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Antiproliferative Activity of Carotenoids, Phenolic Compounds, and Volatile Terpenoids in Carrots (Daucus carota L.)

Brittany Frederick
University of Arkansas, Fayetteville

Antiproliferative Activity of Carotenoids, Phenolic Compounds, and Volatile Terpenoids in Carrots (*Daucus carota* L.)

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by

Brittany Frederick
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This thesis is approved for recommendation to the Graduate Council.

_____
Luke Howard, Ph.D.
Thesis Director

_____
Sun-Ok Lee, Ph.D.
Committee Member

_____
Andrew Bartlett, Ph.D.
Committee Member

Abstract

Epidemiological studies have shown an association between high carrot consumption and low prevalence of cancer. This observation has been thought to be attributed to carrot carotenoids. Despite this, various intervention trials have displayed no changes in incidence or increased incidence of cancer with carotenoid supplementation. It is possible that carrot phenolics are responsible for this association, though this has not been widely accepted. Volatile terpenoids from carrots have not been studied in this regard. Therefore, the primary objective of this study was to compare the antiproliferative effects of carotenoids, phenolics, and volatile terpenoids extracted from carrots on Caco-2 colon cancer cells *in vitro*. Briefly, carrot carotenoids, phenolics, and volatiles were extracted from carrots using liquid-liquid, solid phase, and distillation extraction techniques respectively. 1×10^3 Caco-2 cells were seeded in a 96-well plate, treated with the carrot carotenoid, phenolic, or volatile extract at a dilution of 50X, 100X, or 200X, then counted at 0, 6, and 12 hours after treatment using the MTS assay. The carrot carotenoids, phenolics, and volatile terpenoids did not exhibit a significantly different treatment effect over time compared to control conditions, (p-value = 0.2757), however a significant antiproliferative effect was seen at the 6 hour time point for all treatments except the volatile extract at a dilution of 200X indicating effectiveness after 6 hours of exposure. A secondary objective of this study was to conduct the same MTS assay on Caco-2 cells using the three most predominant individual compounds present in the carrot volatile extract at their inherent concentrations which were γ-terpinene, Terpinolene, and α-phellandrene. None of these compounds exhibited a significantly different treatment effect over time compared to control conditions, (p-value = 0.4975), however all three provided significantly lower mean cell counts compared to control conditions at the 6 and 12 hour time points, indicating them as effective

antiproliferative treatments 6 hours of exposure. Future work is warranted to elucidate mechanisms of action and bioavailability of these experimental treatments.

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Chapter 1: Introduction and Objectives

1.1 Introduction

The leading causes of death in 2015 according to the Center for Disease Control and Prevention lists cancer as the second leading cause of death in the United States (National Center for Health Statistics 2016). Surgery, radiation, or chemotherapy (or any combination thereof) is the most prominently used method of treatment for most types of cancer (American Cancer Society 2014). Because of the commonly-occurring, severe side effects associated with cancer treatments such as cardiotoxicity, bone density loss, cognitive deficits, infertility, pulmonary dysfunction, and more (American Cancer Society 2014), and the fairly low 5 year survival success rate of cancer treatments (Morgan et al. 2004), recent trends in the scientific community for new methods to prevent cancer have leaned towards the use of chemoprevention: a preventative, long-term approach rather than an acute, reactive method of treatment. Chemoprevention focuses on the intake of certain drugs, vitamins, and nutrient-dense foods to prevent or even delay the carcinogenic process. One classic example of a chemopreventative method is the life-long dietary intake of fruits and vegetables.

Since epidemiological and observational studies frequently report that the risk of cancer can often be attributed to diet, it is understandable why a further understanding of the dietary intake of fruits and vegetables as a chemopreventative agent is necessary (Doll and Peto 1981; Tanaka et al. 2012). It is also understandable why advances in chemopreventative techniques would be beneficial because a) chronic diseases such as cancer would be better treated with a chronic, long term preventative approach, and b) traditional cancer treatments are costly and have a fairly low success rate.

One noteworthy, comprehensive review of 206 human epidemiological studies and 22 animal studies regarding the relationship between vegetable and fruit consumption and the risk of cancer showed great potential for carrots to have strong chemopreventative properties (Steinmetz and Potter 1996). In this review, carrots ranked as the 4th highest fruit or vegetable to exhibit an inverse association of consumption and development of cancer (Steinmetz and Potter 1996). Because of carrots' notoriety of high consumption associated with low prevalence and incidence of cancers around the time of this review and their well-known high content of carotenoids, hypotheses began to develop regarding carotenoids' responsibility for this association.

Despite the promising observations from the aforementioned review, primary research regarding carotenoids and their anticancerous effects have not consistently displayed positive results. It is noteworthy to point out that some studies and intervention trials have not displayed a decrease in incidence of cancers, but rather lack of effects on cancer development or even increased incidences of cancers. For instance, increased proliferation of LNCaP and PC-3 prostate cancer cell lines following treatment of β-carotene at concentrations of 3 and 10 μM has previously been demonstrated (Dulinska et al. 2005). Furthermore, a lack of effect of long-term supplementation of β-carotene on the incidence of malignant neoplasms in more than 22,000 participants has also been observed (Hennekens et al. 1996). Lastly, results from two notorious intervention trials entitled "The Effect of Vitamin E and Beta-Carotene on the Incidence of Lung Cancer and Other Cancers in Male Smokers" and "Effects of a Combination of Beta-Carotene and Vitamin A on Lung Cancer and Cardiovascular Disease", otherwise known as the ATBC and CARET studies respectively, showed that smokers and people exposed to asbestos, (i.e. individuals who belong to a high risk population for cancer), had an increased chance and

incidence of obtaining lung cancer after dietary supplementation of β-carotene (The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn et al. 1996). There are several possibilities for the explanation of these inconsistent findings, but one notable possibility is that other bioactive compounds in carotenoid-rich vegetables are responsible for their anti-cancerous effects following consumption rather than the previously-hypothesized carotenoids. This possibility introduces the inspiration of the present study: it is possible that the volatile terpenoid compounds in carrots play a larger role in carrot's largely recognized anticancerous effects instead of the previously hypothesized carotenoids. At the time of this study, no research has been conducted on volatile terpenoid compounds extracted from carrots with respect to anticarcinogenesis specifically, but early literature has suggested that volatile terpenoid compounds in general may have anticancerous effects (Dragsted et al. 1993). Some individual volatile terpenoid compounds found in carrots have previously been proven to display anticancerous effects. For example, d-limonene, (a monoterpene in carrots), and its metabolite perillyl alcohol have displayed chemopreventative efficacy during both the initiation and promotion stages of carcinogenesis in rat skin, liver, and lung cancers (Russin et al. 1989; Crowell 1999). Also, *Myrica rubra* essential oil's dominant volatile terpenoids including β-caryophyllene and α-humulene, (also present in carrots), have been shown to significantly reduce the viability of Caco-2 cells, (a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells), at concentrations as low as 10 µg/mL after 72 hours of treatment (Ambroz et al. 2015). This evidence gives the hypothesis for the present study promising potential: that carrot volatile terpenoid compounds have greater antiproliferative effects on Caco-2 cells, *in vitro* than carrot carotenoids.

1.2 Objectives

1. To extract, identify, and quantify the carotenoid, phenolic, and volatile terpenoid compounds in carrots via HPLC-MS and GC-MS methods.
2. To compare the antiproliferative effects of carrot carotenoid, phenolic, and volatile extracts on Caco-2 cells *in vitro*.
3. To compare the antiproliferative effects of γ-terpinene, Terpinolene, and α-phellandrene on Caco-2 cells *in vitro*.

Chapter 2: Literature Review

2.1 Carotenoids

2.1.1 Function of Carotenoids

Carotenoids are a class of lipid-soluble photosynthetic pigments that yield red, orange, and yellow colors in plants, fruits, and vegetables. Carotenoids in plants serve two key roles; they aid the photosynthetic process and they protect against photo-oxidative damage (Frank and Cogdell 1996). Carotenoids directly aid the photosynthetic process by readily absorbing light and transferring it to chlorophylls to be used to synthesize glucose for energy (Frank and Cogdell 1996). Carotenoids can also act as antioxidants to protect against oxidative and photo-oxidative damage by quenching free radicals or reactive oxygen species produced as a result of the metabolic and pathological processes and can also dissipate excess light energy which they absorb as heat to protect plants from excess UV exposure (Frank and Cogdell 1996; Dembinska-Kiec 2005).

2.1.2 Classification of Carotenoids

Carotenoids are typically classified by their Vitamin A activity, their oxygenated status, and their cyclic status. Cyclic carotenoids can be further characterized as monocyclic or bicyclic.

Carotenoids that do not contain oxygen are referred to as carotenes, and their oxygen containing counterparts are called xanthophylls (Gross 1991). Carotenoids that are necessary for the biotransformation of retinol or Vitamin A in the body exhibit Vitamin A activity, while their counterparts that are not needed in the body for the formation of retinol or Vitamin A have no Vitamin A activity (Tanaka et al. 2012).

Acyclic carotenoids are linear and do not contain a cyclic end-group at the end of the linear hydrocarbon chain structure. Their cyclic counterparts contain either one cyclic end group (monocyclic) or two cyclic end groups; one on either end of the linear hydrocarbon chain structure (bicyclic). Some more commonly found cyclic end groups are the beta, epsilon, gamma, kappa, phi, and chi end groups shown in Figure 2.1. The name of a specific carotenoid hydrocarbon is constructed by adding the appropriate Greek letter respective to the end group that is present as a prefix to the stem name 'carotene' (IUPAC 1974).

Some other more specific classifications exist for carotenoids, but they are not used as frequently as the aforementioned systems. Some carotenoids are allenic or acetylenic, with a C=C=C group or a -C≡C- - respectively located at one end of the molecule. Carotenoids have also been detected with less than or greater than the typical amount of 40 carbon atoms in their structure and are referred to as degraded carotenoids (apocarotenoids), or higher carotenoids (Gross 1991).

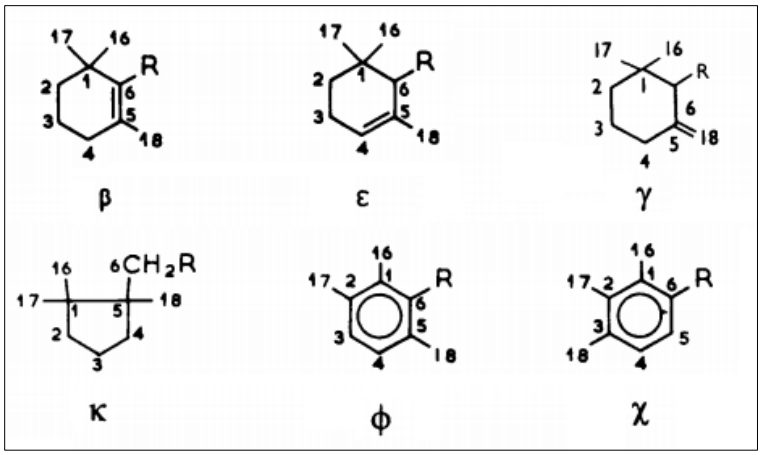

Fig. 2.1 Common cyclic end groups present on carotenoids (IUPAC 1974)

2.1.3 Carotenoid Structures

Carotenoids are synthesized through the isoprenoid pathway in plant plastids and are stored in the chloroplasts (Hager and Howard 2006). All carotenoids contain eight C5 isoprenoid units linked in a head to tail fashion. The formation of these eight isoprene units results in a total of 40 carbon atoms. Because of this, carotenoids are structurally known as C40 isoprenoid polyenes, also known as tetraterpenoids. In the middle of the isoprenoid chain however, the isoprene units are not linked in a head to tail fashion, but rather a tail to tail manner to create a trans isomer and a subsequently symmetrical molecule as shown in Figure 2.2 (Gross 1991). Trans isomers are more stable and more commonly found in natural sources than cis isomers because there is less steric hindrance in the middle of the structure (Berg et al. 2000). Usually, cis isomers of carotenoids are only found during the biotransformation processes leading to more stable, trans isomeric structures (Gross 1991).

There is a direct relationship between the light-absorbing properties of carotenoids and their respective molecular structures. The color of carotenoids changes from yellow to red depending on the number of double bonds in its structure with a minimum of seven double bonds

needed to produce yellow. Interestingly, not all carotenoids impart visible color. To understand this phenomenon, it is important to first understand how carotenoids yield their characteristic yellow, orange, and red hues. Carotenoids appear colored due to their ability to absorb light specifically in the visible region of the light spectrum (between 430 and 480 nm in the visible region), and the structural feature responsible for light absorption is the chromophere, which in carotenoids is typically a long system of conjugated double bonds. The chromopheres of phytoene and phytofluene, (with only three and five conjugated double bonds respectively), are not long enough to impart color via light absorption (Gross 1991; Hui 2006). This unique light-absorption feature native to carotenoids allows the utilization of high performance liquid chromatography- mass spectrometry (HPLC-MS) for the identification and analysis of carotenoids.

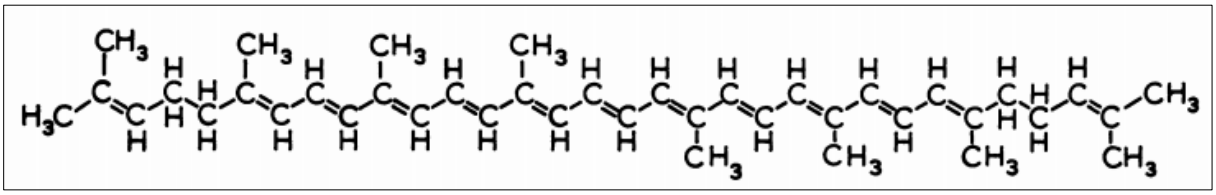

Figure 2.2 The basic structure of a carotenoid: eight isoprene units in the more stable, trans configuration (IUPAC 1974)

2.1.4 Biosynthesis of Carotenoids

Carotenoid compounds are biosynthesized via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastid. The MEP pathway starts with a reaction between pyruvate and glyceraldehyde-3-phosphate, catalyzed by 1-deoxy-D-xylulose-5-phostaphate synthase to form 1-deoxy-D-xylulose 5-phosphate. 1-deoxy-D-xylulose 5-phosphate reductoisomerase then catalyzes an interconversion reaction between 1-deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate. 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR)

then produces isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Geranyl-geranyl diphosphate (GGPP) synthase catalyzes the condensation of three molecules of IPP and one molecule of DMAPP to produce GGPP, which is a 20-carbon molecule. Condensation of two molecules of GGPP by phytoene synthase (PSY) then forms phytoene, the first carotenoid (ACOS Library… c2018). Furthermore, various carotenogenic isomerases, desaturases, cyclases, and hydroxylases are utilized for the transformation of other carotenoids further downstream of the pathway. Figure 2.3 depicts the Carotenoid biosynthetic pathways.

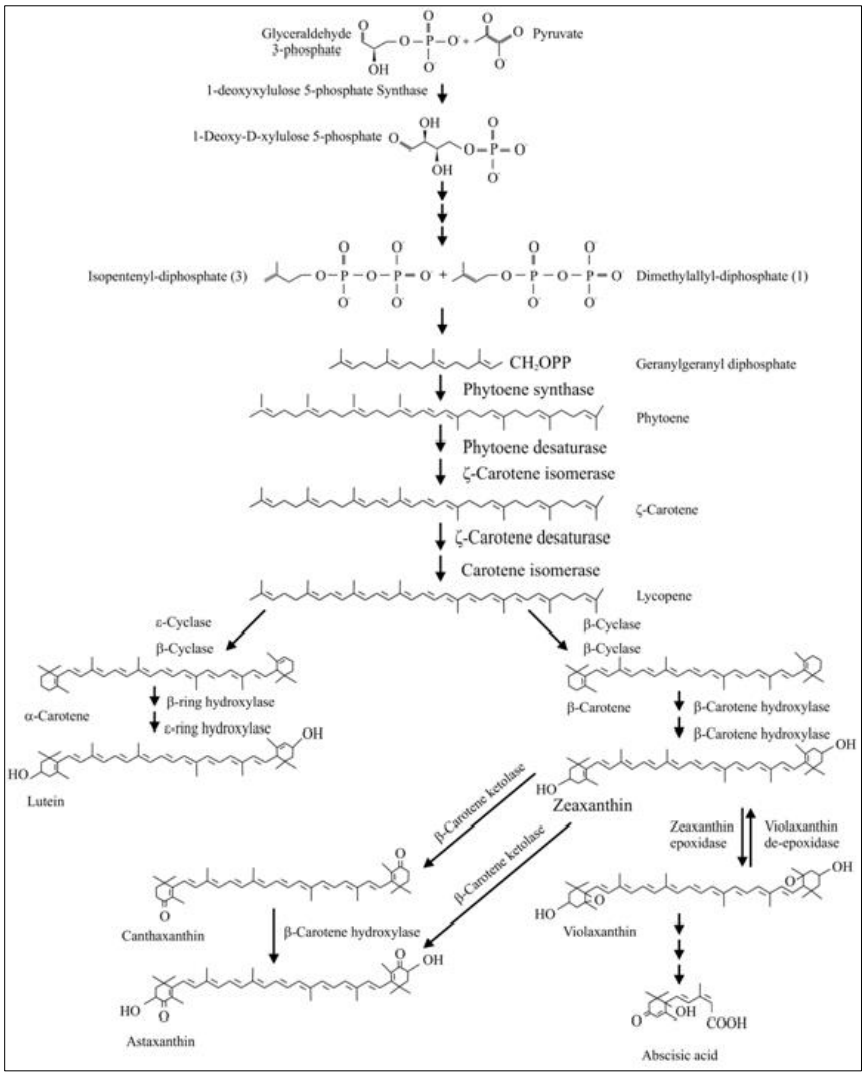

Figure 2.3 Carotenoid biosynthesis pathways (AOCS Library 2018)

2.2 Phenolic Compounds

2.2.1 Function of Phenolic Compounds

Phenolic compounds are highly abundant in plants, fruits, and vegetables. Plant phenolics are generally involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contribution to plants' colors (Dai and Mumper 2010). They are also partially responsible for the overall organoleptic properties of plant foods (Dai and Mumper 2010). Ester forms of phenolic compounds also play an important role in plants as they are generally associated with the cell walls of plants and may have a role in limiting cell wall digestibility (Parr et al. 1997). Ester forms of phenolic compounds are also utilized in various aspects of disease resistance (Parr et al. 1997). The level of phenolic compounds in plant sources depends on several factors such as cultivation techniques, cultivar, growing conditions, ripening processes, processing and storage conditions, and others (Soto et al. 2015). Their content may increase under stressful conditions such as exposure to UV radiation, infection by pathogens and parasites, wounding, air pollution and exposure to extreme temperatures (Soto et al. 2015).

2.2.2 Classification of Phenolic Compounds

Phenolic compounds are typically classified by their molecular structure. Classification of phenolic compounds by their chemical structure depends on how many aromatic rings they contain and what type of functional groups are present. A phenolic compound by definition contains at least one aromatic ring and at least one hydroxyl functional group. The term "Simple Phenol" is used to describe a phenolic compound with only one aromatic ring, while a "Polyphenolic Compound" or "Polyphenol" contains more than one aromatic ring. Further classification of these two groups is based on the type of additional moiety(ies) present in the structure. The classification system used for phenolic compounds is depicted in Figure 2.4.

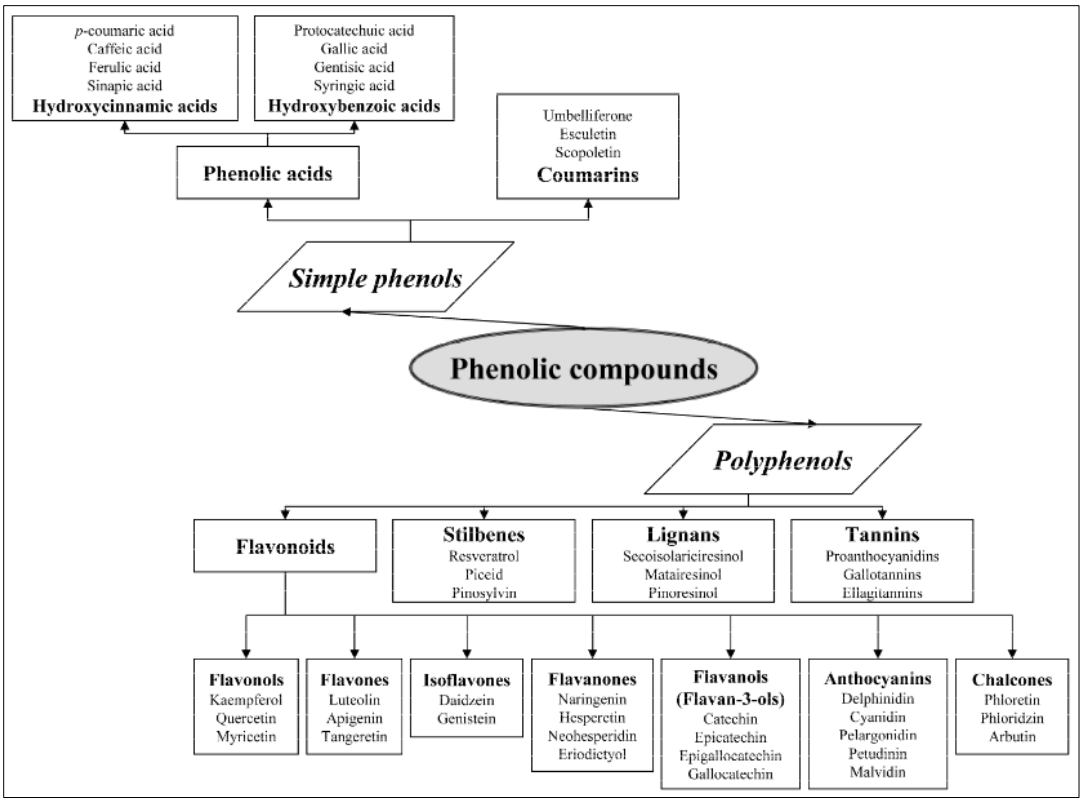

Fig. 2.4 Classification system of phenolic compounds by their chemical structure (Soto et al. 2015)

A major class of phenolic compounds known as “phenolic acids” contains one aromatic ring, but also contains a carboxylic acid functional group. Phenolic acids can be further broken down into two categorical groups; hydroxycinnamic acids (C6-C3) and its congeners, and hydroxybenzoic acids (C6-C1) and its derivatives (Dai and Mumper 2010). An example of a hydroxycinnamic acid derivative is caffeic acid, which is one of the most abundant phenolic acids in many fruits and vegetables (Dai and Mumper 2010). When caffeic acid is esterified with quinic acid, chlorogenic acid is produced; another frequently detected hydroxycinnamic acid derivative in fruits and vegetables. These two examples serve as a representation of the many hydroxycinnamic acid and hydroxybenzoic acid derivatives that are possible in nature.

2.2.3 Biosynthesis of Phenolic Compounds

Hydroxycinnamic acids are synthesized from the shikimate pathway. The shikimate pathway leads to the synthesis of amino acids such as phenylalanine and tyrosine. These are then deaminated by phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase respectively into C_6C_3 units that serve as the core structure for phenylpropanoids further down the shikimate pathway, such as cinnamic acid and *p*-coumaric acid. These can subsequently be transformed into caffeic, ferulic, or sinapic acid (El-Seedi et al. 2012). Many other derivatives can be formed further down the shikimate pathway from these and are depicted in Figure 2.5.

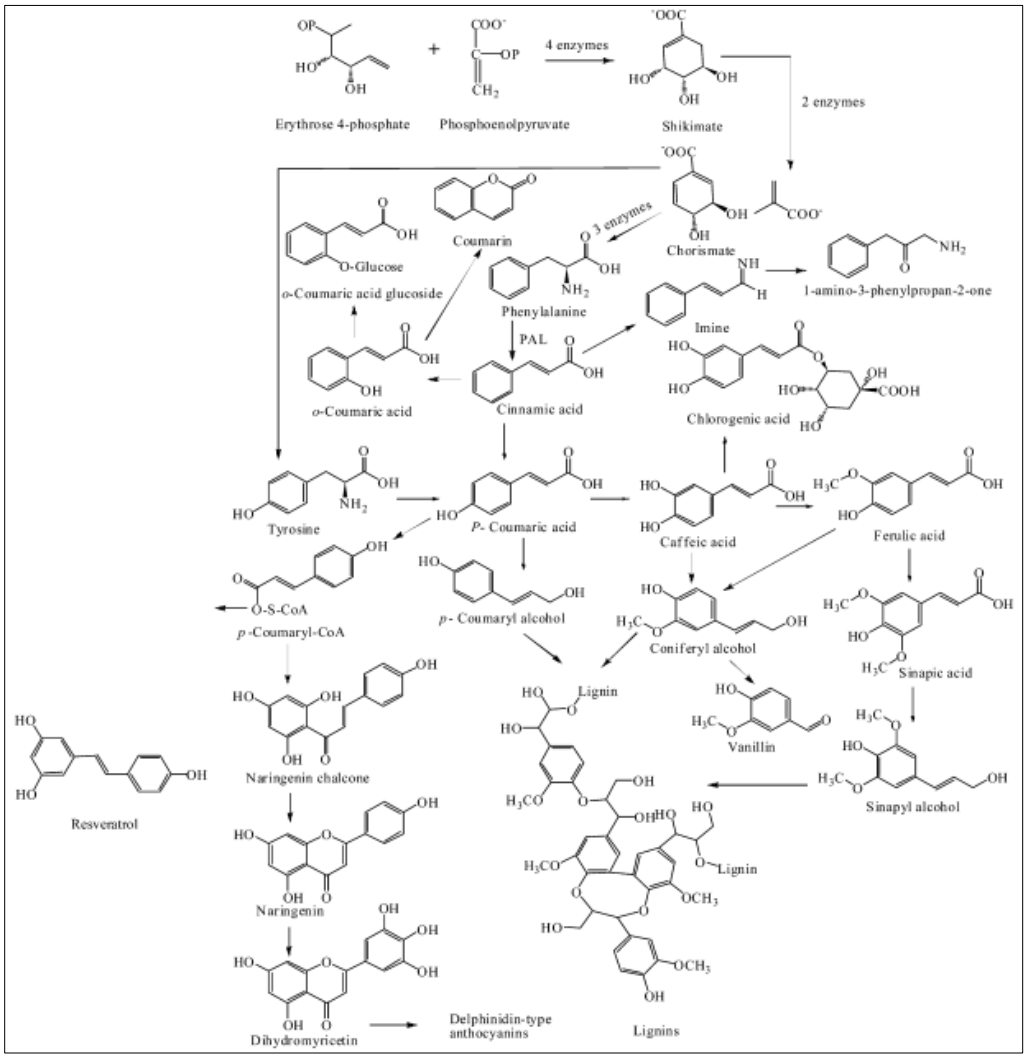

Figure 2.5 Biosynthesis of hydroxycinnamic acid and derivatives (El-Seedi et al. 2012)

2.3 Terpenoid Compounds

2.3.1 Function of Terpenoid Compounds

Volatile terpenoid compounds, (hereinafter referred to colloquially as 'terpenoids'), have a very high vapor pressure (or low boiling point) at room temperature. They are referred to as "volatile" because they are easily evaporated at ambient temperature. Terpenoids in fruits and vegetables are responsible for a large portion of their respective olfactive properties and flavor.

Terpenoids in fruits and vegetables are non-volatile compounds at first but become volatile as a result of enzymatic actions when the tissue of the fruit or vegetable is disrupted by crushing, cutting, or shredding (Gary 1999). This is why the smell of onions proliferates when they are cut, or when you chew on a carrot you can taste and smell their infamous pine-like scent through retronasal olfaction. Plants also release terpenoids as a defense mechanism against mechanical damage from insect feeding. These compounds are not readily available in undamaged plants; they are metabolically activated, synthesized, and released subsequently following insect damage (Pare and Tumlinson 1999). Terpenoids are therefore known as secondary metabolites since they are not necessary for growth or development. Terpenoids also function in plants as hormones, photosynthetic pigments, electron carriers, mediators of polysaccharide assembly, pollination attractors, and structural components of membranes (McGarvey and Croteau 1995).

2.3.2 Classification of Terpenoids

Terpenoids are a sub-group of the complete volatile profile of fruits and vegetables. Terpenoids are a large class of naturally occurring organic molecules consisting of a hydrocarbon chain made of two or more isoprene units. Many variations of this basic structure exist among the terpenoid population; they can be acyclic, monocyclic, bicyclic, oxygen-

containing, or they can also exist as many congeners and isomers. Terpenoids are typically classified by the number of isoprene units they contain. Table 2.1 lists the classes of terpenoids and their respective number of isoprene units and carbon atoms in their structures.

Table 2.1 Classification of Terpenoids (Trudgill 1986)

Terpenoid Class	Number of Isoprene Units	Number of Carbon Atoms
Hemiterpenoids	1	5
Monoterpenoids	2	10
Sesquiterpenoids	3	15
Diterpenoids	4	20
Sesterpenoids	5	25
Triterpenoids	6	30
Tetraterpenoids	8	40
Polyterpenoids	>8	>40

2.3.3 Terpenoid Structure

Terpenoids are hydrocarbon molecules consisting of two or more isoprene units. The general molecular formula for terpenoids is $(C_5H_8)n$, where 'n' is the number of isoprene units present (Yadav et al. 2014). Many oxygenated, hydrogenated and dehydrogenated derivatives extend from this general formula as well. Linking of isoprene units in terpenoids during biosynthesis could occur in three ways; either in a head-to-tail, head-to-head, or tail-to-tail linkage. However, linkage of these isoprene units typically occurs in accordance of the 'isoprene rule'. The 'isoprene rule' states that in most naturally occurring terpenoids, only head-to-tail linkages will occur between isoprene units (Yadav et al. 2014). This rule however can only be used as guiding principle and not as a fixed rule. For example, carotenoids are joined tail-to-tail at the center of their hydrocarbon chain (Yadav et al. 2014).

2.3.4 Biosynthesis of Terpenoids

The formation of terpenoids in plants can occur via two pathways: the mevalonate pathway and the deoxyxylulose phosphate pathway (McGarvey and Croteau 1995). The main function of both pathways is to produce IPP and DMAPP. The mevalonate pathway for the biosynthesis of terpenoids involves the conversion of acetyl coenzyme-a to the "active isoprene unit" referred to as isopentenyl pyrophosphate (IPP) while the deoxyxylulose phosphate pathway involves the conversion of pyruvate and glyceraldehyde 3-phosphate to isopentenyl pyrophosphate (IPP) and/or its isomer dimethylallyl pyrophosphate (DMAPP) (McGarvey and Croteau 1995). These pathways take place in the cytosol and plastid of plants respectively. IPP and DMAPP, along with various prenyl pyrophosphate co-substrates, (e.g. geranyl pyrophosphate [GPP], farnesyl pyrophosphate [FPP], and geranylgeranyl pyrophosphate [GGPP]), are important precursors to a family of enzymes known as terpene synthases. It is these enzymes that make an array of hemi-, mono-, sesqui-, and diterpene volatiles. A single terpene synthase can produce as many as 20 products or as few as one (Baldwin 2010). Figure 2.6 depicts the mevalonate pathway for terpenoid biosynthesis.

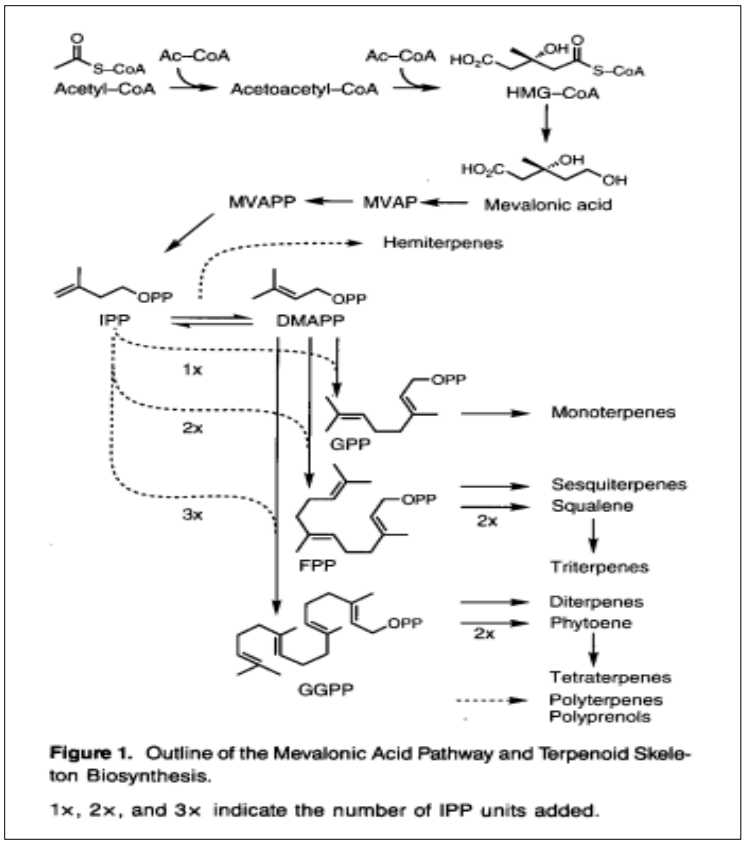

Figure 1. Outline of the Mevalonic Acid Pathway and Terpenoid Skeleton Biosynthesis.

1×, 2×, and 3× indicate the number of IPP units added.

Figure 2.6 Depiction of the mevalonate pathway for biosynthesis of terpenoids (McGarvey and Croteau 1995)

2.4 Carotenoids, Phenolic Compounds, and Terpenoids in Carrots

2.4.1 Carotenoids in Carrots

An average carrot of the Nantes variety in the United States contains approximately 54 µg of carotenoids per gram of fresh weight (Gross 1991). The main carotenoids found in carrots are β- and α-carotene comprising about 60% and 20% respectively of the total carotenoid fraction (Heinonen 1990). The other remaining carotenoids in carrots are typically lycopene (1-5%), and in trace amounts γ-carotene, σ-carotene, phytoene, phytofluene, and β-zeacarotene (Heinonen 1990). The minor carotenoids found in carrots such as γ -carotene, σ-carotene, and β-zeacarotene only constitute approximately 0.1-1% of the total carotenoid content (Rakcejeva et al. 2012). The carotene content of carrots is usually higher in very strong orange-colored carrots

that have had plenty of time to develop during a particular growing season, with paler carrots containing a lower concentration of carotenoids (Heinonen 1990). Carotenoids are also distributed throughout the entire carrot, but the phloem tissue has the highest level followed by the peels and xylem respectively (Howard and Dewi 1996).

2.4.2 Phenolic Compounds in Carrots

The total phenolic profile of carrots is mainly composed of hydroxycinnamic acid derivatives, representing more than 98% of the total phenolic content in the peel, more than 90% in the phloem, and more than 73% in the xylem (Zhang and Hamauzu 2004). Hydroxycinnamic acid synthesis involves the removal of the ammonia group from phenylalanine by phenylalanine ammonia-lyase which results in a compound called cinnamic acid. Furthermore, a hydroxyl group is added resulting in hydroxycinnamic acid (*p*-coumaric acid). It is hydroxycinnamic acid and its many derivatives that can be attributed to the term "hydroxycinnamic acid and derivatives". Chlorogenic acid, (5'-caffeoylquinic acid: a hydroxycinnamic acid derivative), is the largest component of the total phenolic content of carrots and can represent 42.2% to 61.8% of the total phenolic content (Zhang and Hamauzu 2004). Zhang and Hamauzu's findings seem to mirror other literature, such as 52.4% of the total phenolic content in (orange) carrots (Alasalvar et al. 2001). Other less proportionally present hydroxycinnamic acid derivatives in carrots include coumaric acid and its isomers (approximately 4.13% of the total phenolics) as well as ferulic acid and its isomers (approximately 5.43% of the total phenolics) (Zhang and Hamauzu 2004).

The peel of carrots contains the highest proportion of the total phenolic fraction in carrots, followed by the phloem and xylem (Zhang and Hamauzu 2004). Even though carrot peel accounts for only 11.0% of the amount of the carrot fresh weight, it could provide 54.1% of the

amount of the phenolics in 100 g of fresh weight of carrots, with the phloem tissue providing 39.5% and the xylem tissue providing only 6.4% (Zhang and Hamauzu 2004).

While it is consistent among literature that hydroxycinnamic acids are present in carrots, what is inconsistent is detection of hydroxybenzoic acids in carrots. Detection of 4-hydroxybenzoic acid and vanillic acid has been reported, in one case present at 484 µg/g of carrot wall carbohydrate and 16.9 µg/g of carrot wall carbohydrate, respectively (Parr et al. 1997). 4-hydroxybenzoic acid, (1 mg/100 g, 0.26 mg/100 g, and 0.6 mg/100 g of fresh weight in three different varieties of carrot), and its esters, (4.5 mg/100 g, 0.9 mg/100 g, and 1.2 mg/100 g of fresh weight in the same three varieties), has also been reported in freshly shredded carrots, however the presence of these phenolic compounds were not detected until after the first day of storage (Babic et al. 1993). A possible metabolic relationship between hydroxycinnamic acids and both 4-hydroxybenzoic acid and its esters was suggested, since the accumulation of the two latter compounds was associated with a decrease in the former (Babic et al. 1993).

2.4.3 Terpenoids in Carrots

Monoterpenes represent the largest proportion of the total volatile fraction of carrots, (approximately 85.5% of the total volatile fraction), followed by aldehydes (6.9%) and sesquiterpenes (5.2%) (Guler et al. 2015). Alpha-terpinolene constitutes the largest proportion of any other individual terpenoid in carrots, at approximately 62.9% of the total volatile fraction (Alasalvar et al. 2001; Schnitzler et al. 2003; Guler et al. 2015). Other prominent volatile terpenoids include γ-terpinene, limonene, and α-pinene (Simon et al. 1980; Alasalvar et al. 2001; Schnitzler et al. 2003; Guler et al. 2015). Table 2.2 displays the composition of volatile compounds and terpenoids in orange carrots.

Beta-myrcene and β-caryophyllene have also been reported as major terpenoids in carrots, but less consistently than α-terpinolene, γ-terpinene, limonene, and α-pinene (Simon et al. 1980; Alasalvar et al. 2001; Schnitzler et al. 2003; Guler et al. 2015). A few minor terpenoids of lower concentrations have also been detected such as terpinen-4-ol (Simon et al. 1980), α-humulene (Alasalvar et al. 2001; Guler et al. 2015), and camphene (Schnitzler et al. 2003; Guler et al. 2015). Inconsistent detection of terpenoids is most likely due to different methodologies of extraction and analysis of terpenoids from the carrots. Simon and others for example utilized an adaption of the typical gas chromatographic methodology via the implementation of porous polymer traps to collect the volatile compounds (1980).

Table 2.2 The percentages of volatile organic compounds in orange carrots[1]

Compound	Chemical Group	Percentage of Total Volatile Content	Chemical Group's Percentage of Volatile Content
(-)-α-pinene[a]	Monoterpene	2.91	85.49
(-)-ß-pinene[b]	Monoterpene	0.57	
(+)-Sabinen(Thujene)[a]	Monoterpene	0.82	
ß-myrcene[a]	Monoterpene	0.48	
α-phellandrene[b]	Monoterpene	0.29	
d-limonene[a]	Monoterpene	2.02	
γ-terpinene[b]	Monoterpene	10.37	
α-terpinolene[a]	Monoterpene	62.90	
p-cymene[a]	Monoterpene	3.82	
Isobornylacetate[b]	Monoterpene	0.84	
p-cymenene[b]	Monoterpene	0.47	
(-)-ß-caryophyllene[b]	Sesquiterpene	1.50	5.24
α-humulene[b]	Sesquiterpene	0.40	
Trans-γ-bisabolene[b]	Sesquiterpene	2.40	
Cis-α-bisabolene[b]	Sesquiterpene	0.94	
Acetaldehyde[a]	Aldehyde	6.26	6.92
Hexanal[a]	Aldehyde	0.18	
Heptanal[a]	Aldehyde	0.14	
Octanal[a]	Aldehyde	0.34	
Ethanol[a]	Alcohol	1.83	1.83
		Total~99.48*	Total~99.48*

[1]Adapted from Guler et al. 2015.
a=These compounds and their respective quantities were consistent with known standards.
b=Tentatively identified with no standards available to compare.

2.5 Sensory Characteristics of Carrot Terpenoids

Carrots grown in higher temperatures (18° to 21°C) generally result in a higher concentration of terpenoids (Rosenfeld et al. 2002). Interestingly, the concentration of α-terpinolene, (the predominant terpenoid in carrots), significantly decreases with higher growing temperatures (Rosenfeld et al. 2002). Carrots grown in higher temperatures not only have a higher concentration of terpenoids but are also perceived to be less bitter tasting (Rosenfeld et al. 2002). Since α-terpinolene significantly decreases in concentration with higher temperatures during growth, it has been postulated that terpenoids other than α-terpinolene contribute more to bitter taste in carrots (Rosenfeld et al. 2002). One proposed explanation for the higher release of terpenoids with higher growing temperatures is the terpenoids dissolve into the thylakoid membrane and keep chloroplasts from degrading when temperatures exceed the plant's biological optimum (Pare and Tumlinson 1999). Furthermore, the terpenoids evaporate as the temperature rises, and consequently the terpenoid volatilization cools the chloroplasts. However, since the evaporative cooling of terpenoids is relatively small compared with a solvent such as water, this explanation is not universally accepted (Pare and Tumlinson 1999).

2.6 Carrot Consumption

2.6.1 Carrot Consumption Rate and Trends

United States consumption of carrots is approximately 10.1 pounds per year, per capita (U.S. Department of Agriculture Extension Research Service 2016). Fresh cut carrot products and baby carrots have been the most rapidly growing segment of the carrot industry since the 1990s (U.S. Department of Agriculture Extension Research Service 2007). Various factors may affect the rate of carrot consumption including race, age, location, and income. The East section of the United States (Vermont, Pennsylvania, New Jersey, Maryland, Maine, New Hampshire,

Massachusetts, New York, Connecticut, and Rhode Island) consumes the most carrots per capita compared to elsewhere in the country. When race is considered, Non-Hispanic Whites and Asians consume the most carrots when compared to Black and Hispanic individuals. There is also a pronounced positive association between income and both fresh and frozen carrot consumption. Carrot consumption also increases with the age of the household head, apparent in both fresh and processed carrots (U.S. Department of Agriculture Extension Research Service 2007). Carrots rank as the 5th most consumed vegetable (or vegetable product), behind potatoes, lettuce, onion, and tomato (Produce for Better Health Foundation 2015).

2.6.2 Carrot Consumption Benefits

Plant carotenoids constitute the source of all animal carotenoids since they are only synthesized in plants and cannot by synthesized in the human body (Gross 1991). Proportionally, carrots contain the most β-carotene and α-carotene of almost any other vegetable (Bureau and Bushway 1986). Since β- and α-carotene are important precursors to Vitamin A, this consequently makes carrots one of the best dietary sources of Vitamin A. Vitamin A has been reported to play a role in preventing night blindness, improve vitamin A status of lactating women and their infants, improve serum retinol concentrations, and combat vitamin A deficiency (Loganathan et al. 2015).

The carotenoids and phenolic compounds in carrots have the ability to act as antioxidants and free radical scavengers to reduce oxidative damage to DNA, lipid, and proteins in the human body. For example, β-carotene, (the most prominent carotenoid in carrots), has been shown to uniquely be able to quench singlet oxygen without degradation and chemically neutralize free radicals such as the peroxyl, hydroxyl, and superoxide radicals due to its conjugated double bond structure (Agarwal et al. 2012). The phenolic compounds in carrots are also potent antioxidants

and free radical scavengers, especially in the peel. Carrot peel has approximately 78% antioxidant activity and 72% free radical scavenging activity (using the β-carotene–linoleic acid emulsion system and DPPH radical scavenging assay, respectively) (Zhang and Hamauzu 2004). Many individuals peel their carrots prior to consumption, and this may be detrimental to obtaining the full antioxidant free radical scavenging ability of carrots. Because oxidative stress has been known to have a role in the pathogenesis of cardiovascular disease, the aforementioned antioxidant activity of carrot phytonutrients associated with their consumption is also suggested to be beneficial for cardiac health (Agarwal et al. 2012).

Carrots also contain vitamin K in their leaves, pectin in their cell walls, (a beneficial soluble fiber), no cholesterol, insoluble fiber, and vitamin C. They also have a very low glycemic response and are one of the cheapest vegetables available in the United States produce market (U.S. Department of Agriculture Economic Research Service 2011).

2.7 Bioavailability of Carrot Carotenoids, Phenolic Compounds, and Terpenoids

2.7.1 Bioavailability of Carrot Carotenoids

There are several factors that may affect the bioavailability of carotenoids including the matrix in which the carotenoids are incorporated, the content of dietary fat and fiber, the particle size, and the food processing method (Hedren et al. 2002). Since carrot carotenoids are either in the crystalline form or associated with proteins embedded in chromoplasts, their release may be limited during the digestion process (Hedren et al. 2002). Beta-carotene (specifically all-*trans*-β-carotene isomers, 9-*cis*-β-carotene, 13-*cis*-β-carotene, and 15-*cis*-β-carotene) and α-carotene from dietary carrot intake is more bioavailable and bioaccessible from heat-treated carrots compared to raw carrots, with approximately four times as much β-carotene and three times as much α-carotene absorbed from heat-treated carrots compared to raw carrots (Rock et al. 1998;

Aherne et al. 2010). It has been suggested this happens as a result of the β-carotene being released from the protein in chromoplasts via the denaturation of the protein from heat treatment (Ahern et al. 2010). However, the isomerization of β-carotene produced by heat treatment does not negate the enhanced β-carotene uptake associated with consuming heat-treated carrots vs. raw carrots (Aherne et al. 2010). Carotenoids are most readily absorbed in the human body when consumed with dietary fats, and as little as five grams of fat consumed with carotenoid-rich foods results in significantly higher bioavailability (van het Hof et al. 2000).

2.7.2 Bioavailability of Carrot Phenolic Compounds

The bioavailability of hydroxycinnamic acids and hydroxybenzoic acids (the main components of the total phenolic profile of carrots) and their derivatives depends mainly on their structure. Esterification of hydroxycinnamic acids with other conjugates (such as chlorogenic acid, which is caffeic acid esterified with quinic acid) markedly reduces bioavailability when compared to their smaller, non-esterified counterparts (Manach et al. 2005). This notion is confirmed by a recent study that investigated the absorption of chlorogenic acid and caffeic acid in the small intestine of ileostomy patients, where a reported maximum of 33% and 95% of the initial dosage was absorbed respectively (Olthof et al. 2001). Ileostomy patients were the subject of choice for this particular study because absorption of caffeic acid and chlorogenic acid via measurement of fecal excretion can be misleading due to microfloral degradation in the colon. Few bioavailability and pharmacokinetic studies of hydroxybenzoic acids have been conducted due to its low prevalence in foods, but of the few that have it was reported that hydroxybenzoic acids are absorbed extremely well compared to other polyphenols (Manach et al. 2005).

2.7.3 Bioavailability of Carrot Terpenoids

Recent literature is available regarding the bioavailability of terpenoids found in carrots, but its abundance is small compared to phenolic compounds and carotenoids due to their only recent peak of interest in the scientific community. All terpenoids easily enter the human body by oral absorption, dermal penetration, or inhalation very often leading to measurable blood concentrations (Noma and Asakawa 2015). Monoterpenes (the largest component of the total volatile terpenoid profile of carrots) are lipophilic and highly soluble in blood, and inhaled monoterpenes are absorbed almost entirely by the lungs and then delivered to the liver where they are completely metabolized by detoxification enzymes during Phase II metabolism (Aydin et al. 2013). Monoterpenes are predominantly metabolized by cytochrome P450 monooxygenases, epoxide hydrolases, and dehydrogenases to mono- and dihydroxylated compounds, and higher oxidized metabolites are conjugated mainly to glucuronic acids (Schmidt et al. 2013). Oral bioavailability of d-limonene (a major volatile terpenoid in carrots) is somewhat inconsistent in literature. In rats, d-limonene has been reported as highly bioavailable after administration via gavage along with its metabolites in circulation in approximately 20 minutes (Crowell 1999), but has also been reported to be only 43% bioavailable in a different rat study (Chen et al. 1998). In humans, d-limonene is completely absorbed in the small intestine following oral administration, and d-limonene and/or its metabolites distribute throughout the body showing some preference for fatty tissues (Igimi and Nishimura 1974; Crowell et al. 1994).

When considering the nature of the present study, it is also important to consider the bioavailabilities of the metabolites of carrot terpenoids following extensive human metabolism, due to the consideration that the metabolites themselves might be responsible for terpenoids' hypothesized antiproliferative properties. In fact, it has been argued that limonene acts as a

prodrug once ingested, and its metabolites are the active pharmacological agents *in vivo* (Crowell et al. 1994). In humans, significant amounts of d-limonene metabolites including perillic acid, dihydroperillic acid, and limonene-1,2-diol appear in circulation at the 4 hour post-ingesting time point, following a dose of 100 mg/kg of d-limonene (Crowell et al. 1994).

2.8 Antiproliferative and Anticarcinogenic Activity of Carrot Compounds

2.8.1 Antiproliferative and Anticarcinogenic Activity of Carrot Carotenoids

Inconsistent results among intervention trials where β-carotene supplementation was implemented for hypothesized reduced cancer risk have been observed. In studies of this nature where the proportion of high cancer-risk study participants was very high, (e.g. smokers/asbestos exposure), supplementation appeared to be harmful with an increased risk and incidence of cancer (The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn et al. 1996). In other intervention trials where the proportion of high cancer-risk populations was low, (in particular, the Physicians' Health Study had 11% smokers among total subjects while the Women's Health study had 13% smokers), no adverse effects were seen (Hennekens et al. 1996; Lee et al. 1999). It has been proposed that the oxidative environment of the lung created by smoke or asbestos exposure gives rise to unusual carotenoid cleavage products, which are involved in cancer development (Goralczyk 2009). Despite this unusual phenomenon, a body of literature still exists claiming β-carotene to have sufficient antiproliferative and anticarcinogenic effects.

Several animal studies have shown that α-carotene possesses higher activity than β-carotene in suppressing tumorigenesis in the skin, lung, liver, and colorectum (Tanaka et al. 2012). For example, α-carotene has been shown to suppress spontaneous liver carcinogenesis in Male C3H/He mice significantly more than β-carotene when both were supplied in the diet at

0.05% (Murakoshi et al. 1992). This was assessed by counting the number of hepatomas in the mice; the mean number of hepatomas in the control group was 6.3, while the mean number of hepatomas in the mice receiving 0.05% α-carotene and 0.05% β-carotene was 3.0 and 4.7 respectively. In the same study, lung tumors were decreased by 67% in the α-carotene treatment group compared to the control group, while the β-carotene treatment group actually had an increased incidence of lung tumors (Murakoshi et al. 1992).

Some proposed mechanisms of carotenoids in the prevention of cancer include enhancement of the expression of the intercellular communication proteins, induction of the Phase II metabolism enzymes (detoxification enzymes), anti-inflammatory effects, and anti-tumor promoting properties (Meskin et al. 2004).

2.8.2 Antiproliferative and Anticarcinogenic Activity of Carrot Phenolic Compounds

In an investigation of the proliferation rates of HepG2 liver cancer cells following treatment of a phenolic extract from carrots, no antiproliferative activities were observed (Chu et al. 2002). These results are surprising considering phenolic compounds in fruits and vegetables are frequently suggested to be the main phytochemicals responsible for the well-known inverse association of fruit and vegetable consumption and cancer risk/incidence, but also possibly to be expected due to carrots' low concentration of phenolic compounds compared to other vegetables.

Despite this, some studies show promising antiproliferative effects for individual phenolic compounds found in carrots. Chlorogenic acid, (the most prominent phenolic compound present in carrots), in one recent study was found to induce apoptosis of U937 human myelocytic leukemic cells via DNA fragmentation and activation of caspase-3, two well-known mediators of apoptosis (Yang et al. 2012). The antiproliferative effects were seen in a dose-dependent manner, with higher dosages inducing stronger apoptotic effects. Another study

showed promising antiproliferative effects of caffeic acid, another hydroxycinnamic acid in carrots (Lin et al. 2012). Caffeic acid was treated on A549 human lung cancer cells and when proliferation of the cells was assessed following treatment, there was a significant difference between the control group and the cells treated with 150μM of caffeic acid. Caffeic acid had even greater antiproliferative effects when used in combination with paclitaxel, a common chemotherapeutic drug used to treat cancer. However, caffeic acid is not very prominent in carrots and it is noteworthy to point out that only the highest dosage of caffeic acid had a significant effect which is not representative of the caffeic acid content in an average serving size of carrots.

2.8.3 Antiproliferative and Anticarcinogenic Activity of Carrot Terpenoids

There is currently a lack of literature available that investigates the antiproliferative activity of the complete volatile terpenoid profile of carrots, (which gives rise to the purpose of this study), but there is a small body of literature available on individual terpenoids that happen to be present in carrots such as limonene, α-pinene, and β-pinene.

In a recent animal study investigating the effects of α-pinene on the progression of melanoma via a melanoma metastatic assay, C57BL/6 mice were injected with B16F10 murine melanoma cells, and subsequently 100 μL of α-pinene extracted from Brazilian peppertree was injected intraperitoneally into the mice. There was a statistically significant difference between the occurrence of metastatic melanoma nodules in the lungs of the mice treated with α-pinene and the control group (Matsuo et al. 2011).

Another study investigated the apoptosis-inducing effects of the essential oil hydrodistilled from Peruvian peppertree, which contains α- and β-pinene (two terpenoids found in carrots) at 22.7% and 31.1% of the total essential oil fraction respectively (Diaz et al. 2008).

After treatment with the essential oil, three out of five cancer cell lines tested did not show apoptotic activity (HepG2, Hep3B, and EGV-304). Breast cancer cells and leukemic cells however did show apoptotic activity after treatment (EMT-6 and K562 cell lines). A cell proliferation assay using MTT reagent showed that the breast cancer cells and leukemic cells had LD_{50} values of 75.7 mg mL^{-1} at the 48-hour time-point, and 78.7 mg mL^{-1} also at the 48-hour time-point, respectively (Diaz et al. 2008).

Limonene has been proven to have great potential for chemopreventative therapy of cancer and seems to be one of the most widely studied terpenoids found in carrots. Limonene has been proven to inhibit the development of spontaneous neoplasms in mice receiving 1200 mg/kg of limonene orally and to reduce the incidence of spontaneous lymphomas in p53-knockout mice (Crowell et al. 1994). Limonene has also been reported to reduce the rate of tumor incidence using the DMBA-induction method by 72% when fed a diet containing 1% d-limonene (Elegbede et al. 1984). The metabolites of limonene themselves have also been proven to have high antiproliferative abilities. In some studies, limonene metabolites have displayed even greater antiproliferative activities than limonene, specifically in the inhibition of small-G-protein isoprenylation (a mechanism of carcinogenesis), tumor cell proliferation, and chemoprevention of DMBA-induced rat mammary cancer (Crowell et al. 1994). For example, perillyl alcohol (a major limonene metabolite), at a dose of 1 mM completely inhibited the proliferation of human HT-29 colon carcinoma cells (Crowell et al. 1994).

The possibility of different structural varieties of terpenoids having different antiproliferative activities has also been investigated (Russin et al. 1989). Three non-oxygenated cyclic varieties (limonene, α-pinene, β-myrcene) and three oxygenated cyclic varieties (menthol, cineole, linalool) were compared, and the former is of interest since limonene, α-pinene, and β-

myrcene are major terpenoids in carrots. The total number of tumors were significantly lower with limonene and menthol treatments compared to their respective control treatments. This is interesting given their chemical structure; limonene and menthol were the only monoterpenoids to lower the total carcinoma count, and they both happen to be monocyclic (Russin et al. 1989).

It appears no studies have been conducted on other more prevalent terpenoids present in carrots such as γ-terpinene and α-terpinolene with regard to antiproliferative properties. Research on the antiproliferative effects of α-terpinolene would especially be of interest since it is the most prevalent terpenoid in carrots.

Chapter 3: Extraction, Identification, and Quantification of Carrot Carotenoids

3.1 Introduction

To assess the hypothesis of this study, (that carrot terpenoids have stronger antiproliferative effects on Caco-2 cells *in vitro* than carrot carotenoids), the carotenoids needed to be extracted, identified, and quantified from carrots for later use (see Chapter 6 for more details). The selection of an appropriate carotenoid extraction method is based on carotenoids' lipophilic nature, which typically results in the use of a non-polar organic solvent such as chloroform, hexane, petroleum ether, or acetone. However, the potential use of these solvents was met with concern since they are known to be cytotoxic to Caco-2 cells. Other types of extraction methods which use less toxic solvents and more environmentally friendly techniques such as supercritical fluid extraction and low-pressure steam explosion also exist, but due to lack of equipment and materials were not utilized (Butnariu 2016). The issue of using a toxic, organic solvent for carrot carotenoid extraction turned out to be non-problematic because we found that the extract could easily be 'dried' under a stream of nitrogen in an oxygen-free environment to

eliminate toxic solvents, and then resuspended in an appropriate solvent, (in the case of this study, Dimethyl Sulfoxide: see Section 6.3.1), that is non-toxic to Caco-2 cells when ready for *in vitro* experimentation. Because of this phenomenon, the use of the traditional liquid-liquid extraction technique with non-polar organic solvents was used and is described below.

3.2 Materials

Fresh, whole carrots of the brand "Green Giant" were purchased from Harps Food Store (Fayetteville, AR, U.S.A.). Prior to extraction, the carrots were stored intact in original packaging at 4ºC to preserve the compounds of interest. A β-carotene standard was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.).

3.3 Methods

3.3.1 Carotenoid Extraction

Carrots from 4ºC storage were tempered to room temperature. Carrots were washed thoroughly and brushed under running water to remove any dirt or debris. The tail and tops of the carrots were then removed. Approximately 40 g of the washed, cleaned carrots were homogenized with 20 mL of ethanol and 20 mL of acetone using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH, U.S.A.) for about 45 seconds. The homogenate was then filtered under vacuum through Miracloth (CalBioche, LaJolla, Calif., U.S.A.), and then the filtrate was collected for evaluation. The particulate remnants were homogenized with 150 mL of acetone using the Euro Turrax T18 Tissuemizer for about 45 seconds. The homogenate again was filtered under vacuum through Miracloth and combined with the aforementioned filtrate. Homogenization with acetone and subsequent filtration under vacuum through Miracloth was repeated until no orange or yellow color remained in the residue. The Miracloth and final residue was thoroughly rinsed with acetone to collect any remaining carotenoids, then added to the

filtrate. The filtrate was then placed in an evaporator flask and condensed at a temperature of 40 ºC using a Buchi Rotary Evaporator R-114 (Buchi, Flawil, Switzerland) to remove solvents from the carotenoid extract. The carotenoid extract was suspended to a total volume of 200 mL acetone, stored in a plastic specimen cup at -20ºC, and wrapped in aluminum foil until future partitioning of the extract was completed.

3.3.2 Partitioning of the Carotenoid Extract

Partitioning of the carotenoid extract was executed to remove unwanted hydrophilic compounds (mostly water). Since carotenoids are lipophilic, it is imperative to perform this step to eliminate the possibility of the presence of an emulsion between hydrophilic and lipophilic substances in the extract. The carotenoid extract from Step 3.3.1 was added to a portion of a solution of 1:1 (v/v) petroleum ether and hexane in a separatory funnel. Then, a small portion of salted deionized water was added to the mixture. Slight agitation was applied to the mixture, and then it was allowed to rest to form a separation of phases. After the phase separation was visible, the aqueous-rich fraction was drawn off from the separatory funnel, and then added again to another portion of the petroleum ether and hexane solution. The partitioning process was repeated until the aqueous rich phase had no remaining orange coloration. The purified carotenoid extract was then placed in an evaporator flask and condensed using a Buchi Rotary Evaporator R-114 (Buchi, Flawil, Switzerland) to remove solvents. The dried extract was then suspended in 40 mL of hexane to reflect the original starting weight of the extraction process and stored in a sealed glass specimen jar wrapped in aluminum foil at -20ºC until further analysis.

3.3.3 HPLC Analysis

Individual carotenoids were separated by HPLC using a 250 X 4.60 mm YMC S5 C_{30} column (Waters Corp, Milford, MA). Peaks were examined at 470 nm using a Waters Model 996

photodiode array detector (Waters Corp, Milford, MA). The mobile phase was hexane at a flow rate of 1.00 mL/min. The injection volume of the samples was 50 µL. Identification of carotenoids was estimated using their retention time and UV spectra against comparable chromatograms from other studies in our lab, and quantification of carotenoids was calculated using a β-carotene standard curve, with results reported as mg of β-carotene equivalents (BCE) per 100 g of fresh weight.

3.4 Results and Discussion

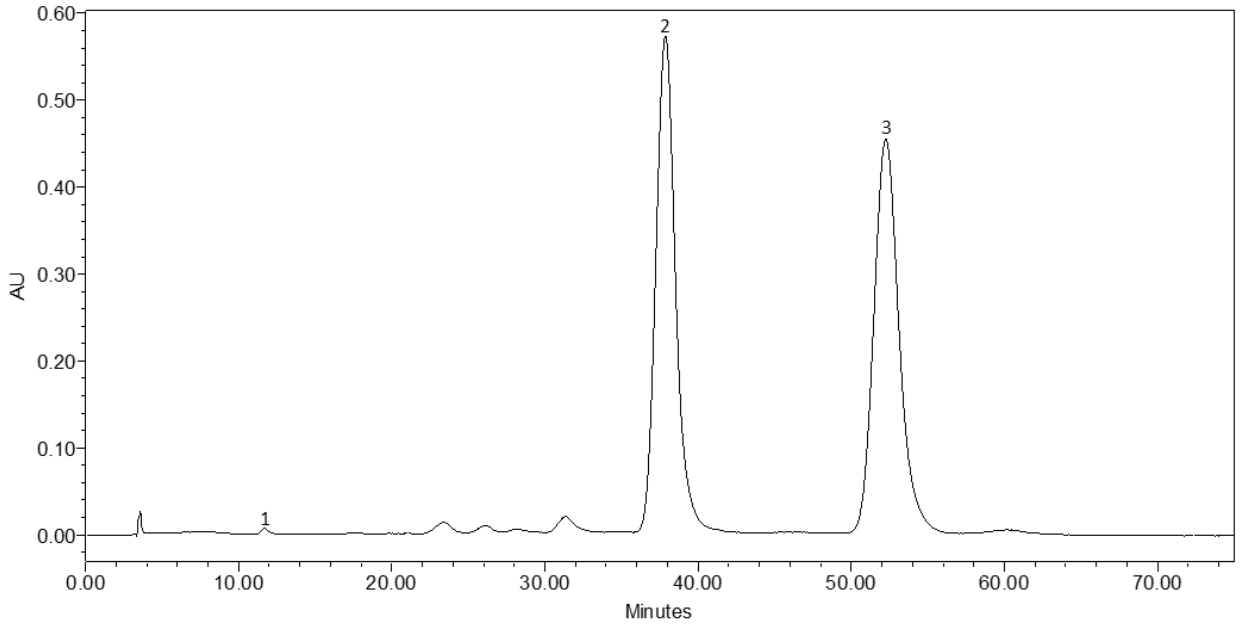

Figure 3.1 HPLC chromatogram of fresh carrot carotenoid extract at 470 nm. Peak identification: (1) Lutein, (2) α-carotene, and (3) β-carotene. Peaks (1) Lutein and (2) α-carotene were tentatively identified based on other similar chromatograms from our laboratory.

Table 3.1 Levels and Identification of Carotenoids in Fresh Carrot (from chromatogram in Figure 3.1)

Peak	Retention Time (min)	Compound	Concentration[1] (mg BCE/100 g)
1	11.70	Lutein	0.03
2	37.87	α-carotene	18.8
3	52.25	β-carotene	18.9
		Total Carotenoids[2]	37.7

All compounds were quantified using a β-carotene standard curve and are expressed as mg of beta-carotene equivalents (BCE) per 100 g of fresh weight.
[1] Concentration was determined on a fresh weight basis.
[2] Total Carotenoids is the sum of Lutein, α-carotene, and β-carotene levels.

The total carotenoid content in the carrots used for this study at first glance appeared to be extremely high, at a level of 37.7 mg BCE per 100 g of fresh weight (Table 3.1). A previous report claims an average orange carrot has 5.4 mg of carotenoids per 100 g of fresh weight (Gross 1991), which is much lower than the carrots used in the present study. However, a variety

of carrots known as ‘high beta-carotene orange’ carrots have been reported to contain 28.3 ± 0.8 mg of total carotenoids per 100 g of fresh weight (Surles et al. 2004). Upon contacting Green Giant, (the producer of our carrots), to obtain more information on the variety of carrots used for this study, that information was not allowed to be released as it was considered proprietary. Therefore, it is impossible to know if varietal or cultivar types could have an influence on the carotenoid levels of our carrots, although this is suspected to be a possible factor.

It is also possible that enzymes involved in carotenoid biosynthesis and their activity had an influence on the carotenoid content of the carrots used for this study. For instance, *Phytoene synthase* (PSY) is an important carotenogenesis enzyme involved in carotenoid accumulation during root development in carrots, and has been observed to be up-regulated with abiotic stressors such as drought and salinity (Nisar et al. 2015). It is conceivable that if these carrots during development were exposed to such stressors, carotenoid levels would be higher than average carotenoid levels in carrots.

Compared to the β-carotene level of 18.9 mg BCE per 100 g of fresh weight, there was a surprisingly high amount of α-carotene, at a similar level of 18.8 mg BCE per 100 g of fresh weight (Table 3.1). Typically, an average orange carrot has much more β-carotene than α-carotene, possibly twice as much β-carotene as α-carotene (Tanaka et al. 2012). This high level of α-carotene in the carrots used for the present study could introduce a possible higher antiproliferative effect during our *in vitro* experimentation on Caco-2 cells that would not have otherwise been observed if the α-carotene concentration was lower, since α-carotene has previously been observed to have a stronger protective effect on the incidence of cancer in preclinical studies and animal models than β-carotene (Tanaka et al. 2012; Nishino et al. 2000).

Chapter 4: Extraction, Identification, and Quantification of Carrot Phenolics

4.1 Introduction

Typically, the carotenoids in carrots have long been suggested to be responsible for carrots' anticancerous properties. Contradicting this common suggestion, the hypothesis of the present study was that carrot terpenoids will have greater antiproliferative effects on Caco-2 cells *in vitro* than carrot carotenoids and as such was investigated. However, the phenolic compounds in carrots were also deemed necessary to analyze for their potential antiproliferative effects on Caco-2 cells *in vitro* compared to the carrot carotenoids and carrot terpenoids since phenolic compounds in fruits and vegetables are frequently conjected to be the major bioactive compounds contributing to anticancerous health effects (Gorenstein et al. 2009). Carrots seem to be an exception to this suggestion since their phenolic compound concentration is commonly quite low, (when no abnormal abiotic stressors are present during development), compared to other vegetables (Hager and Howard 2006; Formica-Oliveira et al. 2017). One might assume a vegetable with low phenolic concentration would not be worthwhile to include in the present investigation as it would be expected to not display any therapeutic effect *in vitro*, however most existing literature at the time of this study involving carrot phenolic compounds' antiproliferative effects *in vitro* were conducted with black carrots, known for their high phenolic compound concentration due to their anthocyanin content. Therefore, it was logical to include the phenolic compounds of orange carrots in this study as information in literature was lacking.

It is typically recommended to extract phenolic compounds from foods with a mixture of organic solvents and more polar solvents such as water, especially for extremely polar phenolic compounds such as cinnamic and benzoic acids which are highly prevalent in carrots (Acosta-

Estrada et al. 2014). This recommendation was adopted for our extraction methodology and is described further in Section 4.3.1.

4.2 Materials

Fresh, whole carrots of the brand "Green Giant" were purchased from Harps Food Store (Fayetteville, AR, U.S.A.). Prior to extraction, the carrots were stored intact in original packaging at 4ºC to preserve the compounds of interest. All standard compounds were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.).

4.3 Methods

4.3.1 Phenolic Compounds Extraction

Carrots from 4ºC storage were tempered to room temperature. Carrots were washed thoroughly and brushed under running water to remove any dirt or debris. The tail and tops of the carrots were then removed. Approximately 25 g of the washed, cleaned carrots were (1) homogenized with 25 mL methanol/water/formic acid (60:37:3 v/v/v) using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH, U.S.A.) for about 45 seconds. The homogenate was then (2) filtered under vacuum through Miracloth (CalBioche, LaJolla, Calif., U.S.A.) and the filtrate was collected for evaluation. The residue remaining after vacuum-treatment was then (3) homogenized with 25 mL of acetone/water/acetic acid (70:29.5:0.5) using the Euro Turrax T18 Tissuemizer for about 45 seconds. The homogenate was then (4) filtered under vacuum through Miracloth and combined with the aforementioned filtrate. Steps (1)-(4) were repeated with the remaining filtrate following vacuum treatment. The Miracloth and final residue was thoroughly rinsed with both solvents previously used in steps (1) and (3) to collect any remaining compounds for analysis to add to the filtrate. The filtrate was then placed in an Evaporator Flask, and then condensed at a temperature of 40ºC using a Buchi Rotary Evaporator

R-114 (Buchi, Flawil, Switzerland) to remove solvents from the phenolic-rich aqueous extract. The sample was then centrifuged at 10,000 rpm for 5 minutes. The supernatant was loaded into a preconditioned Sep-Pak® C18 column cartridge (Waters Corp, Milford, MA) and eluted with 70-100% ethanol until no color remained in the cartridge. The sample that was eluted from the Sep-Pak® C18 column cartridge was then collected as the final phenolic extract, suspended to a total volume of 25 mL using deionized water to reflect the original starting weight of the extraction process, and stored in a sealed plastic specimen cup at -20ºC until further analysis.

4.3.2 HPLC Analysis

All phenolic extracts were passed through 0.45 µm filters (Whatman) prior to HPLC analysis. Individual phenolic compounds were separated by HPLC using a 250 X 4.60 mm Symmetry 5µm C18 column (Waters Corp, Milford, MA). Peaks were examined at 265 nm for the presence of hydroxybenzoic acids and 325 nm for hydroxycinnamic acids using a Waters Model 996 photodiode array detector (Waters Corp, Milford, MA). The mobile phase was solvent A: water acidified with phosphoric acid to pH 2.6 and solvent B: methanol/acetonitrile (3:2 v/v). The flow rate was 1.33 mL/min. The following gradient used in Babic et al.'s study was utilized to obtain the best separation of compounds: at 0 min, 12% B; at 10 min, 12% B; at 16 min, 15% B; at 26 min, 15% B; at 55 min, 60% B; at 65 min, 70% B; and at 70 min, 70% B (1993). The injection volume of the samples was 100 µL. Identification of phenolic compounds was confirmed using LC-MS. Mass spectrometry analysis was conducted in a HP 1100 series HPLC and a Bruker ESQUIRE 2000 (Billerica, MA) quadrupole ion trap mass spectrometer (Bruker, Daltonics Corp., Germany). Mass spectrometry analysis was conducted in negative ion mode with a capillary voltage at 4 kV. A nebulizing gas (N_2) pressure of 32 psi and a dry gas flow of 12 L/min was used. The skim voltage was set to 53.7 V to achieve reasonable in-source

fragmentation for identification and at the same time maintain a reasonable signal from the intact precursor ion Quantification of phenolic compounds were calculated using a chlorogenic acid standard curve, and concentrations were reported as mg chlorogenic acid equivalents (CAE)/100 g of fresh weight.

4.3.3 Total Phenolic Content using the Folin-Ciocalteu Assay

The total phenolic content of the phenolic extract was also analyzed spectrophotometrically using the Folin-Ciocalteu assay. Briefly, 0.1 mL of the phenolic extract was mixed with 0.5 mL of 0.2 N Folin-Ciocalteu reagent. Then, 0.4 mL of a 7.5% $NaHCO_3$ was added to the solution and mixed thoroughly. The solution was placed in triplicate in a 48-well plate and was allowed to stand at room temperature for 2 hours in the dark. After incubation, the samples' absorbances were read at 760 nm. Quantification of total phenolics was assessed by comparing absorbance values of the samples to absorbancies of gallic acid at known concentrations, prepared similarly to the samples. Results are expressed as the average of the triplicates ± standard error of the mean (SEM), in units of mg/100 g gallic acid equivalents (GAE) on a fresh weight basis.

4.4 Results and Discussion

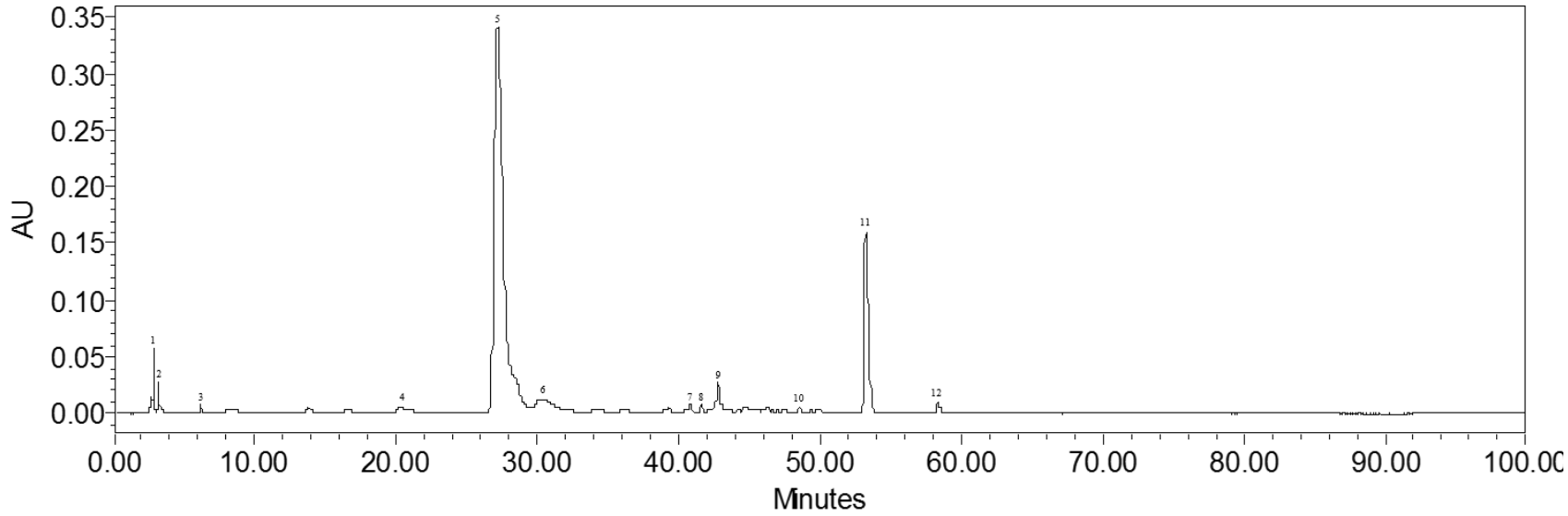

Figure 4.1 HPLC chromatogram of fresh carrot phenolic extract at 325 nm to monitor for hydroxycinnamic acids and derivatives.

Table 4.1 Levels and Identification of Hydroxycinnamic Phenolic Compounds in Fresh Carrot (from chromatogram in Figure 4.1)

Peak	Retention Time (min)	Compound	mg CAE/100 g FW	λmax (nm)	$[M-H]^-$ m/z (parent ion)	$[M-H]^-$ m/z (fragment ion)	Reference[1]
1	2.78	nd	0.07	216	—	—	—
2	3.16	nd	0.04	212	—	—	—
3	6.14	*trans-p*-coumaric acid	0.01	258	292.9	232.7	Wu et al. 1999
4	20.28	nd	0.06	213	—	—	—
5	27.17	5-*O*-caffeoylquinic acid	3.85	325	352.9	190.9	Kammerer et al. 2004
6	30.26	nd	0.12	252	—	—	—
7	40.80	nd	0.01	329	—	—	—
8	41.58	nd	0.02	321	—	—	—
9	42.76	nd	0.1	327	—	—	—
10	48.54	nd	0.02	213	—	—	—
11	53.22	caffeic acid derivative	0.73	330	364.9	202.7	Kammerer et al. 2004
12	58.33	caffeic acid derivative	0.04	330	364.9	202.7	Kammerer et al. 2004
		Total Chlorogenic Acid Equivalents	**5.07**				

All compounds were quantified using a chlorogenic acid standard curve and are expressed as mg of chlorogenic acid equivalents (CAE) per 100 g of fresh weight.
nd, not determined
[1]Mass spectra were matched with previously identified compounds in the references listed.

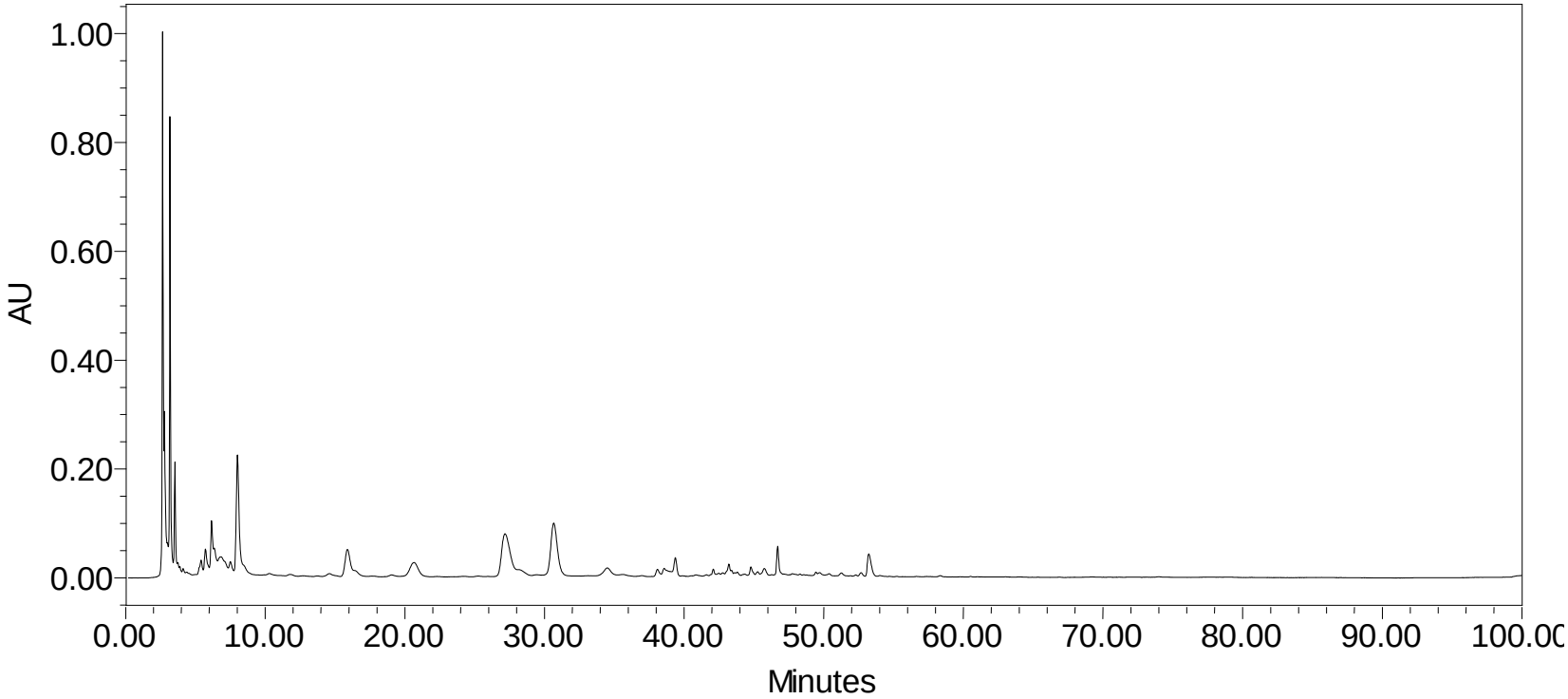

Figure 4.2 HPLC chromatogram of fresh carrot phenolic extract at 265 nm to monitor for hydroxybenzoic acids and derivatives. No hydroxybenzoic acids or derivatives were identified upon completion of HPLC-MS, or after comparison against known standards.

The total phenolic content quantified using the Folin-Ciocalteu assay was 25.8 ± 0.4 mg gallic acid equivalents (GAE)/100 g of fresh weight.

There was no detection of hydroxybenzoic acids and related derivatives upon comparison with known standards following HPLC (Figure 4.2). A lack of hydroxybenzoic acids in carrots is not uncommon, and this may be explained by a previously suggested metabolic relationship between the two (Babic et al. 1993). It has formerly been observed that the concentration of hydroxybenzoic acids increases in concurrence with a decreasing concentration of hydroxycinnamic acids following physical injury to carrots such as shredding as storage time increases (Babic et al. 1993). This proposed metabolic relationship following physical injury to carrots may explain the lack of hydroxybenzoic acids in our carrots due to an absence of physical injury before analysis as the carrots were stored whole and intact.

Following HPLC, 12 hydroxycinnamic acids and derivative compounds were successfully separated (Figure 4.1). All peaks were quantified using a chlorogenic acid standard curve. Mass Spectrometry was then conducted, and 4 of these compounds were identified as *trans*-p-coumaric acid (peak 3), 5-*O*-caffeoylquinic acid (peak 5), and 2 caffeic acid derivatives (peaks 11 and 12) representing 92% of the total peak area (Table 4.1). 5-*O*-caffeoylquinic acid, also known as chlorogenic acid, comprised 76.5% of the total phenolic content of the carrots used in this study. This percentage is slightly higher than other reports of 42.2% to 61.8% of total phenolic content (Alasalvar et al. 2001; Zhang and Hamauzu 2004), however chlorogenic acid has been observed to accumulate with the presence of abiotic stressors during development and as such could explain the high concentration of chlorogenic acid in our carrots if such a phenomenon were subjected to the carrots before harvest (Babic et al. 1993).

Upon comparison of the total phenolic content of 25.8 ± 0.4 mg GAE/100 g of fresh weight using the Folin-Ciocalteu assay to other analyses of the same nature, results seem mixed. Some studies report similar concentrations, ranging from 35.2 ± 5.00 mg GAE/100 g of fresh weight to 21.2 ± 0.4 mg GAE/100 g of fresh weight (Chu et al. 2002; Leja et al. 2013). However, other studies reported higher concentrations such as 84 ± 0.96 mg GAE/100 g of fresh weight and 78.3 mg GAE/100 g of fresh weight (Goncalves et al. 2010; Zhang and Hamauzu 2004). These discrepancies could possibly be attributed to genetic variances across the carrots. Frequently, specific carrot cultivars, (e.g. Imperator 58, Scarlet Nantes), are not considered or described in studies and instead many papers characterize the type of carrot used in the study by color or general overarching cultivar, (e.g. Nantes, Imperator), when in reality many types of orange carrots or Nantes varieties exist and vary in genetic disposition. To further illustrate this point, a study assessing the phenolic content of 15 different varieties of orange carrots using the Folin-Ciocalteu assay found concentrations of phenolic compounds to range from 18.7 ± 1.2 mg gallic acid per 100 g of fresh weight to 53.8 ± 4.4 mg gallic acid per 100 g of fresh weight depending on the variety (Leja et al. 2013).

Chapter 5: Extraction, Identification, and Quantification of Carrot Volatiles

5.1 Introduction

To investigate the hypothesis that carrot terpenoids have a greater antiproliferative effect than carrot carotenoids, the terpenoids needed to be extracted from carrots for *in vitro* experimentation on Caco-2 cells. (At this point in the present document, the whole extract of the volatile organic compounds will be referred to simply as “volatiles”, with the underlying assumption that part of that extract contains the desired terpenoids. This nomenclature is

necessary due to the extraction process including other volatile compounds that are not necessarily volatile terpenoids). Extraction of the volatiles from carrots was also required for further analysis with Gas Chromatography – Mass Spectrometry (GC-MS) to determine what individual terpenoids are present in the extract. Terpenoids are relatively unstable at high temperatures, therefore the most common extraction technique to isolate these compounds is vacuum distillation. This allows for gentle isolation of terpenoids from a food matrix, with rapid cooling to limit potential degradation of these volatile compounds (Belitz et al. 2009).

Analysis and separation of volatiles is typically conducted with GC instead of HPLC, due to their volatile nature. The use of headspace solid phase microextraction (SPME) was also implemented as a step during GC analysis. In food applications headspace SPME is preferred to avoid possible contamination of the headspace system by non-volatile food components and because it is quite sensitive to experimental conditions (Belitz et al. 2009).

One concern regarding the use of the carrot volatile extract for *in vitro* experimentation, (see Chapter 6), was the extract could deteriorate over the course of the length of the cell proliferation assay, (approximately 12 hours), due to the high temperature of the cell culture incubator (37°C). Therefore, GC-MS was also conducted on a sample of the volatile extract that was incubated at 37°C for 24 hours to assess potential compositional changes. Following GC-MS, the identified compounds in both the original carrot volatile extract and the sample that was incubated at 37°C for 24 hours were classified as either an alcohol, ketone, acid, hemiterpene, monoterpene, sesquiterpene, diterpene, ester, lactone, furan, or aldehyde and compared against each other. Results are expressed as the class of compounds' percentage of total volatile content in the sample, and are shown in Figure 5.2.

5.2 Materials

Fresh, whole carrots of the brand “Green Giant” were purchased from Harps Food Store (Fayetteville, AR, U.S.A.). Prior to extraction, the carrots were stored intact in original packaging at 4ºC to preserve the compounds of interest.

5.3 Methods

5.3.1 Volatiles Extraction

Carrots from 4ºC storage were tempered to room temperature. Carrots were washed thoroughly and brushed under running water to remove any dirt or debris. The tail and tops of the carrots were then removed. Carrots were then manually chopped with a knife into pieces approximately one cubic inch in size. 100 g of carrot, 100 mL of deionized water, and 100 g of NaCl were blended to a puree using a Black & Decker Handy Chopper Plus commercial blender. The puree was then placed in an evaporator flask and condensed at a temperature of 65°C using a Buchi Rotary Evaporator R-114 (Buchi, Flawil, Switzerland). During method development for the extraction of the terpenoids, suspicions arose that some of the compounds volatilized and potentially escaped the distillation system too quickly to be condensed into liquid form and collected into the final extract. Because of this suspicion, a ‘cold trap’ was added into the distillation assembly. The cold trap assembly was as follows: a vacuum flask with rubber hosing and a rubber stopper attached was placed in the rotary evaporator chiller, and the other end of the rubber hosing was attached to the condenser of the distillation system. The extremely cold environment of the vacuum flask allowed for quick cooling of escaped volatile compounds that otherwise would have been lost from the system. The first 250 mL of condensate/distillate collected in the receiving flask from the distillation process was collected, (total distillation time was approximately 1.5 hours). To combine the ‘lost’ volatile compounds in the cold trap with the

extract, the 250 mL of distillate was poured into the vacuum flask and gently agitated. The final carrot volatile extract was stored in a sealed glass specimen jar at -20ºC until further analysis.

5.3.2 GC Analysis

Four mL of the thawed carrot volatile extract from Step 5.3.1 (added to four g of NaCl to inhibit enzymatic reactions) was immediately analyzed using Solid Phase Microextraction (SPME) using an 85 µm Carboxen®/Polydimethylsiloxane (CAR/PDMS) fiber (Stableflex, 24 Gauge, Manual). Another four mL sample of the carrot volatile extract was stored at human body temperature (37°C) for 24 hours before SPME analysis to assess any potential changes in the composition of the extract was used. Each four mL sample was incubated for 30 minutes at 55°C, stirred constantly, to achieve partition equilibration of the volatiles between the sample and headspace. After 30 minutes, the SPME fiber was exposed to the headspace of the sample to adsorb the volatile compounds. The fiber was then introduced into a heated gas chromatographic injector (240°C) for desorption for five minutes. For gas chromatography, the following conditions were used: detector temperature was kept at 250°C, flow rate of the carrier gas helium was kept at 1.00 mL / min, and the temperature gradient commenced at 30°C, held for five minutes, ramped to 150°C at 5°C / min, ramped to 250°C at 20°C / min, then held for three minutes. The GC was equipped with a Shimadzu Rtx-5MS column (30 m x 0.25 mm x 0.25 µm thickness). Identification and quantification of carrot volatile compounds were performed using GC-MS on a Shimadzu GC-2010 plus GC equipped with a Shimadzu GCMS-TQ8040 triple quad mass spectrometer.

5.4 Results and Discussion

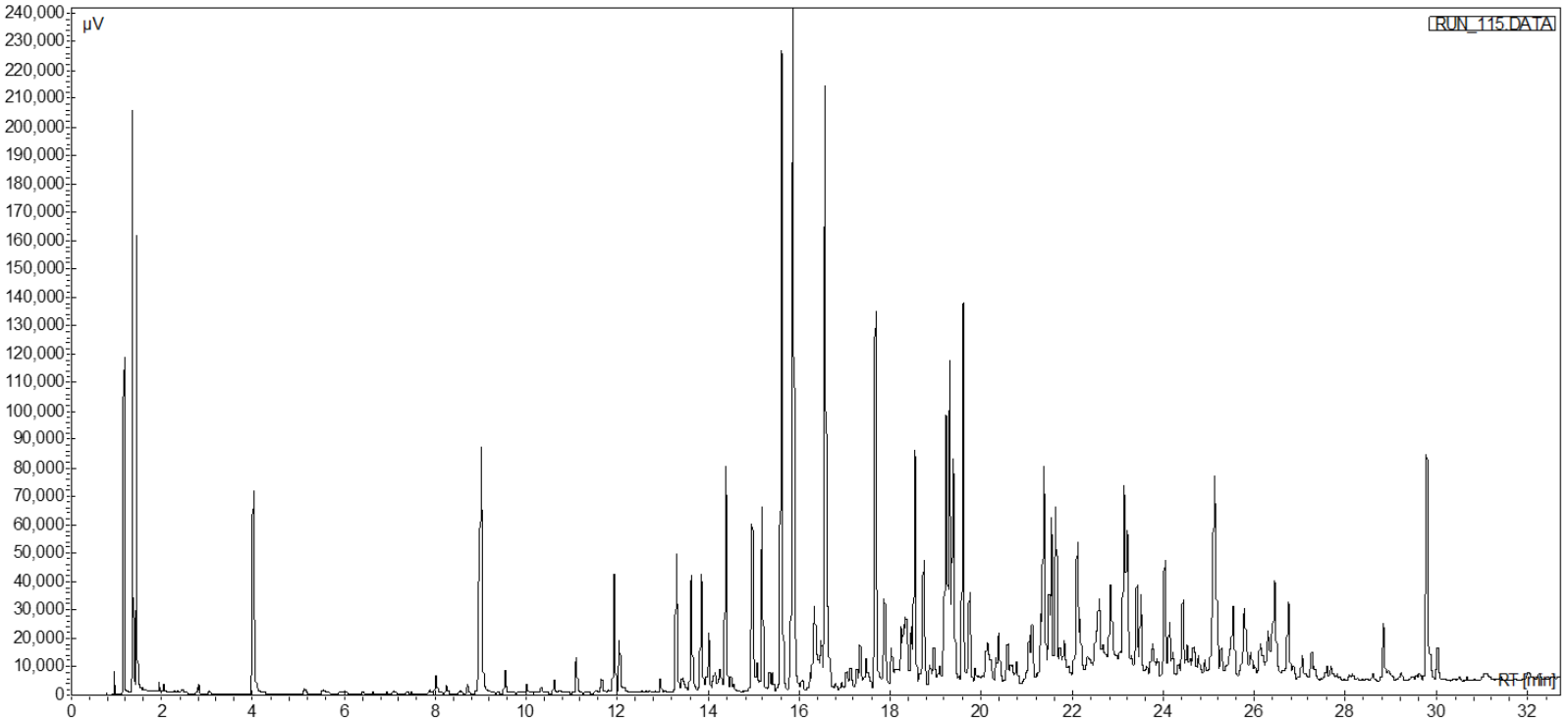

Figure 5.1 A typical GC chromatogram of carrot volatiles collected using Solid Phase Microextraction.

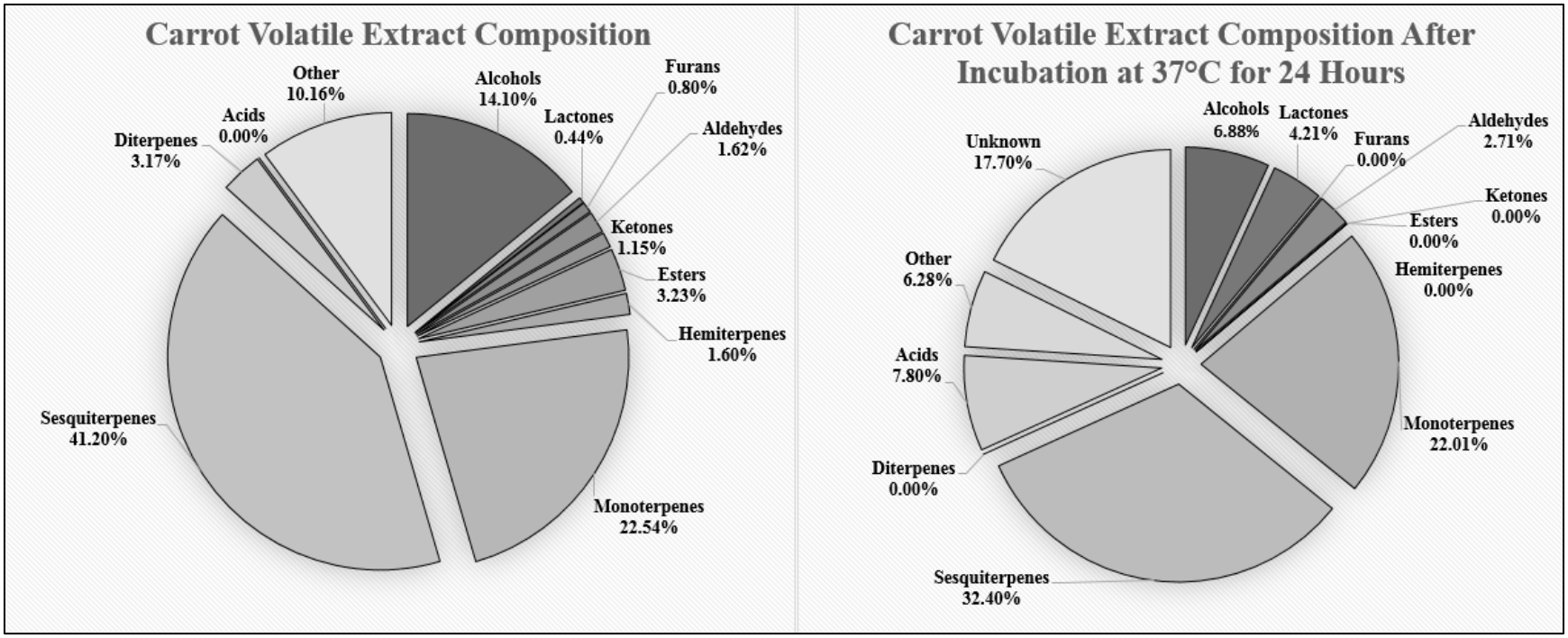

Figure 5.2 Composition changes in the Carrot Volatile Extract at 0 hours and after incubation at 37°C for 24 hours.

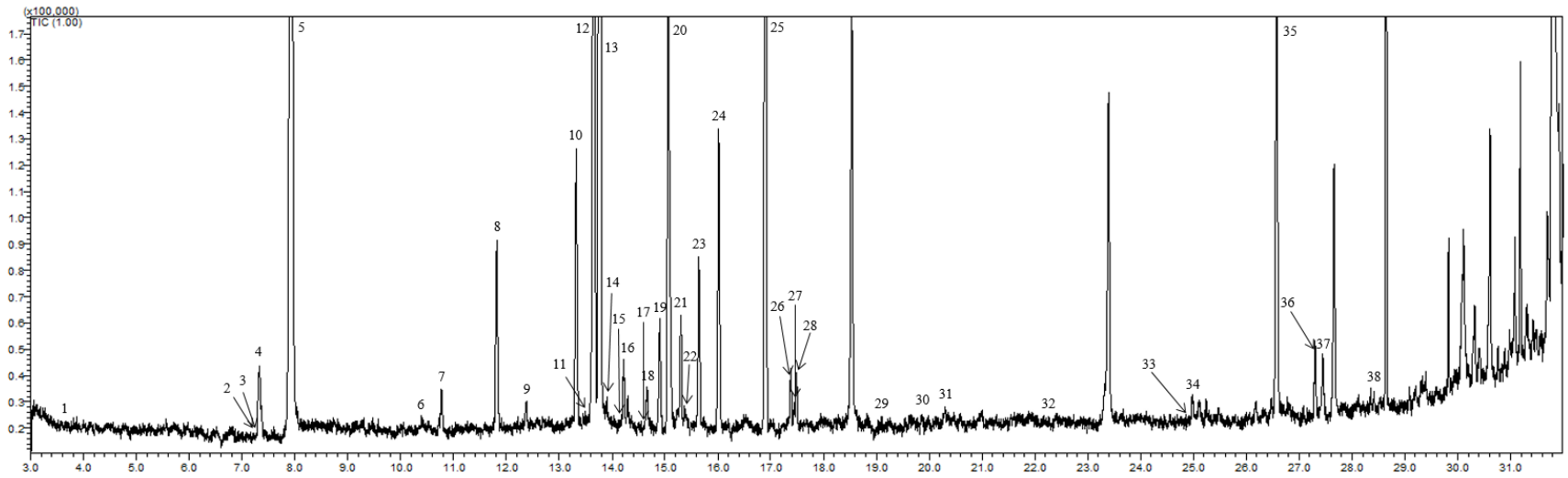

Figure 5.3 GC chromatogram of carrot volatiles from Mass Spectrometry Analysis.

Table 5.1 Compounds identified in the Carrot Volatile Extract via Gas Chromatography coupled with Mass Spectrometry.

Peak Number[2]	Retention Time (min)	Volatile Compound	Concentration (ppb)
1	3.73	Butanol[3]	0.35
2	7.15	Cyclopentanol, 1-methyl[3]	8.04
3	7.21	3-penten-2-one, 4-methyl	nd
4	7.29	Hexanal[3]	72.9
5	7.86	Siloxane	nd
6	10.38	2-heptanone	nd
7	10.75	Heptanal	12.4
8	11.79	α-pinene	34.0
9	12.35	Camphene	nd
10	13.30	β-pinene	nd
11	13.45	1-octen-3-ol[3]	47.7
12	13.65	Siloxane	Nd
13	13.73	β-myrcene	18.3
14	13.89	2-carene	nd
15	14.19	Octanal	9.1
16	14.20	α-phellandrene	150.5
17	14.62	4-terpinyl acetate	nd
18	14.65	Terpinolene	234.5
19	14.84	p-cymene	143.3[1]
20	15.03	D-limonene	143.3[1]
21	15.28	Trans-β-ocimene	61.9
22	15.36	3-octen-2-one	nd
23	15.63	Cis-β-ocimene	14.9
24	16.00	γ-terpinene	904.9
25	16.87	Isoterpinolene	nd
26	17.37	Undecane	nd
27	17.43	Thymol	nd
28	17.48	Nonanal	69.3
29	19.15	2-nonenal	nd
30	19.83	Terpinen-4-ol	52.7
31	20.27	α-terpineol	116.6
32	22.88	Bornyl acetate	19.0
33	24.95	Aristolene	nd
34	25.23	Cis-α-bisabolene	nd
35	26.56	Caryophyllene	22.2
36	27.29	Isocaryophyllene	nd
37	27.43	Humelene	37.1
38	28.50	Trans-α-bergamotene	nd
		Total Volatiles	2172.8

nd, not determined.

[1]p-cymene and D-limonene co-eluted during chromatography, therefore concentrations of these were quantified as D-limonene.

[2]Peak numbers correspond to the peaks in Figure 5.3.

[3]Slope used to quantify these compounds could not be determined from standards. Quantification was estimated using the standard curve of nonanal, a compound of similar structure and chemical properties.

Following GC, a large number of compounds were successfully separated, which is reflective of the complexity of the aromatic composition of carrots (Figure 5.1). Through Mass Spectrometry analysis, 38 volatile compounds that contribute to the aroma of carrots were identified (Table 5.1). This number is higher than other recent studies, with 32, 33, and 31 compounds identified (Alasalvar et al. 1999; Alasalvar et al. 2001; Guler et al. 2015). Trends in more prominent compounds in our volatile extract were similar to previous studies (Alasalvar et al. 1999, Alasalvar et al. 2001). Major compounds consistent across literature and the present study included terpinolene, p-cymene, β-caryophyllene, and γ-terpinene. However, compounds such as γ-bisabolene and myrcene were identified as major compounds in other research but were not even identified through Mass Spectrometry in this study indicating they were not present or present in very low concentration (Alasalvar et al. 1999, Alasalvar et al. 2001). Other trends were also not consistent among literature and the present study. For instance, the total volatile content of the carrots used in this study was 2.2 ppm (Table 5.1), while other sources note total volatile concentrations of 6.7 ppm, and even concentrations as high as 30.9 ppm (Alasalvar et al. 2001, Alasalvar et al. 1999). These comparisons however should be met with caution; for example, Alasalvar et al. did not disclose the color or the variety of the seven types of carrots used in their study, which could present discrepancies due to genetic variation. Also, due to the limitation in this study of not being able to acquire standard compounds to quantify all the compounds identified through Mass Spectrometry, the results of the present study may underestimate the actual concentration of total volatile content as only a portion of all the compounds identified were quantified. Contrasting results in total volatile content between the present study and those previously reported may be attributed to differences in cultivars, genetic

regulation mechanisms, or even microbial deteriorations as volatile compounds in produce have been used as markers for microbial contamination (Alasalvar et al. 1999).

There was concern that the carrot volatile extract to be used for *in vitro* experimentation would deteriorate over the course of the cell proliferation assay due to the high temperature conditions of the incubator (37°C) and the lengthy amount of time to conduct the assay (12 hours). To address this concern, the carrot volatile extract was also analyzed with GC-MS after being incubated at 37°C for 24 hours (Figure 5.2). Overall, monoterpenes remained stable but larger terpenoids including sesquiterpenes and diterpenes seemed to decrease in concentration slightly from 41.2% to 32.4% and 3.17% to 0.00% of the total fraction after 24 hours of incubation at 37°C, respectively.

Once terpenoids are deprived of their protective compartmentation in their original food matrix, they are especially prone to oxidative damage, chemical transformations, or even polymerization (Turek and Stintzing 2013). Polymerization of the volatile terpenoids in our volatile extract seem less likely than the former possibilities, as there was not an increase in large-molecular weight terpenoids observed in our volatile extract observed after 24 hours of incubation at 37°C. However, oxidative degradation does seem likely. Terpenoid compounds, especially those that are highly unsaturated, are particularly prone to oxidative deterioration (Turek and Stintzing 2013). Acids, ketones, lactones, alcohols, aldehydes, epoxides, and peroxides are common degradation products following oxidation of terpenoids and a proposed depiction of this oxidation scheme is depicted in Figure 5.4. Noting the increase in lactones, aldehydes, and acids in conjunction with the decrease in larger terpenoids, it is probable that some oxidation of these terpenoids occurred. What is curious however, is the decrease in alcohols and ketones. These are considered stable secondary products of oxidation and it is

unlikely that any alcohols or ketones produced from such oxidation would have degraded by the time of analysis.

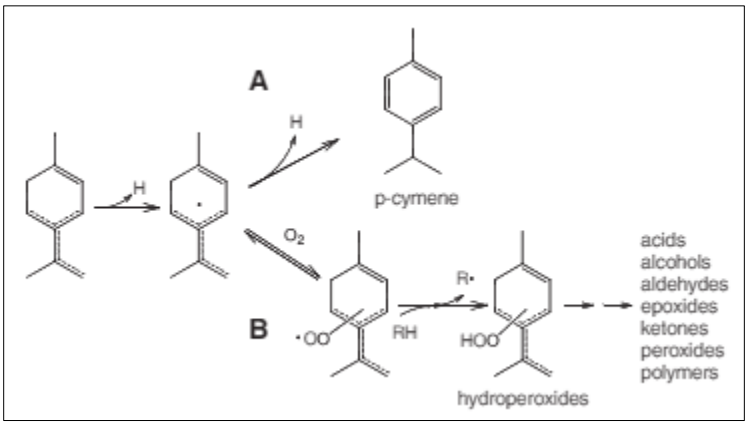

Figure 5.4 Proposed oxidation scheme of terpenoids. (A) Dehydrogenation and possible hydrogen rearrangements. (B) Autoxidation pathway leading to hydroperoxides and subsequent degradation into secondary oxidation products (Turek and Stintzing 2013)

Chapter 6: Effect of the Carrot Carotenoid, Phenolic, and Volatile Extracts on the Proliferation of Caco-2 cells *in vitro*.

6.1 Introduction

To test the antiproliferative potential of carrot terpenoids, an *in vitro* cell proliferation assay was conducted on Caco-2 cells. Cell proliferation assays are often used for screening experimental compounds to determine if they have effects on cell proliferation, whether it be for examining cytotoxic effects of the experimental compound of interest or, (in the case of this study), beneficial antiproliferative effects on cancerous cells (Sittampalam et al. 2014). The principle of this assay is to treat a known number of cells with an experimental substance or compound of interest, allow some time for the experimental substance to potentially undergo its effects on the cells, (e.g. stimulate proliferation or cause cell death), count the cells after the treatment, and compare the two cell counts against each other to assess the viability of the cells following treatment. This principle of the cell proliferation assay was used in the present study to

assess the antiproliferative potential of carrot carotenoids, phenolics, and terpenoids on Caco-2 cells.

Caco-2 cells, (a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells), were selected for the cell proliferation assay because the colon is a major site of cancer thought to be protected by vegetables, and because of carrots' well-known anticancerous effects to colon cancer specifically compared to other sites of cancer (Ferrarini et al. 2012; Guler et al. 2015).

6.2 Materials

The carrot carotenoid, phenolic, and volatile extracts described in Chapters 3, 4, and 5 respectively were used for the cell proliferation assay. Caco-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). All cell culture media components were obtained from Gibco® through Life Technologies (Carlsbad, CA). The CellTiter 96® AQueousOne Solution Cell Proliferation Assay kit was purchased from Promega Corporation (Promega Corp. Madison, WI).

6.3 Method Development

6.3.1 Selecting a Suitable Solvent or Surfactant for Carrot Carotenoid, Phenolic, and Volatile Extracts in Cell Culture Media

There were a few concerns regarding the methodology for the cell proliferation assay. One of these concerns was the solubility and stability of the experimental compounds in the cell culture media over the course of the assay (approximately 12 hours). Phenolic compounds are typically water-soluble, but terpenoids can be lipophilic or hydrophilic and most carotenoids are very lipophilic. Theses chemical properties of the carotenoid and volatile extracts were problematic because the cell culture media used for the cell proliferation assay is a water-based

solution. Because of this, there was a need for a suitable solvent or surfactant as a carrier vehicle to ensure there would be no phase separation between the test compound and the cell culture media. Ideally, the surfactant or solvent would have to be both biocompatible and able to hold the experimental compound in suspension to properly deliver the test compound to the Caco-2 cells for uptake. Tween 80 was deemed a potentially suitable choice due to its proven ability to be cell-culture suitable, hold carotenoids and terpenoids in suspension in cell culture media, and to even improve the uptake of carotenoids and terpenoids in Caco-2 cells (O'Sullivan et al. 2004; Yousefzadi et al. 2014). Tween 80 is a polysorbate nonionic surfactant, and due to its nonionic nature does not readily harm cells *in vitro* at appropriate, therapeutic concentrations.

A cytotoxic evaluation of Tween 80 was conducted on Caco-2 cells to determine (1) the appropriate concentration of Tween 80 to use in the cell culture media for the application at hand, and (2) to determine the tolerable upper limit for Tween 80 concentration in cell culture media on Caco-2 cells. Various concentrations of Tween 80 in cell culture media ranging from 0.001% to 0.10% were applied to a known number of Caco-2 cells, allowed to incubate for 24 hours, and then counted and compared to the known number of cells at the starting point of the experiment. Results from the cytotoxic evaluation showed that Tween 80 at a concentration of 0.01% and lower in the cell culture media had no effect on the Caco-2 cells, and Tween 80 at a concentration of 0.05% had limited effect on the cells. Concentrations of Tween 80 higher than 0.05% were deemed cytotoxic.

Even though existing literature shows high solubility of carotenoids in Tween 80, poor solubility of the carotenoid extract in cell culture media with Tween 80 used as a surfactant was observed in our lab (O'Sullivan et al. 2004). Because of this issue, dimethyl sulfoxide (DMSO) was instead adopted for use as a solvent for the carotenoid extract in cell culture media. A

cytotoxic evaluation of DMSO was also conducted on Caco-2 cells to determine an appropriate concentration of DMSO to use in the cell culture media that both (1) kept the carotenoids in suspension in the cell culture media without any observed precipitation of carotenoids and (2) was not cytotoxic to the Caco-2 cells. Various concentrations of DMSO in cell culture media ranging from 1-4% were applied to a known number of Caco-2 cells, allowed to incubate for 24 hours, and then counted and compared to the known number of cells at the starting point of the experiment. Results from the cytotoxic evaluation showed that DMSO at all concentrations tested (1-4%) were not harmful to the cells.

Because of the results of the cytotoxic evaluations of both Tween 80 and DMSO on Caco-2 cells, a concentration of 0.02% Tween 80 in the experimental media containing the carrot volatile extract and a concentration of 4% DMSO in the experimental media containing the carrot carotenoid extract was utilized during the cell proliferation assay described in Section 6.4.2.

6.3.2 Development of a Protocol for the Cell Proliferation Assay

Another concern for the methodology of the cell proliferation assay was the development and selection of an appropriate protocol. The assay can be executed in many ways, none of which are incorrect but may or may not result in consistent measurements among different experimenters. Typically, the cells are seeded in a 96-well plate, allowed to adhere to the bottom of the plate, and the media is aspirated off the cells and replaced with a fresh media containing the experimental compound of interest. Trial runs of this protocol often resulted in unexplainably inconsistent results. Because of this inconsistency, the existing protocol was amended. Instead of aspirating the old media off the cells after stable attachment, it was left in the 96-well plate and the experimental media containing the experimental compound of interest was added to the

existing media. The plates were then placed in a microplate shaker (VWR International, Radnor, PA) for 3-4 minutes at a speed of 450 revolutions per minute to ensure the experimental media was thoroughly mixed into the existing media.

One proposed theory for the inconsistent results during the trial runs was some cells may have been aspirated along with the old media, which has also been theorized as a problem during this assay among others (Wang et al. 2010). This could potentially explain the inconsistent cell counts that were often observed. When the protocol was amended where no aspiration of media occurred and instead the media containing the experimental compound of interest was added to the wells, more consistent cell counts and results were observed. Therefore, even though the amended protocol is not the typical one used for this cell proliferation assay, it was adopted for the sake of consistent results. The amended protocol is described in Section 6.4.2.

6.3.3 Dosage Selection of the Carrot Carotenoid, Phenolic, and Volatile Extracts

The carrot carotenoid, phenolic, and volatile extracts described in Chapters 3, 4, and 5 respectively were used as experimental treatments on Caco-2 cells during the cell proliferation assay. As a final step during extraction methods, the extracts were brought back up to a volume reflecting the original fresh starting weights as a reflection of ‘as is’ concentrations in a fresh carrot. It would have been desirable to add these extracts directly to the Caco-2 cells, but the cells would have potentially died due to a lack of cell culture media containing vital components such as fetal bovine serum, amino acids, and antibiotic-antimyotic (see Section 6.4.1 for details of the composition of the cell culture media). The goal then became to keep the concentration of the original extracts as high as possible, (i.e. the dilution factor as low as possible), to deliver as much of the carotenoids, phenolics, and volatiles to the Caco-2 cells as possible. However, an issue regarding the solubility of the carotenoid extract in the cell culture media was observed.

DMSO at a concentration of no more than 4% in the cell culture media was already deemed non-cytotoxic to cells, (see Section 6.3.1), however when the carotenoid extract was added to this mixture of the cell culture media containing 4% DMSO, precipitation of the carotenoids was observed indicating only partial solubilization of the carotenoids in the cell culture media. This would be problematic during the cell proliferation assay as it would indicate non-complete delivery of the carotenoids to the Caco-2 cells. After some trial and error, it was found that the lowest dilution factor of the carotenoid extract that could be used in the cell culture media containing 4% DMSO with no observed solubilization issues was a dilution factor of 50 (noted hereafter as 50X). Therefore, dosages of the carotenoid, phenolic, and volatile extracts of 50X, 100X, and 200X were selected for the cell proliferation assay. The selection of three different dosages for each treatment allows for future observation of a dose-response curve as a means of confirming complete delivery of test compounds to the Caco-2 cells. The concentration of the carotenoids, phenolics, and volatiles applied to the Caco-2 cells at the dilutions of 50X, 100X, and 200X each can be found in Table 6.1 for reference.

Treating the Caco-2 cells in this cell proliferation assay with equivalent dilutions of the whole carotenoid, phenolic, and volatile extracts rather than equivalent concentrations of carotenoids, phenolics, and volatiles can be justified as a means of reflecting the actual composition of a carrot since equivalent concentrations of these compounds are not representative of what a person would naturally consume from a carrot.

Table 6.1 Concentrations of the carrot carotenoid, phenolic, and volatile extracts applied to the Caco-2 cells during the cell proliferation assay described in Section 6.4.2.

Extract Treatment	Concentration applied to Caco-2 cells
Carotenoid 50X	1508 µg BCE / mL
Carotenoid 100X	754 µg BCE / mL
Carotenoid 200X	377 µg BCE / mL
Phenolic 50X	645 µg GAE / mL
Phenolic 100X	323 µg GAE / mL
Phenolic 200X	162 µg GAE / mL
Volatile 50X	45 ng / mL (total volatiles)
Volatile 100X	23 ng / mL (total volatiles)
Volatile 200X	12 ng / mL (total volatiles)

BCE = Beta Carotene Equivalents
GAE = Gallic Acid Equivalents

6.4 Methods

6.4.1 Cell Culture

Caco-2 human colon cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) at passage number 18 and maintained in 75 cm^2 cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids, 1% antibiotic-antimyotic, and incubated at 37 °C in a humidified 5% CO_2 atmosphere. All cell proliferation assays with Caco-2 cells were conducted on passage numbers 25-45. All media components and reagents were obtained from Gibco® through Life Technologies (Carlsbad, CA).

6.4.2 CellTiter 96® Aqueous One Solution Cell Proliferation Assay

Assessment of the antiproliferative potential of the carrot carotenoids, phenolics, and terpenoids was conducted using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay, also colloquially known as the MTS Assay (Promega Corp. Madison, WI). Caco-2 cells at 80-100% confluency in 75 cm^2 cell culture flasks were trypsinized in a trypsin/EDTA solution. The trypsinized Caco-2 cells at a cell density of 1 x 10^3 in 100 µL of working media

were seeded in the wells of a 96 well plate and incubated at 37 °C and 5% CO2. After stable attachment (~24 hrs), 100 μL of the experimental medias containing the carrot carotenoid, phenolic, and volatile extracts, (described in Chapters 3, 4, and 5 respectively), at dilutions of 50X, 100X, and 200X with cell culture media were added to the wells with the exception of wells serving as control conditions, where 100 μL of cell culture media containing no experimental compounds was added. For the carotenoid experimental medias at all dilutions, DMSO at a concentration of 4% was added, and for the volatile experimental medias at all dilutions, Tween 80 at a concentration of 0.02% was added to ensure proper solubilization of the test compounds and delivery to the Caco-2 cells, (see Section 6.3.1). Cell count measurements were made at 0, 6, and 12 hours by adding 40 μL of the Cell Titer reagent directly to every well and incubated for 2 hours before measuring the absorbance at 490 nm. After all absorbance readings were made, corrections were made for cell-free background absorbances. Absorbances were then converted into cell counts using an equation from a Caco-2 cell standard curve. Results are reported as the average cell counts ± the standard error of the mean (SEM) for each time point and treatment, and as proliferation percentages relative to the time point of 0 hours ± SEM for each time point and treatment.

6.5 Statistical Analysis

All data analysis was conducted using R Studio version 3.4.3.

6.5.1 Initial Exploratory Data Analysis

Since the design of the cell proliferation assay was one with more than two treatments, and the response variable (cell count) was measured repeatedly over time, the parametric repeated measures ANOVA test is commonly used to statistically analyze the results, depending on whether the original data abides by certain requirements of this method. Requirements for a

repeated measures ANOVA test are as follows: (1) independent observations, (2) normal distribution of residuals calculated from a linear regression model, and (3) equal variances of the differences between any two levels of the within-groups factor, i.e. sphericity (Kabacoff 2015). The requirement of independence of observations was met, as individual cell counts could not influence each other due to their separate placement in wells of the 96-well plate used in the assay. However, it was anticipated due to the relatively small sample size for each treatment at each time point, (n=15), that the requirement of normally distributed residuals calculated from a linear regression model would not be met. To assess this, exploratory data analysis was conducted. First, a linear regression model was created, and data was plotted against the linear output of the regression model. The distance of each data point from the linear output of the regression model, (the residuals), was calculated and plotted on a Q-Q plot found in Figure 6.1. Q-Q plots are used to assess the distribution of two data sets against each other: in this case, it was used to assess the distribution of the residuals against a theoretical normal distribution. If the data points in a Q-Q plot stray from the line representing the distribution of the theoretical data, (in this case a normal distribution), the data is said to not match the theoretical distribution. Looking at the Q-Q plot in Figure 6.1, it is apparent that the residuals were non-normally distributed, which led to a violation of the requirement of normally distributed residuals when using the repeated measures ANOVA analytical method. This was also confirmed with a Shapiro-Wilk test for normality (p-value < 0.001). Because of this violation, and because of the unlikeliness of the requirement of sphericity being met in real-world data (Kabacoff 2015), (considering the possibility of non-sphericity being even more exaggerated due to the small sample sizes), a non-parametric analytical method was used. It is noteworthy to point out that data lacking sphericity can be corrected, for instance with the Greenhouse-Geisser correction.

This correction however was not considered in the present study due to the non-normally distributed residuals.

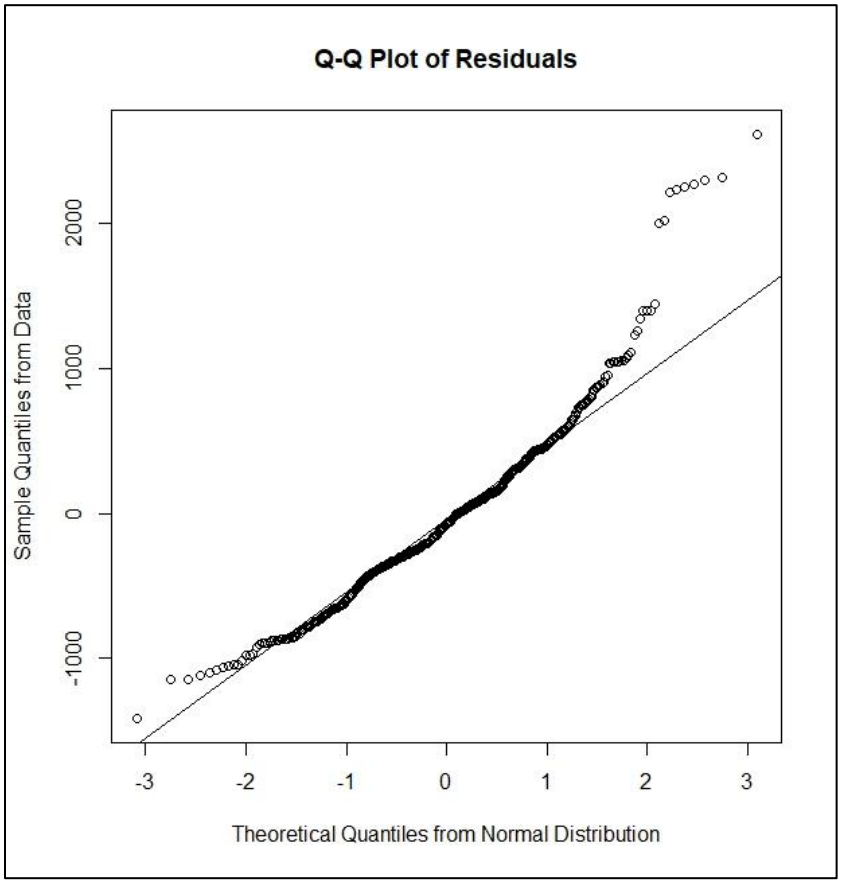

Figure 6.1 Q-Q Plot of residuals from the linear regression model created using the data from the cell proliferation assay described in Section 6.4.2. The line on the plot depicts a theoretical data set reflecting perfectly normally distributed data. The residuals of the data are not normally distributed, as they do not follow the same path as the line in the Q-Q plot.

6.5.2 Non-Parametric Statistical Analysis Methods

Due to the violation of requirements for repeated measures ANOVA, the non-parametric equivalent Friedman's test was adopted for statistical analysis of the cell proliferation assay. The main difference between these two tests is the repeated measures ANOVA relies on data belonging to a normal distribution, while the Friedman's test is a 'distribution-free' test. Another underlying difference between these two tests is the use of a comparison of ranked data in the

Friedman's test rather than the use of a comparison of equality of means of values with the repeated measures ANOVA. This use of ranked data in the Friedman's test alleviates the problem of using a parametric test on non-normally distributed data. The use of a parametric test relies on the assumption that 68% of the data lies within one standard deviation of the mean, but since the data from the cell proliferation assay is not normally distributed the mean would be an inaccurate depiction of the center of the distribution. Therefore, the use of ranked data, (as in the Freidman's test), does not rely on some central tendency of the data, but rather an assessment of whether or not data points are typically higher or lower than each other.

The Friedman's test also depends only on the ranks of the observations within blocks. The use of these blocks is to account for some potentially related data. The Friedman's test can also be thought of as a two-way ANOVA; with regard to this cell proliferation assay, the two factors being considered are the experimental treatments, and the variable of time. The use of analyzing ranked data blocked by some potentially related variable allows the effect of some experimental treatment to be compared with each other without an excess of unwanted effects confusing the results of the experiment (Conover 1999). The assumptions of the Friedman's test are as follows: (1) independent observations and (2) observations may be ranked according to some criterion of interest (Conover 1999). These two assumptions were met. The data to be ranked during the Friedman's test was represented by the mean cell counts for each treatment at each time point.

First, a primary Friedman's test was conducted to assess for an overall significant treatment effect over time, blocking for the variable of time since it was hypothesized that cell counts might be dependent on the variable of time (with blocks for the 0 hour, 6 hour, and 12

hour time points). The following null and alternative hypotheses were used for the primary Friedman's test:

H_0: Each ranking of the mean cell counts for each experimental treatment within the data blocked for the 0, 6, and 12 hour time points are equally likely.

H_1: At least one of the experimental treatments tends to yield larger ranks of the mean cell counts than at least one other experimental treatment.

If significance was detected, post hoc analysis according to the method described by Conover was completed using the following equation:

$$\left|R_j - R_i\right| > t_{1-\alpha/2}\left[\frac{2\left(bA_1 - \sum R_j^2\right)}{(b-1)(k-1)}\right]^{\frac{1}{2}} \quad (1)$$

Where R_j and R_i represent the sum of ranks for each treatment type, b represents the number of time points, k represents the number of treatment types, and $t_{1-\alpha/2}$ is the $1-\alpha/2$ quantile of the t distribution and where A_1 is calculated with the following equation:

$$A_1 = bk(k+1)(2k+1)/6 \quad (2)$$

(1999). Treatment types i and j were considered significantly different from each other if the inequality in equation 1 was satisfied.

Furthermore, if significance was not found following the primary Friedman's test then a secondary Friedman's test was conducted to assess if non-significance in the primary test was due to a non-random dependency of time. The following null and alternative hypotheses were used for the secondary Friedman's test:

H_0: Each ranking of the mean cell counts for each time point within the data blocked for the experimental treatments are equally likely.

H_1: At least one of the time points tends to yield larger ranks of the mean cell counts than at least one other time point.

If significance was detected in the secondary Friedman's test, then post hoc analysis was conducted using equations 1 and 2, (but inversing the values of b and k, i.e. blocking for treatment types instead of blocking for time points).

Further analysis was completed on each time point separately, (i.e. ignoring the variable of time), using the Kruskal-Wallis test to obtain more information about significantly effective antiproliferative treatments if the primary Friedman's test resulted in a lack of significance. The following null and alternative hypotheses were used for each Kruskal-Wallis test at each time point:

H_0: All of the population distribution functions for the experimental treatments are identical.

H_1: At least one of the populations tends to yield larger observations than at least one of the other populations.

If significance was found at any time point following the Kruskal-Wallis test, then post hoc analysis was completed using the Mann-Whitney test for every possible pair of treatment and control to assess which treatments were significantly lower than the control, (as an indication of a significantly effective antiproliferative effect). The following null and alternative hypotheses were used for each Mann-Whitney test:

H_0: The population distribution functions from the experimental treatment group and the control group are identical.

H_1: The probability of an observation from the population of the experimental treatment group exceeding an observation from the population of the control group is lower than the probability of an observation from the population of the experimental treatment group being lower than an observation from the population of the control group.

For proliferation percentages, statistical analysis was conducted at the 6 and 12 hour time points using the Kruskal-Wallis test and Dunn's test for post hoc analysis. The Bonferroni correction was also utilized for multiple comparisons. The null and alternative hypotheses for the Kruskal-Wallis test is mentioned above, and the null and alternative hypotheses for the Dunn's test is similar to those for the Mann-Whitney test, also mentioned above. The Kruskal-Wallis, Mann-Whitney, and Dunn's tests are appropriate for use on samples of unequal sizes, but it is worth noting that because of this caveat the power of those tests will potentially be reduced. A significance level of $\alpha = 0.05$ was used for all statistical analyses.

6.6 Results and Discussion

Table 6.2 Mean cell counts [± standard error of the mean (SEM)] of Caco-2 cells after treatment of the carrot carotenoid, phenolic, and volatile extracts at 0, 6, and 12 hours following treatment.

		Time Point		
		0 hours[a]	**6 hours**[b,d]	**12 hours**[c]
Treatment	Carotenoid 50X	1182 ± 116	722 ± 84[e]	1469 ± 104
	Carotenoid 100X	1078 ± 114	704 ± 69[e]	1520 ± 131
	Carotenoid 200X	1326 ± 90	876 ± 103[e]	1436 ± 162
	Phenolic 50X	1064 ± 113	676 ± 95[e]	1416 ± 158
	Phenolic 100X	1282 ± 117	829 ± 119[e]	1616 ± 152
	Phenolic 200X	1533 ± 157	982 ± 114[e]	1818 ± 191
	Volatile 50X	1058 ± 145	976 ± 151[e]	1987 ± 212
	Volatile 100X	1111 ± 150	960 ± 114[e]	1884 ± 243
	Volatile 200X	1109 ± 177	1029 ± 125	1582 ± 164
	Control	1024 ± 56	1196 ± 68	2063 ± 231

Caco-2 cells were treated with the carrot carotenoid, phenolic, or volatile extracts at 3 dilutions each of 50X, 100X, and 200X. n = 15 for all treatments at all time points, n = 36 for control conditions at all time points. No significance was detected following the primary Friedman's test to assess for a significant treatment effect over time. [a,b,c]Denotes a significant difference between time points following the secondary Friedman's test, indicating a non-random dependency of time. [d]Denotes detection of significance following the Kruskal-Wallis test. [e]Denotes a significantly lower cell count from the control at the 6 hour time point following the Mann-Whitney post hoc test. A graphical representation of this data can be found in Figures 6.2 and 6.3.

Table 6.3 Mean proliferation percentages [± standard error of the mean (SEM)] of Caco-2 cells after treatment of the carrot carotenoid, phenolic, and volatile extracts at 6 and 12 hours following treatment.

		Time Point	
		6 hours	**12 hours**
Treatment	Carotenoid 50X	61 ± 7	124 ± 9
	Carotenoid 100X	65 ± 6	141 ± 12
	Carotenoid 200X	66 ± 8	108 ± 12
	Phenolic 50X	63 ± 9	133 ± 15
	Phenolic 100X	65 ± 9	126 ± 12
	Phenolic 200X	64 ± 7	119 ± 12
	Volatile 50X	92 ± 14	188 ± 20
	Volatile 100X	86 ± 10	170 ± 22
	Volatile 200X	93 ± 11	143 ± 15
	Control	117 ± 7	201 ± 23

Caco-2 cells were treated with the carrot carotenoid, phenolic, and volatile extracts at 3 dilutions each of 50X, 100X, and 200X. n = 15 for all treatments at all time points, n = 36 for control conditions at all time points. A graphical representation of this data can be found in Figure 6.4.

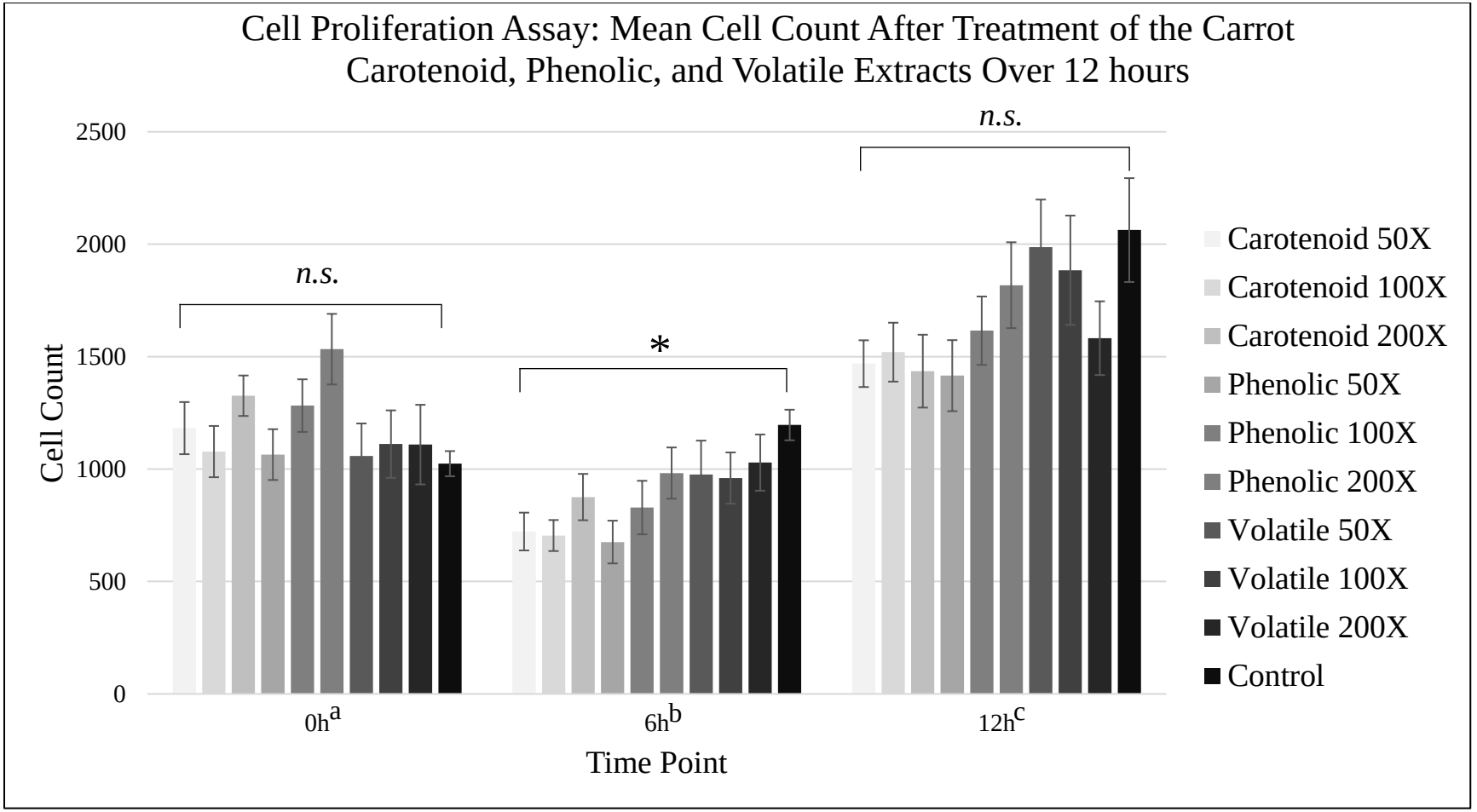

Figure 6.2 Mean cell counts [± standard error of the mean (SEM)] of Caco-2 cells after treatment of the carrot carotenoid, phenolic, and volatile extracts at dilutions of 50X, 100X, and 200X at 0 hours, 6 hours, and 12 hours. n = 15 for all treatments at all time points, n = 36 for control conditions at all time points. No significance was detected following the primary Friedman's test to assess for a significant treatment effect over time. [a,b,c]Denotes a significant difference between time points following the secondary Friedman's test, indicating a non-random dependency of time. [*]Kruskal-Wallis tests at each time point revealed significance only at the 6 hour time point, (*n.s.* = no significance). Post hoc analysis at the 6 hour time point can be found in Figure 6.3. Data corresponding to graph can be found in Table 6.2.

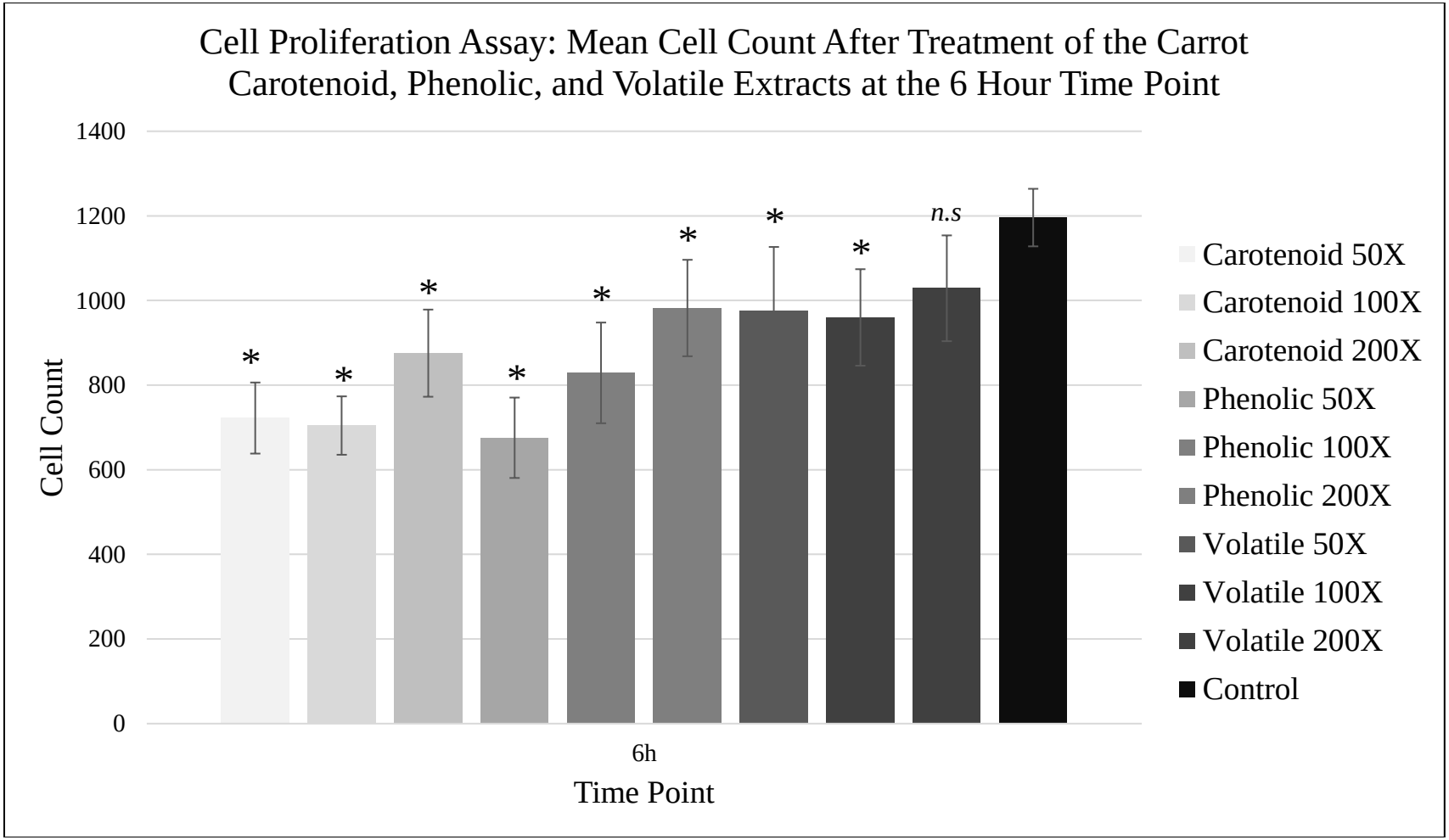

Figure 6.3 Mean cell counts [± standard error of the mean (SEM)] of Caco-2 cells after treatment of the carrot carotenoid, phenolic, and volatile extracts at dilutions of 50X, 100X, and 200X at the 6 hour time point. n = 15 for all treatments, n = 36 for control condition. *Denotes a significantly lower cell count from the control following the Mann-Whitney test. *n.s.* not significantly different from control condition following the Mann-Whitney test. Data corresponding to graph can be found in Table 6.2.

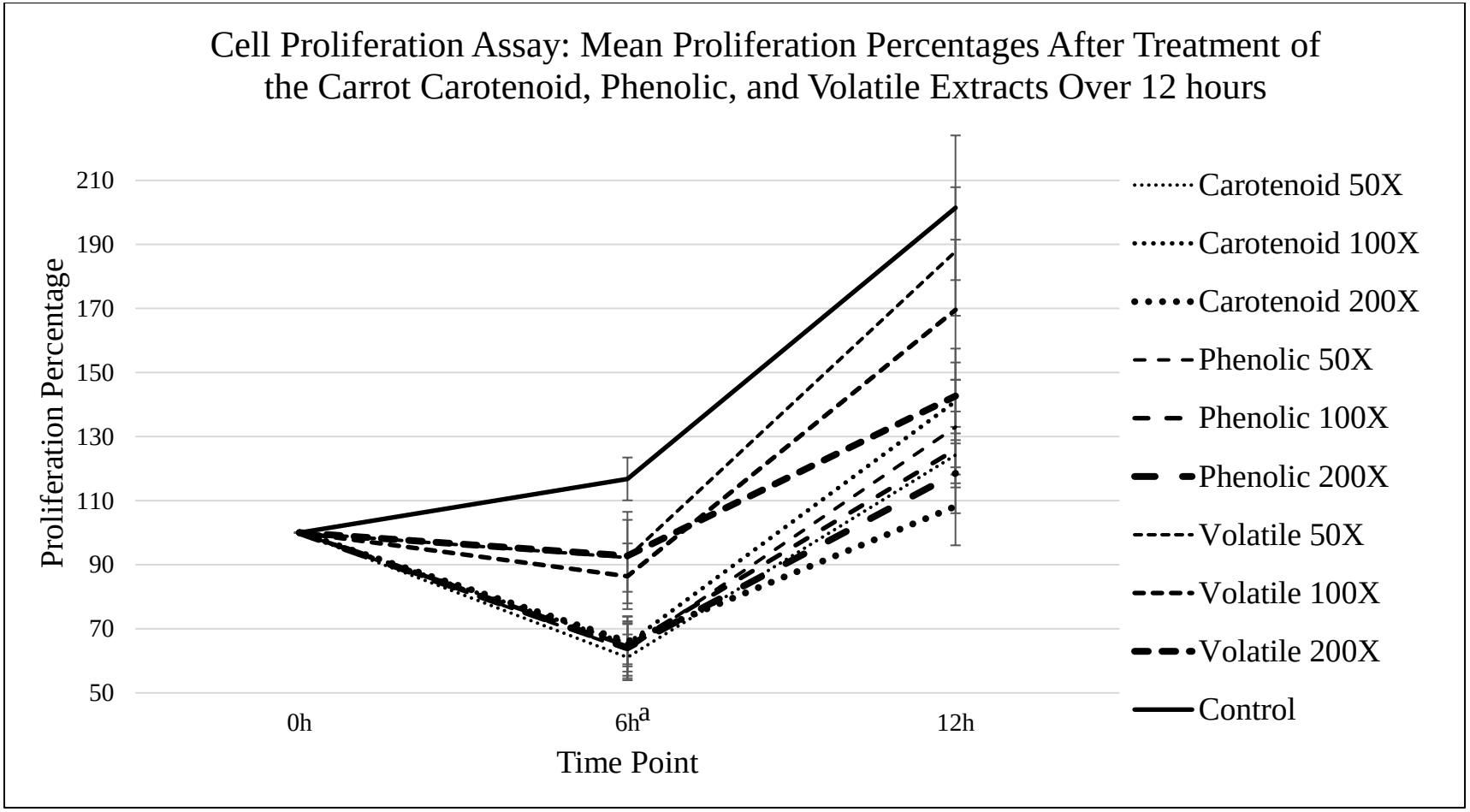

Figure 6.4 Mean proliferation percentages [± standard error of the mean (SEM)] of Caco-2 cells after treatment of the carrot carotenoid, phenolic, and volatile extracts at dilutions of 50X, 100X, and 200X. Data represented in graph can be found in Table 6.3. [a]Denotes significance following the Kruskal-Wallis test for significantly different proliferation percentages (post hoc analysis can be found in Table 6.4).

Table 6.4 Post hoc analysis of proliferation percentages of Caco-2 cells at the 6 hour time point after treatment of the carrot carotenoid, phenolic, and volatile extracts at dilutions of 50X, 100X, and 200X.

Comparison	Unadjusted p-value	Adjusted p-value
Carotenoid 100X - Carotenoid 200X	0.771	1.000
Carotenoid 100X - Carotenoid 50X	0.560	1.000
Carotenoid 200X - Carotenoid 50X	0.771	1.000
Carotenoid 100X - Control	**<0.001**	**0.012**
Carotenoid 200X - Control	**<0.0001**	**<0.01**
Carotenoid 50X - Control	**<0.0001**	**<0.001**
Carotenoid 100X - Phenolic 100X	0.570	1.000
Carotenoid 200X - Phenolic 100X	0.782	1.000
Carotenoid 50X - Phenolic 100X	0.988	1.000
Control - Phenolic 100X	**<0.0001**	**<0.001**
Carotenoid 100X - Phenolic 200X	0.745	1.000
Carotenoid 200X - Phenolic 200X	0.974	1.000
Carotenoid 50X - Phenolic 200X	0.796	1.000
Control - Phenolic 200X	**<0.0001**	**<0.01**
Phenolic 100X - Phenolic 200X	0.808	1.000
Carotenoid 100X - Phenolic 50X	0.423	1.000
Carotenoid 200X - Phenolic 50X	0.611	1.000
Carotenoid 50X - Phenolic 50X	0.828	1.000
Control - Phenolic 50X	**<0.0001**	**<0.001**
Phenolic 100X - Phenolic 50X	0.816	1.000
Phenolic 200X - Phenolic 50X	0.634	1.000
Carotenoid 100X - Volatile 100X	0.253	1.000
Carotenoid 200X - Volatile 100X	0.151	1.000
Carotenoid 50X - Volatile 100X	0.084	1.000
Control - Volatile 100X	0.022	1.000
Phenolic 100X - Volatile 100X	0.087	1.000
Phenolic 200X - Volatile 100X	0.142	1.000
Phenolic 50X - Volatile 100X	0.052	1.000
Carotenoid 100X - Volatile 200X	0.127	1.000
Carotenoid 200X - Volatile 200X	0.069	1.000
Carotenoid 50X - Volatile 200X	0.035	1.000
Control - Volatile 200X	0.067	1.000
Phenolic 100X - Volatile 200X	0.036	1.000
Phenolic 200X - Volatile 200X	0.064	1.000
Phenolic 50X - Volatile 200X	0.020	0.897
Volatile 100X - Volatile 200X	0.701	1.000
Carotenoid 100X - Volatile 50X	0.265	1.000
Carotenoid 200X - Volatile 50X	0.160	1.000
Carotenoid 50X - Volatile 50X	0.090	1.000
Control - Volatile 50X	0.020	0.913
Phenolic 100X - Volatile 50X	0.093	1.000
Phenolic 200X - Volatile 50X	0.150	1.000
Phenolic 50X - Volatile 50X	0.056	1.000
Volatile 100X - Volatile 50X	0.976	1.000
Volatile 200X - Volatile 50X	0.679	1.000

Boldface comparisons denote significantly different proliferation percentages for the two respective treatments. Post hoc analysis was conducted using the Dunn's Test with the Bonferroni adjustment for multiple comparisons. An adjusted p-value of <0.05 was considered significant.

Following the primary Friedman's test to potentially identify an overall significant treatment effect over time, no significance was detected (p-value = 0.2757). Because of this non-significance, a secondary Friedman's test was conducted to assess if this was due to a non-random dependency of time. Following the secondary Friedman's test, significance was detected indicating a significant time effect was present (p-value = 0.0001117). Post hoc analysis also revealed detection of significance for all three pairs of time points using equation 1 detailed in Section 6.5.2 (p-value < 0.05). Since there was a lack of significance following the primary Friedman's test, the Kruskal-Wallis test was conducted at each individual time point to assess for significantly effect antiproliferative treatments. Significance using the Kruskal-Wallis test at each time point was only detected at the 6 hour time point, (p-value = 0.1704, p-value = 0.0003803, p-value = 0.75 for the 0 hour, 6 hour, and 12 hour time points respectively) (Figure 6.2). Post hoc analysis was conducted at the 6 hour time point to reveal which treatments had a significant antiproliferative effect compared to the control condition using the Mann-Whitney test for every possible pair of treatment with control. At the 6 hour time point, post hoc analysis revealed that the all experimental treatments resulted in a significantly lower mean cell count than the control condition, with the exception of the carrot volatile extract diluted 200X (p-value = 0.0001127, p-value = 0.00006019, p-value = 0.003964, p-value = 0.0000541, p-value = 0.002583, p-value = 0.03758, p-value = 0.03489, p-value = 0.03983 respectively) (Figure 6.3). For the proliferation percentages, significance was only detected at the 6 hour time point (p-value = 0.0000009233) but the 12 hour time point was borderline significant following the Kruskal-Wallis test (p-value = 0.06439) (Figure 6.4). Post hoc analysis at the 6 hour time point for proliferation percentages can be found in Table 6.4.

Unfortunately, these results did not follow the *a priori* hypothesis of this study; that the carrot terpenoids would have a significantly greater antiproliferative effect than the carrot carotenoids and phenolics on Caco-2 cells *in vitro*. However, there were many characteristics of the carotenoid, phenolic, and volatile extracts that could have swayed results away from the hypothesized results. Firstly, the antiproliferative effect of the volatile extract could have been stronger if the total volatile concentration was higher. The volatile extract used in this cell proliferation assay had a concentration of 2.2 µg / mL total volatiles, which is much lower than what has been previously found in carrots (Alasalvar et al. 1999; Alasalvar et al. 2001; Guler et al. 2015). Additionally, the strong antiproliferative effect of the phenolics are surprising as the total phenolic content was relatively low but within the range of other published concentrations in carrots (Zhang and Hamauzu 2004; Goncalves et al. 2010). However, the specific composition of the phenolic extract may explain these surprising findings. In the phenolic extract, chlorogenic acid was identified in majority, along with caffeic acid derivatives and trace amounts of *trans*-p-coumaric acid. Previously, a mix of chlorogenic acid and caffeic acid have been shown to have excellent antiproliferative effects on Caco-2 cells *in vitro* (Ektaban et al. 2018). This mixture of chlorogenic acid and caffeic acid is representative of the metabolic mechanisms *in vivo*; chlorogenic acid is mainly de-esterified, (approximately 70%), in the colon forming caffeic acid metabolites. Therefore, a mixture of caffeic acid and chlorogenic acid (remaining 30%), is left in the colon to exert therapeutic effects (Ektaban et al. 2018). Lastly, the strong antiproliferative effect of the carotenoid extract may be explained by the atypically high concentrations of total carotenoids as well as the abnormally high concentration of α-carotene; almost as much in concentration as β-carotene, when β-carotene usually makes up as much as 80% of the total carotenoids in carrots (Tanaka et al. 2012). This high concentration of α-carotene may have

introduced a stronger antiproliferative effect than what would be typical from carrot carotenoids, as α-carotene has been found to be a stronger anticancerous protective agent than β-carotene *in vitro* (Tanaka et al. 2012). This possibility is well supported by the high prevalence of studies that show strong antiproliferative effects of a mixture of carotenoids rather than β-carotene alone which has been associated with lack of a therapeutic chemopreventative effect or even adverse effects *in vivo* and *in vitro* (The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn et al. 1996; Hennekens et al. 1996; Dulinska et al. 2005).

One curious finding from this cell proliferation is the apparent inverse dose response effect for the volatile extract. Especially at the 12 hour time point, it is evident that lower doses of the volatile extract induced greater antiproliferative effects than the higher doses of the volatile extract. This phenomenon has been observed in our laboratory before with volatile extracts from other fruits and vegetables unexplainably. Although no other literature to our knowledge has discussed similar findings regarding volatile extracts from fruit, vegetables, or other plants, this inverse dose response relationship has previously been observed with substantial frequency with endocrine disrupters (Lagarde et al. 2015). It may seem logical to deem such an unusual dose response effect as implausible. However, one theory that has been proposed to explain the inverse dose response frequently observed for endocrine disrupters could potentially also help to explain the observed inverse dose response in the present study. It has been suggested that at low doses, an endocrine disrupter stimulates a metabolic pathway producing metabolites that contribute to observed effects. But at higher doses, an endocrine disrupter may completely saturate this metabolic pathway resulting in an opposite effect, i.e. the upper limit of metabolites produced from the pathway has been reached resulting in a lower observed effect than anticipated (Lagarde et al. 2015). Borrowing from this theory, it is possible

that in the present study therapeutic antiproliferative effects of the volatile extract are from metabolites of the Caco-2 cells, and not from the parent treatment. Furthermore, it is possible that at higher concentrations, the metabolic pathway that produces these metabolites is saturated, resulting in lower production of therapeutic metabolites.

The potential mechanisms of actions for the treatments are unclear, as only a proliferative or antiproliferative effect from the MTS Assay can be confirmed, i.e. whether cells died or did not die. However, a similar study using the volatile extract of *Plectranthus hadiensis*, (a perennial shrub), containing terpenoids treated on HCT-15 colon cancer cells for assessment of potential chemopreventative effects and evaluation of associated mechanisms found the extract to induce apoptosis via DNA fragmentation, upregulation of caspase-3 and proapoptotic Bax, and downregulation of antiapoptotic Bcl-2 and COX-2 (Menon and Gopalakrishnan 2015). Similar findings were also found elsewhere; after treatment of a *Boswellia sacra,* (a tree from which frankincense is harvested), volatile extract on several human pancreatic cell lines, apoptosis was also found to be induced via DNA fragmentation and upregulation of caspase-3 (Ni et al. 2012). Similar mechanisms to this may have occurred in the present study following treatment of the carrot volatile extract, although discrepancies may be present between studies due to differences in composition of volatile extracts and cell lines. Phenolic compounds have been known to have an antiproliferative effect against colorectal carcinoma cells mostly due to their antioxidant capacity, which may explain their strong antiproliferative effect in the present study (Ekinci et al. 2016). The mechanisms by which carotenoids suppress carcinogenesis in humans have been widely discussed. Some of these mechanisms include anti-inflammation effects, immune modulation, induction of cell differentiation, antioxidative effects, and more (Tanaka et al. 2012). However, the protective mechanisms of carotenoids for *in vitro*

experiments such as the present study design are less clear. Carotenoids have often been observed to induce apoptosis on many carcinomic cell lines *in vitro*, but the mechanisms behind this warrant clarification (Tanaka et al. 2012). Molecular biological assays would have also been beneficial to conduct alongside the MTS Assay, as more information behind the mechanisms of actions of the carotenoids, phenolics, and terpenoids would have been elucidated.

There were limitations to this cell proliferation assay that are noteworthy to consider. Firstly, the doses of the carrot carotenoid, phenolic, and volatile extracts were very low due to the use of high dilution factors before being treated to the Caco-2 cells. The utilization of the 50X, 100X, and 200X dilution factors were necessary in the assay because of inherent solubility issues of the carrot carotenoids in the carrier vehicle of DMSO as described in Section 6.3.3. Following method developments for this assay, the dilution factor of 50X was the most concentrated dilution factor that still allowed proper solubility of the carotenoids in cell culture media, ensuring proper delivery of test compounds to the Caco-2 cells. Conversely, even after being highly diluted before application to the Caco-2 cells some of the carotenoid, phenolic, and volatile extract treatments were observed to have a significant antiproliferative effect on the cells compared to the control; in particular, at the 6 hour time point. When considering the fact that people consume fresh carrots 'as-is', (i.e. carrots without dilution as required in this assay), it is entirely possible that an even stronger antiproliferative effect could be observed *in vivo*.

A second limitation to this cell proliferation assay is a possible discrepancy between *in vitro* and *in vivo* effects, particularly what would be observed in the colon *in vivo* if an equivalent study was to be designed using humans. Data is currently lacking about the ability of terpenoids to reach the colon following ingestion, absorption, distribution, and metabolism from a food matrix such as carrots. One review noted the major route of excretion of terpenoids to be via the

kidneys in urine and lungs as exhaled air, with only minor amounts reaching the colon and trace amounts of unmetabolized terpenoids being excreted altogether (Kohlert et al. 2000). Future work involving a cell proliferation assay of this type on a lung carcinoma cell line such as the A549 adenocarcinomic human alveolar basal epithelial cell line may be beneficial, as a major route of absorption for terpenoids has been reported to be via the lungs during inhalation (Kohlert et al. 2000). However, pulmonary absorption of terpenoids has mostly been reported via inhalation of essential oils, and data regarding pulmonary absorption of terpenoids via nasal or oral inhalation during mastication is lacking. One study on retronasal transportation of terpenoids showed many compounds to be present in exhaled air with good retention of original dosage concentrations following oral consumption, but caution should be met when considering these results due to the delivery method of the terpenoids. The terpenoids were delivered in an aqueous solution rather than a food matrix, presenting discrepancies due to lack of bolus interactions with salivary components and reduction of air volume in the mouth when a bolus is present (Linforth et al. 2002).

Another limitation of this assay is the use of only one type of method to assess cell proliferation (the MTS Assay). The principle of the MTS Assay is the reagent containing tetrazolium salt is bioreduced to purple formazan salt by mitochondrial succinate dehydrogenase in viable cells, and cell proliferation can subsequently be indirectly assessed via color change. This principle is vulnerable to the fact that some phytochemicals have been known to change the activity of mitochondrial succinate dehydrogenase or even react with the tetrazolium salt directly, leading to an overestimation of cell counts (Wang et al. 2010). Injured cells sometimes increase activity of mitochondrial succinate dehydrogenase in an attempt to repair which would also lead to an overestimation of viable cells when they may soon die after obtaining

measurements (Wang et al. 2010). It would have been beneficial to use other assays that also measure biomarkers of cell proliferation such as the lactate dehydrogenase assay, or measurement of caspase-3 levels using western blotting techniques. In addition to this, it also would have been beneficial to conduct the same MTS Assay on healthy colon cells to ensure treatments would not cause them harm.

Accompanying the limitations of this cell proliferation assay are substantial strengths worth discussing. Firstly, the use of several varying concentrations of the carotenoid, phenolic, and volatile extracts to establish a dose-response was beneficial. Dose response curves are important to establish in cell proliferation assays as they help to clarify the minimum, (or maximum), threshold concentrations of experimental treatments that exhibit desirable effects. Secondly, the use of physiological relevant concentrations of experimental treatments was a strength of this assay. The use of appropriate dosages of experimental treatments in cell proliferation assays are extremely important. If a dose of an experimental treatment is too high and significant antiproliferative effects are observed, it can be difficult to consume equivalent amounts of the parent food containing the experimental compounds to exhibit the same effects *in vivo*. In addition to the strength of using physiologically relevant dosages of the carotenoid, phenolic, and volatile extracts in this cell proliferation assay, the use of 'as is' concentrations of these found originally in carrots and not equivalent concentrations of the indicated extracts were also considered a strength as they represent the real experience of eating a carrot and subsequent effects *in vivo* after consuming carrots. Lastly, the use of shorter time points and lower cell densities to prevent contact inhibition was also a strength of this study. Contact inhibition is a phenomenon where cells grow to the point of contacting each other, leaving no room for growth and subsequently slowing growth rates possibly leading to biased results. The use of shorter time

points also prevents depletion of vital nutrients in the cell culture media which would also prevent potentially biased results.

Chapter 7: Effect of γ-terpinene, Terpinolene, and α-phellandrene on the Proliferation of Caco-2 cells *in vitro*.

7.1 Introduction

A secondary objective of the present study was to conduct a cell proliferation assay on Caco-2 cells using the three most predominant terpenoid compounds present in the carrot volatile extract, (described in Chapter 5), which were γ-terpinene, terpinolene, and α-phellandrene. The purpose of this objective was to assess the antiproliferative potential of these terpenoids individually rather than as a whole volatile extract, with the aim of elucidating which individual compounds are more responsible for *in vitro* biological activity. Doses of 905 ppb, 235 ppb, and 151 ppb for γ-terpinene, terpinolene, and α-phellandrene respectively were selected for use in this assay as those were the concentrations found in the original volatile extract. Although these dosages are not equivalent, they were selected as they are representative of their concentration in actual carrots and subsequently more representative of a human eating carrot.

7.2 Materials

The standard terpenoid compound of γ-terpinene was purchased from Sigma Aldrich (St. Louis, MO) and the standard terpenoid compounds of terpinolene and α-phellandrene were purchased from Penta International Corporation (Livingston, NJ). The CellTiter 96® AQueousOne Solution Cell Proliferation Assay kit was purchased from Promega Corporation (Madison, WI).

7.3 Methods

7.3.1 Cell Culture

Caco-2 human colon cancer cells were purchased from the American Type Culture Collection (Rockville, MD) at passage number 18 and maintained in 75 cm^2 cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids, 1% antibiotic-antimyotic, and incubated at 37°C in a humidified 5% CO_2 atmosphere. All cell proliferation assays with Caco-2 cells were conducted on passage numbers 25-45. All media components and reagents were obtained from Gibco® through Life Technologies (Carlsbad, CA).

7.3.2 CellTiter 96® Aqueous One Solution Cell Proliferation Assay

Assessment of the antiproliferative potential of γ-terpinene, terpinolene, and α-phellandrene was conducted using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay, also colloquially known as the MTS Assay (Promega Corp. Madison, WI). Caco-2 cells at 80-100% confluency in 75 cm^2 cell culture flasks were trypsinized in a trypsin/EDTA solution. The trypsinized Caco-2 cells at a cell density of 1 x 10^3 in 100 µL of working media were seeded in the wells of a 96-well plate and incubated at 37°C and 5% CO_2. After stable attachment (~24 hrs), 100 µL of the experimental medias containing γ-terpinene, terpinolene, and α-phellandrene at concentrations of 905 ppb, 235 ppb, and 151 ppb respectively with cell culture media were added to the wells with the exception of wells serving as control conditions, where 100 µL of cell culture media containing no experimental compounds was added. Tween 80 at a concentration of 0.02% was added to ensure proper solubilization of the test compounds and delivery to the Caco-2 cells, (see Section 6.3.1). Cell count measurements were made at 0, 6, and 12 hours by adding 40 µL of the Cell Titer reagent directly to every well and incubated for two

hours before measuring the absorbance at 490 nm. After all absorbance readings were made, corrections were made for cell-free background absorbances. Absorbances were then converted into cell counts using an equation from a Caco-2 cell standard curve. Results are reported as the average cell counts ± the standard error of the mean (SEM) for each time point and treatment, and as proliferation percentages relative to the time point of 0 hours ± SEM for each time point and treatment.

7.4 Statistical Analysis

7.4.1 Initial Exploratory Data Analysis

Similarly to the cell proliferation assay described in Chapter 6, the results of this cell proliferation assay are also commonly analyzed statistically using repeated measures ANOVA due to the presence of more than two experimental treatments and the fact that the response variable (cell count) was measured repeatedly over time. Assumptions for repeated measures ANOVA are described in Section 6.5.1. To assess for the assumption of normally distributed residuals, a linear regression model was created and data points were plotted against the linear output of the model. The residuals, (the distance of the data points from the linear output), were calculated and plotted in a Q-Q plot, shown in Figure 7.1.

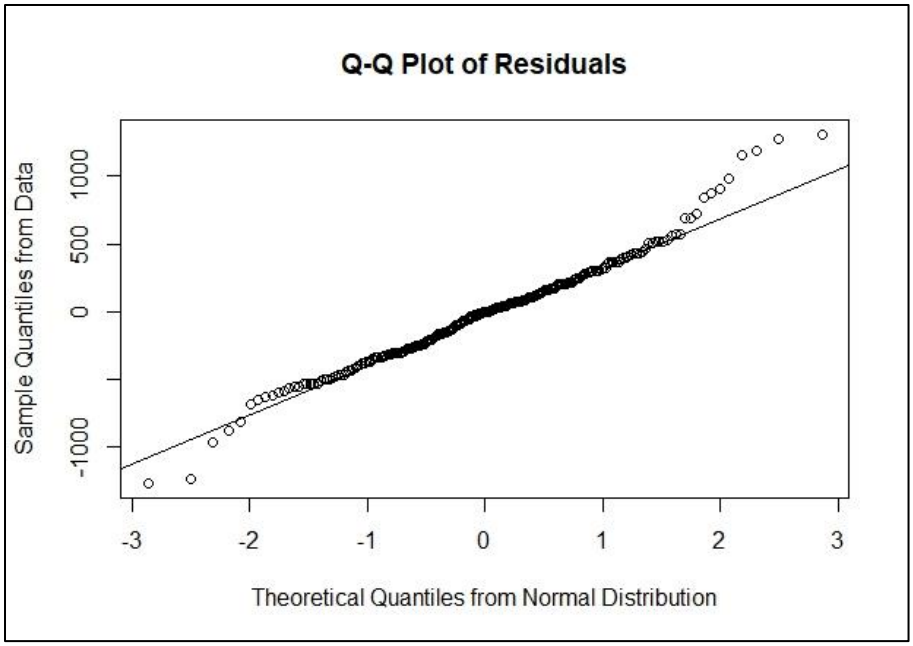

Figure 7.1 Q-Q Plot of residuals from the linear regression model created using the data from the cell proliferation assay described in Section 7.3.2. The line on the plot depicts a theoretical data set reflecting perfectly normally distributed data. The residuals of the data are not normally distributed, as they do not follow the same path as the line in the Q-Q plot.

It is evident from observing the Q-Q plot in Figure 7.1 that the residuals were not normally distributed, and this was confirmed with a Shapiro-Wilk test for normality (p-value < 0.001). This lack of normality led to a violation of the requirement of normally distributed residuals when using the repeated measures ANOVA test.

7.4.2 Non-Parametric Statistical Analysis

Due to the violation of the requirement of normally distributed residuals, non-parametric statistical analyses were instead used to analyze the results of this cell proliferation assay. Specifically, the non-parametric equivalent of the repeated measures ANOVA test was used which is the Friedman's test. Assumptions and details regarding this test are described in Section 6.5.2.

First, a primary Friedman's test, (using mean cell counts for ranking), was conducted to assess for a significant treatment effect over time, blocking for the variable of time since it was

hypothesized that cell counts might be dependent on the variable of time. The following null and alternative hypotheses were used for this primary Friedman's test:

H_0: Each ranking of the mean cell counts for each experimental treatment within the data blocked for the 0, 6, and 12 hour time points are equally likely.

H_1: At least one of the experimental treatments tends to yield larger ranks of the mean cell counts than at least one other experimental treatment.

If significance was detected, post hoc analysis according to the method described by Conover was completed using equation 1, where R_j and R_i represent the sum of ranks for each treatment type, b represents the number of time points, k represents the number of treatment types, and $t_{1-\alpha/2}$ is the $1-\alpha/2$ quantile of the t distribution and where A_1 is calculated using equation 2 (1999). Treatment types i and j were considered significantly different from each other if the inequality in equation 1 was satisfied.

If significance was not found following the primary Friedman's test then a secondary Friedman's test was conducted to assess if non-significance in the primary test was due to a non-random dependency of time. This secondary Friedman's test also used mean cell counts for ranking. The following null and alternative hypotheses were used for the secondary Friedman's test:

H_0: Each ranking of the mean cell counts for each time point within the data blocked for the experimental treatments are equally likely.

H_1: At least one of the time points tends to yield larger ranks of the mean cell counts than at least one other time point.

If significance was detected in the secondary Friedman's test, then post hoc analysis was conducted using equations 1 and 2, (but inversing the values of b and k, i.e. blocking for treatment types instead of blocking for time points).

Further analysis was completed on each time point separately, (i.e. ignoring the variable of time), using the Kruskal-Wallis test to obtain more information about significantly effective antiproliferative treatments if the primary Friedman's test resulted in a lack of significance. The following null and alternative hypotheses were used for each Kruskal-Wallis test at each time point:

H_0: All of the population distribution functions for the experimental treatments are identical.

H_1: At least one of the populations tends to yield larger observations than at least one of the other populations.

If significance was found at any time point following the Kruskal-Wallis test, then post hoc analysis was completed using the Mann-Whitney test for every possible pair of treatment and control to assess which treatments were significantly lower than the control, (as an indication of a significantly effective antiproliferative effect). The following null and alternative hypotheses were used for each Mann-Whitney test:

H_0: The population distribution functions from the experimental treatment group and the control group are identical.

H_1: The probability of an observation from the population of the experimental treatment group exceeding an observation from the population of the control group is lower than

the probability of an observation from the population of the experimental treatment group being lower than an observation from the population of the control group.

For proliferation percentages, statistical analysis was conducted at the 6 and 12 hour time points using the Kruskal-Wallis test and Dunn's test for post hoc analysis. The Bonferroni correction was also utilized for multiple comparisons. The null and alternative hypotheses for the Kruskal-Wallis test is mentioned above, and the null and alternative hypotheses for the Dunn's test is similar to those for the Mann-Whitney test, also mentioned above. The Kruskal-Wallis, Mann-Whitney, and Dunn's tests are appropriate for use on samples of unequal sizes, but it is worth noting that because of this caveat the power of those tests will potentially be reduced. A significance level of $\alpha = 0.05$ was used for all statistical analyses.

7.5 Results and Discussion

Table 7.1 Mean cell counts [± standard error of the mean (SEM)] of Caco-2 cells after treatment of γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb at 0, 6, and 12 hours following treatment.

		Time Point		
		0 hours[a]	**6 hours**[a]	**12 hours**[a]
Treat-ment	γ-terpinene 905 ppb	1158 ± 76	792 ± 64[b]	1125 ± 100[c]
	Terpinolene 235 ppb	1208 ± 75	900 ± 72[b]	1100 ± 91[c]
	α-phellandrene 151 ppb	725 ± 99	792 ± 79[b]	1175 ± 89[c]
	Control	924 ± 95	1660 ± 113	1993 ± 87

Caco-2 cells were treated with γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb. n = 15 for all treatments at every time point, n = 36 for control conditions at every time point. No significance was detected following the primary Friedman's test to assess for a significant treatment effect over time, or after the secondary Friedman's test to assess if non-significance in the primary test was due to a non-random dependency of time. [a]Denotes detection of significance following the Kruskal-Wallis test. [b]Denotes a significantly lower mean cell count than the control condition at the 6 hour time point following the Mann-Whitney test. [c]Denotes a significantly lower mean cell count than the control condition at the 12 hour time point following the Mann-Whitney test. A graphical representation of this data can be found in Figure 7.2.

Table 7.2 Mean proliferation percentages [± standard error of the mean (SEM)] of Caco-2 cells after treatment of γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb at 6 and 12 hours following treatment.

		Time Point	
		6 hours[a]	**12 hours**[a]
Treat-ment	γ-terpinene 905 ppb	68 ± 6	97 ± 9
	Terpinolene 235 ppb	74 ± 6	91 ± 8
	α-phellandrene 151 ppb	109 ± 11	162 ± 12
	Control	180 ± 12	216 ± 9

Caco-2 cells were treated with γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb. n = 15 for all treatments at each time point, n = 36 for control conditions at each time point. A graphical representation of this data can be found in Figure 7.3.

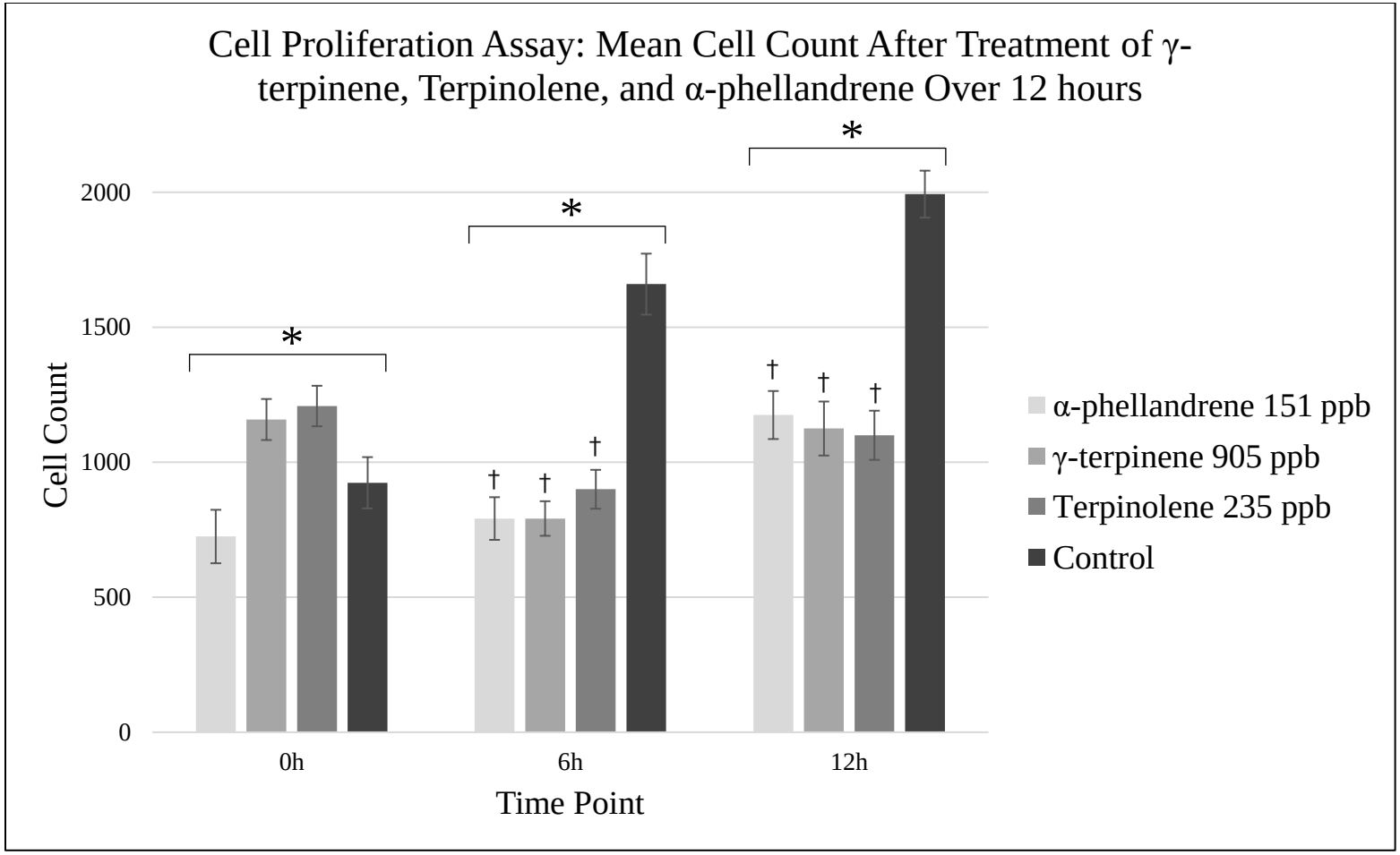

Figure 7.2 Mean cell counts [± standard error of the mean (SEM)] of Caco-2 cells after treatment γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb at 0 hours, 6 hours, and 12 hours following treatment. n = 15 for all treatments at all time points, n = 36 for control conditions at all time points. No significance was detected following the primary Friedman's test to assess for a significant treatment effect over time, and no significance was detected following the secondary Friedman's test to assess if non-significance in the primary test was due to a non-random dependency of time. *Kruskal-Wallis tests at each time point revealed significance at all time points. †Denotes a significantly lower mean cell count than control condition at respective time point. Data in graph can be found in Table 7.1.

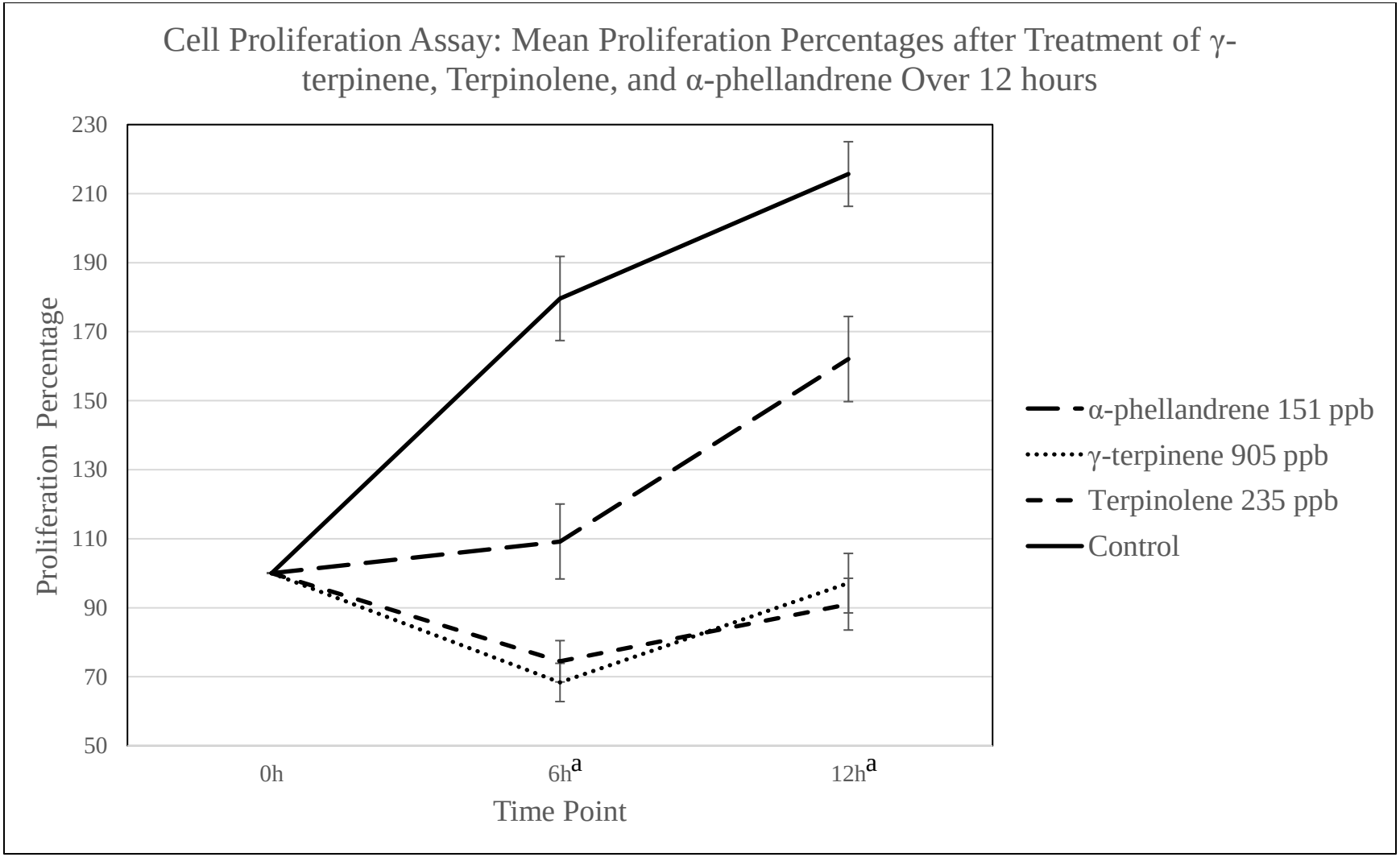

Figure 7.3 Mean proliferation percentages [± standard error of the mean (SEM)] of Caco-2 cells after treatment of γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb. Data represented in graph can be found in Table 7.2. [a]Denotes significance following the Kruskal-Wallis test for significantly different proliferation percentages (post hoc analysis can be found in Tables 7.3 and 7.4 for the 6 and 12 hour time points respectively).

Table 7.3 Post hoc analysis of proliferation percentages of Caco-2 cells at the 6 hour time point after treatment of γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb.

Comparison	Unadjusted p-value	Adjusted p-value
α-phellandrene - Control	**0.003**	**0.017**
α-phellandrene - γ-terpinene	0.015	0.092
Control - γ-terpinene	**<0.0001**	**<0.0001**
α-phellandrene - Terpinolene	0.033	0.196
Control - Terpinolene	**<0.0001**	**<0.0001**
γ-terpinene - Terpinolene	0.774	1.000

Boldface comparisons denote significantly different proliferation percentages for the two respective treatments. Post hoc analysis was conducted using the Dunn's Test with the Bonferroni adjustment for multiple comparisons. An adjusted p-value of <0.05 was considered significant.

Table 7.4 Post hoc analysis of proliferation percentages of Caco-2 cells at the 12 hour time point after treatment of γ-terpinene at a concentration of 905 ppb, terpinolene at a concentration of 235 ppb, and α-phellandrene at a concentration of 151 ppb.

Comparison	Unadjusted p-value	Adjusted p-value
α-phellandrene - Control	0.009	0.057
α-phellandrene - γ-terpinene	0.023	0.139
Control - γ-terpinene	**<0.0001**	**<0.0001**
α-phellandrene - Terpinolene	**0.004**	**0.023**
Control - Terpinolene	**<0.0001**	**<0.0001**
γ-terpinene - Terpinolene	0.537	1.000

Boldface comparisons denote significantly different proliferation percentages for the two respective treatments. Post hoc analysis was conducted using the Dunn's Test with the Bonferroni adjustment for multiple comparisons. An adjusted p-value of <0.05 was considered significant.

Following the primary Friedman's test to potentially identify an overall significant treatment effect over time, no significance was detected (p-value = 0.4975). Because of this non-significance, a secondary Friedman's test was conducted to assess if this was due to a non-random dependency of time. Following the secondary Friedman's test, significance was not detected (p-value = 0.3679). Since there was a lack of significance following the primary Friedman's test, the Kruskal-Wallis test was conducted at each individual time point to assess for significantly effect antiproliferative treatments. Following the Kruskal-Wallis tests at each time point, significance was detected at every time point (p-value = 0.00581, p-value =0.000000008511, and p-value = 0.0000001681 for the 0 hour, 6 hour, and 12 hour time points respectively) (Figure 7.2). Post hoc analysis using the Mann-Whitney test was conducted at each time point to assess which treatment conditions were significantly lower than the control conditions. At the 0 hour time point, no experimental treatments resulted in a significantly lower mean cell count than the control condition, (p-value = 0.9832, p-value = 0.9618, and p-value = 0.0779 for γ-terpinene, terpinolene, and α-phellandrene respectively) (Figure 7.2). Significance from the Kruskal-Wallis test at this time point was most likely due to some experimental treatment resulting in a significantly higher mean cell count than the control, as indicated by the very high p-values. However, at the 6 hour and 12 hour time points, every experimental treatment resulted in a significantly lower mean cell count than the control condition (p-value = 0.0000007626, p-value = 0.00001321, and p-value = 0.000001902 for γ-terpinene, terpinolene, and α-phellandrene respectively at the 6 hour time point and p-value = 0.0000125, p-value = 0.000005807, and p-value = 0.00001105 for γ-terpinene, terpinolene, and α-phellandrene respectively at the 12 hour time point). For proliferation percentages, significance was detected at both the 6 and 12 hour time points following the Kruskal-Wallis test (p-value =

0.0000000000656 and p-value = 0.0000000000829 respectively) (Figure 7.3). Post hoc analysis for both of these time points can be found in Tables 7.3 and 7.4 respectively.

The results of this cell proliferation assay show promising potential for γ-terpinene, terpinolene, and α-phellandrene as antiproliferative agents against Caco-2 cells. The hypothesis of this cell proliferation assay was all three of the indicated compounds would have a significant antiproliferative effect, (significantly lower mean cell counts than control conditions), on Caco-2 cells as they were the most predominant individual terpenoids in the carrot volatile extract. While none of the treatments provided a significant treatment effect over time, at the 6 and 12 hour time points all treatments resulted in significantly lower mean cell counts implicating them as effective antiproliferative treatments after 6 hours of exposure.

Terpinolene exhibited excellent antiproliferative effects in this cell proliferation assay. Although this effect was not significantly different throughout the entirety of the assay compared to control conditions, at the 6 and 12 hour time points mean cell counts after being treated with terpinolene were significantly different from control conditions resulting in 54% and 55% of mean cell counts from control conditions respectively. Currently there is no literature that reports effects of terpinolene on Caco-2 cells for comparison, but previously the antiproliferative effect of terpinolene treated on K562 human leukemia cells was investigated with positive results. It was found in this study that after treatment of 0.01% terpinolene, cell growth was significantly reduced (Okumura et al. 2011). Additionally, it was found that after treatment of 0.05% terpinolene, expression of the protein kinase AKT1 was reduced by over 95% in K562 cells (Okumura et al. 2011). AKT is a central protein in many cellular pathways such as cell survival, proliferation, glucose uptake, metabolism, angiogenesis, as well as radiation and drug response, with the AKT1 isoform hypothesized to be most essential for cell survival (Sahlberg et al. 2016).

Previously, elevated expression and levels of AKT have been implicated with development of many types of cancer, including colon cancer (Sahlberg et al. 2016). In an *in vitro* colon cancer model, AKT knockout DLD-1 colon cancer cells were observed to have lower cell migration rates than parental cells (Sahlberg et al. 2016). These results have an implication in the metastatic stage of colon cancer since cancer cells become more mobile with increased motility rates. In the present study it is possible that levels of AKT were lowered and downregulated after treatment of terpinolene, but discrepancies between the two different cell types warrant discretion when comparing these findings.

The experimental treatment of α-phellandrene also provided a substantial antiproliferative effect on Caco-2 cells in this assay. To date there is no other published literature investigating the antiproliferative effect of α-phellandrene alone on Caco-2 cells, but a recent investigation of the cytotoxic activity of the essential oils of *Schinus mole L.* and *Schinus terebinthifolius Raddi*, (two varieties of evergreen trees), on MCF-7 human breast cancer cells provides considerable results for reference. These two essential oils containing 46.52% and 34.38% α-phellandrene as the most predominant terpenoid exhibited IC_{50} values of 54 and 47 ppm respectively on MCF-7 human breast cancer cells (Bendaoud et al. 2010). These findings are confirmed by another recent investigation of the antiproliferative effect of a volatile extract from the leaves of *Solanum erianthum*, (also known as potatotree), containing α-phellandrene as the second-most predominant terpenoid in the extract. This extract was found to kill over 90% of Hs 578T human breast cancer cells and PC-3 human prostate cancer cells when treated with 250 ppm and 100 ppm of the volatile extract, (containing 17.5 ppm and 43.75 ppm of α-phellandrene respectively) (Essien et al. 2012). These findings may indicate α-phellandrene as an excellent anticancer treatment when treated in isolation rather than as part of a volatile extract or essential oil due to

the fact that in our study cell counts were almost reduced by 50% at a low treatment concentration of 151 ppb by the 12 hour time point. However, it is again worth nothing that discrepancies between findings are likely due to differences in cell lines and extract compositions.

The experimental treatment of γ-terpinene also exhibited exceptional antiproliferative effects in this assay. Previously, this compound as the predominant terpenoid in the essential oil of *Satureja intermedia* and the second-most predominant terpenoid in the essential oil of *Satureja boissieri*, (two varieties of a shrub species related to rosemary and thyme), has been observed to have cytotoxic effects against cell lines such as HeLa cervical cancer cells, Hep-G2 hepatocellular carcinomic cells, and MCF-7 human breast cancer cells (Oke-Altuntas et al. 2015; Sharifi-Rad et al. 2015). A concentration as low as 100 µg / mL of the *Satureja boissieri* essential oil applied to HeLa cells, (containing 22.84 µg / mL of γ-terpinene), exhibited excellent antiproliferative effects while IC_{50} values of ≥ 50 µg / mL for the essential oil of *Satureja intermedia* was observed against MCF-7 and Hep-G2 cells (Oke-Altuntas et al. 2015; Sharifi-Rad et al. 2015). Discrepancies are probable across studies due to differences in cell types and essential oil compositions, in addition to the fact that in the present study γ-terpinene was applied to cells in isolation while elsewhere it was applied as part of an essential oil.

Mechanisms of actions in this cell proliferation assay for α-phellandrene and γ-terpinene are difficult to hypothesize as no other studies were found that investigated this on any type of carcinomic cell line for reference. Future work regarding the mechanisms of action for all three of the indicated compounds in this assay is warranted.

There were limitations of this cell proliferation assay worth discussing. Firstly, it is difficult to compare the experimental treatments of γ-terpinene, terpinolene, and α-phellandrene

with each other as they were treated on the Caco-2 cells at different concentrations. However, this was deemed necessary to be as representative as possible of their inherent concentrations in an average carrot. Even so, terpinolene at a low concentration of 235 ppb was just as effective as γ-terpinene at a higher concentration of 905 ppb (see Figure 7.3). Furthermore, a range of concentrations for each compound would have been desirable to apply to the cells as the effect of doing so would establish a dose-response effect. This dose-response effect is typically used to detect the minimum concentration of experimental treatments necessary to induce effects.

Another limitation to this assay is the difficulty of translating the results of this *in vitro* assessment back to humans; particularly what would be observed in the human colon. The ability of γ-terpinene, terpinolene, and α-phellandrene to reach the colon following ingestion is currently unknown, and future work is warranted to clarify this. A recent review noted that the major route of excretion for terpenoids is via the kidneys in urine and lungs as exhaled air, with only minor amounts reaching the colon and trace amounts of unmetabolized terpenoids being excreted altogether (Kohlert et al. 2000). However, this review did not include findings from primary research on γ-terpinene, terpinolene, and α-phellandrene but rather other terpenoids.

Other limitations of this cell proliferation assay include the inability to confirm mechanisms of action of the experimental treatments, and a lack of other assays conducted alongside the MTS assay to confirm measurements. It has previously been discussed that there are some potential issues to the MTS assay to be wary of, including the possibility that experimental treatments themselves may react with the tetrazolium salt in the reagent leading to an overestimation of cell counts, or an increase in the activity of mitochondrial succinate dehydrogenase of injured cells attempting to repair which would also lead to an overestimation of viable cells when they may soon die after obtaining measurements (Wang et al. 2010).

Even though there were inherent limitations to this cell proliferation assay, there were also complementary strengths. An advantage of this cell proliferation assay compared to the one described in Section 6.4.2 was the ability to apply the experimental compounds directly to the Caco-2 cells at their original concentration in the volatile extract without the need of dilution factors. This was because there were no solubility issues observed with the experimental compounds in the cell culture media. The use of shorter time points and low cell densities was also a strength, as there was an observed reduction of contact inhibition of cells compared to longer time points. Contact inhibition is a phenomenon where cells proliferate to the point where they are contacting each other which markedly reduces the rate at which they grow leading to the possibility of biased results. The use of these shorter time points also ensures no depletion of nutrients in the media over the course of the assay which could also potentially lead to biased results.

Chapter 8: Final Conclusions

There were three objectives for this study: (1) extract, identify, and quantify the carotenoid, phenolic, and volatile compounds from carrots, (2) compare the antiproliferative effects of the carotenoid, phenolic, and volatile extracts from carrots on Caco-2 human colon cancer cells *in vitro*, and (3) compare the antiproliferative effects of α-phellandrene, terpinolene, and γ-terpinene treated on Caco-2 human colon cancer cells *in vitro.*

Regarding the first objective, a total carotenoid content of 37.7 mg β-carotene equivalents (BCE) per 100 g of fresh weight was observed in our carrots. β-carotene, α-carotene, and lutein were identified as individual carotenoids in the carotenoid extract and were present at concentrations of 18.9, 18.8, and 0.03 mg of BCE per 100 g of fresh weight respectively. These

concentrations were higher than reported in most literature, although higher carotenoid levels in carrots have previously been associated with "high beta-carotene" varieties of carrots and exposure to abiotic stressors during development. The ratio of β-carotene to α-carotene was also a unique finding, as α-carotene was present at much higher concentrations than what is typical. Phenolic compounds were quantified using the Folin-Ciocalteu assay and traditional HPLC methods. Total phenolics using the Folin-Ciocalteu assay in our carrots were 25.8 ± 0.4 mg gallic acid equivalents (GAE) per 100 g of fresh weight. Other reports of total phenolics in carrots using this assay seem to range widely in literature and discrepancies seem to be attributed to genetic variety, but total phenolics in our carrots fell within the range of these reports. Quantification of phenolics using typical HPLC methods was also conducted, and concentration of total phenolics was found to be 5.07 mg of chlorogenic acid equivalents (CAE) per 100 g of fresh weight. Identification of phenolic compounds was conducted using LC-MS. Although 12 peaks in the chromatogram were quantified, identification of only four of these were confirmed following LC-MS, (trans-*p*-coumaric acid, 5-*O*-caffeoylquinic acid, and two caffeic acid derivatives). However, this was deemed a success as the identification of these four compounds reflected almost the entirety of the phenolic compounds at approximately 92% of the total peak area in the chromatogram. Volatile compounds in our carrots were lower in concentration than other reports, at a total concentration of 2.2 ppm. Following GC-MS, we were able to identify 38 individual volatile compounds. Some of the more prevalent compounds in the total volatile fraction were γ-terpinene, terpinolene, α-phellandrene, limonene, and *p*-cymene at concentrations of 905 ppb, 235 ppb, 151 ppb, 143 ppb, and 143 ppb respectively which seemed parallel to other reports. Gamma-bisabolene was not detected in our carrots but has previously been detected at very high concentrations in carrots in other reports.

The second objective of this study was to conduct a cell proliferation assay on Caco-2 cells using a carotenoid, phenolic, and volatile extract from carrots. The findings from this assay did not follow the *a priori* hypothesis; that the volatile extract would have significantly greater antiproliferative effects on the Caco-2 cells than both the carotenoid and phenolic extracts. In fact, there was not a significantly different antiproliferative treatment effect over time for any of the extracts at any dose compared to control conditions. At each individual time point however, only a significant antiproliferative effect was observed at the 6 hour time point, with post hoc analysis revealing that all treatments with the exception of the volatiles diluted 200X were significantly lower than the control. These findings may be due in part to the fact that the original concentration of total volatiles in the volatile extract was low compared to other reports. This caveat combined with the need to use high dilution factors to ensure proper solubilization of the carotenoids in the cell culture media resulted in a very low concentration of volatile compounds being treated to the Caco-2 cells. Specifically, doses of only 45, 23, and 12 ng / mL of total volatiles were treated to the cells. However, a slight antiproliferative effect was observed after treatment of the volatile extract. Even though this effect was not statistically significant, it can be presumed that this effect might be stronger with higher doses of the test compounds applied to the cells, and with the use of carrots containing higher concentrations of total volatiles which is typical for normal carrots. Over the course of the 12 hours of the cell proliferation assay, the carotenoid extract at all dilutions had the strongest antiproliferative effect. These findings may be due to the abnormally high concentration of α-carotene present in the carotenoid extract, since α-carotene has been previously observed to have much stronger anticancerous effects *in vitro* and *in vivo*.

Lastly, the third objective of this study was to conduct a cell proliferation assay on Caco-2 cells using the three most predominant individual volatile compounds at their original concentration in the volatile extract. These compounds and their respective concentrations in the volatile extract were γ-terpinene at 905 ppb, terpinolene at 235 ppb, and α-phellandrene at 151 ppb. There was not a significant treatment effect over time for any of these treatments compared to the control, however at the 6 and 12 hour time points each treatment resulted in a significantly lower mean cell count than control conditions indicating them as effective antiproliferative treatments after 6 hours of exposure. Surprisingly, terpinolene exhibited the strongest antiproliferative effect at the low concentration applied to the cells of 235 ppb, and comparable results were seen for γ-terpinene at 905 ppb. Alpha-phellandrene at a concentration of 151 ppb provided the weakest antiproliferative effect, but this is understandable as the concentration of it was extremely low. However, impressive effects of α-phellandrene at even lower concentrations on carcinomic cell lines have been observed elsewhere.

Future work is warranted to elucidate mechanisms of action for experimental treatments, particularly for γ-terpinene, terpinolene, and α-phellandrene as they exhibited impressive effects. Establishing an understanding behind the mechanisms of action for effective cancer therapeutics can aid in the discovery of novel treatments. In addition to this, future work is also necessary on assessing the bioavailability of carrot volatile compounds as it is currently unclear whether they can reach the colon in sufficient quantities to induce the effects observed in the present study. However, terpenoids have been observed to reach the lungs following oral ingestion in sufficient quantities and because of this knowledge these cell proliferation assays will soon be conducted on A549 human lung cancer cells in our laboratory. It is also recommended to perform the same cell proliferation assay on non-carcinomic healthy cell lines of equivalent tissue types as it

essential to ensure the experimental treatments in the present study would cause them no harm. Other recommendations include the execution of this study using other types of cell proliferation assays such as the lactate dehydrogenase assay to confirm cell count measurements as limitations to the MTS assay have recently been identified.

In summary, the results from this study provide valuable insight on alternative avenues of research for novel chemopreventative therapies. Some of these alternative avenues that might soon be observed in research include exploration of terpenoids from carrots as potential adjuvant agents to typical methods of treatment for cancer, or as a form of preventative medicine. These possibilities are exciting to the future of cancer therapy as carrots are commercially available to a wide demographic, low in cost to produce or purchase at the retail level, and currently widely consumed in the United States. These possibilities are extended even further when one considers that these alternative research avenues do not have to be confined only to carrots, but to the vast amount of fruits, vegetables, and plants currently available for future investigation.

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