL MeOH (0.01% BHT) and 6 mL tetrahydrofuran (THF) (J.T. Baker, Phillipsburg, NJ) (0.01% BHT) followed by 5 minutes of vortexing after each addition. The MeOH/THF extract was transferred into a 25 mL volumetric flask after a 5 minute centrifugation at 3000 rpm to precipitate the vegetable particles. Each extract was brought to volume by adding MeOH:THF (1:1, 0.01% BHT) and was mixed by inverting several times. An 0.5 mL aliquot of the MeOH/THF extract was transferred to a 15-mL screw-capped test and combined with 1.0 mL 40% potassium hydroxide (KOH) in MeOH containing 0.1 M pyrogallol. Each sample tube was flushed with argon and tightly capped for a 3 minute saponification with vortexing. Two milliliters of ultrapure water (Milipore, Billerica, MA) were added and vortexed for 30 seconds. β-Apo-8'-carotenal (Fluka, Buchs, Switzerland) and tocol (Sigma, St. Louis, MO) in MeOH were added to each sample as internal standards for carotenoids and tocopherols, respectively. Four milliliters of hexane (EMD, Gibbstown, NJ)/methylene chloride (Fisher Scientific, Fairlawn, NJ) (5:1 by vol containing 0.01% BHT) were added and thoroughly mixed by a 60 second vortex. Samples were centrifuged for 5 min at 700 × *g* to separate the organic phase from water phase. The analytes containing organic phase was transferred to a 16 × 100 mm test tube and evaporated to dryness under vacuum. The dried lipid extract was reconstituted with 1000 µL methyl-tert-butyl ether (MTBE) and 1000 µL MeOH. A 25 µL aliquot was injected into the HPLC-ECD system with cell potentials set at 100 mV, 300 mV, 450 mV, 550 mV and 600 mV. The dominant channels were 300 mV and 450 mV for tocopherols and carotenoids, respectively.

The phylloquinone in the salad vegetables was extracted using 2-propanol (EMD, Gibbstown, NJ)/hexane according to the method of Koivu et al. (Koivu et al., 1997). Lipoxygenase activity in the vegetables was prevented by adding 2-propanol (0.01% BHT) (2:1, 2-propanol:sample weight) to the frozen sample immediately after opening. Menaquinone-4 (MK4) was added to the spinach, romaine lettuce and carrot samples as an internal standard; whereas vitamin $K_{1(25)}$ was used as the internal standard for cherry tomato samples due to the presence of an interfering peak at the retention time of MK-4. Ten milliliters of 2-propanol were added to each one gram of homogenized vegetable sample followed by a 2-minute vortex. Fat-soluble analytes were extracted twice with the addition of 5 mL of n-hexane followed by a 2 min vortex. Five milliliters of ultrapure water were

added followed by vigorous shaking. Samples were centrifuged for 5 min at 700 × *g* to separate the organic phase from water phase. For romaine lettuce and spinach, the total volume of the hexane layer was recorded and 0.5 mL was evaporated to dryness under vacuum. Due to the low contents of phylloquinone in cherry tomatoes and carrots, the entire hexane layer (~ 10 mL) was evaporated to dryness under vacuum. The dried extract was reconstituted with 100 µL methyl-tert-butyl ether (MTBE) and 100 µL MeOH. A 25 µL aliquot was injected into the HPLC-ECD system. The cell potentials were set at -800 mV, -800 mV, 50 mV and 200 mV. The 50 mV channel was used for the quantification of the vitamin K compounds.

Analysis of tocopherols in the soybean oil

The concentrations of α-tocopherol and γ-tocopherol in the soybean oil were analyzed according to a modification of a rapid saponification method (Granado et al., 2001) by using HPLC-ECD. A 0.1-g aliquot of soybean oil was weighed into a 50 mL screw capped glass test tube and vortexed with 6 mL methanol (0.01% BHT) followed by addition of 6 mL tetrahydrofuran (0.01% BHT) and 60 s vortex. A 2 mL aliquot was transferred into a 15 mL screw capped glass test tube. Then 1 mL of freshly prepared 40% potassium hydroxide in methanol with 0.1 M pyrogallol was added into the test tube. The test tube was flushed with argon and immediately capped before vortexing for 2 min. The saponification step was followed by a washing step in which 2 mL HPLC grade water were added and vortexed for 30 s. Next, 4 mL of hexane:methylene chloride (5:1 by volume, 0.01% BHT) was added and vortexed prior to a 5 min centrifugation at 700 × *g*. The organic phase was collected and evaporated to dryness under vacuum. The dried extract of the soybean oil was reconstituted with 800 µL of methanol:MTBE (1:1 by volume). The reconstituted extract was filtered using a 0.2 µm syringe filter and 100 µL was injected into the HPLC-ECD system. Solvents were HPLC grade; methanol and MTBE were purchased from EMD (Fairlawn, NJ), whereas ammonium acetate was purchased from Fisher Scientific (Chicago, IL).

Calibration curves

Internal and external standard calibration curves were generated for carotenoids, tocopherols and *trans*-phylloquinone, as noted above. α-Carotene, lutein, zeaxanthin and lycopene were purchased from Carotenature (Lupsingen, Switzerland), and β-carotene from

Fluka. α-, γ- and δ-Tocopherols, as well as phylloquinone, menatetrenone (MK-4) and retinyl palmitate were purchased from Sigma (St. Louis, MO). Tocol was purchased from Matreya (Pleasant Gap, PA). Vitamin $K_{1(25)}$ was purchased from GL Synthesis Inc. (Worchester, MA).

3.3.7 Statistical analyses

Statistical analyses were performed using SAS software (version 9.1.3; SAS Institute Inc., Cary, NC). The amounts of carotenoid, phylloquinone and tocopherol absorbed were represented by the absolute areas under the curves (AUCs) of the plasma chylomicron content versus time curves calculated by trapezoidal approximation. The plasma chylomicron carotenoid, phylloquinone, and tocopherol data was analyzed by repeated measures analysis of covariance (ANCOVA) using the baseline value as a covariate. Statistical analyses were performed on the Intent-to-treat (ITT) population and the Per Protocol (PP) population. Data from the 12 subjects were categorized into two populations: the intent-to-treat (ITT) population consisted of all subjects randomized in the study and having completed at least one intervention, whereas the Per Protocol (PP) population excluded subjects who were non-compliant or had an adverse event on the test day that could affect the absorption of fat-soluble compounds.

3.4 RESULTS

3.4.1 Subject characteristics

The average age of the twelve female subjects was 24.0 ± 5.9 y; the mean (± SD) body mass index (BMI) was 23.60 ± 3.43 kg/m^2. Non-compliances with the study protocolwere reported by two subjects; the use of a single-dose of 3 mg drospirenone/ethinyl estradiol during the third intervention period and the daily use of Orthocyclin for two weeks preceding the test salad consumption during the fifth intervention period. Both hormone treatments may have influenced plasma chylomicron clearance (Berr et al., 1986). Results were excluded for the PP statistical analyses and included in the ITT analyses.

3.4.2 Carotenoid, phylloquinone and tocopherol composition of the test salad

The total amounts of carotenoids, phylloquinone and tocopherols in the test salad were 29.44 mg, 0.23 mg, and 4.138 mg respectively (**Table 7 & 8**). The carotenoid, phylloquinone and tocopherol contents in the individual salad vegetables were comparable to the amounts reported in the USDA National Nutrient Database for Standard Reference (USDA, 2009). The variation in nutrient contents of the test salads across study periods was minimized by obtaining prepackaged vegetables from the same brands. In addition, spinach and romaine lettuce were sorted according to leaf coloration to maintain uniformity in carotenoid content. As reported in our previous salad study (Brown et al., 2004), zeaxanthin had the most variable content among the measured carotenoids. α- and γ- Tocopherol varied the most among all analyzed nutrients in the test salad, which may be due to the duration of the study that included weeks from June to September. The tocopherol content in vegetables is substantially affected by the growing conditions (weather, growing season, intensity of sunlight, and soil state) (Bauernfeind, 1980). Analysis of the stripped soybean oil used in the firt, third and fifth study periods showed consistent concentrations of α-tocopherol and γ-tocopherol (**Table 6**).

Table 6. Tocopherol levels in short path distillation stripped soybean oil.

Concentration in oil sample		
α-Tocopherol	γ-Tocopherol	δ-Tocopherol
mg/kg	mg/kg	mg/kg
3.752 ± 0.25	12.650 ± 0.50	7.236 ± 0.18

Table 7. Carotenoid contents of the vegetables in the test salad[1]

Ingredient	Weight	α-Carotene	β-Carotene	Lutein	Zeaxanthin	Lycopene
	g	mg	mg	mg	mg	mg
Carrot, grated	66	6.688 ± 0.581[2]	7.094 ± 0.505	0.304 ± 0.017	0.007 ± 0.000	-
Lettuce, romaine	48	-	1.127 ± 0.063	1.441 ± 0.070	0.011 ± 0.002	-
Spinach, leaf	48	-	2.177 ± 0.031	3.925 ± 0.065	0.047 ± 0.001	-
Tomato cherry	85	-	1.142 ± 0.080	0.287 ± 0.008	0.009 ± 0.001	4.485 ± 0.400
Total	247	6.688 ± 0.581	11.693 ± 0.702	6.497 ± 0.186	0.078 ± 0.004	4.485 ± 0.400

Table 8. Tocopherol and phylloquinone contents of the vegetables in the test salad[1]

Ingredient	Weight	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Phylloquinone
	g	mg	mg	mg	mg
Carrot, grated	66	0.536 ± 0.039[2]	0.000 ± 0.000	0.000 ± 0.000	0.005 ± 0.000
Lettuce, romaine	48	0.298 ± 0.021	0.182 ± 0.025	0.011 ± 0.001	0.085 ± 0.003
Spinach, leaf	48	1.237 ± 0.165	0.112 ± 0.019	0.000 ± 0.000	0.109 ± 0.006
Tomato cherry	85	1.340 ± 0.086	0.422 ± 0.018	0.000 ± 0.000	0.010 ± NA[3]
Total	247	3.411 ± 0.311	0.716 ± 0.062	0.011 ± 0.001	0.230 ± NA

[1] n=5. A representative sample of each vegetable from each of the 5 study periods was analyzed in duplicate

[2] x± SEM (all such values)

[3]Value estimated according to USDA Nutrient Database for Standard Reference, Release 22, 2009

3.4.3 Changes in carotenoid, phylloquinone and tocopherol levels in plasma chylomicrons

To evaluate inter-assay precision, an aliquot of a plasma triacylglycerol-rich lipoprotein pool was analyzed each day with each sample set.The inter-assay precision of the plasma chylomicron analyses was confirmed by the consistent levels of the analytes in the plasma triacylglycerol-rich lipoprotein pool (CV%: γ-tocopherol, 7.10; α-tocopherol, 5.77; lutein, 9.80; retinyl palmitate, 10.18; α-carotene, 10.93; β-carotene, 8.45; lycopene, 9.53).

The mean changes from baseline in the carotenoid, phylloquinone and tocopherol contents in the plasma chylomicron fractions were calculated and plotted for each analyte. After salad ingestion with 0 g added oil, the absorption curves were essentially flat and close to the baseline for all analytes, reflecting negligible absorption. Negative changes from baseline in tocopherol concentrations in plasma chylomicrons were observed after ingestion of 0 g added oil. In general, greater amounts of oil led to greater mean changes of the carotenoid, retinyl palmitate, phylloquinone and tocopherol contents in the plasma chylomicron fraction (**Figures 2, 3, 4 and 5**). The peak changes in the chylomicron content increases with increasing amount of added oil for all analytes. A similar trend is observed for all analytes as shown in Figures 3, 4 and 5; the change in the chylomicron content of carotenoids, phylloquinone and tocopherols increased and peaked at 3.5 h or 5 h followed by a return back to the baseline at 9.5 h. This trend is prominent particularly when the salad was ingested with 32 g of oil; the chylomicron content peaked at the 5 h time point for carotenoids, phylloquinone and tocopherols. The greatest mean changes were observed for all analytes after ingestion of the test salad with 32 g of added oil.

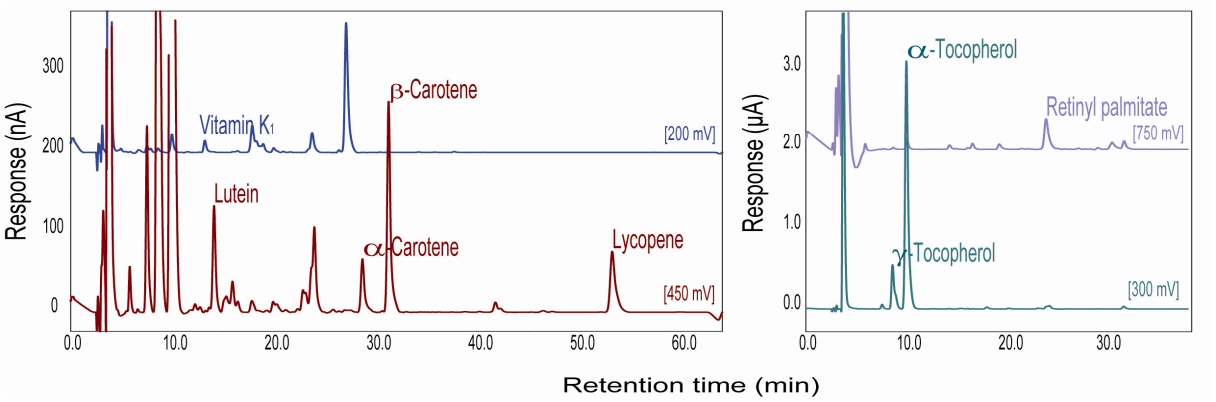

Figure 1. HPLC−ECD chromatograms of an extract of a chylomicron fraction collected 5 h after the salad was ingested with salad dressing containing 32 g oil. Vitamin K_1 was first reduced at -1000 mV on a downstream channel.

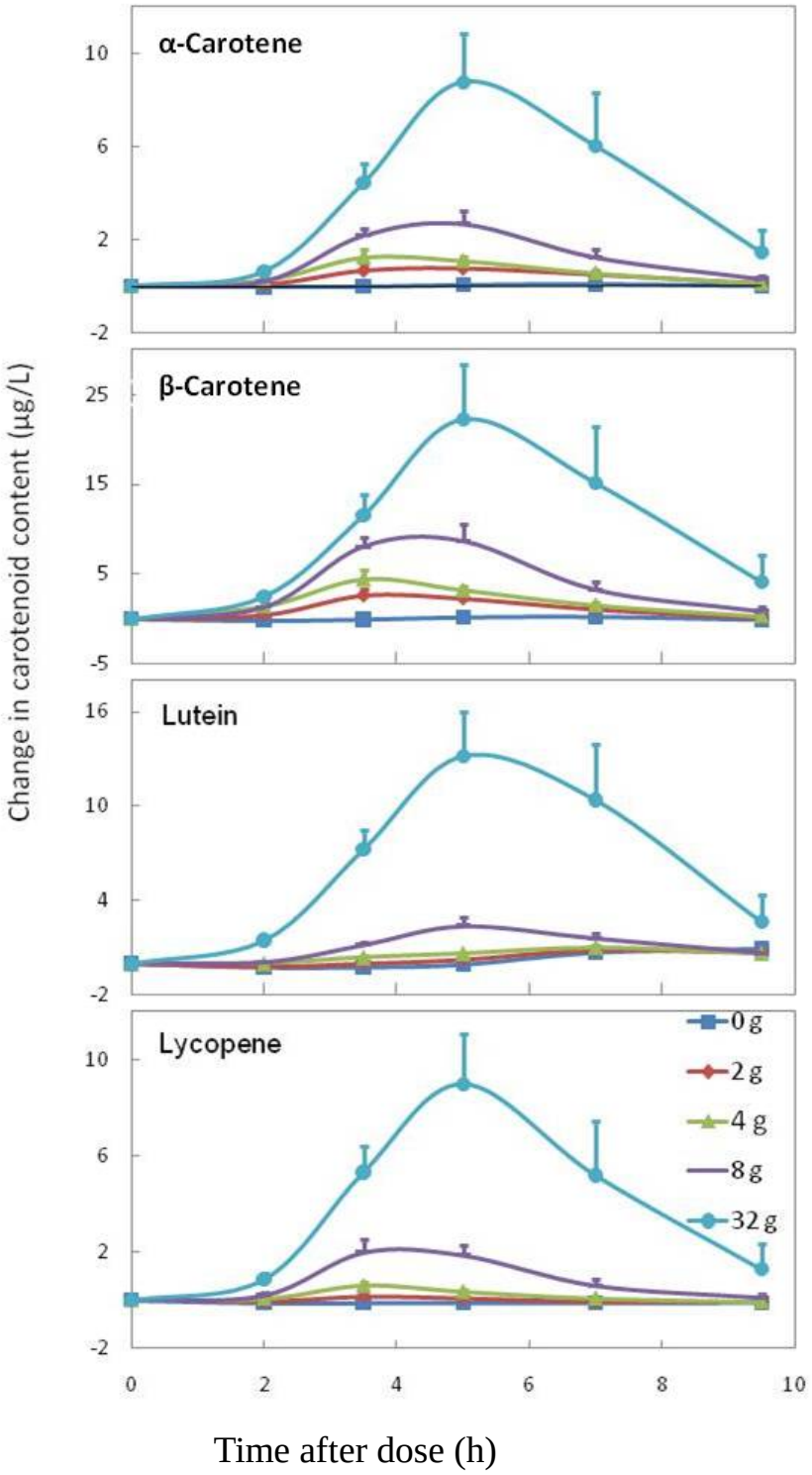

Figure 2. Mean (±SE) changes from baseline (fasting) in the content of the carotenoids α- and β-carotene, lutein and lycopene in the plasma chylomicron fraction expressed per liter of plasma.

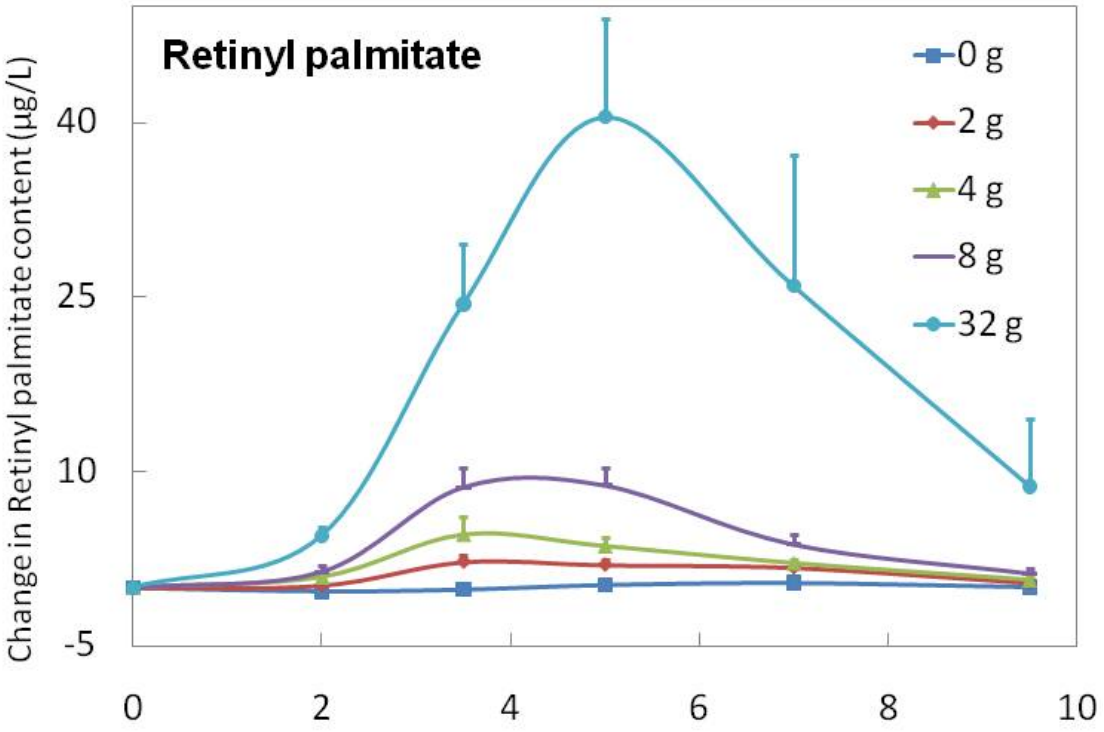

Figure 3. Mean (±SE) changes from baseline (fasting) in the content of the retinyl palmitate in the plasma chylomicron fraction expressed per liter of plasma.

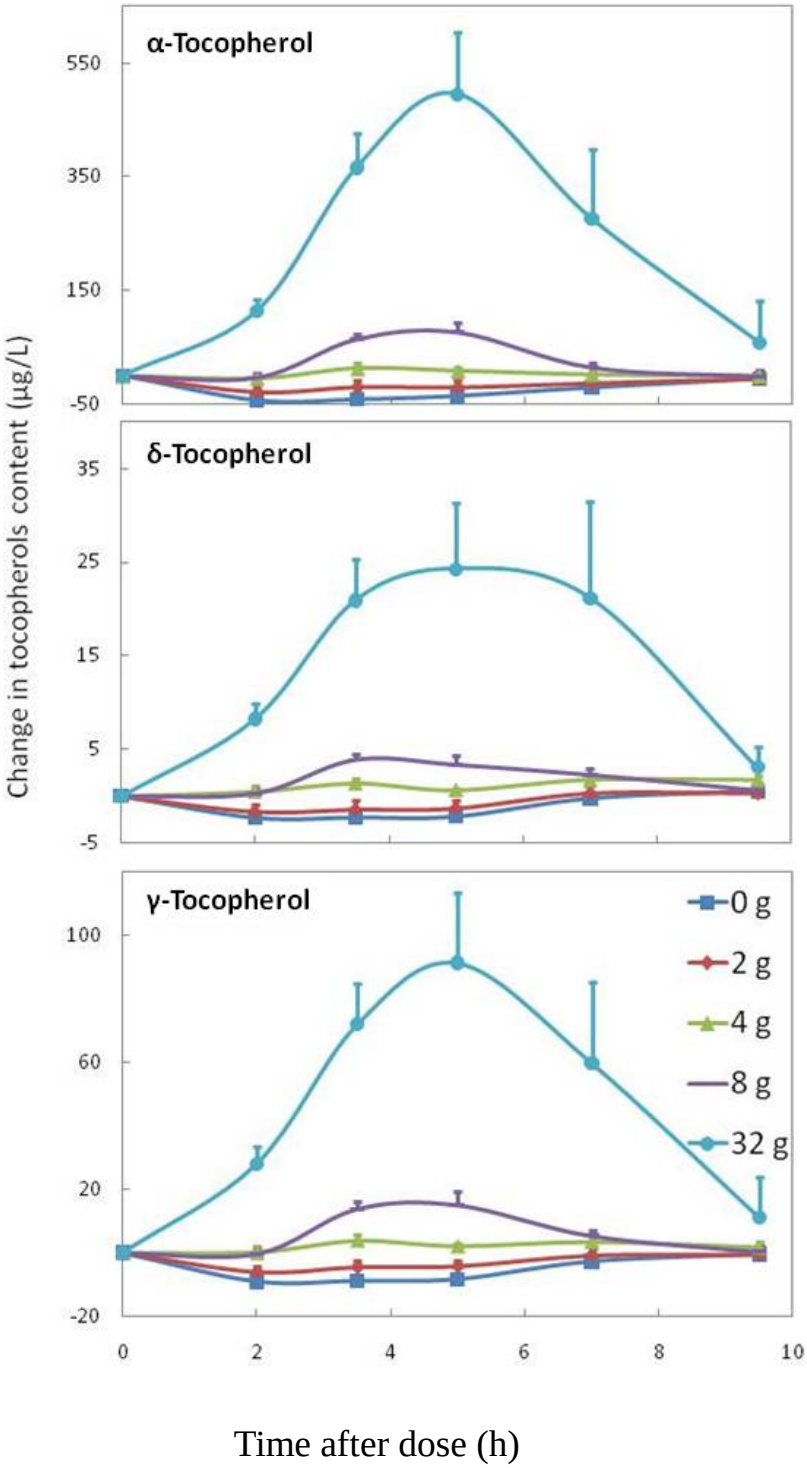

Figure 4. Mean (±SE) changes from baseline (fasting) in the content of the tocopherols, α-, δ- and γ-tocopherol in the plasma chylomicron fraction expressed per liter of plasma.

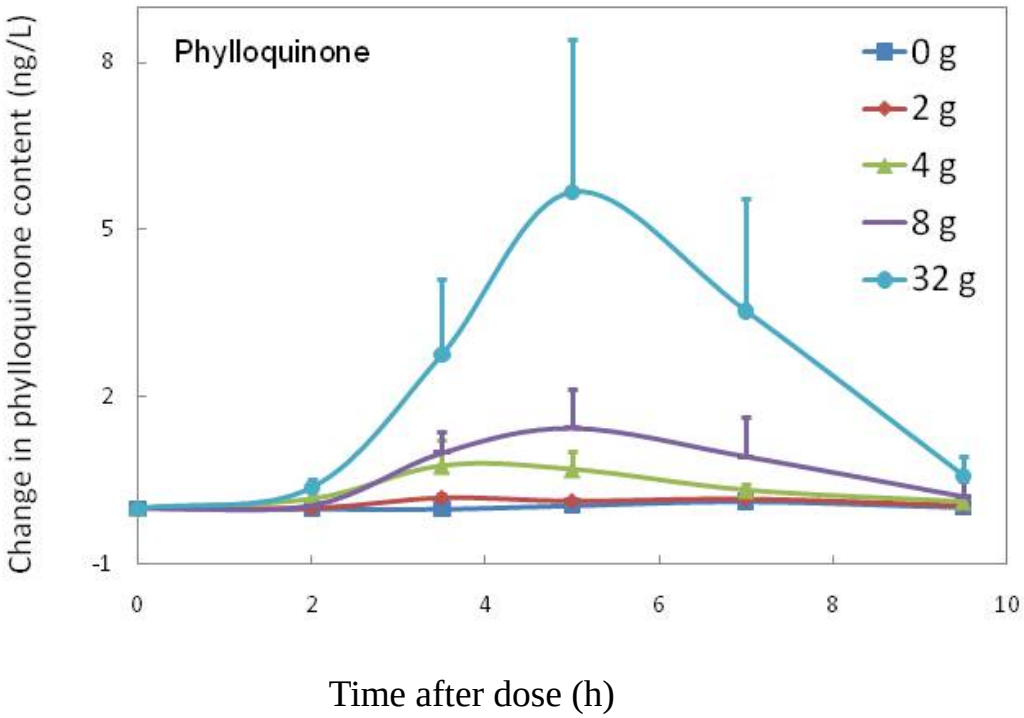

Figure 5. Mean (±SE) changes from baseline (fasting) in the content of the phylloquinone in the plasma chylomicron fraction expressed per liter of plasma.

3.4.4 Comparison of the analyte levels after ingestion of the test salad with different amounts of added oil

Most comparisons between the different oil levels for the various analytes were statistically significant in both the ITT population and PP population (**Tables 10 and 11**). The only difference between the ITT and PP populations was the 0 g and 2 g fat comparison for α-tocopherol, where the difference was significant in the ITT group (P = 0.045), but not in the PP group (P = 0.051). Further analyses used the more complete ITT data given that there was only a minor difference between the two data sets. The absorption of all analytes were higher at greater amounts of oil (e.g. 4, 8 and 32 g) compared with 0 g oil (**Table 9**). Ingestion of the salad with 32 g of oil showed significantly greater absorption of all analytes compared with the lower amounts of added oil (e.g 0, 2, 4 and 8 g). The absorption of β-carotene, *trans*-lycopene, retinyl palmitate and α-tocopherol varied in accordance with the amount of added oil as shown by significant P-values for all comparisons among all five

treatments (**Table 9 and 10**). The absorption of all analytes was significantly higher after ingestion of 2 g of oil compared with 0 g of oil, with the exception of lutein (P = 0.15), 13-*cis*-lycopene (P = 0.29) and phylloquinone (P = 0.47). Absorption of those three analytes (lutein P = 0.13, 13-*cis*-lycopene P = 0.17-, and phylloquinone P = 0.52), as well as α-carotene (P = 0.06) and γ-tocopherol (P = 0.06) was not significantly different at 2 g of oil compared with 4 g of oil. Phylloquinone absorption was not significantly different after salad ingestion with 4 g compared with 8 g of added oil (P = 0.55).

Table 9. Mean AUC values from the ITT population for the investigated analytes at different levels of ingested oil in the salad dressings.

Analyte	0 g fat	2 g fat	4 g fat	8 g fat	32 g fat
	nmol•h/L plasma				
α-Carotene	0.72	2.29	3.27	6.20	14.32
β-Carotene	1.29	6.13	10.14	18.56	36.10
α-Tocopherol	120.3	150.4	191.6	277.3	955.5
γ-Tocopherol	20.1	27.7	37.3	56.7	181.8
Lutein	2.48	3.23	4.29	6.64	26.47
Retinyl palmitate	2.24	6.95	11.85	19.41	64.99
Vitamin K_1	0.31	0.45	0.63	0.88	2.31
13-*Cis*-lycopene	0.29	0.35	0.45	0.96	2.57
Trans-Lycopene	0.53	1.04	1.68	4.44	15.26

Table 10. P-values for all oil level comparisons of the investigated analytes from the ITT population

Comparisons		ITT population P-values								
Fat 1	Fat 2	AC	BC	LU	13C-lyco	*Trans*- lyco	Vit-A	Vit-K1	α-Toco	γ-Toco
0	2	<0.0001	<0.0001	0.1535	0.2943	<0.0001	<0.0001	0.4704	0.0450	0.0293
0	4	<0.0001	<0.0001	0.0003	0.0011	<0.0001	<0.0001	0.0136	<0.0001	<0.0001
0	8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001
0	32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2	4	0.0586	0.0254	0.1276	0.1724	0.0079	0.0073	0.5233	0.0355	0.0633
2	8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0434	<0.0001	<0.0001
2	32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
4	8	0.0001	0.0042	0.0042	<0.0001	<0.0001	0.0153	0.5491	0.0003	0.0026
4	32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
8	32	<0.0001	0.0013	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	<0.0001

AC: α-Carotene; BC: β-Carotene; LU: Lutein; 13C-lyco: 13-*Cis*-lycopene; Trans- lyco: Trans-lycopene; Vit-A: Retinyl palmitate; Vit-K1: Phylloquinone; α-toco: α-Tocopherol; γ-toco: γ-Tocopherol.

Table 11. P-values for all oil level comparisons of the investigated analytes from the PP population

Comparisons		PP population P-values								
Fat 1	Fat 2	AC	BC	LU	13C-lyco	*Trans*- lyco	Vit-A	Vit-K1	α-Toco	γ-Toco
0	**2**	<0.0001	<0.0001	**0.1714**	**0.3170**	0.0001	<0.0001	**0.4777**	**0.0508**	0.0346
0	**4**	<0.0001	<0.0001	0.0007	0.0044	<0.0001	<0.0001	0.0174	<0.0001	<0.0001
0	**8**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	<0.0001
0	**32**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2	**4**	**0.0960**	0.0437	**0.1498**	**0.2928**	0.0387	0.0065	**0.5800**	0.0390	**0.0701**
2	**8**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0495	<0.0001	<0.0001
2	**32**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
4	**8**	0.0004	0.0099	0.0112	<0.0001	<0.0001	0.0219	**0.5783**	0.0012	0.0086
4	**32**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
8	**32**	<0.0001	0.0018	<0.0001	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	<0.0001

AC: α-Carotene; BC: β-Carotene; LU: Lutein; 13C-lyco: 13-*Cis*-lycopene; Trans- lyco: Trans-lycopene; Vit-A: Retinyl palmitate; Vit-K1: Phylloquinone; α-toco: α-Tocopherol; γ-toco: γ-Tocopherol.

3.5 DISCUSSION

In the current study, we showed that the high sensitivity of coulometric array ECD applied to measure the absorption of carotenoids in the previous salad study (Brown et al., 2004) was also effective in measuring the absorption of tocopherols and phylloquinone, as well as the absorption of retinyl palmitate formed from the absorbed provitamin A carotenoids.. This achievement in measuring the absorption of nine fat-soluble nutrients/bioactives from a realistic serving of salad vegetables was a valuable breakthrough in studying factors affecting the bioavailability of fat-soluble nutrients.

The minimal amount of dietary fat necessary to optimize the bioavailability of fat-soluble nutrients from vegetables is currently controversial. Inclusion of as little as 3 g of fat was needed for optimal absorption of a carotenoid and tocopherol supplement ingested in the form of a margarine spread (Roodenburg et al., 2000). In a 12 week intervention study in Phillipino children, optimal absorption of provitamin A carotenoids from cooked carrots, squash and green leafy vegetables was achieved with only 2.4 g of fat per meal (Ribaya-Mercado et al., 2007). Data from our study show for the first time that consumption of raw salad vegetables with as little as 2 g of oil compared with 0 g of oil is adequate to show significant increases in the absorption of α-carotene, β-carotene, α-tocopherol, γ-tocopherol, retinyl palmitate, and *trans*-lycopene. Compared with 0 g of added oil, phylloquinone absorption was significantly greater when 4 g of oil or more (8 g and 32 g) was added to the salad dressing. In agreement with these results, our previous salad study showed significantly greater absorption of carotenoids with full fat (28 g of canola oil) compared with reduced fat (6 g of canola oil) salad dressing. Increasing the amount of oil in the salad dressings from 0 g up to 32 g resulted in dose-dependent increases in the absorption of carotenoids, tocopherols and phylloquinone from salad vegetables. In contrast, Unlu et al. (Unlu et al., 2005) reported no significant differences in carotenoid absorption from salads ingested with 12 g or 24 g of fat from avocado or avocado oil suggesting a “nonlinear” effect on carotenoid bioavailability. This might be due to the different source of fat used. Roodenberg et al. (2000) showed that different carotenoids require different amounts of fat to be optimally absorbed. Lutein esters required a higher amount of fat (36 g) for optimal absorption compared with α-carotene and β-carotene (3 g) (Roodenburg et al., 2000).

The mechanisms by which co-ingested fat affects the bioavailability of fat-soluble nutrients have been investigated in both *in vivo* and *in vitro* studies (Huo et al., 2007). The amount, type and composition of co-ingested fat have been shown to affect a number of steps in the digestion and absorption of fat-soluble nutrients. First, lipid is necessary for the release of carotenoids and fat-soluble vitamins from plant matrices. The presence of dietary fat stimulates secretion of digestive enzymes and also solubilizes fat-soluble nutrients from the diet for absorption. Moreover, *in vitro* digestion showed that both amount and type of co-ingested lipid affect micellarization and chylomicron secretion (Huo et al., 2007). Micellarization refers to incorporation of fat-soluble compounds into micelles for transport across the water layer bringing them to close proximity of enterocytes for uptake (Huo et al., 2007). A low amount of oil (0.5-1%) was needed for *in vitro* micellarization of α-carotene, β-carotene and lycopene (Huo et al., 2007). Addition of triacylglycerol (TAG) improved the micellarization of carotenes dependent upon fatty acid chain length, but independent of the degree of unsaturation or position of the double bond (Huo et al., 2007). The efficiency of micellarization or bioaccessibility of carotenoids was reported to be inversely proportional to their hydrophobicity (LUT+ZEA > AC and BC) (Huo et al., 2007). Long chain fatty acids were more effective in improving micellarization compared with short chain fatty acids. Hu et al (Hu et al., 2000) showed the different effects of sunflower oil and beef tallow on postprandial plasma triacylglycerol response and in turn on the absorption of fat-soluble nutrient, β-carotene. Ingestion of β-carotene with a meal rich in sunflower oil resulted in lower appearance of β-carotene and greater appearance of triacylglycerol in triacylglycerol-rich lipoproteins compared to a meal rich in beef tallow. The high content of polyunsaturated fatty acid (PUFA) and saturated fatty acid in sunflower oil and beef tallow, respectively, may likely have contributed to the different effects.

The time progression of the emergence and the half life of triglycerol and carotenoids in chylomicrons were reported to be analogous (Erdman et al., 1993). Studies on postprandial lipid metabolism revealed the effects of co-ingested fat on blood triglycerol levels (Lairon et al., 2007). Triacylglycerol can be included to form the lipid-rich portion of the prechylomicron (Mansbach & Siddiqi, 2010). Evans et al. (Evans et al., 1998) reported that chylomicrons are pre-formed rather than newly formed before they are released into the

circulation (Evans et al., 1998), which explains how an increased amount of triglyceride improves the absorption of fat-soluble nutrients perhaps via enhancing the size of chylomicron particles in enterocytes. Moreover, the presence of lipid on the luminal side of the endoplasmic reticulum of enterocytes prevents the ubiquitin-proteasome degradation of apoB48 (Sakata et al., 1993), which is a crucial component of the chylomicron. Thus, it is reasonable to speculate that the absorption of fat-soluble nutrients and the postprandial triglycerol level share similar trends in their responses to different amounts of dietary fat in a meal.

The type of co-ingested fat may influence the bioavailability of fat-soluble nutrients by affecting the plasma triglycerol level and chylomicron formation. An in vivo study investigating the influence of different types of fat on postprandial triglyceride-rich-lipoproteins revealed an increase in apoB containing lipoproteins after short-term consumption of an olive oil-rich diet (Perez-Martinez et al., 2009; Jackson et al., 2002). ApoB48 can be used to identify, quantify and estimate the size of mature chylomicrons since each chylomicron only contains one apoB48 (Phillips et al., 1997). Monounsaturated fatty acids, the primary fatty acids in olive oil, have been reported to elicit higher postprandial lipemia compared with polyunsaturated (PUFAs) and saturated fatty acids (van Greevenbroek et al., 1996). Moreover, short and medium fatty acids are transported as membrane complexes via albumin through the portal route, whereas long fatty acids are utilized in the chylomicron formation leading to an elevated triglycerol level in the lymph and thus more efficient transport of fat-soluble nutrients. Different types of fat also influence the size of micelles, which in turn affects the absorption of fat-soluble nutrients. This may account for the discrepancies in the amount of fat necessary for optimum bioavailability of fat-soluble nutrients across studies that used different types of fat. In addition, the size of fat-soluble components in the emulsion affects the diffusion rate through enterocytes. Linoleic or eicosapentanoic acid form larger micelles resulting in reduced β-carotene absorption compared with oleic acid (Raju et al., 2006). Oil rich in oleic acid, such as olive oil, was shown to enhance the bioavailability of lutein by promoting incorporation into micelles (Laksminarayana et al., 2006). Released fatty acids from hydrolysis of dietary triglycerides play a significant role in emulsifying carotenoids and other fat-soluble nutrients for intestinal

uptake (Borel et al., 1996). Moreover, the composition of free fatty acids has an effect on chylomicron secretion (van Greevenbroek et al., 1996). Ingestion of a meal with medium-chain fatty acids did not promote chylomicron secretion (Borel et al, 1998), while unsaturated fatty acids, especially oleic acids facilitated the formation of lipoproteins (Jackson et al., 2002; van Greevenbroek et al., 1996). The comparison among different fat types was not the focus of this study. Soybean oil as the only fat type used in this study is rich in polyunsaturated fatty acids.

Collection of blood samples at different time points (immediately before consumption of the test salad, and then 2, 3.5, 5, 7, and 9.5 h after salad consumption) allows monitoring of the postprandial fluctuation of fat-soluble nutrients levels in postprandial chylomicrons. Previous studies showed predictable elevations in the appearance of fat-soluble nutrients in chylomicrons; a moderate increase 2-3 h after test meal consumption and a substantial increase at 5 h (Gartner et al., 1997; O'Neill et al., 1998; Brown et al., 2004). In this study, absorption of α-carotene, β-carotene, α-tocopherol, γ-tocopherol, lutein, retinyl palmitate, phylloquinone, 13-*cis*-lycopene, and *trans*-lycopene increased over time and reached a maximum at 3.5 to 5 h. At the 9.5 h time point, the fat-soluble nutrients in the chylomicron fraction returned to the baseline level. This trend was not observed when 0 g of oil was added to the salads. Negligible absorption of fat-soluble micronutrients was observed after ingestion of the test salad with 0 g of added oil highlighting the importance of co-ingested fat for carotenoids and phylloquinone absorption. The absorption and transport mechanism unique for tocopherols may account for the negative changes from baseline in tocopherol concentrations in plasma chylomicrons observed after ingestion of 0 g added oil. Incoming tocopherols can either be absorbed through intracellular vesicular trafficking for incorporation into chylomicron or protein-mediated trafficking for transport into HDL (Anwar et al., 2006); whereas chylomicron serves as the primary carrier for newly absorbed carotenoids and phylloquinone. A study using the CaCo-2 cell model showed that the proportion of vitamin E absorbed as HDL versus chylomicrons seems to be regulated by fatty acid availability (et al., 2006). In the absence of dietary fat, more tocopherols might be transported in HDL than in chylomicron.

Small levels α-tocopherol and γ-tocopherol have been found in the stripped oil, but

phylloquinone was not in the oil. δ-Tocopherol was found in the stripped oil, but not in the salad vegetables with the exception of a trace amount in carrots. Consequently, the amount of δ-tocopherol in plasma chylomicron samples can be used to assess whether the tocopherols originate from the salad vegetables or the oil.

The correlation between fat metabolism and absorption and absorption of fat-soluble nutrients infers the necessity to control factors that influence lipid metabolism in the subjects prior and during the study. Gender, physiopathological status, and polymorphisms of genes involved in lipid metabolism affect chylomicron production and clearance (Lairon et al., 2007). The composition of a previous meal, eating frequency and dietary pattern also influence chylomicron production and clearance; while age, baseline triglyceride, hydration status and alcohol consumption affect the blood triglyceride level (Lairon et al., 2007).

A review by Lairon et al. (Lairon et al., 2007) states the importance of combining solid and liquid components in a test meal in order to simulate normal eating conditions, gastric emptying, GI tract activities, as well as physiological and hormonal response for studying postprandial lipid metabolism. In this study, the test meal was accompanied with 240 mL of water and each subject was instructed to simulate a normal eating behavior.

In comparison with the previous salad study conducted in our lab, this present study expanded the questions regarding the effects of fat level on the bioavailability of fat-soluble nutrients in salad vegetables through expanding 1) the number of factors that were covered, such as the levels of added oil to include low amounts of oil (i.e. 2 g and 4 g); 2) the number of investigated analytes, including phylloquinone and tocopherols. In addition, the quantification of retinyl palmitate in the chylomicron fraction provided insights on vitamin A bioconversion from provitamin A carotenoids in salad vegetables.

APPENDIX A. FOODS TO BE AVOIDED BY SUBJECTS DAYS 1-3 OF EACH STUDY PERIOD

CAROTENOID-RICH FOODS

Fruits:

Apricots
Cantaloupe
Mango
Papaya

Vegetables:

Beet greens
Broccoli
Brussels sprouts
Carrots
Collard greens
Corn
Endive
Kale
Lettuce
Mustard greens
Peas
Peppers, red
Pumpkin
Spinach
Sweet potatoes, yams
Swiss chard

Tomatoes, tomato juice, tomato sauce
Turnip greens
Vegetable soup
Winter squash

Allowed foods include: apples, bananas, blueberries, celery, cherries, cucumbers, grapefruit, grapes, honeydew melon, mushrooms, olives, onions, pineapple, plums, raspberries, strawberries, wax beans, white beans, white potatoes

VITAMIN A-RICH FOODS

Animal products:

Eggs
Butter
Cheese
Liver
Milk (limit; 4 ounces/day)
Milk products, including ice cream

Other products:

Margarine (limit: 1 teaspoon/day)

Fortified breakfast cereals, e.g. Total®

VITAMIN E-RICH FOODS

Plant products:

Nuts and seeds

Peanut butter (limit: 1 tablespoon/day)

Vegetable oils, mayonnaise (limit: 1 tablespoon/day)

Wheat germ

VITAMIN K-RICH FOODS

Plant products:

Green leafy vegetables and vegetable oils as listed above

Allowed foods include: meat, fish, poultry, bread, pasta, rice

APPENDIX B. EXPERIMENTAL DIET

Breakfast	
Food/Beverage	**Amount**
AE YoLite Yogurt	170.1 g (6 oz container)
Sara Lee Classic 100% Whole Wheat Bread	28 g
Jif Creamy Peanut Butter	21.3 g
Smucker's Strawberry Preserves	12 g
Thomas Plain Bagel	95 g
Welch's White Grape Juice	170 g
Lunch	
Sara Lee Classic 100% Whole Wheat Bread	56 g
Oscar Mayer Oven Roasted Turkey Breast (Natural)	60 g
Kraft Miracle Whip Light Dressing	14 g
Country Fare Pineapple Chunks in juice (drained)	75 g
Minute Maid Apple Juice	10 oz. Bottle
Afternoon snack	
Keebler Vienna Cookies	60 g (4 cookies)
Minute Maid Apple Juice	10 oz. Bottle
Quaker Chewy Peanut Butter Granola Bar	28 g (1 bar)

Dinner	
Food/Beverage	**Amount**
Baked chicken breast	
• Boneless, skinless chicken breast	120 g (raw)
• Butter	2.4 g (½ tsp)
• Lemon juice	1.4 g (¼ tsp)
• Salt	1 g
• Ground black pepper	0.2 g
• Minute White Rice	30 g with 85 g of water
• Del Monte Wax Beans	70 g
• Hyvee Unsalted Sweet Butter	4.7 g
Kemp's Lemon Sherbet	86 g
Evening Snacks	
Del Monte Lite Sliced Pears in Extra Light Syrup (drained)	150 g
Keebler Honey Grahams	28 g

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