AN ABSTRACT OF THE DISSERTATION OF

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Purple tomatoes (*Lycopersicon esculemtum*) were bred by incorporating genes that result in anthocyanin production in the skin of fruits. These genes originally came from related wild species as no naturally occurring genes encoding fruit anthocyanin expression are known within the cultivated species. By combining *Aft* or *Abg* with *atv* or *hp-1*, expression in the fruit was enhanced. Light affected intensity of anthocyanin expression because shaded portions of the fruit lacked purple color. The issue of allelism between *Aft* and *Abg* was not resolved. Tomato lines that combined anthocyanin genes with red (wild type) orange (*B*) and yellow (*r*) carotenoid pigments were developed. The anthocyanins responsible for the purple color were characterized by HPLC-DAD and HPLC-MS. The predominant anthocyanin was petunidin-3-(p-coumaryl)-rutinoside-5-glucoside, with detection of five additional forms of petunidin, malvidin, and delphinidin in lower amounts. Up to 300 mg g^{-1} FW of anthocyanin in skin of fruits was detected with an average of about 14 mg g^{-1} FW. Flavonoid precursors to anthocyanin were also upregulated in the fruit. Anthocyanin- and total phenolic contents were related to changes in antioxidant activity using the Oxygen Radical Absorbance Capacity assay. The introduction of anthocyanin-expression inducing genes to the tomato significantly increased antioxidant activity in both the hydrophilic and lipophilic

fractions of the tomato fruit. Tomato lines with similar genetic background but different carotenoid profiles conditioned by og^c, *Del*, *r*, and *B* genotypes were characterized for antioxidant capacity. These genes alter expression of carotenoids to produce elevated levels of β-carotene, all-trans-lycopene, prolycopene, δ-carotene, or with diminished total carotenoid. Tomato juice was made from each of these lines and fed to two human volunteers, following which blood plasma was drawn and evaluated for antioxidant capacity. Although differences in antioxidant activity of plasma measured by Total Antioxidant Performance and Ferric Reduction Assay of Plasma assays were observed, the contribution of the carotenoids was limited relative to the effects of other antioxidants present in the tomato. A major finding of this research was that the water-soluble antioxidants contribute significantly more to overall antioxidant activity compared to the lipid-soluble antioxidants. Future breeding efforts can capitalize on these discoveries to produce a tomato with unprecedented levels of antioxidant activity and nutrient content.

Breeding Tomatoes for Improved Antioxidant Activity

by
Peter J. Mes

A DISSERTATION

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APPROVED:

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Head of the Department of Horticulture

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Peter J. Mes, Author

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CONTRIBUTION OF AUTHORS

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Dedication

To my sons, Abraham, Micah, and Samuel, who inspire me to do my best, to teach and be taught, and to love without reservation.

Breeding Tomatoes for Improved Antioxidant Activity

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

TOMATO-CAROTENOID SIGNIFICANCE TO NUTRITION

Tomatoes are an important part of the human diet as shown by the United States estimated per capita consumption of 39-41 kg fresh weight in 1999 (Gann and Kahachik, 2003; ERS, 2004). No other vegetable except potato exceeds this level of consumption. Not only are they important in the United States, but also are produced and consumed worldwide. Tomatoes are an important source of carotenoids, vitamins, and other nutrients. Specifically, tomatoes have a relatively high content of β-carotene, a precursor to vitamin A. In the last ten years the perceived nutritional value of tomatoes has been enhanced by reports of the presence and importance of the vitamins C and E, ubiquinone-10, lutein, and lycopene (Abushita et al., 1997; Lin and Chen, 2003).

Relative to other consumed fruits and vegetables, tomatoes are high in carotenoids. Because consumption is high, they are a primary source for carotenoids in the diet of many human populations. Lycopene, a red carotenoid, is not unique to tomatoes, but tomato accounts for about 80% of lycopene intake (Bowen, 2003). The role many carotenoids play in the human diet is not fully understood. To date there is scant evidence that carotenoids are actively absorbed in the gastrointestinal tract (Traber et al., 1994), but are believed to be absorbed passively when they are consumed with fats (Sies and Stahl, 1998). Only recently

has the possibility of a carotenoid-specific epithelial transporter in the intestine been proposed (During and Harrison, 2004). The reason carotenoids are not absorbed as well from fresh vegetables consumed without fat is that carotenoids are highly lipophilic, being almost completely insoluble in water (Institute of Medicine, 2000). Brown et al. (2004) demonstrated that carotenoids absorption is improved following processing and when consumed in conjunction with a high fat dressing. Lycopene and β-carotene levels were previously thought to be unchanged in the short term (eight hours) in human plasma following single doses of either raw or cooked tomatoes (Salucci et al., 2002), but that both lycopene and β-carotene levels would increase following repeated consumption over two weeks (Riso et al., 1998). Brown et al., (2004) demonstrated that previous findings might be misleading due to the limitations of the low-end detection threshold of the photodiode arrays (PDA) used in the past. Another reason for past failures to detect changes in carotenoid content *in vivo* is the very small immediate changes in plasma carotenoids following consumption. To be absorbed, carotenoids must first be dissolved and absorbed in micelles. Dietary fat stimulates release of bile acids, which in turn stabilizes micelles and increases their size, facilitating increased carotenoid absorption (Institute of Medicine, 2000). Mucosal cells are thought to absorb micelles by passive diffusion (Hollander and Ruble, 1978). Once in mucosal cells, carotenoids must be packaged into chylomicrons and released into the lymphatics prior to the mucosal cell being sloughed off into the intestine (Institute of Medicine, 2000). Chylomicrons are processed in the liver, and the carotenoids are packaged into low density lipoproteins (LDLs), high

density lipoproteins (HDLs), and very low density lipoproteins (vLDLs). The timeframe for this processing is approximately 4 hours for integration into chylomicrons (Brown et al., 2004), and between 24-48 hours for conversion into the lipoprotein fractions (Rock and Swendseid, 1992).

Once carotenoids are in the bloodstream, several fates await them. Carotenoids with provitamin A activity may be cleaved to produce retinol, also known as vitamin A. β-carotene has the highest provitamin A activity, followed by α-carotene and β-cryptoxanthin (Institute of Medicine, 2000). Provitamin A activity is based on the presence of an unsubstituted β-ionone ring. Carotenoids with provitamin A activity can undergo central and eccentric cleavage: central cleavage of β-carotene produces two molecules of retinal, while eccentric cleavage produces β-apo-carotenals of varying chain length, some of which may still be converted to retinal. Provitamin A carotenoids other than β-carotene can only produce one molecule of retinal from central cleavage, and have reduced efficiency of conversion of the eccentric cleaved products to retinal (During and Harrison, 2004). Both the provitamin A carotenoids and non-β-ionone ring carotenoids are also detected intact and uncleaved in various parts of the body without a proven function or activity associated with their presence. The carotenoids are distributed in almost every tissue in the body, although there is preferential distribution, which differs for different carotenoids. β-carotene levels are higher in the liver and adrenal glands, while lycopene levels are higher in the testes (Kaplan et al., 1990). Both β-carotene and lycopene levels are low in the lungs and kidneys (Schmitz et al., 1991).

Despite the lack of a known direct or active function, carotenoids have become the focus of intense study because of an association with decreased cancer incidence and other health benefits, including prevention of atherosclerosis and other cardiovascular diseases (Willcox et al., 2003). The most prominent candidate for beneficial effects is lycopene. Lycopene is effective by itself and synergistically with α-tocopherol to decrease oxidation *in vivo* (Balestrieri et al., 2004; Césarini et al., 2003; Fuhrman et al., 2000). It is the antioxidant property that has elevated lycopene to new heights within the public awareness, with lycopene recently being incorporated into over-the-counter vitamin supplements and promoted for its perceived benefits associated with antioxidant activity. Of all the carotenoids, lycopene is the most efficient singlet oxygen quencher (Di Mascio et al., 1989; Sies and Stahl, 1998), can scavenge peroxynitrite (Yokota et al., 2004), and has the highest trolox equivalence antioxidant capacity (TEAC) score (Miller et al., 1996). These characteristics are measures of *in vitro* antioxidant activity, though, and may not necessarily reflect *in vivo* benefits or activity, although there is accumulating evidence that singlet oxygen is generated endogenously in humans (Wagner et al., 1993). Current thinking is that this elevated antioxidant activity makes lycopene the best candidate of the carotenoids to have beneficial antioxidant effects. This theory has been and continues to be tested, with most studies providing support for an *in vivo* antioxidant role of lycopene. For example, reduced lymphocyte DNA damage associated with increased tomato product consumption was observed following three weeks of tomato purée consumption (Riso et al., 1998). This study demonstrated that

tomato products, and not necessarily lycopene, reduce oxidative damage to lymphocyte DNA. However lycopene is one of the compounds nearly unique to tomatoes versus other foods. In another study using an *ex vivo* test, plasma was exposed to chemically generated singlet oxygen, and oxidation of LDLs was monitored. Lycopene and β-carotene quenched 40% of singlet oxygen radicals, while apo-B and α-tocopherol quenched 59% (Wagner et al., 1993). Studying LDL oxidation *in vivo* following tomato juice consumption, Maruyama et al. (2001) found that α-tocopherol was the major determinant in reducing measured oxidation, while lycopene was associated with a reduction in oxidant propagation rate in phospholipids, but not elsewhere. This is not entirely unexpected, as lycopene, like most hydrophobic compounds, is located almost exclusively within the non-polar hydrocarbon portion of the phospholipid bilayer (Gruszecki and Sielewiesiuk, 1990). Radicals are not frequently generated in the lipoprotein membrane, although they do permeate into the bilayer. Another study found that under *ex vivo* conditions, tomato product consumption extended the lipoprotein oxidation lag period (Hadley et al., 2003). The increase in lag was explained by an increase in lipophilic antioxidants such as α-tocopherol, β-carotene, and lycopene (Esterbauer et al., 1989). This increase occurred in all tomato product groups studied, including condensed tomato soup, ready to serve tomato soup, and tomato juice, all of which had high lycopene content. Interpreted collectively, these studies offer evidence, but not proof, for an antioxidant role of lycopene *in vivo* in human LDLs. The proposed mechanism for this *in vivo* antioxidant activity of lycopene is through quenching of singlet oxygen radicals (Oshima et al., 1996),

which is similar to the antioxidant mechanism reported *in vitro* (Stahl and Sies, 1996).

MECHANISMS OF OXIDANT PROTECTION

LYCOPENE

Recent research indicates that beneficial effects of lycopene and β-carotene are not always detected when the compounds are consumed in purified form. In some cases, the beneficial effects associated with lycopene or β-carotene are only detected or increased when the purified carotenoid lycopene or β-carotene is consumed together with α-tocopherol (vitamin E) and other lipophilic tomato extracts (Balestrieri et al., 2004; Nakanishi-Ueda et al., 2002), as opposed to the lack of observed benefits when taken as purified supplements (Hininger et al., 2001; Gaziano et al., 1995). Lycopene, together with α-tocopherol, reduces oxidation of LDLs and LDL phospholipids by enhancing the biosynthesis of platelet-activating factor (Balestrieri et al., 2004, Maruyama et al., 2001). This is good news for tomato consumers interested in preventing atherosclerosis, a disease believed to be caused by the oxidation of LDLs (Higdon and Frei, 2003; Morrow, 2003), because red tomatoes contain both lycopene and α-tocopherol. Indirect support for the hypothesis that lycopene is most active in the presence of α-tocopherol also comes from a study on the effects of purified lycopene or tomato extract on chemically-induced oxidant stress in rats livers (Kim et al., 2004). Lycopene alone did not reduce liver injury; but lycopene in conjunction with other

tomato lipophilic compounds significantly reduced injury caused by chemically-induced oxidation. Synergistic activity of α-tocopherol and β-carotene reduces the *ex vivo* oxidation of LDLs and *in vivo* lipid peroxidation in cystic fibrosis (CF) patients (Winklhofer-Roob et al., 2003). In those patients, supplementation of β-carotene and α-tocopherol reduced oxidation stress status to normal levels compared to the elevated levels normally observed in CF patients. The model of interactions between lycopene, β-carotene, or any other carotenoid, and α-tocopherol within the lipophilic fraction of plasma shown in Figure 1.1, (adapted from Krinsky and Yeum, 2003; Schafer et al., 2002; Viana et al., 1996; and Yeum et al., 2004), reveals the synergistic activity of the components in cycling radicals out to the aqueous environment. The difference between the methods employed by α-tocopherol and carotenoids to effectively dissipate the energy of the free radical electron is highlighted in this figure. α-tocopherol chemically transfers the electron out of the lipid particle to ascorbic acid, while carotenoids such as lycopene and β-carotene, when reacting with singlet oxygen physically return the oxygen to ground state and dissipate energy through non-radical interaction with the solvent environment (Schafer et al., 2002; Stahl and Sies, 2003), and produce non-radical reaction products when reacting with peroxynitrite (Yokota et al., 2004). The carotenoids also cycle radicals to α-tocoperol within the lipid particle or possibly directly to ascorbic acid if the carotenoid has a hydrophilic end. This synergistic effect explains the increase in antioxidant activity above the activity of either compound when analyzed in isolation. The synergistic effect is greatest when lycopene or lutein is present (Stahl et al., 1998).

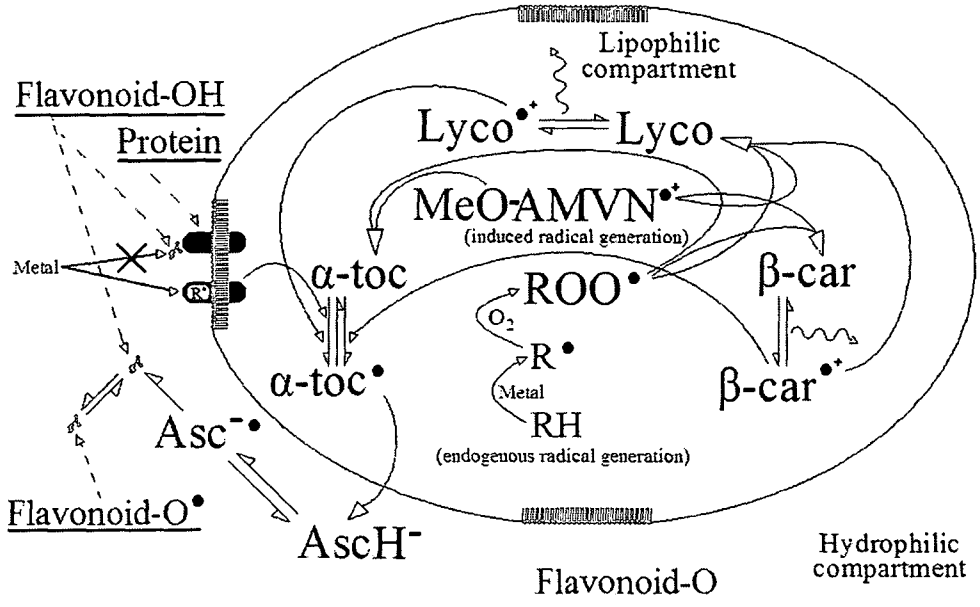


Figure. 1.1. Radical interactions of an aqueous/lipophilic environment. R•: carbon-centered lipid radical; ROO•: peroxyl lipid radical; MeO-AMVN•: 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile)-derived radical; Lyco: lycopene; β-car: β-carotene; α-toc: α-tocopherol; AscH: Ascorbic acid

It is not always necessary to have α-tocopherol together with lycopene to observe benefits. In a study of prostate cancer cell lines, α-tocopherol supplementation increased apoptosis of cells following gamma-irradiation, while lycopene had the opposite effect, increasing cell survival and preventing DNA damage (Rossinow et al., 2003). Another study using lycopene supplementation of prostate cancer patients produced similar results. Lycopene was absorbed by prostate tissue, reducing observed DNA damage in leukocyte and prostate tissue (Bowen et al., 2002). These finding have profound implications for men preparing for, or undergoing radiation treatment for prostate cancer. Whether it is beneficial for men known to have prostate cancer to consume tomatoes or lycopene remains

to be determined. However, these results provide encouraging evidence of benefits to men seeking to avoid prostate cancer through reduction of DNA damage caused by oxidation. In addition, lycopene inhibits the proliferation of normal prostate epithelial cells *in vitro* in a dosage dependant manner (Obermuller-Jevic et al., 2003). This reduction is evident at 1 μM dosage, within the range of physiological relevance. Thus lycopene may lower the risk of prostate cancer onset. No *in vivo* work has yet been reported on this effect. This effect is not restricted to prostate cells. Cell culture work with human mammary cancer cells has indicated that lycopene causes a disruption of cell-signaling, that inhibits progression of the cell-cycle (Karas et al., 2000; Levy et al., 1995; Nahum et al., 2001).

Oxidation inhibition is not the only proposed mechanism for lycopene to prevent prostate cancer. Lycopene may also cause cell apoptosis. Apoptosis is a natural method to select certain cells in the body for programmed cell death (Kerr et al., 1972). Cancerous cells are resistant to apoptosis, giving them a reproductive advantage (Tang et al., 1998). While lycopene may indirectly prevent apoptosis through prevention of DNA damage following gamma-irradiation as previously mentioned, it may also stimulate apoptosis directly in cancerous cells. At the physiologically relevant dosage of one μM and at a higher dosage of five μM lycopene, *in vitro* cancerous prostate cells exhibited both slowed growth and a significant increase in apoptosis compared to placebo treated cancer cells (Hwang and Bowen, 2004). Of the carotenoids found in tomato, the reduction in prostate cancer cell viability is caused by lycopene, ζ-carotene, α-carotene, β-carotene,

lutein and phytofluene, but not phytoene, all at dosages of 20 μM. At the lowest dosage reported by Kotake-Nara et al. (2001) (five μM and closest to physiological relevance), only lycopene consistently decreased human prostate cancer cell viability. This may explain why out of all foods consumed, only tomato consumption is associated with decreased prostate cancer risk, because the primary source of lycopene in the human diet is tomatoes (Giovannucci et al., 1995).

Of the carotenoids and tocopherols, only lycopene has been associated with a decrease in prostate cancer incidence (Miller et al., 2002), although ten major carotenoids are found in significant quantities in prostate tissue (Paetau et al., 1998). The decrease in prostate cancer incidence of 15% and 35% was achieved by increasing tomato servings from 8 to 16 and 14 to 28 servings per week respectively (Cohen et al., 2000; Tzonou et al., 1999). From an epidemiological standpoint, lycopene was the only antioxidant at significantly lower levels in prostate cancer patients (Gann et al., 1999; Rao et al., 1999). While this is not direct evidence of beneficial activity of lycopene, the correlation of decreased prostate cancer incidence with increased tomato product consumption is significant and bears consideration.

Lycopene may not be directly responsible for all of the observed benefits to human health. For example: the autoxidation cleavage products of lycopene cause apoptosis in promyelocytic leukemia cells; all-*trans*-lycopene does not have this effect (Zhang et al., 2003). This is a relatively new finding, and one that warrants further study, as other research indicating lycopene as the active component did not investigate the autoxidation products produced. Even if the oxidation products

are exclusively responsible for the beneficial effects currently associated with lycopene, continuing lycopene research remains justified because lycopene is the only substrate for reactions producing beneficial products *in vivo*.

PHYTOENE

The carotenoid phytoene is often omitted from studies of nutrition and antioxidant activity. Phytoene is the first true carotenoid in the carotenoid biochemical pathway, and is the only compound in the pathway without color; absorbing light with a λ-max of about 286 nm, well into the UV-spectrum of light. Phytoene is a relative newcomer to our awareness of human nutrition/antioxidant research, with little published information available regarding its bioavailability and bioactivity. One of the first reports on phytoene activity indicated a correlation of phytoene injections to delayed skin tumor appearance after UV-light irradiation in mice (Mathews-Roth, 1982). This finding is particularly interesting given the author's simultaneous report that β-carotene had no inhibitory effect on UV-induced tumors, indicating that provitamin A activity is not the mechanism of prevention. The mechanism of tumor inhibition by phytoene was not determined. Nishino et al., (2002) reported that transgenic mammalian phytoene-expressing cells acquired resistance to carcinogenesis. The mechanism of prevention appeared to be antioxidative in nature, because the phytoene-expressing cells had lower detectable phospholipid hydroperoxidation.

In transgenic phytoene-expressing mice cells, phytoene induced *connexin-*

26 gene expression, evidence that phytoene may affect cell-to-cell communication (Satomi et al., 2004). This is of particular interest, as it is a similar activity to that reported for α-carotene, β-carotene, and lycopene, all of which have been implicated in inducing *connexin-43* gene expression and cell gap junction communication in precancerous cells at low doses (Chang et al., 1992; Krutovskikh et al., 1997; Stahl et al., 2000). The benefits of inducing the connexin genes are from comparison of normal and cancerous cells: tumor cells communicate poorly via connexins compared to normal cells. Connexins allow inter-cell communication, facilitating the transmission of growth-inhibition signals (Hix et al., 2004). Tissues of phytoene-expressing mice do not exhibit an increase in induction of *connexin-43* (Satomi et al., 2004). This raises the possibility that different carotenoids act synergistically to prevent the loss of cell-to-cell communication, with phytoene inducing one set of connexin genes and other carotenoids inducing other connexin genes.

Phytoene is bioavailable and detectable in human plasma (Nishino et al., 2002), accumulating preferentially in lung and liver tissues (Khachik et al., 2002). Exact figures for phytoene bioavailability in humans have not yet been published, but phytoene is a significant carotenoid in tomatoes. Unlike lycopene, which is found in tomatoes and watermelons, phytoene is found in all fruits and vegetables containing carotenoids, making it a far more common carotenoid, although content is usually low because it is a precursor to downstream products. Phytoene content in tomatoes varies widely among cultivars within a species, although it commonly compromises approximately 15% of the total carotenoids (Jones, 2000).

β-CAROTENE

In terms of human nutrition, β-carotene is the most controversial of the carotenoids. It is best known for provitamin A activity. Vitamin A is necessary for low-light vision, bone growth, and maintenance of healthy skin and mucous membranes. Of the provitamin A carotenoids, β-carotene is the predominant contributor to vitamin A because cleavage of the symmetrical β-carotene molecule results in two molecules of vitamin A, whereas other carotenoids convert to vitamin A in a 2:1 ratio at best.

β-carotene is associated with benefits of reducing oxidation as measured by susceptibility of LDL to *ex vivo* oxidization, plasma peroxide concentrations, and urinary γ-oxo-dG concentrations when body stores are repleted following depletion due to a carotenoid-restricted diet (Paiva et al., 1998; Winklhofer-Roob et al., 2003). When consumed in natural vegetable sources, β-carotene produces a time-delayed increase in tumor necrosis factor-α (TNFα) secretions, thereby modulating immune function in a beneficial manner (Watzl et al., 2003). However, dietary supplementation with purified β-carotene is associated with increased lung cancer incidence in smokers (Goodman et al., 2003; Omenn et al., 1996). This negative effect has been attributed to β-carotene having pro-oxidant activity at high oxygen partial pressure (Krinsky and Yeum, 2003), a condition met in certain oxidant-stress conditions such as found in smokers. It seems that β-carotene stores must be maintained in a moderate and steady state, unlike the current hypotheses for other carotenoids where increasing amounts have increasing

health benefits.

β-carotene content in tomatoes is highly variable, and is often directly related to lycopene content. Because β-carotene is made from lycopene, the two are inversely related: high lycopene tomatoes have low β-carotene content, while high β-carotene tomatoes have low lycopene content. Lincoln and Porter (1950) first explored this relationship by incorporating the dominant *B* allele from *L. hirsutum* into *L. esculentum*. The *B* allele has another effect besides increasing β-carotene: total carotenoids are reduced in *B*-gene fruit. A possible explanation for this phenomenon is that β-carotene acts as a feedback inhibitor for the carotenoid pathway (Fraser and Bramley, 2004). Proof of such inhibition in the tomato carotenoid biosynthetic pathway has not been published. However there are many reports of tomato variety carotenoid profiles of β-carotene, lycopene, and total carotenoids that support the hypothesis. This hypothesis will be discussed later.

LUTEIN

Lutein is often overlooked in reports of tomato carotenoids, despite its presence in the fruit. Lutein is a xanthophyll, or oxygenated carotene. Its presence in the human body is most notable in the macula, where lutein is preferentially accumulated over other xanthophylls or carotenes, resulting in the yellow color of the macula. The activity of lutein in the macula has been reviewed extensively elsewhere (Davies et al., 2004; Alves-Rodrigues et al., 2004). In the eye, lutein filters blue-green light, and may act as an antioxidant by quenching photo-induced

reactive oxygen species (ROS). Like lycopene, lutein has strong antioxidant properties. It also has promise as a chemopreventive agent. In a study of rat hepatic preneoplastic lesions, lutein and lycopene both contributed to a reduction in number and size of glutathione-S-transferase-positive preneoplastic lesions (Toledo et al., 2003). Lutein, like lycopene, also had a positive effect by protecting against DNA strand breakage. Although lutein concentrations are far less than lycopene in tomato, it may still contribute significantly to antioxidant activity, and must not be overlooked when studying tomato antioxidants. Other xanthophylls are also found in trace quantities in tomato fruit including neoxanthin, violaxanthin, *cis*-violaxanthin, α-cryptaxanthin, and β-cryptaxanthin (Ben-Amotz and Fishler, 1997), although their contribution is negligible for the purposes of this research.

BIOAVAILABILITY OF TOMATO CAROTENOIDS

Tomatoes are the primary source for lycopene in the human diet. It is bioavailable at a very low rate; more so in unprocessed tomatoes than in processed tomato products. About 79 percent of consumed tomatoes are eaten as processed products (ERS, 2004). The difference in bioavailability is due at least in part to the lipophilic nature of the carotenoids. Lipophilic compounds must first leave the food matrix and be incorporated in micelles to be bioavailable (Stahl et al., 2002). In aqueous systems, lycopene will precipitate to form crystals (Shi, 2002). Lycopene and β-carotene are in crystalline form in tomato fruit (Harris and Spurr,

1969 a and b), making them particularly difficult to dissolve, even in many organic solvents (Britton et al., 1995). It follows that even in the fat phase of the intestine, very little carotenoid is expected to dissolve. This is indeed the case, as less than 1% of lycopene and about 1-3% β-carotene is absorbed by the human digestive system (Stahl et al., 2002). The bioavailability of lycopene and all other carotenoids can be increased in several ways: the food can be heat processed; it can be consumed with fat, or both (Agarwal et al., 2001; Brown et al., 2004; Rock et al., 1998). Despite low bioavailability, once carotenoids enter the body, they persist both in tissues and in plasma LDLs (Agarwal and Rao, 1998; Porrini et al., 1998; Rao and Agarwal, 1998). Washout periods to reduce carotenoids in the blood can be several weeks long, and even then total carotenoids may be reduced, but are still detectable (Porrini et al., 1998; Brown et al., 2004; Institute of Medicine, 2000). Because β-carotene, α-carotene, and β-cryptoxanthin have pro-vitamin A activity, their persistence is considered a positive characteristic because it allows for extended periods between carotenoid consumption without reaching harmfully low levels of vitamin A.

Complicating the issue of carotenoid bioavailability is the enduring problem that carotenoids, other than those with pro-vitamin A activity, have no demonstrated biologically relevant function to justify classification as an essential nutrient by the Institute of Medicine, the governing body that releases recommendations for daily intake (Institute of Medicine, 2000). This means that at present, based on recommended daily intake (RDI) values, the claim that fruits and vegetables with increased carotenoid content are "better" is not true. While

there is mounting evidence that the xanthophylls lutein and zeaxanthin are important to the health of eyes, and that lycopene and possibly other carotenoids may play a role in prostate cancer prevention and increase total antioxidant protection (Miller et al., 2002), there is no determined level of consumption which provides these benefits. This leads to another complication in terms of human nutrition: not only are carotenoids bioavailable in low amounts, different types compete with one another for absorption (Tyssandier et al., 2002). On the other hand, once absorbed, carotenoids do not displace each other (Riso et al., 2004). It is critical to know which carotenoids are most relevant and significant to human nutrition. If several carotenoids are important for human nutrition, then it is important to determine how to elevate bioavailability of all carotenoids to prevent reduced absorption of any carotenoid. It is also important to assess synergistic effects that carotenoids may have with other compounds. If RDI values are ever established, the problem of most important carotenoids will be easier to assess.

Red-fruited tomatoes sold fresh and processed have carotenoid profiles typically dominated by lycopene, although fruit with other colors and different carotenoid profiles are also marketed. Tomato breeders have selected processed cultivars for uniform, intensely red fruit color, which appears to have increased lycopene at the expense of β-carotene (Jones, 2000). A trend of decreasing β-carotene could be a concern in human populations that derive a significant portion of their dietary β-carotene from tomatoes. Fresh market and home garden tomato breeders have developed cultivars with other fruit colors, ranging from yellow, to orange, to green and brown. Orange and yellow tomatoes are common in fresh

markets and some grocery stores. The antioxidant activity and nutritional benefits of these fruit types are nearly as well characterized as that of red fruit. Ferruzzi (2001) reported on the absorption and detection of prolycopene in humans after consumption of tomatoes containing that carotenoid. Nevertheless, the bioavailability of prolycopene remains undetermined, as is the situation for several other carotenoids found in tomatoes.

CAROTENOID BIOSYNTHESIS AND CHARACTERIZATION IN TOMATOES

While lycopene is the dominant carotenoid in red tomatoes, other carotenoids may be present. Naturally occurring genetic mutants alter carotenoid biosynthesis at various steps in the carotenoid pathway (Table 1.1). These mutants can be broadly grouped into those that block production within the carotenoid pathway, increase the production of carotenoids other than lycopene and beta-carotene, or affect levels of beta-carotene and/or lycopene.

In tomato, the biosynthetic pathway governing the production of carotenoids has been completely characterized, although some genes remain unmapped and have not been confirmed as to function. The first proposed carotenoid pathway in tomatoes (Lincoln and Porter, 1950) had five steps: a colorless precursor (since identified as phytoene), phytofluene, ζ-carotene, lycopene, and ending with β-carotene. The variety of colors found in tomato fruit made for easy selection of mutations within the pathway, because each carotenoid

Table 1.1. Carotenoid pathway mutants in tomato

Gene	Description	Fruit Color
r [1]	phytoene synthase non-functional/not expressed: minimal carotenoid production	yellow
t [2]	carotenoid isomerase: no cis-trans isomerization results in prolycopene production	light orange
B [3]	Lycopene Beta-cyclase: over-expressed enzyme increases β-carotene at the expense of lycopene	orange
og [c 3]	Lycopene Beta-cyclase: non-functional enzyme results in increased lycopene and a reduction in β-carotene	crimson-red
Del [4]	Lycopene-epsilon ring hydroxylase: 30 fold increase in enzyme results in delta-carotene production	burnt orange
hp-2/dg [5]	Deetiolated-1: increases photoresponse, elevating pigment content	red
hp-1 [6]	DNA damage binding protein1: increases photoresponse, elevating pigment content	red

[1]Fray and Grierson, 1993; [2] Isaacson et al., 2002; [3] Ronen et al., 2000; [4] Ronen et al., 1999; Mustilli et al., 1999; [5] Levin et al., 2003; [6] Liu et al., 2004 and Lieberman et al., 2004.

i

n the pathway has a distinctive color and spectral absorption pattern. These mutations have been bred into near-isogenic backgrounds, and are currently maintained as germplasm at the C.M. Rick Tomato Resource Center, University of California, Davis, CA. In mutants with non-functional enzymes, subsequent carotenoids in the pathway are not produced or are generated at very low levels. This results in the tomato primarily accumulating the last carotenoid in the pathway prior to the stop, with a color shift in the direction of that particular carotenoid. The carotenoid pathway (Figure 1.2) begins with an accumulation of double bonds along the length of the carbon chain following production of phytoene from geranylgeranyl diphosphate (GGDP), derived from the mevalonic

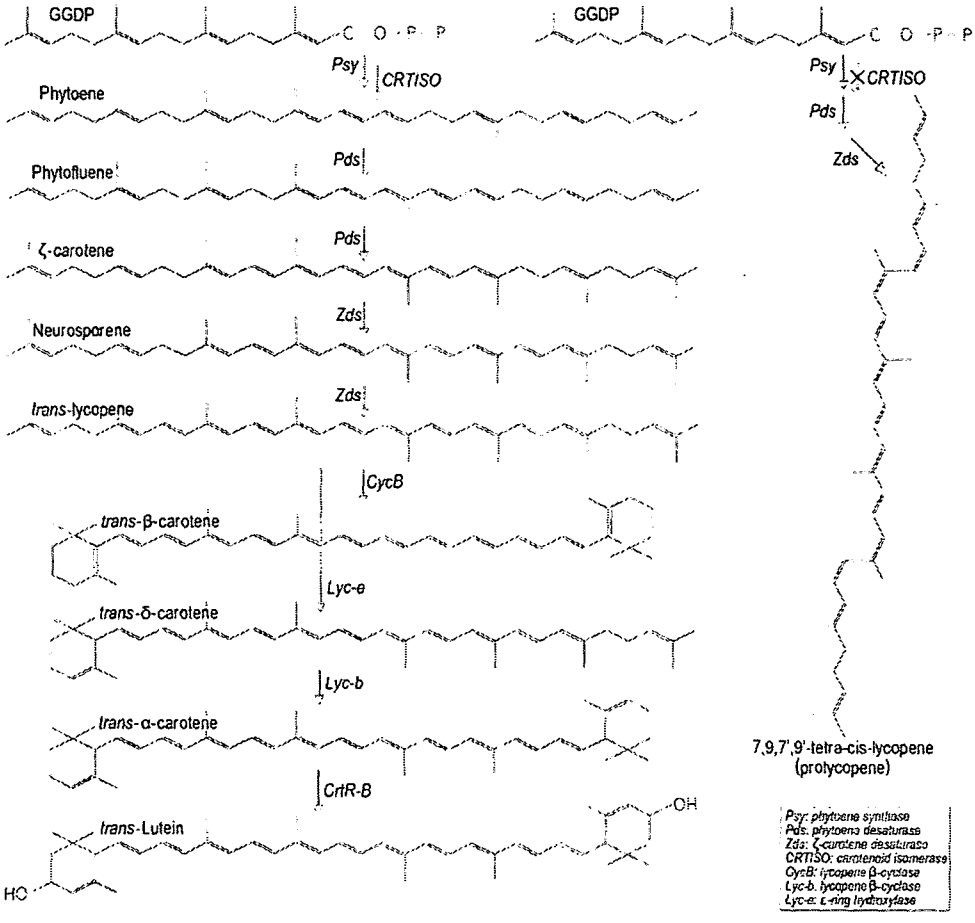


Figure. 1.2. Tomato carotenoid biosynthetic pathway.

and methylerythritol phosphate (non-mevalonic) pathways (Bramley et al., 2002; Fraser and Bramley, 2004; Rodriguez-Concepcion et al., 2003). The biochemical pathway splits after the formation of lycopene with various cyclizations and oxygenations. Each additional desaturation (accumulated double bond) lengthens the chromophore and results in a shift in the λ_{max} (Goodwin, 1976). Lycopene, with the most conjugated double bonds, has the highest λ_{max} of 476 nm in hexane (Goodwin, 1976). This corresponds to a light yellow color in hexane, but when lycopene crystals are formed, the λ_{max} increases further to produce the characteristic red color seen in tomatoes. When cyclization is introduced in α- and

β-carotene there is a significant shift down in λ_{max} to 450nm in hexane (Goodwin, 1976), resulting in orange crystals in fruit. When oxygenation is introduced in lutein, the λ_{max} undergoes bathychromatic shift to 458nm, resulting in yellow crystals (Goodwin, 1976). These differences in λ_{max} allow for identification of carotenoids in liquid chromatography using a photodiode array detector (PDA). In tomatoes with complicated carotenoid profiles, the λ_{max} absorption, in conjunction with column retention time, is used to identify and quantify carotenoid content.

There are several branches within the carotenoid biosynthetic pathway. The first known branching point is *carotenoid isomerase* (*CRTISO*) (Isaacson et al., 2002). When this enzyme is nonfunctional as in the *tt* genotype, carotenoids downstream in the pathway continue to be produced but with altered carotenoid profile. *Cis-* isomers of phytoene, phytofluene, ζ-carotene, neurosporene, lycopene (including prolycopene, a tetra-*cis*-isomer), and β-carotene are produced, rather than the all-*trans* forms found in at tomato with functional *CRTISO*. This demonstrates that trans-isomerization is not a necessity for enzymes to act on their respective substrates within the carotenoid biochemical pathway. The next branch in the pathway follows the production of lycopene. Lycopene can be cyclized to produce β-carotene, α-carotene, and δ-carotene. α-carotene can subsequently be oxygenated to produce lutein (Fraser and Bramley, 2004). δ-carotene is only produced in tomatoes containing the gene *Del*, which encodes a functional carotene cyclization enzyme lycopene ε-cyclase (CrtL-e) (Ronen et al., 1999). α-carotene is produced from γ-carotene by lycopene β-cyclase (CrtL-b) or from δ-carotene when *Del* is present (Pecker et al., 1996, Ronen et al., 1999). This is not

observed in tomato fruit that do not have *Del*. β-carotene is produced from lycopene, and at its expense, a fact first hypothesized 60 years before the biochemical proof could be provided (Kohler et al., 1947; Lincoln and Porter, 1950). Lycopene β-cyclase (Cyc-B) is the enzyme which cyclizes both ends of lycopene to form two β-ione rings (Ronen et al., 2000). When *Cyc-B* is in its wild-type form in *L. esculentum*, the enzyme has limited activity, resulting in limited β-carotene production. In fruit carrying the og^c allele of *Cyc-B*, the enzyme is non-functional, resulting in little to no production of β-carotene and elevated lycopene content. When the *B* allele of *Cyc-B* is present, the enzyme is highly active, resulting in the bulk of lycopene being converted to β-carotene (Ronen et al., 2000).

The carotenoid pathway in tomato is tightly regulated both in timing of production and in quantities of products. This is evident when comparing tomato lines with different mutations within the pathway, because their total carotenoids are never the same. Regulation of the pathway is best exemplified in the comparison of high β-carotene tomatoes with high lycopene tomatoes, where the single gene mutation (*B*) halves the total carotenoid amounts. Feedback inhibition caused by accumulation of either β-carotene or one of its metabolic products is responsible for the changes in profiles (Fraser and Bramley, 2004). The carotenoid pathway is regulated at the phytoene synthase (*Pds*) step, which is where feedback inhibition appears to regulate the pathway. It is thought that β-carotene, abscissic acid, or a xanthophyll activates feedback inhibition (Corona et al., 1996). Evidence for this activity by β-carotene comes from comparison of

high β-carotene lines which generally do not accumulate elevated levels of any precursor carotenoids to other tomato mutant or wild-type lines (Corona et al., 1996; Fraser et al., 1994; Mes, unpublished results; Ronen et al., 1999). The inhibition is likely activated above a certain threshold, as high-β-carotene lines have up to 37 μg g^{-1} FW β-carotene (Hanson et al., 2004) and less than 70 μg g^{-1} FW total carotenoids, while lines with normal Cyc-β or reduced Cyc-β can have up to 146 μg g^{-1} FW lycopene (Thompson, 1956; Thompson, 1957; Jones, 2000) or even 246 μg g^{-1} FW in material recently developed by M. Foolad (Hyman et al., 2004). At the same time, β-carotene in the fruit remains at or well below 10μg g^{-1} FW (Hyman et al., 2004; Wann and Jourdain, 1985). This indicates that the β-carotene inhibition threshold concentration is above 10 μg g^{-1} FW, if β-carotene is the triggering element of the feedback mechanism.

One way to bypass the carotenoid regulatory elements in tomato fruit is to express the *hp-1* or *hp-2* mutants. The *hp-2* mutation of the *DE-ETIOLATED1* (*DET1*) prevents negative regulation in the light signal transduction pathway (Levin et al., 2003), while *hp-1* is a mutant of *UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*), which interacts with *DET1* (Liu et al., 2004). As a result, all plant tissues expressing these mutant genes behave as if they are shaded, increasing plastid density, but tissues also perceive the need for increased antioxidant protection, resulting in global increases to the carotenoids, anthocyanins, and chlorophylls, unlike the carotenoid mutants that primarily affect one or several structural carotenoids (Mochizuki et al., 1987; Kendrick et al., 1997; Peters et al., 1992). This phenotype is the result of an increase in plastid

number (Yen et al., 1997; Bramley, 1997), and not an upregulation of the chlorophyll or carotenoid pathways. These mutants, despite their benefits in terms of carotenoid content, remain largely absent from commercial cultivars because of negative pleiotropic effects on plant morphology caused by alterations in the gibberelic acid pathway. It is possible that some of these effects may be alleviated through selection of high-gibberelic acid-expressing lines (Van Wann, 1995). However, to date no high GA mutants have been reported in tomato.

TOMATO WATER SOLUBLE NUTRIENTS

Tomatoes are important for human nutrition for many components beyond the visually dominant carotenoids. Also present in significant quantities are the water soluble nutrients. The most well know of these is ascorbic acid, commonly called vitamin C. Ascorbic acid acts as an antioxidant, recycling α-tocopherol and carotenoids following oxidative events (Krinsky and Yeum, 2003). Tomato ascorbic acid content varies widely, depending on the genetic background of the variety and the amount of sunlight exposure (Singh et al., 2004 Abushita et al., 2000). A single serving (235 grams, or 1 cup) of a tomato will contain approximately 45 mg ascorbic acid based on an average value of 19 mg ascorbic acid $100g^{-1}$ FW (ERS, 2004). This fulfills half of the daily dietary intake recommendation of 90 mg (Buettner, 1990).

Tomatoes also contain a class of water soluble compounds called flavonoids. These heterocyclic compounds are derived from chalcone (Figure 1.3)

(De Jong et al., 2004), and have varying color and antioxidant properties, the latter of which positively corresponds with increasing the number of free OH substitutions, while the former is affected by substitutions of the 3',4', and 5' positions of the B ring, and the moieties attached at the 3rd carbon of the C ring (Rice-Evans et al., 1996; Rodriguez-Saona and Wrolstad, 2001). Some flavonoids do not absorb in the visible spectrum, while others such as the anthocyanins (derived from Greek *antos* for flower, *kyanos* for blue) are highly pigmented. Flavonoids have antioxidant activity *in vitro*, although their absorption and antioxidant activity *in vivo* have only recently begun to be characterized. Antioxidant activity measured *in vitro* showed that of the flavonoids found in tomatoes, the flavones have the highest antioxidant activity as measured by oxygen radical absorbance capacity (ORAC) assay, followed by the anthocyanins (Wang et al., 1997). Of these, the anthocyanins petunidin, delphinidin, and malvidin, the flavonoids, rutin, quercetin, naringenin, kaempferol, myricetin, and the phenolic acids chlorogenic acid and caffeic acid are all present in significant quantities in tomato fruit (Jones et al., 2003; Minoggio et al., 2003; Wang et al., 2003; Tokusoglu et al., 2003). Reported values include 38 μg g^{-1} FW quercetin, 7 μg g^{-1} kaempferol, 6.8μg g^{-1} naringenin, 7.4 μg g^{-1} rutin, 0.5 μg g^{-1} FW myricetin, and 0.02 μg g^{-1} chlorogenic acid (Bahorun, 2004; Minoggio et al., 2003; Tokusoglu et al., 2003). Hanson et al. (2004) reported that tomato phenolics make a major contribution to the total antioxidant activity in lines that had no anthocyanins. Increasing the anthocyanins may significantly increase the tomato antioxidant capacity.

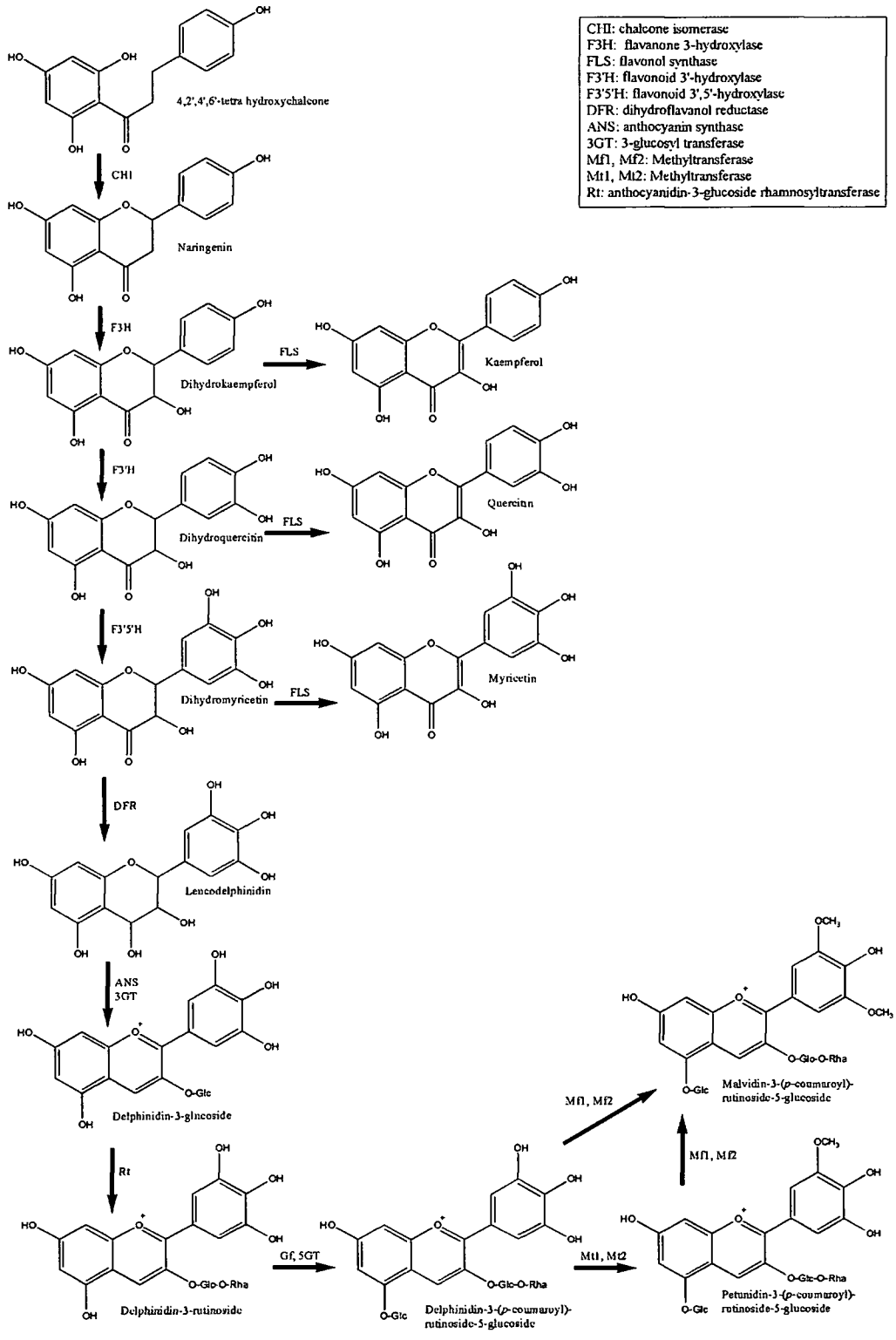


Figure. 1.3. Tomato anthocyanin biosynthetic pathway.

Like the carotenoids, increased consumption of the antioxidant flavonoids correlates to a decrease in LDL oxidation (Saute-Gracia et al., 1997). This discovery came as an indirect result of studying the French paradox (Renaud and de Lorgeril, 1992), a surprising finding that the French, with their generally high cholesterol intake, have a lower risk for atherosclerosis and coronary heart disease than their American counterparts. As high cholesterol intake is associated with an increase in cardiovascular disease, the postulated explanation for the low disease incidence in the French is that they consume a significantly greater amount of wine. Wines contain many complex phenolics and flavonoids, including anthocyanins, and thus, the interest in the relationship of flavonoids to human health began.

Flavonoids are not reported to be in LDL particles in plasma, although they associate with liposomal systems (Tsuda et al., 1996), *in vitro* microsome suspensions (Kravchenko et al., 2003), and may bind with apolipoprotein B (Satue-Gracia et al., 1997) as shown in Figure 1.1. Flavonoids are most soluble in alcohol or water, and are not highly lipophilic, although with increasing methylation, their polarity decreases. This puts them largely outside the LDL complex, which is highly lipophilic, though flavonoids are known to bind with proteins or form complexes with them, which can result in their being closely associated with membranes. The inhibition of LDL oxidation by flavonoids is thus likely to occur prophylactically. Oxidants are neutralized by the flavonoids near the membrane surface, prior to reaching proteins, LDLs, or other lipid soluble macromolecules susceptible to oxidative damage (Viljanen et al., 2004; Kahkonen

and Heinonen, 2003). A second method of preventing LDL oxidation is through steric hindrance. Flavonoids bind to membrane-bound proteins at sites susceptible to metal-induced oxidation, making the site inaccessible (Viljanen et al., 2004). Metal chelation is also a candidate mechanism of flavonoid oxidation inhibition (Rice-Evans et al., 1996), preventing the metal from undergoing the Fenton reaction to produce radicals (Hou, 2003). If flavonoids enter the LDL particle, they may further facility the antioxidant activity by recycling oxidized tocopherol (Rice-Evans et al., 1996; Viana et al., 1996). The presence of α-tocopherol in the prevention of LDL oxidation is a requisite for flavonoid antioxidant activity. Once the α-tocopherol in the LDL is consumed, a broad spectrum of flavonoids tested ceased to prevent LDL oxidation (Viana et al., 1996).

The antioxidant activity of flavonoids has been tested in both an *in vitro* and *ex vivo* rat model system, where the flavonoids provided significant protection against induced lipid peroxidation (Kravchenko et al., 2003). The flavonoid quercetin inhibits *in vivo* LDL oxidation (Chopra et al., 2000). However, it is not free quercetin, but small intestine and liver metabolites such as quercetin-7-glucuronide and quercetin-3'-sulfate which are found *in vivo* after consumption of quercetin (Janisch et al., 2004). Of these, the small intestine metabolites such as quercetin-7-glucuronide exhibit antioxidant activity greater than that of quercetin, while the liver metabolites such as quercetin-3'sulfate have significantly reduced activity. Thus, the metabolism of the consumed free quercetin in the small intestine is beneficial in two ways: the antioxidant activity is increased, and the quercetin is made available for absorption. When the quercetin metabolites are

bound with serum albumin, the most common protein found in plasma, the antioxidant effect is reversed: the liver metabolite quercetin-3'sulfate increases the lag period for copper-induced lipid oxidation, while no other albumin-bound quercetin conjugate causes a similar increase (Janisch et al., 2004). Under these conditions, quercetin is not acting as an antioxidant, but is assisting albumin in binding copper.

While the antioxidant activity of flavonoids is important for human nutrition, there are additional benefits from consuming them. Quercetin and kaempferol, both found in tomatoes, affect cell signaling in cancerous cells, inhibiting cell proliferation and reducing cancer cell resistance to apoptosis (Wang et al., 2003). The flavonols quercetin, kaempferol, and myricetin also protect against lymphocyte DNA oxidation (Lean et al., 1999). These findings combined with that of Rossinow et al. (2003) and Bowen et al. (2002), which report that lycopene reduces DNA damage during chemotherapy treatment of prostate cancer, is evidence in support of the recommendation to increase tomato consumption prior to prostate cancer treatment. All prostate tissue will benefit from the DNA-protective effect of lycopene, while the flavonoids may assist specifically within cancerous cells, disrupting and terminating the cell growth cycles. This hypothesis has yet to be tested fully, but the evidence to support the activities of the various individual compounds involved has already been demonstrated.

Anthocyanins are the end-product of the flavonoid pathway. They are bioavailable as intact glucosides, with estimates of availability consistently below 2%, although the exact level is significantly affected by the sugar moiety and type

of anthocyanidin (Bub et al., 2001; Cao et al., 2001; Cooney et al., 2004; Matsumoto et al., 2001; Milbury et al., 2002). Absorption of anthocyanins at this level is nutritionally relevant, although absorption may be significantly higher than current estimates. This is possibly due to the chemical structure of anthocyanins, which are detected by color at low pH in HPLC/MS, but exist in colorless form *in vivo*. It is possible that metabolic modification *in vivo* prevents the anthocyanin from returning to the colored flavilium cation, resulting in low bioavailability estimates (McGhie et al., 2003). Anthocyanins may also form complexes or bind with other components in the epithelium, preventing their subsequent detection by PDA analysis (Manach and Donovan, 2004).

The estimated half-life of anthocyanins in serum is about 130 minutes, following first-order kinetics (Hou, 2003; Galvano et al., 2004). Thus, benefits from consumption of anthocyanins are limited to the short term, unless the diet regularly contains anthocyanins at every meal to replenish elimination. The mechanism of absorption is an area of continued research. There is no reported evidence that anthocyanins are actively transported across the mucosal epithelium following consumption. In other words, anthocyanin aglycones are thought to be passively absorbed by diffusion across intestinal membranes. However, quercetin glycosides have been reported to interact with glucose transport receptors (Gee et al., 2000; Gee et al., 1998; Walgren et al., 2000; Wolffram et al., 2002). Because quercetin-glycosides and anthocyanin-glycosides are structurally similar, this raises the possibility that anthocyanins may also interact with glucose-transport receptors, particularly because it is the glucosyl moiety that influences the

bioavailability. Bioavailability of the phenolics naringenin and chlorogenic acid is increased by cooking tomatoes (Bugianesi et al., 2004), and so it may be that anthocyanins are also made more available, again due to their similar structure and location in the tomato fruit. This effect is likely due to changes in the food matrix, although it may also be attributable to cleavage of glucosyl moieties (Manach and Donovan, 2004).

Toxicity of anthocyanins was tested in single doses, and the LD_{50} was found to be 2000mg kg^{-1} in mice and rats, while in dogs a single dose of 3000 mg kg^{-1} had no adverse effects (Morazzoni and Bombardelli, 1996). Over a two month period of consumption of 160 mg twice daily, human subjects tolerated the anthocyanins with minimal side effects. An estimate of anthocyanin consumption for humans is between 180-200 mg day^{-1}, certainly below the toxicity threshold (Galvano et al., 2004; McGhie et al., 2003).

A caveat to the low flavonoid bioavailability, including anthocyanins, is that they may not need to be bioavailable to impart nutritional, antioxidant, or other benefits. Gonthier et al. (2003) studied the fate of flavonoids that persist in the intestinal system until they reach the colon. There, resident microbes metabolize the flavonoids to produce smaller phenolics. These phenolics retain the active reducing group responsible for the antioxidant activity of the flavonoids. Absorption of these phenolics in the colon is high, and thus it may be that some beneficial effects associated with flavonoids are actually the result of their metabolic products. This idea is continuing to be investigated. In the mean time, whether the flavonoids are directly or indirectly responsible for benefits to human

health is not so much of an issue for the horticulturist, for increasing flavonoid levels should enhance health benefits.

Benefits of anthocyanins continue to be discovered in addition to the antioxidant effects on LDLs, including antiproliferative effects on human tumor cell lines, anti-inflammatory effects on lungs, and beneficial modulation of transcriptional activity (Rossi et al., 2003; Seeram et al., 2004; Tsuda et al., 2003). Anthocyanins act as antioxidants in several ways: they inhibit the Fenton reaction, an action believed to be caused by chelation of iron (Tsuda et al., 1996), and they scavenge active oxygen species (AOS) including superoxide radicals, hydroxyl radicals, and singlet oxygen (Tsuda et al., 2000 a and b; Tsuda et al., 1996). These functions of anthocyanins are similar to those reported in the precursor flavonoids. Of particular relevance is the finding that the anthocyanin glucosides of delphinidin, petunidin, and malvidin are the three most reactive towards singlet oxygen (Ichiyanagi et al., 2003). These three anthocyanins are known to be expressed in tomato fruit possessing the *Anthocyanin fruit* (*Aft*) gene (Jones et al., 2003).

One anthocyanin had a unique nutritional benefit: cyanidin-3-*O*-β-D-glucoside rich purple corn ameliorated induced hyperglycemia in mice. This effect was noted in mice fed high-fat diets, with the anthocyanin-rich corn supplemented diet having a significant reduction in the advent of obesity. The cause of this effect was not initially clear, but has since been explained by cyanidin-3-glucoside enhancement of adipocytokine secretion and expression of adiponectin in rats (Tsuda et al., 2004). The significance of anthocyanins in

reducing the incidence of obesity and hyperglycemia in humans remains to be studied. However, it is evident that anthocyanins can affect cell signaling and gene expression, although the mechanisms involved are not known.

Anthocyanins are produced in tomato in many vegetative parts and in the fruit. The anthocyanidins petunidin-, delphinidin-, and malvidin-3-(p-coumaroyl)rutinoside-5-glucoside have been characterized in the vegetative tissue of tomatoes (Mathews et al., 2003). Jones (2000) reported on the detection of anthocyanins in the fruit, with the identified anthocyanidins consistent with those found in vegetative tissues (Jones et al., 2003). It is thought that the anthocyanins are produced to mitigate photooxidation, shielding chloroplasts from excess sunlight (Close and Beadle, 2003; Field et al., 2001; Gould, 2003; Lee and Gould, 2002). Anthocyanins may work synergistically with ascorbic acid in plants to regulate AOS. Plant anthocyanin and ascorbic acid production increases following an increase in flux of AOS (Nagata et al., 2003). Presumably the same synergistic activity will be observed in humans. However, anthocyanins also work independently of ascorbic acid, chelating copper ions, scavenging lipid alkoxyl and peroxyl radicals, and regenerating α-tocopherol (Rice-Evans et al., 1996). Whether AOS mitigation is the function of anthocyanins in tomato fruit has not yet been determined. Regardless, the expression of anthocyanins and increase in precursor flavonoids in the fruit represents a potential increase in the fruit antioxidant profile.

THE ANTIOXIDANT PRINCIPLE IN HUMAN NUTRITION: BREEDING A BETTER TOMATO

With marketing and scientific efforts placing emphasis on beneficial antioxidants, it is crucial to understand what makes antioxidants important. From a nutritional standpoint, the definition of an antioxidant is "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1995). Dietary antioxidants are believed to facilitate the reduction or prevention of oxidative stress *in vivo*. Oxidative stress is a condition of metabolic imbalance of AOS and antioxidants resulting in damage to biological macromolecules, including DNA, proteins, and membranes. AOS are naturally generated within the body and in plants, occur in the atmosphere, and can be produced under certain cooking conditions. The human body has natural defenses to prevent a buildup of AOS. These defense systems include enzymatic proteins, non-enzymatic proteins, small water soluble and lipid soluble molecules. While diet may affect the first two systems indirectly, it has a profound effect on the levels of small water and lipid soluble antioxidants present in the bloodstream. Known antioxidants such as β-carotene, ascorbic acid, and α-tocopherol fall into the category of small molecules important to the human diet (Nagata et al., 2003).

There are many antioxidants that are part of the diet that may provide equal or greater antioxidant protection than α-tocopherol, ascorbic acid, and β-carotene. In 2003 there were 17 compounds present in tomato fruit listed as antioxidants

with possible anti-cancer activities, including the carotenoids, tocopherols, phenolic acids, flavonoids, and a glycoalkaloid (Bowen, 2003). Already, there are more candidates to add to this list. In order to breed a 'high-antioxidant' tomato, it will be necessary to determine the contribution of each known antioxidant compound, investigate any incompatibilities or synergistic activities that may exist, and finally to select fruit based on their elevated content of the antioxidants having the most significant beneficial effects. We report here on such testing, preformed both *in vitro* and *ex vivo*.

Three antioxidant assays were employed in the analysis of the lipophilic antioxidants in tomatoes. Most previous reports of antioxidant activity of the carotenoids use variations on a common theme, using a water-borne radical generator or metal-induced LDL oxidation. Such assays include the copper-induced LDL oxidation assay and subsequent measurement of malondialdehyde or lipid hydroperoxides, the ferric reducing/antioxidant power (FRAP) assay, the thiobarbituric acid reactive substances (TBARS) assay, the total antioxidant activity (TAA) assay, the oxygen radical absorbance capacity (ORAC) assay, and the trolox-equivalent antioxidant capacity (TEAC) assay (Cao et al., 1993 ;Djuric et al., 2003; Esterbauer et al., 1989; Fuhrman et al., 2000; Gaziano et al., 1995; Hininger et al., 2001; Minoggio et al., 2003; Nakanishi-Ueda et al, 2002; Roberts and Gordon, 2003; Samman et al., 2003; Zhang et al., 1994). Other than the TAA assay, these methods are unsuitable for determination of lipid antioxidants like the carotenoids because the assays and their radical generators or initiators of oxidation are water based (Del Rio et al., 2002). The $ORAC_{FL}$ assay used here, as

first reported by Prior et al. (2003), is similar to many of these assays with respect to the use of an aqueous radical generator 2,2'-azobis(2-amineпropane dihydrochloride (AAPH). The assay is made relevant to lipids using randomly methylated cyclodextrone, a complex that suspends lipids within an aqueous environment. The PhotoChem assay, a proprietary antioxidant measuring system of AnalyticJena (Konrad Zuse Straße 1, Jena, Germany) also uses an aqueous, light sensitive radical generator. The third assay, recently named the Total Antioxidant Performance (TAP) assay, uses the lipophilic radical initiator 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) (Aldini et al., 2001). This ensures that the carotenoids have ample opportunity to act directly as antioxidants, rather than indirectly when water-soluble radical generators are used. Reported here is an overview and comparison of the various tomato antioxidants and their activity based on these three antioxidant assays.

This purpose of this work is foremost to explore the potential avenues for improvement of tomato fruit antioxidant capacity. Based on the hypothesis that increased anthocyanin content will increase antioxidant capacity, it is anticipated that an intensely purple pigmented tomato will elevate the fruit antioxidant capacity beyond that of conventional tomatoes. The effects of the lipid-soluble antioxidant will also be considered both in the conventional tomatoes as well as the anthocyanin-expressing tomatoes. Since lycopene is the most active and prominent antioxidant present in tomatoes, the red high lycopene tomatoes are expected to have higher lipophilic antioxidant status than any other colored tomato. This hypothesis will be tested both *in vitro* and *ex vivo*.

Chapter 2.

CHARACTERIZATION AND BREEDING OF THE TOMATO FRUIT WITH ANTHOCYANIN-EXPRESSION INDUCING TOMATO GENES

INTRODUCTION

Tomatoes are often classified by market type and fruit color in seed catalogues. Typical classifications include processing, fresh market or home garden, red, orange, yellow, green, and black/purple. It is unfortunate that 'black' is a description that has been attached to heirloom varieties exhibiting a pink, brown, or dark green fruit color. These colors are the result of many varying genetic factors, usually with one common underlying gene, which is most probably green flesh (*gf*) (Butler, 1962). Kerr (1956) described the *gf* color as "dirty purplish brown", and the classification of *gf* fruit as purple began. The trait has been around in uncharacterized form since at least the 19th century. Vilmorin's (Vilmorin –Andrieux, 1885) Vegetable Garden lists several tomatoes ('Tomate Pomme Rose', 'Tomate, Pomme Violette', 'Criterion', and 'Tomate Belle de Leuville') that are rose or pink colored that ripens to an "almost violet" hue. The descriptions are strikingly similar to certain heirloom varieties grown today that have a green flesh like phenotype. Allelism tests remain to be done to verify that the phenotype in the heirloom varieties is a product of *gf*. The phenotype produced by *gf* is similar to the pepper (*Capsicum annuum*) gene *cl*, which prevents the normal breakdown of chlorophyll, and results in a brownish-green color upon ripening (Butler, 1962; Smith, 1950; Hornero-Mendez and Minguez-Mosquera, 2002). In the normal ripening process of tomatoes, chlorophyll concurrently breaks down while carotenoids accumulate (Butler, 1962). In fruit

with the *gf* gene, the chlorophyll does not break down, although carotenoids accumulate normally (Kerr, 1956). Jones (2000) used HPLC to examine heirloom tomato fruit with putative *gf* alleles, and found pheophytin, chlorophyll lacking the magnesium molecule, accumulating in significant amounts. Ripe *gf* fruit are an olive brown color as a result of the combined pigments of pheophytin and lycopene. The color is not a true purple in terms of light absorption, showing λ-max peaks at about 440 and 660 nm (Jones, 2000) rather than ~520 nm. 'Black' varieties such as 'Black Krim' and 'Black Plum' have been postulated to contain anthocyanin accumulation to produce their muddied color (Cox et al., 2003). It is possible that when grown elsewhere under higher light intensity that these black varieties may accumulate anthocyanins. To clarify the issue, the purple fruit containing anthocyanins described in this report, although sometimes black in appearance, will always be called purple fruit, and 'black' will be used to describe only heirloom green flesh varieties.

To achieve true purple color in fruit requires the accumulation of anthocyanins, a trait associated with the genes *Anthocyanin Fruit* (*Aft*) and *Aubergine* (*Abg*). *Aft* was first described as *Af* by Georgiev (1972), with the gene causing anthocyanin expression in the subepidermal tissue in both green and ripe fruit. The dominant allele, later characterized by Jones (2000) and renamed *Aft* (to prevent confusion with *anthocyanin free* of *af*; Jones et al., 2003), initiates anthocyanin expression in immature green fruit with continued accumulation throughout development. Environmental effects that enhance gene expression include exposure to cold temperatures and particularly by high light intensity.

Abg was described by Rick (1994). Like *Aft*, *Abg* causes anthocyanin accumulation in the fruit, and is also enhanced by high light intensity. Unlike *Aft*, *Abg* has variegated expression. This is apparent as a blotchy, flecked appearance that may form a solid, intensely pigmented sheet by maturity as long as the fruit receives at least partial or even indirect light exposure. It is this intense coloring which led to the allele name *Aubergine*, a reference to the purple color found in eggplant (*Solanum melongena*) fruit.

Another gene in tomato that influences anthocyanin accumulation is *atroviolaceum* (*atv*). This gene causes purple pigmentation in foliage and fruit of *L. pimpinellifolium*, although the purple fruit phenotype was not been recovered in *L. esculentum* (Rick, 1964). Cool temperatures enhance the *atv*-induced anthocyanin accumulation. Kerckhoffs et al. (1997) postulated that *atv* encodes a repressor within the phytochrome signal amplification pathway. When *atv* is present in homozygous recessive condition, the phytochrome signaling pathway perceives a high red irradiance response, resulting in an elevated anthocyanin response throughout the plant. This anthocyanin response is twice as strong in red light compared to blue light (Kendrick et al., 1997). *Aft* and *Abg* do not appear to work within this pathway, although *Aft* does produce characteristics typical of a high-photoperiod response mutant. The *atv* gene differs from *Abg* and *Aft* in that it is inherited as a single recessive, whereas the latter two are inherited as single dominant genes. It may be possible to increase the anthocyanin response in fruit through the combination of *atv* with either *Aft* or *Abg*. The effects of such genetic combinations are explored in this work.

Aft and *Abg* produce an anthocyanin response in the cell layer directly beneath the epidermis. The intensity and timing of the response varies among the genes, although they share the property that no other layers are affected despite the fact that other layers of leaf, stem, and fruit tissue have the capacity for plastid development (Kendrick et al., 1994). Regulation of the flavonoid pathway that leads to the production of anthocyanin pigments is tissue- and developmental-specific. The flavonoid and flavonols naringenin chalcone, quercetin-glycosides, and kaempferol-glycosides accumulate almost exclusively in the peel during fruit ripening (Krause and Galensa, 1992; Muir et al., 2001). Analysis of enzyme transcripts revealed that *chalcone synthase* (*chs*), *chalcone isomerase* (*chi*), and *flavonols synthase* (*fls*) are below detection levels in fruit pericarp, while in the peel *chs*, *flavonoid-3 hydroxlyase* (*f3h*), and *fls* are expressed in abundance (Verhoeyen et al., 2002). Thus, it is unlikely that the three-way mutant gene combination of *Aft*, *atv*, and *Abg* should produce an anthocyanin response in tissue layers of tomato fruit other than the peel. To produce anthocyanin in other layers will require another gene or genes that regulate the flavonoid pathway specifically in the pericarp and placental tissues. To date, no gene has been described in tomato that produces an anthocyanin response in the internal tissues of vegetative or reproductive organs.

Flavonol expression in the fruit peel varies 10-fold among commercially available cultivars (Verhoeyen et al., 2002). However, significant increase to these levels can also be achieved through the expression of transgenic *chi*. An increase in flavonoids can be accomplished by the simultaneous expression of *CHS* and

FLS genes. Verhoeyen et al. (2002) postulated that a downstream "pull" in the pathway is required to increase flavonoid production in addition to the expression of *CHS*, and that *FLS*, but not *CHI* or *F3H* acts as such a carbon "pull". This is the case in both peel and pericarp tissues, as shown by an increase in flavonoid production following the transgenic expression of *CHS*, *CHI*, and *FLS*.

The *hp-1* mutant allele has pleiotropic effects resulting from the gene interactions with the photoresponse pathway (Lieberman et al., 2004; Peters et al., 1998; Peters et al., 1992). While the most prominent effects observed are an increase in carotenoids and ascorbate, a more subtle effect is an increase in anthocyanin in the subterranean portion of the hypocotyl. Anthocyanin expression is also elevated in other plant tissues. The anthocyanins from the hypocotyls, identified as petunidin, malvidin, and delphinidin, were identical to those identified in wild type tomatoes (Ibrahim et al., 1968). The primary function of the *hp-1* gene is to produce a damaged-DNA binding protein (Lieberman et al., 2004). This protein is important for DNA repair, and is known to have a high affinity for UV-damaged DNA (Fu et al., 2003). The protein interacts with *DEETIOLATED1*, a gene in which mutations such as *dg* and *hp-2* result in exaggerated photomorphogenic de-etiolation (Levin et al., 2003; Schroeder et al., 2002). *DET1* also has the pleiotropic effect of increasing anthocyanin production, likely a result of its causing an upregulation of *CHS* (Peters et al., 1998). Combining the *dg* or *hp-2* gene alleles with the *hp-1* gene does not further increase anthocyanin expression in the plant or fruit (Chris Bowler, Personal communication). However, by combining the high pigment genes with *atv*, *Aft*, or

Abg, it may be possible to produce elevated anthocyanin production in the fruit tissues. In the case of *hp-1*, the anthocyanin expression inducing genes should elevate anthocyanin production due to the increased the pool of precursors.

It is common to observe purple stripes on ripening fruit of wild tomato species *L. hirsutum* and *L. peruvianum* (Young, 1954; Roger Chetalat, personal communication). This characteristic is particularly visible in the *L. hirsutum* accession LA2099. Anthocyanin production is evident in the stems as a vivid purple color, and as a thin purple line along the longitudinal axis of the green fruit. The gene or genes responsible for this expression have not yet been described.

Anthocyanins in tomato vegetative tissues have been previously characterized by Ibrahim et al. (1968). Jones et al. (2003) found the anthocyanidins produced in *Aft* as similar to those from vegetative tissues in composition and quantity. Further characterization of anthocyanin composition of other tomato fruit genes is needed. In the present work, *Abg* and *Aft* were combined with the *atv* and *hp-1* genes to examine their effect on anthocyanin expression and quality. Our hypothesis was that when combined, these genes would produce fruit with elevated anthocyanin content and total phenolic content, and that anthocyanin composition would be similar. We also expected that *Aft* would produce fruit with increased carotenoids, but the effects of *Abg* and *atv* on carotenoid content were unknown.

MATERIALS AND METHODS

TOMATO BREEDING PROGRAM

A breeding program was initiated to generate fruit carrying the combined genes *Abg-atvatv* and *Aft-atvatv*. Parents lines LA3668 (*Abg*), LA1996 (*Aft*), LA3736 (*atv*), LA0797 (*atv*), LA3538 (*hp-1*), LA2099 (*L. hirsutum* accession with purple foliage and fruit), as well as carotenoid mutant lines LA3311 (og^c), LA3532 (*r*), LA2996A (*Del*), LA3183 (*t*), and LA2374 (*B*) were acquired from the C.M. Rick Tomato Resource Center, University of California, Davis, CA, with their genetic backgrounds shown in Table 2.1. The anthocyanin mutant gene combinations were generated by making reciprocal crosses of the parents LA3668 with LA3736, LA1996 with LA0797, and LA2099 with LA1996. F_1 progeny were self-pollinated for the *L. esculentum* crosses, and F_2 progeny were selected based on the purple stem phenotype for *atv* and the accumulation of anthocyanin in the fruit for both *Aft* and *Abg*. F_1 progeny of the *L. esculentum* x *L. hirsutum* cross were backcrossed to the *L. esculentum* parent prior to selfing to overcome the self-incompatibility barrier contributed by the *L*

Table 2.1. Tomato accession genetic background

Accession	Gene	Genetic background	Species of origin
LA0797	*atv*	VF36	*L. cheesmanii*
LA1996	*Aft*	Vigoroz?	*L. chilense*
LA2099		wild	*L. hirsutum f. glabratum*
LA2374	*B*	Caro Red	*L. hirsutum*
LA2996A	*Del*	Rutgers	*L. esculentum*
LA3183	*t*	Ailsa Craig	*L. esculentum*
LA3311	og^c	Ailsa Craig	*L. esculentum*
LA3532	*r*	Ailsa Craig	*L. esculentum*
LA3538	*hp-1*	Ailsa Craig	*L. esculentum*
LA3668	*Abg*	not reported	*S. lycopersicoides*
LA3736	*atv*	Ailsa Craig	*L. cheesmanii*

hirsutum parent.

To evaluate the combined effects of *Aft* and *atv* with some of the carotenoid mutant genes, a breeding scheme was devised to generate material which would allow comparison of total phenolic content of the *Aft* and *atv* genes in different carotenoid backgrounds. An *Aft-atvatv* double mutant parent line with normal lycopene and β-carotene expression was crossed to orange and yellow tomato accessions. The yellow and orange tomato lines used were LA3532 (*r*) and LA2374 (*B*) respectively. The most advanced material for both lines produced in the summer of 2004 were in the F_2 generation, producing F_3 seeds. The parent used to produce the *B-Aft-* double mutant did not carry the *atvatv* gene, so the effects of homozygous recessive *atvatv* in combination with *B* and *Aft* were not available for study. Segregants with both *Aft* and the carotenoid mutant alleles *B* or *rr* combined with *atvatv*, were analyzed for carotenoid content, total phenolics, total anthocyanins, and tocopherol content, as were the parents to these mutants and lines carrying the *Abg* and *Abg-atvatv* gene combinations.

ANTHOCYANIN EXTRACTION AND PURIFICATION

Anthocyanins were extracted from the tomato skins using the basic protocol 1 of Rodriguez-Saona and Wrolstad (2001). Replicate extracts were made using ~20g of cryogenically milled tomato peel, and were volumetrically adjusted. To correct for the low total anthocyanin scores obtained when using a fresh skin peel method, the procedure was modified to facilitate more accurate anthocyanin content determinations. Fully ripe fruit were frozen at -23°C and

stored until anthocyanin extraction. The fruit were removed from the freezer and immersed in 50°C water for approximately 10 seconds. This caused the skin to pull away from the pericarp, allowing for complete and clean skin removal. The skin was quickly removed, blotted dry, weighed, and placed in liquid nitrogen for immediate cryogenic milling. This method excludes more of the cell layers found immediately beneath the skin. While in some breeding lines, anthocyanin is expressed in the cell layers beneath the skin, the majority of material had no expressed anthocyanin, and inclusion of the subepidermal tissue diluted overall anthocyanin and flavonoids amounts. This method appeared best for extracting skin anthocyanins, giving a repeatable and uniform extraction of skin tissues across fruit sizes and genotypes.

ANTHOCYANIN SPECTROPHOTOMETRY AND HPLC ANALYSIS

After isolating the anthocyanins in acidified water, the samples were purified by passage through a C_{18} solid phase Sep-Pak mini-column (Waters, Milford, MA, USA) to remove sugars and acids, using basic protocol 2 of Rodriguez-Saona and Wrolstad (2001). A subsample was analyzed by a Shimadzu UV-Vis recording spectrophotometer UV160A (illuminant C) to determine the anthocyanin spectral absorption by transmission. One sample of *Aft-atvatv* fruit was also analyzed for color using a Hunterlab CT1100 ColorQuest transmission spectrophotometer (illuminant C). A sample diluted 50% was placed in a quartz cuvette and analyzed by spectrophotometer from 250 nm to 800 nm. Sample hydrolysis was accomplished using the Basic Protocol 1 of Durst and Wrolstad

(2001) to determine the anthocyanidins. The samples were saponified using the Basic Protocol 3 of Durst and Wrolstad (2001). The HPLC protocol was used as reported by Durst and Wrolstad (2001), with slight modification: Solvent A was Acetonitrile, and Solvent B consisted of 10% Acetic Acid and 1% Phosphoric Acid in water. Chromatographic conditions were initially 2%A, 98%B, with a linear gradient over 25 minutes to 20%A, 80%B, a ten minute gradient to 40%A, 60%B, and a 2 minute hold at 40%A, 60%B before returning to the start conditions, at a flow rate of 1 ml min^{-1}. Simultaneous detection at 280, 320, and 520 nm was recorded using a Hewlett-Packard 1090 photodiode array detector (DAD) (Agilent Technologies Inc, Wilmington, Del.). Twenty µl samples were injected into this system that was equipped with a Prodigy 5µm ODS (3) 100Å (250 x 4.6 mm) column fitted with a 4.0x3.0 mm i.d. guard column (Phenomex, Torrance, CA).

ANTHOCYANIN MASS SPECTROSCOPY (MS)

Samples were analyzed by HPLC-MS to determine the number of anthocyanin groups and their respective masses present. HPLC-MS data were produced using a using a Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan) equipped with DAD and a Perkin-Elmer SCIEX API III triple-quadrupole mass spectrometer (MS) (Toronto, Canada) equipped with an ion spray source (ISV=5500, orifice voltage=50) in positive ion mode. Multiple reaction monitoring mode was used, scanning the mass of the molecular (parent) ions in the first quadrupole (Q1), and scanned for the fragmented ions and anthocyanidins

(daughter ions) in the third quadrupole (Q3). Argon was used as the collision gas, and the nitrogen was used for the nebulizer gas and orifice curtain. The system was equipped with a Synergi 4μ Hydro-RP 80Å (250x2mm) column fitted with a 4.0x3.0 mm i.d. guard column (Phenomex, Torrance, CA). Solvent A was 5% formic acid, 80% acetonitrile (v:v); solvent B contained 5% formic acid. Chromatographic conditions were initially 10%A, 90%B, with a linear gradient 30%A, 70%B in 30 minutes, using a 0.2 ml min^{-1} flow rate. 20 μl sample were injected into this system.

ANTHOCYANIN QUANTIFICATION BY pH DIFFERENTIAL

To determine the total anthocyanin and calculate the differences between *Aft* and *Aft-atvatv* fruit monomeric anthocyanin content was measured in 2003 field grown fruit using the pH differential method (Guisti and Wrolstad, 2001). Replicate anthocyanin extracts were diluted 10 fold in pH 1 and pH 4.5 buffers, allowed to stand for 10 minutes, and scanned for absorption at 520 nm and 700 nm using a Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan). This was done prior to analyzing the purified anthocyanins spectophotometrically, and so 520 nm was used as a estimate for maximum absorption based on the published values for petunidin-3,5-diglucoside in 0.1N HCl (Niketic-Aleksic et al, 1972), given that "petunidin-3-*p*-coumaric-rhamoside" (sic) has been identified in the tomato family of *Solanum* (Rao, 1978).

DETERMINATION OF TOTAL PHENOLICS

Total phenolics were determined using the Folic-Ciocalteau (F-C) method (Singleton and Rossi, 1965) from the ethyl acetate and methanol fractions produced in the anthocyanin extraction procedure. One-half ml of each of the sample fractions was added to 7.5 ml ddH_2O, 0.5ml F-C Reagent and allowed to stand for ten minutes. 1.5ml 20% Na_2CO_3 solution was added and vials kept at 40°C in a heat block for 20 minutes. Vials were placed on ice and immediately measured for absorbance at 755 nm using a Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan). A standard curve was prepared using serial dilutions of gallic acid monohydrate (Sigma).

ANALYSIS OF TOMATO TOCOPHEROL CONTENT

Analysis of tomato tocopherol content was based on the method by Podda *et al.* (1996). Samples of homogenized tomato fruit were saponified with alcoholic KOH. The tocopherols were extracted with hexane and dried under nitrogen. They were then resuspended in 1:1 ethanol:methanol for injection into the HPLC system, and detected by electrochemical oxidation at 500 mV potential using a LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). A Phenomenex RP-Aqueous C-30 10 x 4.6 mm guard column mated to a Phenomenex Develosil 5u RP-Aqueous C-30 150 x 4.6 mm column was used. Isocratic liquid chromatography conditions were used for a six minute run, with the mobile phase consisting of 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate.

EXTRACTION OF TOMATO CAROTENOIDS

Carotenoids were extracted from tomatoes using a modified version of the method described by Ferruzzi et al (2001). All the extractions were completed under gold lamps (Sylvania F40/GO) to reduce light-degradation. Six replicates were made of five ripe tomato fruits that were homogenized in a Waring blender for 2 minutes. Ten g of the resulting tomato purée were added to 1 g $CaCO_3$ and 25 ml methanol in a glass vial with a teflon-lined cap. Samples were vortexed for 2 minutes, followed by centrifugation at 730 xg for 5 minutes. The supernatant was collected in a separate sealed flask, and the juice solids were re-extracted with 25 ml 1:1 acetone-hexane solution. The vials were vortexed and centrifuged, and supernate added to the sealed flask. The tomato juice was extracted again with 25 ml 1:1 acetone-hexane, but not centrifuged after the final extraction. The collected supernate was vacuum filtered through Whatman #1 qualitative paper, and the remaining solid material in the vial was also vacuum filtered into the same flask. The vial and supernate flask were both triple rinsed with ~1-2 ml 1:1 acetone-hexane, pouring the solutions into the vacuum flask. Filtrate was added to a separatory funnel together with 2% NaCl solution, and allowed to stand, sealed, for 20 minutes. Next, the lower aqueous layer was decanted, and the upper hexane layer saved. The hexane layer was placed in a 50 ml flask with 1 g $NaSO_4$ to remove residual water. This solution was then pipetted into a 50ml glass vial with a Teflon-lined cap. The $NaSO_4$ mixture was triple rinsed with 1:1 acetone:hexane, the wash solution added to the glass vial. The solution was then dried under

nitrogen in a water bath at 35°C, and the contents sealed under nitrogen when dry. Ten ml methylene chloride was added to the vial to dissolve the carotenoids as well as any other lipophilic compounds present. Two ml samples of this solution were filtered through a 0.45 μm PTFE membrane Acrodisc filter (Waters, Milford, MA, USA) into an amber vial for carotenoid analysis by HPLC.

DETERMINATION OF TOMATO CAROTENOID CONTENT

Two solvents were used for HPLC: solvent A was 70:30 acetonitrile: 1-butanol, and solvent B 100% methylene chloride. HPLC conditions were based on the method of Lin and Chen (2003), shown in Table 2.2, with linear gradients used for all changes in solvents. A YMC Carotenoid S-5 4.0 x 20 mm guard cartridge (Waters, Milford, MA, USA) was mated to a YMC C_{30} carotenoid column, 250 mm x 4.5 μm (Waters, Milford, MA, USA). A Waters 996 PDA detector (Waters, Milford, MA, USA) scanned between 250-550nm wavelengths at 1.2 nm intervals every 0.5 seconds. all-*trans*-lycopene, all-*trans*-β-carotene, prolycopene, α-carotene, δ-carotene, ζ-carotene, neurosporene, lutein, and *E/Z*-phytoene standards were purchased from CaroteNature (Lupsingen, Switzerland). Standards were dissolved in chloroform. Subsamples were diluted in hexane for purity testing using a UV-

Table 2.2. Linear gradients for solvents A and B used for HPLC of tomato carotenoids. (See text for solvent composition)

Time	A%	B%
0	99	1
1	99	1
20	96	4
50	90	10
55*	88	12
65	99	1

*End of useful data

vis spectrophotometer. Absorption was measured and compared to published λ max and extinction coefficients (Lin and Chen, 2003; Goodwin, 1976). Carotenoid identification was based on retention time and comparison of spectra with standards and with published values for carotenoids in the same or similar solvent systems (Appendix 1).

Extraction efficiency was determined by repeated extractions of *rr* genotype tomatoes. The yellow tomatoes were ideal for determining extraction efficiency because exogenous carotenoids introduced into the extract other than β-carotene will not co-elute with any endogenous carotenoids of the tomato. Lycopene and β-apo-8'-carotenal were added to separate glass vials. The carrier solvent for these carotenoid standards was evaporated under nitrogen. Next, *rr* tomato purée or juice was added to the vial for extraction and the vials were extracted using the previously described procedure. The calculated extraction efficiency using this method was 83.1% for lycopene and 85% for β-apo-8'-carotenal. This indicates that carotenoids were lost during glassware transfers, despite repeated rinses. Degradation was also a factor contributing to the loss of carotenoid standard. All carotenoid data presented in this report are the raw values, and thus represent an underestimate of the carotenoids present.

RESULTS

The color readings of the Minolta 45/0 reflectance spectrometer taken on round whole fruit tomatoes, and the transmittance spectrophotometer readings of the anthocyanin extract gave similar hue angles of ~330-340°. Plotting the

Minolta values on a chromaticity coordinate plot revealed that there is no dominant wavelength, due to the color falling well into the range of the Line of Purples. However, the complimentary wavelength was determined to be 523 nm.

The initial spectral scan of a sample of tomato phenolics by transmission spectrophotometer is shown in Figure 2.1. Peak integration showed major peaks at 311 nm and 549 nm. The peak at 311 is due to the presence of multiple flavonoids other than anthocyanin, compounds which do not contribute to absorption at around 500 nm but which have strong absorption in the UV range. The λ-max peak at 549 nm is far higher than the expected 520 nm of the anthocyanidin petunidin, and as such is evidence of acylation of the anthocyanins present in purple tomatoes.

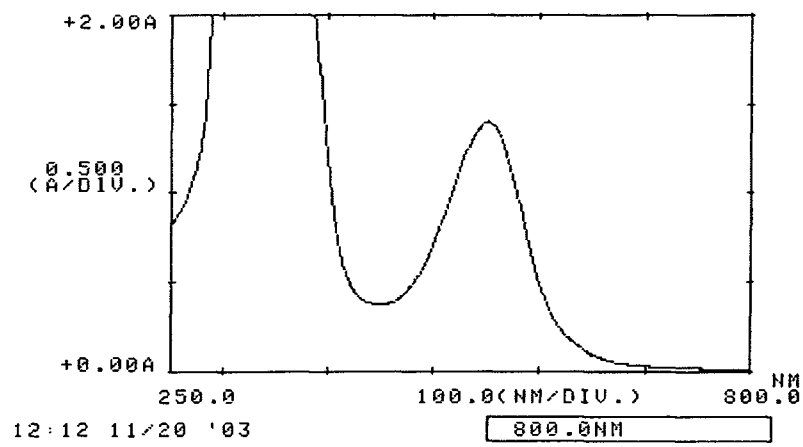


Figure. 2.1. Spectrophotometer spectral curve of *Aft-atvatv* tomato fruit extract anthocyanins in acidified water.

Initial analysis of *Aft*, *Aft-atvatv* and *Abg-atvatv* by HPLC (Figures 2.2, 2.3 and 2.4) revealed near identical peak elution times and respective peak sizes.

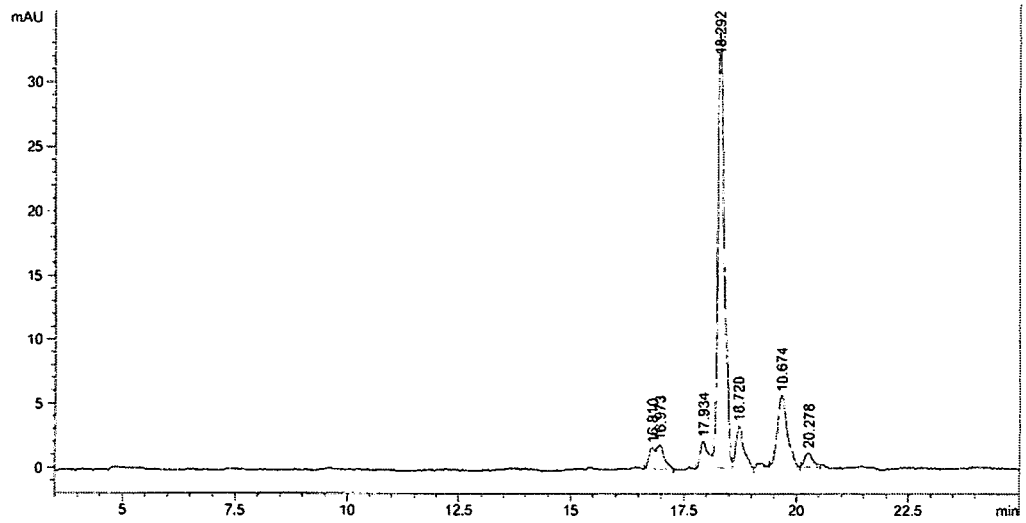


Figure 2.2. *Aft* tomato fruit anthocyanin HPLC chromatogram, 520 nm.

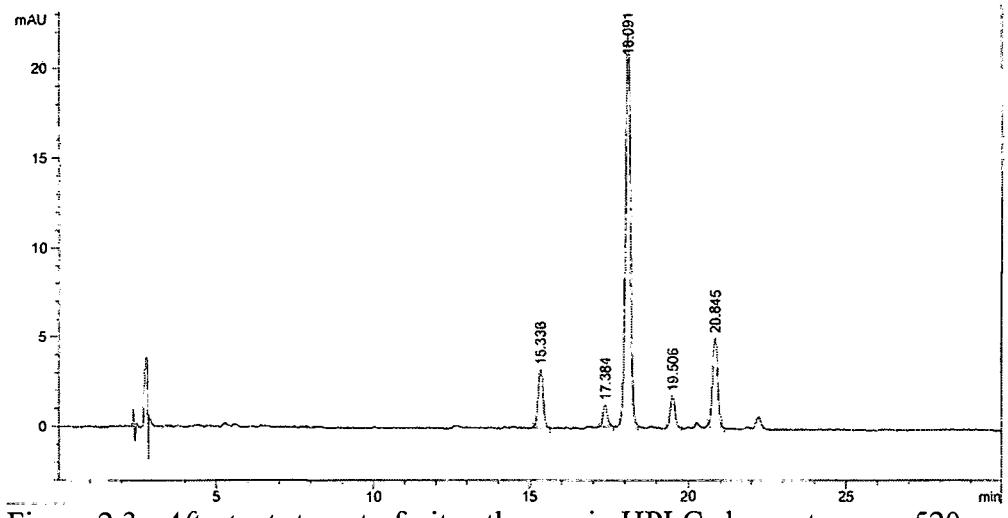


Figure 2.3. *Aft-atvatv* tomato fruit anthocyanin HPLC chromatogram, 520 nm.

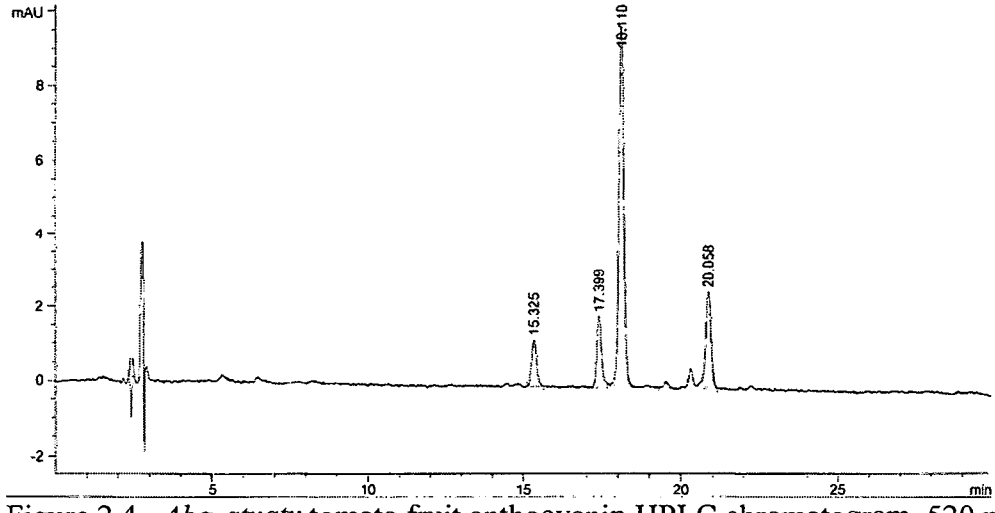
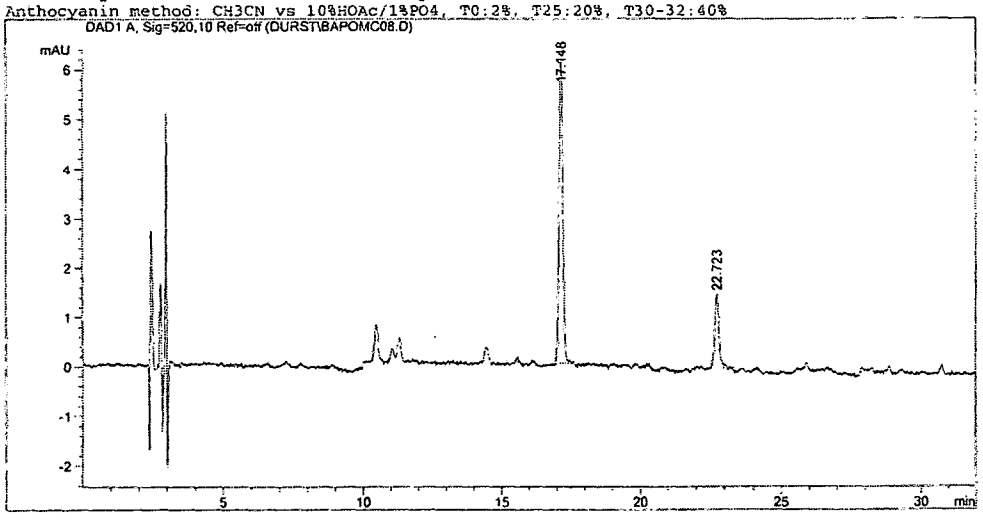


Figure 2.4. *Abg-atvatv* tomato fruit anthocyanin HPLC chromatogram, 520 nm.

Without standards for comparison the peaks were not positively identified. The results are consistent among the three genotypes in that there was one predominant anthocyanin and four minor anthocyanins present in tomato fruit. Due to their similarity, only *Aft* genotype samples were used for subsequent analysis. First, a sample was hydrolyzed to remove the acyl- and glucosyl-moieties to simplify the chromatogram. Peak elution times of the hydrolyzed sample were compared with standards produced from hydrolyzed Welch's Concord Grape Juice (Appendix 3). The results of the chromatogram from hydrolyzed tomato line 02-126-3-3 (*Aft-atvatv*) (Figure 2.5) were consistent with previous reports: the primary anthocyanidin in tomato fruit is the petunidin, with malvidin and delphinidin also



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=520,10 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.148	BB	0.1398	56.40691	6.10506	78.8959
2	22.723	BB	0.1481	15.08848	1.47593	21.1041
Totals :				71.49539	7.58099	

Figure 2.5. HPLC chromatogram of hydrolyzed anthocyanins of *Aft-atvatv* tomato fruit.

present. The ratio of petunidin:malvidin:delphinidin was estimated to be 78:20:~1 based on area under the curve integration of peaks. Other peaks in the chromatogram were not positively identified, although it is unlikely that they were anthocyanidins due to the lack of absorption in the 480-550 nm range.

To determine the moieties attached to the anthocyanin, samples were injected into an HPLC-MS system, allowing spectral analysis followed by mass determination. Prior to this, to provide an initial idea of the masses to expect, a saponified sample of *Aft-atvatv* anthocyanins was analyzed by HPLC, (Figure 2.6). This did not provide any definitive proof of the moieties, although the elution time was consistent with that of a diglycoside. For this reason MS scans were made up to 1100 mass/charge (m/z), since most diglycosides have an m/z of 600-700, and

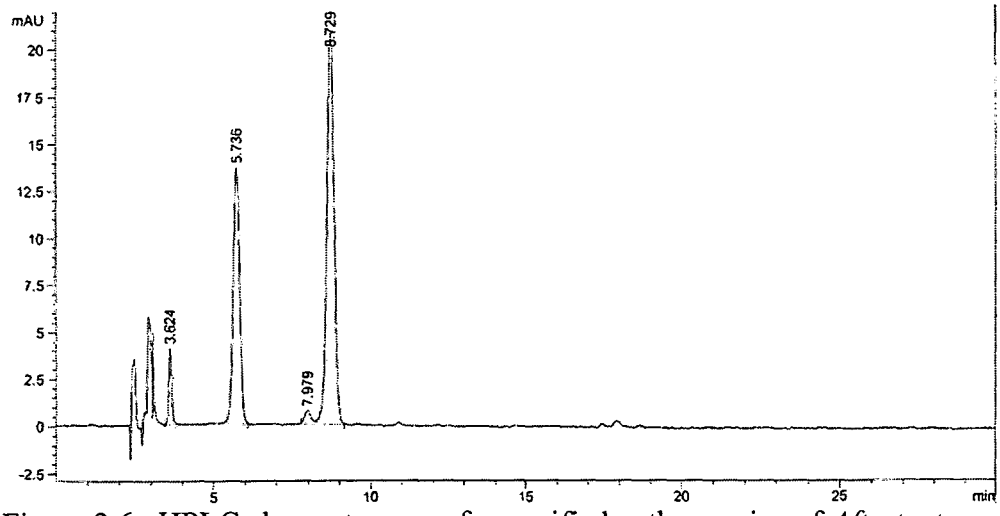


Figure 2.6. HPLC chromatogram of saponified anthocyanins of *Aft-atvatv*.

single acyl moieties adds between 90-160 mz. Parent peaks were identified at 933, 947, 919, 625, 632, and 611 m/z, with daughter peaks at 331, 317, and 303 m/z (Figure 2.8). A subsequent direct injection MS-ES also detected these peaks, as well as several others in lesser quantities (Figure 2.7, Table 2.3). These masses

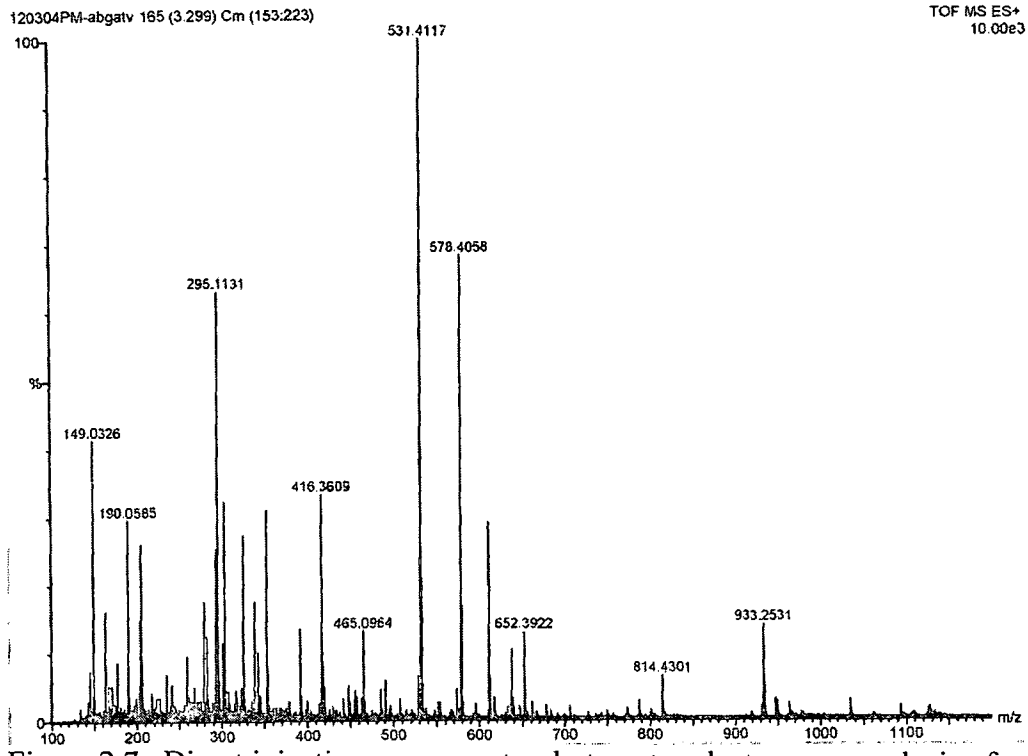


Figure 2.7. Direct injection mass spectrophotometry electrospray analysis of *Abg-atvatv* tomato fruit methanol fraction extract.

Table 2.3. Detected masses and corresponding tentative identification from mass spectrophotometry analysis of *Abg-atvatv* tomato fruit methanol fraction extract.

Functional groups	Anthocyanidin		
	delphinidin	petunidin	malvidin
	303	*317*	331
glycoside	**465**	*479*	*493*
rutinoside	**611**	625	*639*
p-coumaroyl+rutinoside	**757**	*771*	*785*
p-coumaroyl+rutinoside+glycoside	**919**	**933**	**947**
caffeic acid+rutinoside	**773**	**787**	**801**
caffeic acid+rutinoside+glycoside	**935**	**949**	**963**
p-coumaroyl+rutinoside+glycoside+glycoside	1081	*1095*	*1109*
caffeic acid+rutinoside+glycoside+glycoside	1097	*1111*	*1125*

Bold indicates a detected mass
Italics indicates detected in trace quantities
Grey indicates no detection at mass

were compared with all combinations of known anthocyanidins and glycosyl and acyl moieties. The most predominant acylated anthocyanin was the peak at 933

m/z, consistent with petunidin-3-(p-coumaryl)-rutinoside-5-glucoside. The most predominant non-acylated anthocyanin was the peak at 611 m/z, consistent with delphinidin-3-rutinoside. Based on the HPLC-MS relative peak intensities, delphinidin was the dominant anthocyanidin. However, peak intensities do not necessarily correspond to sample concentration due to differences in ionization. More reliable quantitative data came from the HPLC of the hydrolyzed anthocyanins that indicated that petunidin was the predominant anthocyanidin. Analysis of hydrolyzed samples is ongoing. To date, repeat experiments have

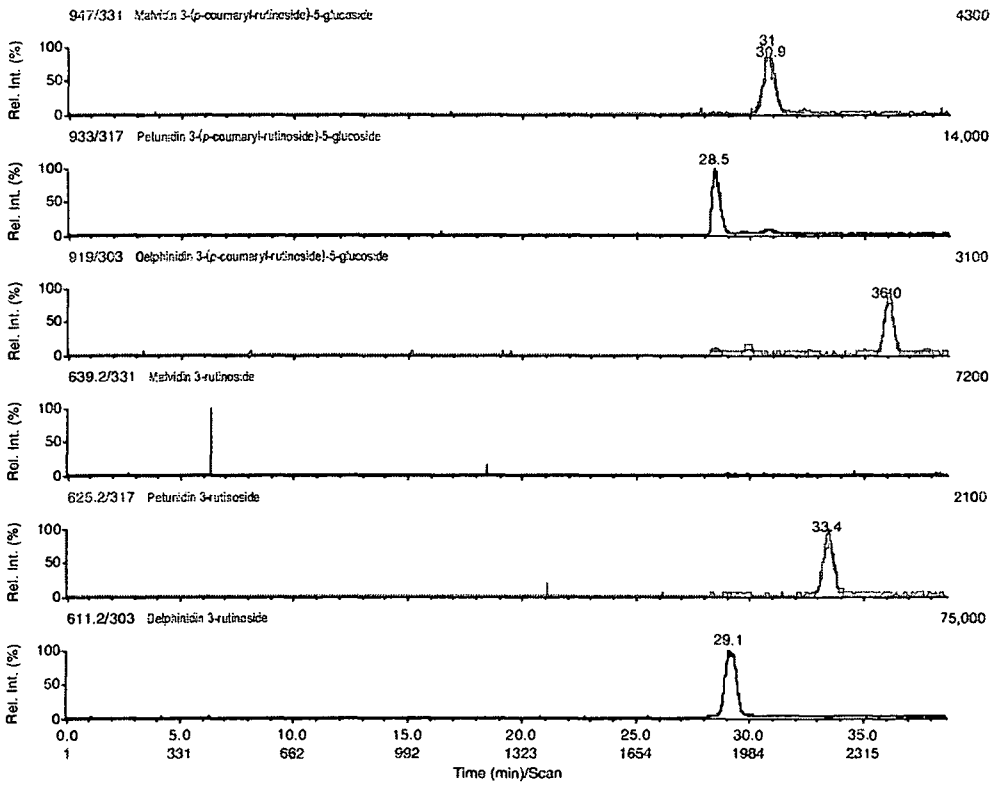


Figure 2.8. HPLC-mass spectrophotometry analysis of *Aft-atvatv* tomato fruit methanol fraction extract.

failed to completely hydrolyze the samples, and so our confidence in the reported ratio of anthocyanins is limited.

ANTHOCYANIN QUANTIFICATION

Petunidin 3-(p-coumaroyl-rutinoside)-5-glucoside was used as the primary anthocyanin present for mass and extinction coefficients in calculating the total monomeric anthocyanin content (Table 2.4). The replicate average value of 13.07 mg $100g^{-1}$ FW is low compared with the previously published value in *Aft* fruit, where fruit have ~ 20-60 mg $100g^{-1}$ FW (Jones et al., 2003). The discrepancy can be explained by the skin removal procedure used for this experiment. The tomatoes had been frozen prior to removing the skin and pericarp for extraction. Without the use of a razor, much green, unpigmented tissue remained and was used in the extraction, thereby diluting the detected anthocyanin on a per weight

Table 2.4. Total monomeric anthocyanin in *Aft-atvatv* tomato fruit

Petunidin 3-(p-coumaroyl-rutinoside)-5-glucoside						extinction	17000		mol wt	933.6
				pH 1.0	pH 1.0	pH 4.5	pH 4.5			
Sample ID	Sample wt	Final vol	Dilution Factor	700 nm	520 nm	700 nm	520 nm	Absorb-ance	Anthocyanin Conc. mg/ml extract	mg/100g FW
Aftatv extract 2	22	50	10.0	0.0090	0.1220	0.0000	0.0130	0.100	0.0549	12.48
Aftatv extract 1	18	25	10.0	0.0010	0.2050	0.0020	0.0270	0.179	0.0983	13.65
Average										13.07

basis. Also, after this analysis it was determined that the tomato anthocyanins have a λ-max at 538 nm, not 520 nm, both factors contributing to the lower-than-expected absorption values and subsequent total monomeric anthocyanin calculated values. Although lower than previously published values, 13.07 mg $100g^{-1}$ FW tissue indicates a high concentration of anthocyanin in the skin tissues. This is consistent with expectations based on visual inspection, as the tomato skins

were intensely pigmented.

An absorption maxima of 538 nm was used when analyzing 2004 field

Table 2.5. Total monomeric anthocyanin content in tomato fruit grown in the field in 2004.

Genotype	Fruit Size, g	Total Anthocyanins, mg 100g^{-1} FW skin [z]		
		Ethyl Acetate	Methanol	Total
Abg-atvatv	37.18	24.03 ± 1.94	390.95 ± 3.4	414.97 [a]
Aft-hp-1hp-1	162.37	2.56 ± 0.24	86.58 ±1.21	89.14 [c]
Aft-atvatv	87.60	4.41 ± 0.01	111.70 ± 0.41	116.11 [b]
Aft-	115.23	1.08 ± 0.31	71.24 ± 0.61	72.32 [d]
Aft-B-	124.37	0.53 ± 0.38	23.24 ± 0.56	23.77 [e]
Aft-atvatvrr	62.83	0.17 ± 0.23	17.70 ± 0.23	17.86 [e]

[a-e] letters indicate significant difference (P<0.05) determined by Fishers' LSD

[z]: Mean of 3 replicate pH differential tests

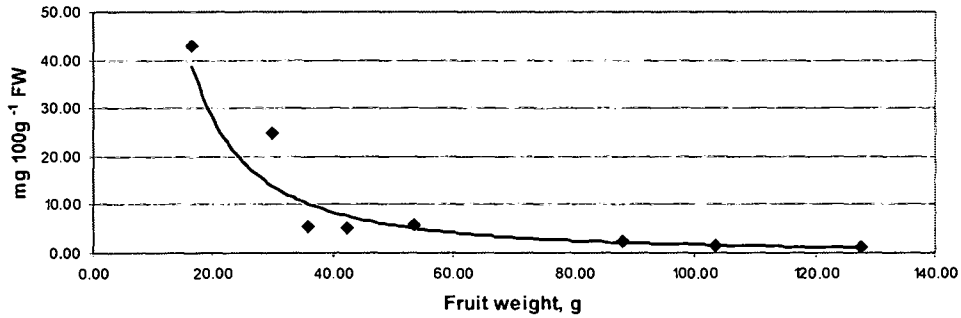


Figure 2.9. Whole fruit anthocyanin versus fruit weight in tomato lines grown in the greenhouse in 2004.

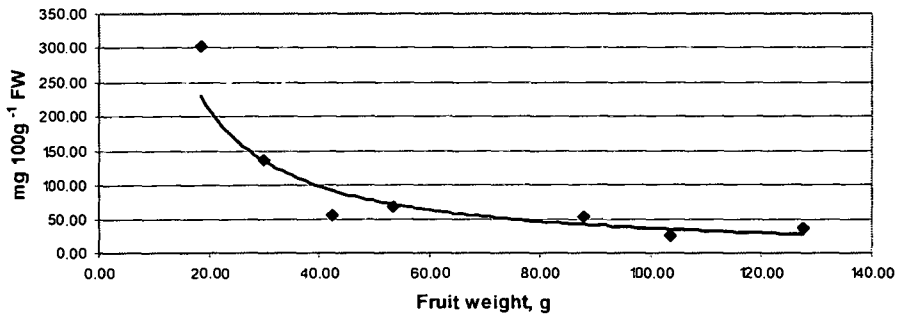


Figure 2.10. Skin anthocyanin versus fruit weight in tomatoes grown in the greenhouse in 2004.

grown fruit, based on the observed maxima from spectrophotometrically scanned 2004 material (Table 2.5). The genotypes tested varied widely in visually observed pigment expression and in fruit size, both having significant effect on total anthocyanins. Figures 2.9 and 2.10 plot the inverse-logarithmic relationship between total anthocyanin content and fruit size for both skin and whole fruit extractions. The figures are the result of multiple extractions of greenhouse grown *Aft-atvatv* genotype fruits of varying sizes. In whole fruit the relationship was primarily due to anthocyanin expression being restricted to the skin, specifically partitioned within the vacuoles of the third and fourth cell layers of subepidermal

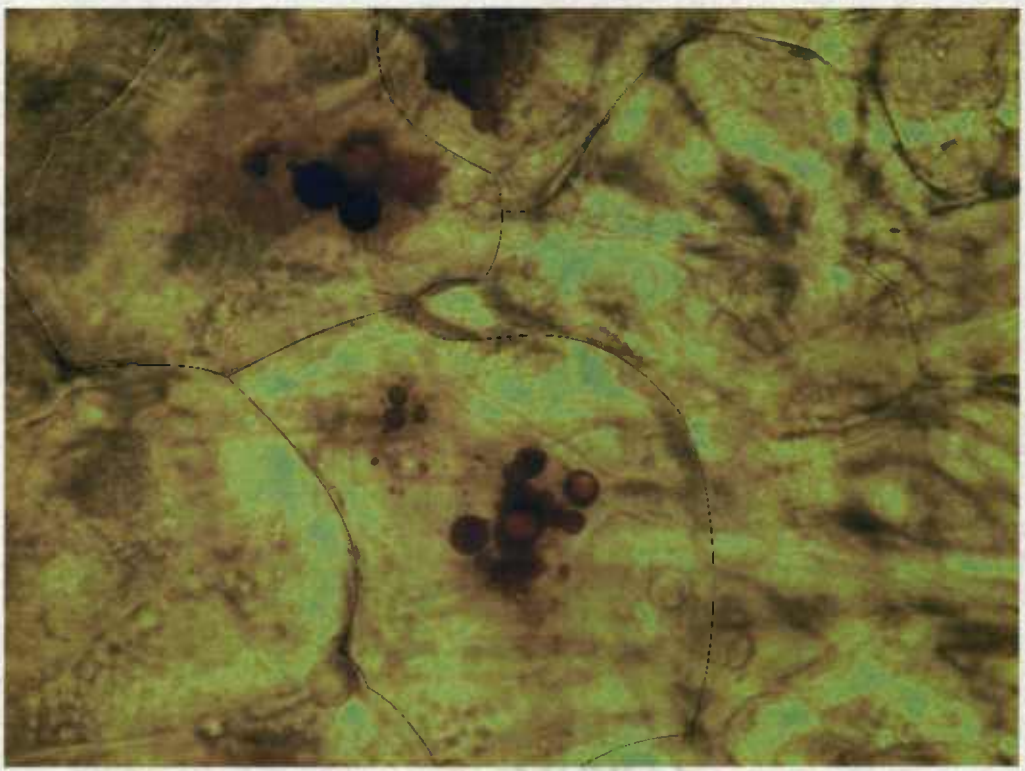


Figure 2.11. Excised tomato fruit skin showing cellular anthocyanin expression in the third subepidermal cell layer (approximate cell walls shown in black).

tissues in tomatoes (Figure 2.11). As fruit size increased, the proportion of skin to

fruit volume decreased exponentially, resulting in lower total anthocyanin per fruit weight. Under such conditions, if anthocyanin expression remained constant in the skin regardless of size, a larger sized tomato will produce lower total anthocyanins per g FW extracted tissue due to the difference in volume. In skin extractions the increase in volume and mass were not a factor in calculating total anthocyanins, because the extracted skin tissue volume is the equivalent of the anthocyanin-expressing tissue volume. The cause of the inverse relationship in skin extractions is instead due to the regulation of anthocyanin expression. Light incidence is a requirement for anthocyanin expression. In smaller fruit, more skin tends to be exposed to light, and there is less self-shading. This results in a greater fraction of the skin expressing anthocyanin. Thus, skins from smaller sized tomatoes have higher total anthocyanins than do larger tomatoes. For this reason, maximizing anthocyanin expression without genetic change in the anthocyanin biosynthetic or regulatory pathways requires that fruit size must be minimized and light incidence maximized.

BREEDING FOR INCREASED FRUIT ANTHOCYANIN PIGMENTATION

The initial goal of the breeding scheme to produce a tomato with increased anthocyanin expression was to combine *Aft*, *Abg*, *atv*, *hp-1*, and the unknown gene or genes in LA2099. To achieve this, *atv* or *hp-1* with *Aft* and *Abg* were concurrently crossed, and recessive genes were selected in the F_2 generation. In the case of the *L. hirsutum* line with the unknown gene(s), interspecific crosses

were made with LA1996, the *L. esculentum* line carrying the *Aft* gene, and Legend, a cultivar developed by the Oregon State University breeding program with no purple color.

An immediate problem arose in the *Abg* material: many lines segregated for sterility and for intensity of anthocyanin expression. Because we were restricted in the number of F_1 and F_2 plants that could be grown in the greenhouse, elevated anthocyanin expression in the *Abg-hp-1hp-1* double mutant was lost. The problem of sterility in crosses using the *Abg* parent line accession LA3668 was also persistent. For example, an advanced line in the F_6 generation still segregated for 1 sterile plant out of 10. Whether this segregation is due to linkage drag of a recessive lethal, a cryptic chromosomal aberration, or due to persistent incompatibility-induced sterility resulting from combining *S. lycopersicoides* with *L. esculentum* remains to be determined. While *Abg* can be combined and expressed with other anthocyanin mutants, we are uncertain as to whether *Abg* has been fixed in the homozygous condition.

Both *Abg-atvatv* and *Aft-atvatv* double mutants were generated, with very similar phenotypes. Figure 2.12 shows the phenotypes of *Aft* and *Abg* individually and when combined with *atv*. The phenotypes of the two combinations were exceptionally difficult to distinguish without knowing the pedigree, although subtle differences were observed during the progression of fruit ripening. The anthocyanin response in both *Aft* and *Abg* mutants was obviously enhanced by the presence of *atv*, although the increase was more striking in *Aft* material. In immature green fruit, the *Aft-atvatv* mutants began forming purple crowns, in

contrast to the *Aft* parent LA1996. In *Aft* material, a speckling pattern opposite the sepals of the calyx was often observed. During breaker and ripening in *Aft* lines, anthocyanin expression was confined to blotchy patches of pigment appearing in a topographical pattern indicative of light incidence and shading patterns. In rare instances, this blotchy pattern accumulated where speckled stripes persisted: the reason for this variable in expression is not clear.

Fig. 2.12. Anthocyanin expression of tomato fruit mutant gene combinations. Left to right, from top to bottom: *Abg, Aft, Abg-atvatv,* and *Aft-atvatv*.

Upon ripening, fruit with both *Aft-atvatv* and *Abg-atvatv* mutant combinations appeared in red fruit with completely purple crowns. However, the *Abg-atvatv* lines tended to have greater coverage of the skin with anthocyanin expression, although the intensity of the color was similar in both mutants. In well

exposed fruit, *Abg-atvatv* double mutant fruit expressed anthocyanin uniformly over the entire skin surface to produce a nearly black fruit due to the intensity of the purple pigmentation.

Crosses to generate a triple mutant with *Abg*, *Aft*, and *atv* are difficult to score visually due to the similarity of phenotypes of the two double mutant combinations. F_3 progeny of an *Aft-atvatv* x *Abg* were scored as 100% *Aft-atvatv* based on the collection of fruit and plant characteristics, including purple stems, purple crowns but not blossom ends, and large dark green leaves. By comparison, an *Abg* phenotype would exhibit purple color on the crowns that would on occasion extend all the way to the blossom end, small pale yellow leaves, and green stems. That all the F_3 plants had the purple stems indicates that at least *atv* was fixed in the selection of the F_2 parent. It is possible that the *Abg* allele was inadvertently discarded in the F_2, although this is unlikely given the dominant nature of the allele. Scoring the double mutants of *Abg* and *Aft* was equally problematic. Working on the hypothesis that *Abg* and *Aft* were alleles of separate and distinct genes, we predicted that the two mutant genes would combine additively to express anthocyanin pigment in excess of either parent. This hypothesis was found to be false from examination of F_2 materials. Segregation in the F_2 was difficult to assess in the field, as entire plots from reciprocal crosses exhibited an *Aft*-striped pattern of expression. While this might indicate that the mutants were in fact allelic, under greenhouse conditions, segregants had fruit exhibiting both *Aft* striping and *Abg* induced late-development purple crown. When grown in the field, these segregants were phenotypically scored as all *Aft*.

This is particularly puzzling because we expected to observe the much stronger phenotype of *Abg* in the F_1, F_2 and F_3 generations. Because *Abg* is dominant, and assuming normal transmission of the gene, the *Abg* phenotype should have been observed in about ¾ of the F_3 progeny. In addition, 2004 field grown backcross segregants of [(*Aft* x *Abg*) x *Abg*] (BC_1F_1) were scored as *Abg*, and no *Aft* traits were observed. It is possible that in the greenhouse, the material was phenotyped incorrectly, and that *Aft* and *Abg* are indeed allelic. However, the segregation numbers observed in the greenhouse did not support this either, since F_2 plants should have segregated 25% *Aft*, 50% dominant allele, and 25% *Abg*, but were not observed to do so. An alternative possibility is that *Aft* and *Abg* are distinct, non-epistatic genes that should segregate 9:3:3:1 (or 15:1 if heterozygotes are indistinguishable) in the F_2 generation. While this ratio of segregation was not observed in the greenhouse, growing a greater number of F_2 progeny in the field would improve the odds of generating all four genotypes. At present, there are two working hypotheses of this program: *Abg* and *Aft* are neither allelic nor complimentary in their putative regulatory role in the anthocyanin pathway, or they are allelic, with dominance determined by the environment. A repeat of crossing the two parents, with all progeny generations grown in both greenhouse and field conditions will be necessary to determine which hypothesis is correct.

Incorporating the *L. hirsutum* purple color trait progressed slowly due to the self-incompatibility barrier from *L. hirsutum*, slow greenhouse growth of the *L. hirsutum* parent line and the interspecific F_1, and slow fruit maturation in the F_2, (72 days later than the Legend, and 50 days later than LA1996). It was not until

Figure 2.13. (LA2099xLA1996) x LA1996 BC_2F_1 fruit. From top to bottom: 93 days and 120 days after seeding.

the summer of 2004 that the BC_2F_2 progeny of the Legend and LA1996 crosses

were grown. This is fully two generations behind other crosses initiated at the same time (with three generations grown per year). Of the evaluated segregating material, the progeny of the Legend cross exhibited light speckled fruit skin pigmentation, while the progeny of the LA1996 cross exhibited the most intense pigmentation observed of any material evaluated in the breeding program (Figure 2.13). The *Aft* phenotype was clearly visible in the early development of skin pigmentation. This was masked 30 days later in some segregants; the still-unripe fruit became completely purple. Other segregants maintained the *Aft* phenotype only, and were considered to have lost the genetic contribution of the *L. hirsutum* parent. Still other segregants exhibited a previously undescribed phenotype shown in Figure 2.14, with a uniform speckling pattern of anthocyanin

Figure 2.14. New anthocyanin color and pattern of expression in (LA2099xLA1996) x LA1996 BC_2F_1 fruit.

expression with a color not seen in either *Aft* or *Abg* material. Due to the lateness of maturation of this fruit, the anthocyanin responsible for this color was not characterized by HPLC, although visual evaluation suggests that there is a

difference in either anthocyanidins or glycosyl-/acyl- moieties due to the apparent difference in spectral absorbance and resulting shift in observed color.

COMPARING THE EFFECTS OF ANTHOCYANIN EXPRESSION ENHANCING GENES IN DIFFERENT CAROTENOID BACKGROUNDS

Stable, non-segregating plant selections were obtained for the *atv* and *Aft* combination, resulting in types with attractive pigmentation. In addition to increasing anthocyanin pigment expression, this gene combination also increased fruit phenolic content. To evaluate the combined effects of *Aft* and *atv* with some of the carotenoid mutant genes, a breeding scheme was devised to generate material which would allow comparison of total phenolic content of the *Aft* and *atv* genes in different carotenoid backgrounds. An *Aft-atvatv* double mutant parent line with normal lycopene and β-carotene expression was crossed to a yellow tomato accession, while an *Aft-* mutant was crossed to a high β-carotene mutant. The progeny of these crosses, shown in Figure 2.15, exhibited anthocyanin

Fig. 2.15. Tomato fruit of *rratvatvAft-* and *B-Aft-* triple and double mutants.

expression with little indication of interaction between the mutations in the carotenoid pathway having influence on anthocyanin accumulation.

Analysis of the different tomato mutant genotype carotenoid profiles is shown in Table 2.5, total phenolics and anthocyanins in Table 2.6 and Figure 2.16, tocopherol content in Figure 2.17, and total carotenoids in Figure 2.18.

Table 2.5. Carotenoid content of fresh tomatoes, μg g^{-1} FW.

	Genotype									
Carotenoid, μg/g FW	*og*^{c}*og*^{c}	Wild type	*rr*	*Aft-*	*Aft-atvatv*	*Aft-B-*	*Aft-rr*	*Aft-hp-1hp-1*	CaroRich	*B-*
trans-Lutein + 9-cis lutein	0.37	0.45	0.52	0.49	0.49	0.49	0.45	0.55	0.57	0.55
Phytoene, E/Z	12.22	6.40		6.61	4.79	4.90		9.42	6.52	4.66
Phytofluene	7.80	3.40		3.83	2.65	2.48		6.86	2.66	2.36
unknown_1									0.57	
9-cis-beta-carotene	1.39	1.14				1.92				1.82
Zeta-carotene	0.71	0.69		0.54	0.50	0.59		0.70	6.44	0.56
Beta-carotene	6.90	7.68	0.24	4.63	3.96	21.10	0.18	16.58	24.51	20.50
unknown_2								0.77	2.67	
cis-beta-carotene	0.58	0.38		0.17		0.61			2.94	0.58
15-cis-lycopene	0.86			1.08	0.67			0.65		
cis-delta-carotene ?									0.15	
unknown_7				5.14					2.12	
13-cis-lycopene ?	2.37			0.93	2.93	0.61		1.34	0.72	
unknown_9									1.09	
9,13-di-cis-lycopene	5.24	4.13		1.96	0.72	2.30		2.76	1.24	0.91
unknown_11	1.54	0.68		1.96		1.10			1.66	1.05
9-cis-lycopene	0.94	0.63		0.79	0.56				1.06	
5-cis-lycopene	6.03	1.71							0.76	
all-trans Lycopene	64.25	24.00		32.88	15.48	11.43		55.63	8.72	5.20
Total	111.21	51.29	0.77	61.00	32.74	47.53	0.63	95.26	64.58	36.43

Table 2.6. Effect of genotype on tomato skin phenolic content and total monomeric anthocyanin content of tomato lines varying in flavonoids and carotenoid profiles.

	Gallic Acid Equivalents, mg g^{-1} FW skin [x]			Total Anthocyanins, mg $100g^{-1}$ FW skin [y]		
Extract	Ethyl Acetate	Methanol	Total	Ethyl Acetate	Methanol	Total
Abg-atvatv	166.2 ± 4	284.7 ± 1.5	451 [a]	24.03 ± 1.94	390.95 ± 3.4	414.97 [a]
Aft-atvatv	71.7 ± 1.9	147.3 ± 6.2	219 [c]	4.41 ± 0.01	111.70 ± 0.41	116.11 [b]
Aft-hp-1hp-1	98.8 ± 0.9	182.3 ± 1.2	281.2 [b]	2.56 ± 0.24	86.58 ±1.21	89.14 [c]
Aft-	67.2 ± 1	83.9 ± 8.6	151.1 [d]	1.08 ± 0.31	71.24 ± 0.61	72.32 [d]
Aft-B-	86.1 ± 1.3	133.7 ± 5.2	219.8 [c]	0.53 ± 0.38	23.24 ± 0.56	23.77 [e]
Aft-atvatvrr	48.9 ± 0.1	49.1 ± 1.3	98 [e]	0.17 ± 0.23	17.70 ± 0.23	17.86 [e]
rr	77.3 ± 6.5	74.4 ± 2	151.7 [d]	0.00	0.00	0 [f]
CaroRich (*B*)	100.3 ± 3.7	112.9 ± 1.6	213.2 [c]	0.00	0.00	0 [f]

[a-e] letters indicate significant difference (P<0.05) determined by Fishers' LSD

[x]: Mean of 3 replicate Folin-Ciocalteau reactions

[y]: Mean of 3 replicate pH differential tests

Observed effects of *Aft* were similar to those reported by Jones (2000) in red tomatoes, with an associated increase in total carotenoids in *Aft* compared to

wild type tomatoes ('Caro Red' background). This carotenoid increase was prevented when the *r* gene is present, as observed in the *Aft-rr* line. The effect of combining *Aft* with *atv* is unclear from this material. Tomatoes with both these genes have the lowest total carotenoids of all the carotenoid expressing tomato lines tested.

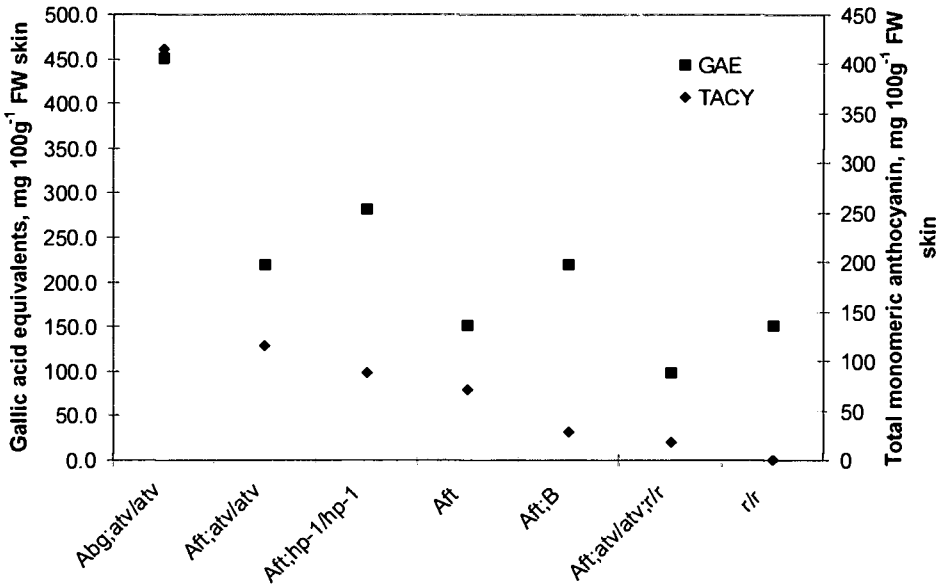


Figure 2.16. Relationship of phenolics and anthocyanins in tomato skins. ■: gallic acid equivalents, a measure of phenolic content (left scale), ♦: total monomeric anthocyanins (right scale).

Because the *atv* parent (LA0797) was not analyzed for comparison, it's genetic contribution to total carotenoids cannot be determined. When *Aft* was combined with *B*, total carotenoids were less than observed in the wild type or *Aft* parent tomatoes, but show a putative increase over the *B* parent. It should be noted that values for *B* came from material grown in 2003 because the 2004 planting was discovered to be wild type, not *B* at the CycB locus. Assuming no environmental effect, *Aft* causes an increase in total carotenoids and in β-carotene when combined

with *B*. The 'CaroRich' variety, another *B* gene line, is also shown for comparison. Although the genetic background of this variety is quite different from the other lines tested, it shows a similar carotenoid profile to L2374 (*B*). It also had a greater total carotenoid content than either the *Aft-B-* mutant or L2374.

DISCUSSION

It has been reported that mutations in the carotenoid pathway and subsequent changes in the carotenoid profile may also cause changes in phenolic content (Cox et al., 2003; Martinez-Valverde et al., 2002; Singh et al., 2004; Minoggio et al., 2003). In all three reports the phenolic content is elevated in high lycopene content tomatoes, and Cox et al (2003) also reported that it was lower in yellow tomatoes than in orange or red, while Minoggio et al (2003) report that high phenolics correlated with high lycopene but not with high β-carotene lines. This correlation was not borne out in the current data. While lines with low lycopene did have low total phenolics, the lines with highest total phenolics were not the lines with highest lycopene content. This is not surprising due to the near complete independence of the flavonoid and carotenoid biosynthetic pathways.

When the effects of the mutant alleles on both total anthocyanins and phenolics are considered, several trends become evident, although comparisons of these genotypes are of inherently limited value, as the breeding lines are not isogenic. An obvious genetic difference not related to anthocyanin and carotenoid genes is fruit size, which varied significantly among the lines. As previously discussed, this had a major impact on the anthocyanin expression. It is safe to

assume that this same effect will carry through to all phenolics by means of the regulation of the entire flavonoid pathway of which anthocyanins are a part. That said, useful observations can still be made from the data. *Aft* does not necessarily increase phenolics, because the *Aft-B-* double mutants have increased phenolics while *Aft-rr* double mutants have lower total phenolic content. Likewise, *atv* does not appear to enhance phenolics in the *rr* mutant. This suggests that the yellow tomato (*rr*) has other regulatory mechanisms in place that override possible increases caused by either *Aft* or *atvatv*. When combined with *Aft* or *Abg*, *atvatv* produces tomato skins with the highest measured phenolic content and high total anthocyanins, with the significantly different GAE values likely being due to the effects of fruit size. The *Aft-hp-1/hp-1* double mutant also produced elevated phenolics, despite a relatively low total anthocyanin content compared with the other high phenolic scoring genotypes. One may speculate that this effect is due to the light-sensitivity of the *hp-1* gene causing an increase in the flavonoid pathway without channeling metabolites all the way to the anthocyanin pathway. This may be due to timing of flavonoid pathway activation. Anthocyanins expression requires induction early in fruit development in both *Aft* and *Abg* fruit as well as in double mutants with either *hp-1* or *atv*. The mutant allele *hp-1* may upregulate the flavonoid pathway throughout fruit development after the effects of *Aft* on the flavonoid pathway seem to diminish. This would result in increased flavonoids later in fruit development without anthocyanin accumulation, while also increasing anthocyanin accumulation during early fruit development when the *Aft* phenotype is expressed. Further evidence to support this hypothesis is not provided here,

although this explanation agrees with the reports of *hp-1* increasing the light response in all plant tissues. Another more global observation across the genotypes is that there is a trend of increased total phenolics correlating positively with increased anthocyanin content (R^2=0.78). Such a relationship is not unexpected. Increasing anthocyanins by unspecific upregulation of the flavonoid biosynthetic pathway will also result in an increase in other flavonoids, subsequently observed as an elevated GAE score. Although the biochemical function or activity of *Aft* and *Abg* is not known, it is unlikely that either gene is a biosynthetic gene in the flavonoid pathway, because tomatoes without these genes express anthocyanins in other plant tissues. More plausible is the possibility that the genes act as direct or indirect regulators of the flavonoid and anthocyanin pathway. Apart from the cause of the increase, it is worth noting that the size of the increase is significant: a previous report of total phenolics of many red tomato cultivars found no tomato with above 33 mg/100g FW total phenolics as gallic acid equivalents using the Folin-Ciocalteau method as applied in this work (Singh et al., 2004). Introduction of the anthocyanin-expression inducing pathway increased the total phenolics as high as 457 mg/g FW. As phenolics correlate highly to total antioxidant capacity of tomatoes, the observed increases in phenolics reported here are expected to have a significant impact on antioxidant activity.

The effects of both carotenoid and anthocyanin mutant genes on fruit tocopherol content have not been reported previously. Carotenoids and tocopherols are not reported to compete directly for metabolites, although they

share a common precursor in isopentenyl diphosphate. Comparing the tocopherol content with total carotenoids (Figure 2.17), a positive correlation is evident, with a few exceptions (R^2=0.62, excluding the low carotenoid mutant lines containing *r*). At present, given the genetic variability among the sampled material, the possible explanations for the apparent relationship include: 1) the lines are not completely isogenic: genetic factors other than the carotenoid mutants and anthocyanin-enhancing mutants are responsible for the differences in tocopherols; 2) upregulation of the carotenoid pathway results in increased tocopherol production by way of increased production of precursors, and thus the relationship between the two is not based on feedback between the two pathways, but stems from both drawing on the same pool of precursors; 3) a signaling pathway triggers a response in tocopherol production when the carotenoid content increases due to perceived or realized increase in oxidant/energy flux. While possible, the first explanation is not likely because it requires several cases of spontaneous association between tocopherol and carotenoid profile. Concerning the second explanation, an apparent weakness of the carotenoid-tocopherol common pool relationship is shown by the *r* and *Aft-rr* material, because they have reduced carotenoids but maintain their tocopherol content.

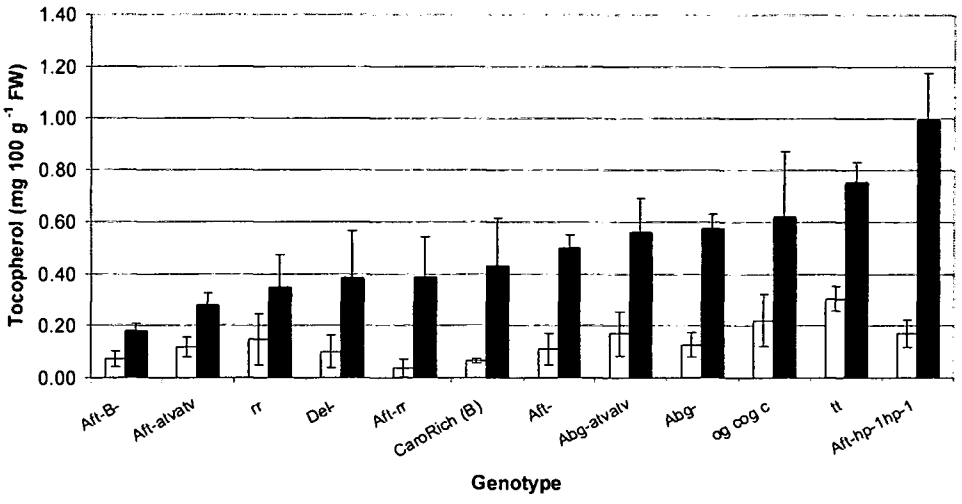


Fig 2.17. Tocopherol content of fresh tomato fruit of lines with different flavonoid and carotenoid profiles. □:γ-tocopherol, ■:α-tocopherol.

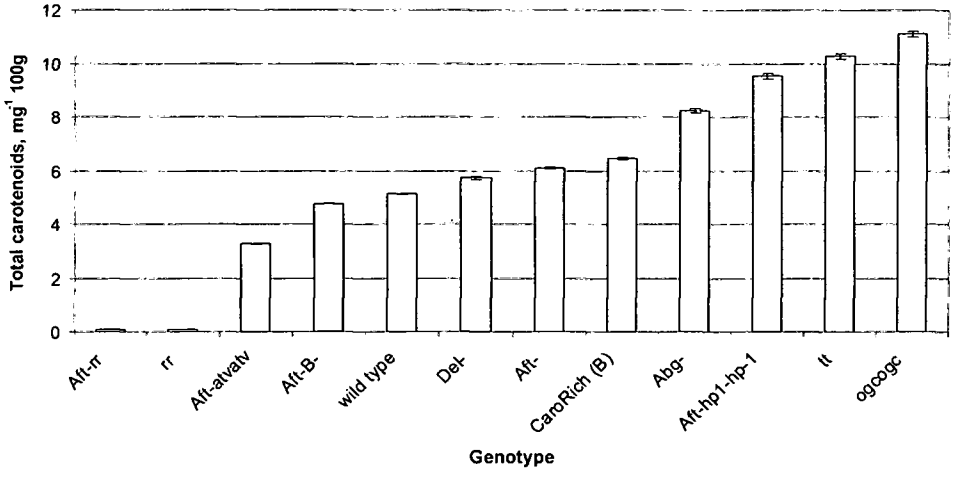


Figure 2.18. Total carotenoids of fresh tomato fruits of lines with different flavonoid and carotenoid profiles.

Between these two lines, the *Aft-rr* genotype had slightly lower though not significantly different total carotenoids (Table 2.5, Figure 2.18), but significantly higher tocopherols ($P<0.05$) compared to the *rr* genotype material (Figure 2.17), and so the relationship of increased tocopherols with concurrent increases in carotenoids holds within the reduced carotenoid material. Concerning the

anthocyanin mutants, it appears that the *Aft* and *atv* genes have limited effects on the tocopherols given the spread of tocopherol content in the various combinations of with these two genes. Not having carotenoid mutants in the *Abg* background made it difficult to assess the effects of the mutant gene: however, as the tocopherol content of both the *Abg* and *Abg-atvatv* lines are similar to that of the *Aft* line, it is likely that the *Abg* mutant also has a limited or negligible effect on tocopherol production.

GENE FUNCTION

Aft, and likely also *Abg* are involved in a signaling pathway in response to UV light. In other plant species, the anthocyanin pathway is upregulated by UV light (Harborne and Williams, 2004; Shirley, 1996). Tomato fruit do not normally produce anthocyanin in the skin, but fruit with the *Aft* gene do so as long as a UV-light threshold is met. A similar response has been demonstrated in *Arabidopsis*, with flavonoid response to UV-light being tightly regulated to irradiated cells (Lois, 1994). The observed accumulation of anthocyanins in *Aft* fruit suggests that the gene is involved in the response to UV-light. This could be a direct response in the phytochrome pathway, although there have been no published reports of such activity of *Aft*. It is also possible that, similar to *hp-1*, *Aft* causes an exaggerated response to UV-induced DNA damage, signaling an increase in protective flavonoid and anthocyanin production in tissues with high light incidence. However, unlike *hp-1*, *Aft* requires UV-light incidence, and thus there is no ubiquitous signaling change throughout the plant and in all tissues regardless

of light incidence. This suggests that the gene is involved in either the perception of UV light or in the response pathway to indicators of increased UV incidence. Because the response is similar with *Abg*, although different in magnitude, it is possible that *Abg* is part of the same regulatory element as *Aft*, but is a stronger mutation of the same allele (if the two are allelic) or is an up- or down-stream element with a greater effect on the regulation of the flavonoid pathway. Both phytochrome and cryptochrome are involved in the photoregulation of anthocyanin production in tomatoes. Cryptochrome has been postulated to enhance sensitivity to the phytochrome-far red species responding system (Mancinelli et al., 1991). A mutation of within this system could explain the enhanced anthocyanin response to UV light incidence observed in *Aft* and *Abg* tomatoes.

POSSIBILITIES OF BREEDING PURPLE TOMATOES

Combining the tomato fruit anthocyanin-expression enhancing genes produced fruit with intense purple pigmentation not previously observed in *L. esculentum*. Incorporation of other wild species genes may also enhance the degree of pigmentation. Given the increases in water- and lipid-soluble nutrients and antioxidants resulting from *Aft*, *atv*, *Abg*, and *hp-1*, there is potential for significant nutritional improvement of tomatoes over conventional varieties through the incorporation of other genes affecting the flavonoid biosynthetic pathway. We expect that genetic diversity in wild species that can be incorporated to further increase the antioxidant potential of the tomato.

Chapter 3.

ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF FLAVONOIDS FOUND IN TOMATOES

INTRODUCTION

Anthocyanins and their precursor flavonoids function as strong antioxidants through inhibition of the Fenton reaction (Tsuda et al., 1996) and through reactive oxygen species (ROS) scavenging, particularly superoxide and peroxide (Tsuda et al., 2000 a and b; Tsuda et al., 1996; Ichiyanagi et al., 2003). By producing a purple tomato with increased flavonoids and expressing anthocyanins where previously there were none, it is to be expected that the antioxidant activity of the tomato will increase. To test this hypothesis, flavonoids and anthocyanins (where present) were extracted and concentrated from various yellow, orange, red, and purple tomatoes.

Anthocyanins as well as other phenolic compounds have been implicated in various antimicrobial roles. Aglycon-, monoglycoside-, and diglycoside-anthocyanidins inhibit the growth of *Aspergillus flavus* as well as inhibit the formation of the pathogen's toxin aflatoxin B_1 (Norton, 1999). The proanthocyanidins in strawberry are thought to inhibit growth of the fungus *Botrytis cinerea* (Chung et al., 1998; Jersch et al., 1989). Chlorogenic acid, a phenolic compound found in tomato fruit, inhibits *Pseudomonas syringae* (Niggeweg et al, 2004). Many phenolic compounds thus far not tested may also exhibit antimicrobial properties: the antimicrobial effect is reportedly associated with the ester linkage between gallic acid and polyols (Chung et al., 1998), though

gallic acid itself was not responsible for the effect (Chung et al., 1993). As strawberries ripen, the ester bond of tannic acid is broken by hydrolysis, and antimicrobial activity is lost (Chung and Murdock, 1991). We hypothesized that the purple tomatoes would have increased inhibition of fruit microbial growth due to the elevated phenolic content. To test the efficacy of tomato phenolics in inhibition of microbial growth purple and yellow tomatoes were used in a pilot study to determine the effect of phenolic extracts on *Botrytis cinarea* spore germination and mycelial growth.

MATERIALS AND METHODS

ANTHOCYANIN AND PHENOLIC SAMPLE PREPARATION

Anthocyanins, flavonoids, and other phenolics were extracted using the basic protocol 1 of Rodriguez-Saona and Wrolstad (2001) and basic protocol 2 of Rodriguez-Saona and Wrolstad (2001). Extracts were made using ~5g of cryogenically milled tomato peel (weights were recorded individually to 0.01g for concentration adjustments), and after extraction were volumetrically adjusted to maintain a consistent ratio of extract weight to solvent volume. Extracts were applied to a C_{18} solid phase column. Sugars, acids, and other water soluble components were washed away with acidified water. Phenols and a fraction of anthocyanins were eluted using ethyl acetate, and the remaining anthocyanins and flavonoids were eluted in acidified methanol.

CHARACTERIZATION OF TOTAL ANTHOCYANINS AND PHENOLICS

The ethyl acetate fractions and the methanol fractions were analyzed for anthocyanin content by pH differential using the method of Guisti and Wrolstad (2001) as described in chapter 2. Total phenolics in the two fractions were measured using the Folin-Ciocalteau (F-C) method as described in chapter 2.

MEASUREMENT OF ANTIOXIDANT ACTIVITY

The ethyl acetate and methanol fractions were tested for antioxidant activity using the oxygen radical absorbance capacity (ORAC) assay, described by Cao et al., (1993). The ORAC assay measured prevention of oxidation of β-phycoerythrin by the sample, using the water-soluble radical generator 2,2'-azobis(2-amineпropane dihydrochloride (AAPH) (Wako Chemicals USA). A SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA) recorded fluorescence at 2 minute intervals for 2 hours at 485 nm excitation wavelength and 585 nm emission wavelength.

RESULTS

Tomato extract total phenolics, expressed as gallic acid equivalents (GAE), are shown in Table 2.6 (Field 2004) and Table 3.1 (Greenhouse 2004). ORAC of field grown summer 2004 fruit extracts is shown in Figure 3.1, and ORAC of greenhouse grown spring 2004 fruit extracts is shown in Figure 3.2. As expected, combining the anthocyanin mutant genes results in an increase in both total

anthocyanin content and in total phenolics. There was an increase in ORAC

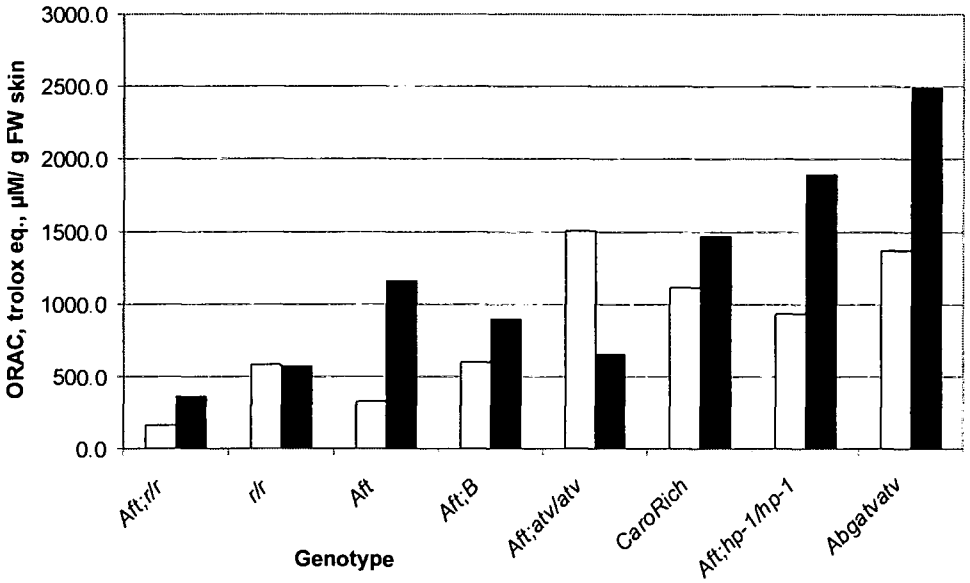


Figure 3.1. Field grown tomato antioxidant capacity of ethyl acetate (□) and methanol fractions (■).

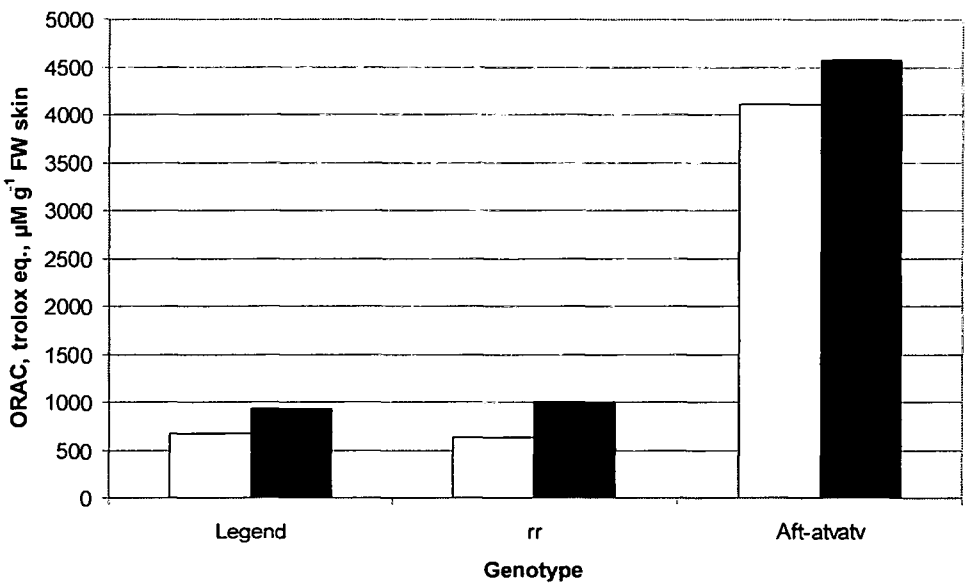


Figure 3.2. Greenhouse grown tomato antioxidant capacity of ethyl acetate (□) and methanol fractions (■).

scores in conjunction with the increase in total monomeric anthocyanins and total

phenolics. R-square coefficient between the ORAC antioxidant activity and total phenolics (Figure 3.3) or monomeric anthocyanin content was 0.93 and 0.68, respectively in the field grown material. This is consistent with previous reports that total phenolics play a significant role in determining antioxidant activity, while the anthocyanins certainly contribute, but are not the primary antioxidants

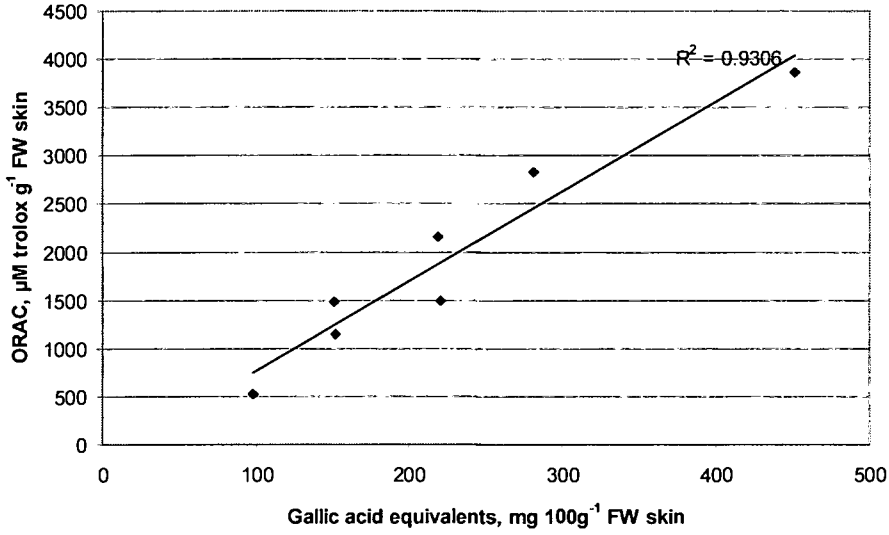


Figure 3.3. Relationship tomato fruit skin antioxidant capacity (ORAC) to total phenolic content (GAE).

present. Primary contribution notwithstanding, significant increases of tomato phenolics were produced through the use the anthocyanin-expression inducing gene mutants. Without resorting to transgenic manipulation, the way to increase the water soluble antioxidant content of tomatoes is to breed for increased anthocyanins and flavonoids. It is possible that the use of the available anthocyaninless mutants, when combined with the fruit anthocyanin-inducing genes, may also result in fruit with increased flavonoids without the production of the purple anthocyanins, although this hypothesis was not explored in this work. It

is also worth noting that, as expected, the addition of the *hp-1* gene increased the antioxidant capacity of the fruit extract, most likely due to the increases in total phenolics, including the anthocyanins over wild type and *Aft* genotypes.

The increase in antioxidant capacity was consistent in both the field and greenhouse grown fruit. The scores of *rr* and *Aft-atvatv* differed between the two environments, although the rank was the same in both environments. An unanticipated result was that the greenhouse material had higher ORAC scores than the field grown material. Differences in total phenolics do not explain this, either, because the greenhouse-grown fruit had lower total phenolics than the field-grown fruit for both *rr* and *Aft-atvatv* genotypes. It is likely that there were differences in the phenolic profiles of the greenhouse- and field-grown fruit, with stronger antioxidant phenolic compounds produced in the greenhouse.

Table 3.1. Spring 2004 greenhouse tomato genotype total phenolics and total monomeric anthocyanins of skin extracts.

	Fruit	Gallic Acid Equivalents, mg/100g FW			Total Anthocyanins, mg/100gFW		
Extract	Weight, g	Ethyl Acetate	Methanol	Total	Ethyl Acetate	Methanol	Total
Legend	107	24.4 ± 1.8 [c]	51.4 ± 3.4 [c]	75.8	-0.28 ± 0.64	0 ± 0.73	0 [c]
rr	43	25.4 ± 3.6 [c]	47.9 ± 8.8 [c]	73.3	0.44 ± 0.76	0.15 ± 0.67	0 [c]
Aft-atvatv	53	303.7 ± 49.7 [b]	268.8 ± 8.1 [b]	572.5	7.19 ± 1.73	118.66 ± 11.56	125.85 [b]
Aft-atvatv	24	871.3 ± 1.6 [a]	928 ± 4 [a]	1799	36.85 ± 1.05	459.57 ± 2.62	496.42 [c]

[a-c] letters indicate significant difference (P<0.05) determined by Fishers' LSD

[y]: Means of 2 replicate extractions with 3 pH differential tests each

[yy]: Mean of 4 replicate extractions with 2 pH differential tests each

[z]: Mean of 2 replicate extractions with three Folin-Ciochalteau reactions each

EFFECT OF TOMATO ANTHOCYANINS AND PHENOLICS ON FRUIT ROT PATHOGENS.

Comparison of the purple-colored fruit material in the field and in the greenhouse to red, yellow, and orange fruit led to an interesting observation: purple fruit do not rot as readily as fruit without the purple pigment. Figure 3.4

shows the difference between one of the purple selections versus a red and a

Figure 3.4. Possible antimicrobial effect of purple versus conventional tomatoes. Top to bottom: *Aft-atvatv*, *rr*, *og*^{c}*og*^{c}

yellow breeding line. The photographs were taken at 35 days after maturity. The red and yellow fruits were placed as shown at maturity and left in the field, as was a purple line (not shown). The purple tomatoes exhibited no pathogen activity upon visual inspection. The most severe cases of fruit rot were selected from the purple breeding line (021-8-11-1-1-3, *Abg-atvatv*) for comparison. Although not a completely objective observation, the photo shows a lack of infections in the purple portion of the fruit. What the photo cannot show is the differences in fruit integrity. The whole yellow tomatoes and red tomatoes were soft throughout, and yielded to pressure. This was not the case for the purple tomatoes. Even the purple tomatoes with rot damage maintained their solidity in the uninfected portions of the fruit.

While resistance to microbes conditioned by anthocyanins or related compounds has been reported in other species, the effects of anthocyanins have not been reported in tomatoes. We believe that the high-flavonoid purple portion of the tomato skin inhibited microbial growth. An attempt was made to develop a microplate assay that would allow us to evaluate the effect of flavonoids and anthocyanins on spore germination of fruit rot organisms. However, the initial assay used to measure inhibition of *B. cinarea* spore germination and mycelial growth by tomato fruit extracts did not produce conclusive results. Adjustment of the method will be necessary to develop a protocol that reliably correlates with field observations of fruit rot resistance. Results of the pilot study are shown in Appendix 4.

Chapter 4.

TOMATO GENOTYPES EFFECTS ON CAROTENOIDS AND ANTIOXIDANT ACTIVITY

INTRODUCTION

Of commercially available fruit juices, tomato juice has the third highest oxygen radical absorbance capacity (ORAC) antioxidant activity, ranked only below grape and grapefruit juices (Wang et al., 1996). Of the antioxidants in tomatoes, vitamin C accounts for less than 15% of the antioxidant activity (Prior and Cao, 1999). The remaining candidate antioxidants are the flavonoids and other phenolics, the tocopherols, and the carotenoids.

In the carotenoids, antioxidant activity varies greatly based on isomerization and addition of double bonds (Bohm et al., 2002). Antioxidant activity is affected by changes in the electron displacement across the conjugated double bond backbone of the carotenoid molecule (Stahl and Sies, 2003). Increasing the number of conjugated double bonds increases the antioxidant activity of the carotenoid. Thus lycopene, with 11 conjugated double bonds, has the highest antioxidant activity of the carotenoids determined by measurement of singlet oxygen quenching. Tomatoes come in many different colors, but it is primarily red tomatoes and red tomato processed products that have been tested for antioxidant effects. Red tomatoes, having high lycopene content, presumably have the highest antioxidant content.

Total carotenoids and carotenoid profiles vary widely among tomatoes with different genotypes. The antioxidant contribution of carotenoids from tomatoes

with different carotenoid profiles is expected to vary depending on the carotenoid makeup of the tomato. In the present work, we evaluated antioxidant differences of tomato accessions with different carotenoid profiles, based on the genes *r* (*yellow flesh*), *t* (*tangerine*), *og* ^{c}(*old gold crimson*), *B* (*Beta*), *Del* (*Delta*), *hp-1Aft* (*high pigment-1/Anthocyanin fruit*), *Aft* (*Anthocyanin fruit*), *Aft/rr* (*Anthocyanin fruit/yellow flesh*), and *Aft/B* (*Anthocyanin fruit/Beta*).

MATERIALS AND METHODS

TOMATO PRODUCTION

Tomato seed lines LA1996 (*Aft*), LA0797(*atv*), LA3311(og^{c}), LA3532(*r*), LA2374(*B*), LA3183(*t*), and LA2996a(*Del*) were acquired from the C.M.Rick Tomato Resource Center, University of California, Davis, CA. LA1996 was crossed to LA0797. F3 seed were produced from this line in the greenhouse in the winter of 2002-2003, as well as increased seed lots of the other lines. Tomato fruit of these lines were produced at the Oregon State University Vegetable Farm during the summer of 2003. Plots of each line were 50 m long, with 1 m interplant spacings and 2 m row spacing. These tomatoes were analyzed for carotenoids and tocopherol content. The method employed for both carotenoid and tocopherol extraction and detection are described in Chapter 2.

TOMATO JUICE PROCESSING PROTOCOL

Tomato juice was made from tomatoes with the genes *r*, *t*, *og* ^{c}, *B*, or *Del*. The tomatoes were grown during the summer of 2003 at the Oregon State

University Vegetable Research Farm, Corvallis, OR. Eighteen kg (40 lbs) of ripe fruit were harvested in a once over picking of each genotype, and were immediately processed into juice at the OSU Food Science and Technology Pilot Plant. The process used to make the juice was developed jointly with Jeff Clawson (OSU) and Dennis Anderson (Green & Green, Inc) to ensure that the juices were safe for storage and subsequent human consumption. After an initial wash in chlorine, the tomatoes were disintegrated using an Intelligent Drive Disintegrator (AC Technology Corporation, Uxbridge, MA) into a container maintained at 195° C. After 10 minutes of mixing at 195°C, the juice was run through a Reeves VariSpeed MotoDrive pulper/finisher (Reeves Pulley Co., Columbus, IN) to remove particulates and seeds. The screened juice was then placed in one quart jars using an AS-1 filler (Simplex Filler Co., Hayward, CA). The unsealed jars were placed in a custom-built belt blancher steam exhaust tunnel for sterilization, and were capped with sterilized lids prior to exiting the tunnel to maintain sterility. Juice was stored at 4°C in the dark to reduce carotenoid degradation until consumption or analysis.

ANTIOXIDANT ANALYSIS OF TOMATO LIPOPHILIC EXTRACTS

Antioxidant capacity of the lipophilic carotenoids was tested using the extraction method employed for carotenoids with subsequent analysis using the $ORAC_{FL}$ assay method described by Prior et al., (2003). Instead of resuspending the carotenoids (and tocopherols also carried through in the extraction procedure) in methylene chloride as was done in the last step of preparation for HPLC

analysis, the 1/10 aliqout extracts from 10g fruit extract were dissolved using 300 µl of acetone followed by 9.7ml 1:1 aqueous 7% randomly methylated β-cyclodextrin (RMCD) (Cyclodextrin Technologies Development Inc., High Springs, FL):acetone. Forty µl of this solution was added to 200 µl 7.5 nM fluorescein solution and 75 µl of fresh-prepared 10.67 mg ml^{-1} 2,2'-azobis(2-aminepropane dihydrochloride (AAPH) solution (Wako Chemicals USA). Plates were read in a Spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) at 485 nm excitation, 520 nm emission at two minute intervals for 120 minutes. Area under the curve calculations were compared to trolox standards, and antioxidant protection of fluorescein was calculated using the Gemini SoftMax software package.

During initial runs, undissolved crystals were observed using this procedure. Although no definitive analysis of the undissolved crystals was made, lycopene was the reasonable candidate for two reasons: the crystals were intensely red, and were observed in increasing quantities in extracts known to contain elevated levels of lycopene. It appeared that lycopene did not dissolve efficiently in either acetone or RMCD solution. Allowing the extract to stand for 24 hours in a shaker did not dissolve the crystals. The procedure was modified by adding a drop of methylene chloride, followed by 0.2 ml DMSO to extracts prior to adding the acetone and RMCD solutions to ensure the lycopene would dissolve.

Juice made in 2003 from *og* ^{c}, *Del*, *r*, and *B* genotype tomatoes was also tested using the $ORAC_{FL}$ system, with analysis of ascorbic acid content, carotenoids, and tocopherols. Both the lipophilic and hydrophilic fractions of the

juices were tested using the $ORAC_{FL}$ method. Lipophilic extracts were generated using the carotenoid extraction procedure described previously. The hydrophilic antioxidants were extracted using a modified method based on that of Prior et al. (2003). One g of tomato juice was extracted three times with hexane to remove the lipophilic compounds. After removal of the hexane layer, the remaining juice was partially dried under nitrogen to remove residual hexane. The residue was then extracted with 10 ml of a mixture of acetone/water/acetic acid (70:29.5:0.5 v/v/v). The extract was vortexed for 1 minute and sonicated for 10 minutes at 37° C. Following this, the tube was vortexed briefly and immediately centrifuged at 2200 rpm for 10 minutes. The supernatant was removed and volumetrically adjusted to 50 ml for subsequent testing using the $ORAC_{FL}$ method. Carotenoids and tocopherols were determined using the methods described previously.

TOMATO JUICE ASCORBATE ANALYSIS

Ascorbic acid content of the tomato juice was measured by paired-ion reverse phase HPLC with electrochemical detection. Samples of tomato juice were acid-precipitated using 5%(w/v) metaphosphoric acid containing 1 mM of the metal chelator (DTPA). Following centrifugation at 10,000 xg, 20µl of the supernatant was removed. Twelve µl of 2.59 M K_2HPO_4 buffer, pH 9.8, was added together with 148 µl of the HPLC mobile phase. The chromatographic solvent used was 40 m sodium acetate, 0.54 mM DTPA, 1.5 mM dodecyltriethylammonium phosphate, 7.5% (v/v) methanol, pH 4.5. Twenty µl of the sample was injected into an LC-8 column (100 mm x 4.6 mm I.D.) (Supelco,

Bellefonte, PA, USA) with a 20 mm x 4.6 mm i.d. guard column. Flow rate was set at 1.0 ml min^{-1}. An LC 4C amperometric electrochemical detector equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode (Bioanalyticaal Systems, West Lafayette, IN, USA) with an applied potential of +600 mV was used to detect ascorbate. Serial dilutions of ascorbate were prepared in phosphate buffer solution (PBS), pH 7.4, containing 20 µl 1 mM DTPA for use as standards.

THE PHOTOCHEM SYSTEM

In order to analyze the carotenoid contribution to antioxidant activity using as many radical generators and measurement methods as possible, the tomato juices were tested using the PhotoChem system from AnalytikJena AG (Konrad Zuse Straße 1, Jena, Germany). To make use of the full spectrum of tests available on this machine, both the water and lipid soluble antioxidants were extracted and tested. Extraction of the lipid soluble compounds was done using the ACL kit, and the water soluble compounds were extracted using the ACW kit. Juice samples were diluted 1:100 initially in the dilution reagent 1 (containing methanol and chelating agents in ACL; water and chelating agents in ACW). The diluted samples were then prepared using the protocol described in the AnalyticJena methods papers 'Protocol:ACL-kit' and 'Protocol:ACW kit' supplied with the instrument. Briefly, reaction solutions containing 100µl 1:100 diluted sample were added to 2.3ml of kit diluent, 200 µl reaction buffer (reagent 2), and 25µl photosensitizer working solution.

RESULTS AND DISCUSSION

CAROTENOID PROFILES

Differences in the carotenoid profiles of the genotypes tested were consistent with previous reports: highest total carotenoids were observed in the crimson (*og ^{c}og ^{c}*) genotype tomatoes, followed by the *tt* and *hp-1hp-1* genotype tomatoes (Table 4.1). Total carotenoids were greatly diminished in *B* genotypes, and were almost completely eliminated in the *rr* genotypes. All the total carotenoid estimates were slightly lower than expected based on extraction efficiency calculations (see chapter 2) compared with previously reported findings using a similar extraction protocol (Lin and Chen, 2003). Furthermore, it became evident that certain minor carotenoids were missed during peak integration. This was first noted in the *rr* genotypes, because their chromatographic signals were very low. Signal magnification and noise reduction through peak integration at wavelengths other than 450 nm indicated the presence of several unidentified xanthophylls other than lutein co-eluting very early in the HPLC run. Co-elution complicated the characterization and quantification of the compounds, though the three-peak λ-max values were certainly within the typical range of absorption for xanthophylls. Xanthophyll content of all the tested genotypes was certainly low based on the total absorption observed during the chromatographic runs, though the method used in this work prevents further interpretation or characterization of the xanthophylls. However, as the xanthophylls are known antioxidants, their presence, while in trace quantities, must not be overlooked.

As has been reported previously, the mutated alleles of the carotenoid pathway genes cause dramatic shifts in carotenoid profiles (Fray and Grierson, 1993; Isaacson et al., 2002; Lieberman et al., 2004; Liu et al., 2004; Mustilli et al., 1999; Ronen et al., 1999, 2000). While most lines express all-*trans*-lycopene in at least limited amounts, there were several notable exceptions. The *tt* genotype produces mostly prolycopene, and only ~1/100th of the all-*trans*-lycopene found in the *og* ^{c}*og* ^{c} genotype and ~1/36th that found in the wild type tomato tested. The other exception was the *rr* genotype, which has no detected all-*trans*-lycopene. The *rr* genotype did contain β-carotene and lutein, although no other carotenoids were detected.

Another genotype with a significant difference in carotenoid profiles is the *Delta* genotype. This tomato genotype exhibited an increase in delta-carotene synthesized by lycopene-epsilon ring hydroxylase enzyme. A second consequence of this gene was that α-carotene was also produced, a carotenoid normally not detected in tomatoes, and lutein production was increased. The production of α-carotene is the result of the lycopene β-cyclase (*Crtl-b*) enzyme that is expressed in fruit but has no substrate on which to act in wild type fruit. The increase in lutein is also a consequence of the *Del* allele because lutein is derived from α-carotene by β-ring hydroxylase (*CrtR-b*), and is the immediate precursor of lutein using the *CrtR-b* enzyme. That lutein is detected in all genotypes of tomatoes, not just *Del* genotypes, suggests that both lutein and β-carotene (in the *rr* genotype) are synthesized through an independent biochemical pathway.

Table 4.2. Tomato genotype carotenoid profile, µg g^{-1} FW

Carotenoid	Retention time	λ (nm) reported				µg g^{-1} fruit											
						og[c]	*t*	Wild type	*r*	*Del*	*Aft*	*Aftatv*	*AftB*	*Aftr*	*Abg*	*Afthp-1*	CaroRich
trans-Lutein + 9-cis lutein	4.6		424.5	447.2	478.2	0.37	0.43	0.45	0.52	1.20	0.49	0.49	0.49	0.45	0.48	0.55	0.57
Phytoene, E/Z @ 286.8	5.3			286.8		12.22	27.49	6.40		4 18	6.61	4.79	4.90		14.32	9.42	6.52
Phytofluene @ 348.8	8.2		332.5	348 8	366.4	7.80	13.41	3.40		3.069512	3.83	2 65	2.48		8.34	8.86	2.66
unknown_1	7.7	295.1	378.5	398.9	423												0.57
9-cis-beta-carotene	8.3	341.6		449.6	473	1.39		1.14		2.625109			1.92				
Zeta-carotene @ 400	9.4		379.8	401.3	426.8	0.58	3 11	0.54		0.64	0.21	0 16	0.29		1.29	0.78	8.43
Beta-cerotene	10.25			455.8	483.5	6.90		7.68	0.24	4.27221	4.63	3.96	21.10	0.18	10.26	16.58	24.51
Prolycopene	10.7		(421)	442.3	(465.5)		44.30										
unknown_2	11.7			449.6	476.2											0.77	2.67
cis-beta-carotene	12.8		424.6	452	477.4	0.58		0.38		1.359477	0.17		0.81		0.54		2 94
(CaroRich only)	13.1	348.8	441	480.5	487												0.20
unknown_3	13.4		418	441.1	466.5		0.98										
unknown_4	14.8		418	441 1	466.5		1 23										
15-cis-lycopene	15.5	361	447	472.6	502.9	0.86					1.08	0.67			0.88	0.65	
Delta-cerotene	18.8		433.9	459.3	490.8					18.08966					1.684708		
cis-delte-cerotene ?	18.7	296.3	432.5	456.9	487.2												0.15
unknown_6	18.7		420.6	442.3	471.4		1.56										
unknown_7	19.8	360	441.1	446.6	498.1						5.141868						2.12
13-cis-lycopene ?	20.4	361	442.3	469	498.1	2.37				2.98	0.93	2 93	0.61		4.44	1.34	0.72
Neurosporene	20.9		(421.9)	448	471.4		5.17										
Neurosporene, cis/trans	22.9		418.2	442.4	472.6					1.33							
unknown_9 gamma carote	23.1		438.7	462.9	493.2												1.09
unknown_10	23.2			450.8	472.8		3.24										
9,13-di-cis-lycopene	24.6	361	442.3	467.8	499.3	5.24		4.13		2.00	1.96	0.72	2.30		1.22	2.78	1.24
unknown_11	27		447.5	466.5	489.6	1.54		0.68			1.96		1.10				1.66
9-cis-lycopene	34.9	350	443.5	471.4	500.5	0.94		0.63			0.79	0.56			0.71		1.06
5-cis-lycopene	37	362.1	443.5	470.2	501.7	8.03		1.71									0.76
unknown_12	41.1				499.3		0.63										
unknown_13	45.9			471.4	505.4		0.68										
all-trans Lycopene	51		449.8	476.2	507.8	84.25	0.67	24.00		12.97835	32.88	15.48	11.43		38.23	55.63	8.72
Alpha-carotene			425.4	448.4	479.2					2.625109							
Total						111.07	102.88	51.13	0.77	57.35	60.68	32.41	47.23	0.63	82.37	95.31	66.57

As expected, the *Beta* (*B*) allele had the effect of decreasing total carotenoids and increasing β-carotene at the expense of lycopene. There was an increase in cis-β-carotene as well, although its ratio to β-carotene content was not consistent among the lines tested. The lack of consistency may be significant as it indicates the possibility of altering β-carotene content without significantly affecting the production of cis-β-carotene.

The unique carotenoid profile resulting from *tt* mutation in the carotenoid biosynthetic pathway included elevated phytoene content. Other tomato genotypes possessed 10-15% of total carotenoids in the form of phytoene, compared with 27% in the *tt* genotype. The cause of this increase was possibly due to the decrease in phytoene desaturase (*PDS*) efficiency due to the persisting cis-isomerization. It was unfortunate that no other tomato genotype had a similar increase in either phytoene or prolycopene as it hampered interpretation of which carotenoid was responsible for changes in antioxidant activity.

The genes that enhance anthocyanin expression may cause limited changes in the carotenoid profile (see Chapter 2), although the primary change is an increase in total carotenoids, not a change in specific carotenoid expression. The data suggested that the carotenoid and anthocyanin pathways do not compete for metabolites. The two pathways use different biosynthetic precursors, and they are mostly active in different tissues. In tomato fruit the anthocyanins are expressed in the third and fourth layers of epidermal cells based on repeated tissue scoring under a microscope, while the bulk of the carotenoids are expressed in the

pericarp, with relatively limited epidermal expression. If the anthocyanin expression did reduce carotenoid expression within the same cells, the effect was not detected using the bulk-tissue carotenoid extraction procedure applied in this research. Thus, the ability of a cell to simultaneously express anthocyanins and carotenoids and the possible metabolite competition between the two pathways is not clear from the present research. The data do show that the anthocyanin-expressing tomatoes do not exhibit a coincident inhibition of carotenoid expression in internal tissues. This could be very important to tomato processors interested in using anthocyanin expressing lines without the purple pigment: carotenoid expression may be enhanced by the *Aft* or *Abg* genes, while the unwanted anthocyanins could be excluded from product by removing the peels.

ANTIOXIDANT ANALYSIS OF TOMATO LIPOPHILIC EXTRACTS

$ORAC_{FL}$ determined from extracts with undissolved crystals and the DMSO-blank corrected extracts are shown in Figure 4.1. Comparison of the two methods demonstrated the contribution of lycopene to the antioxidant activity of these extracts. Changes in rank of the genotypes were greatest in lines with high lycopene content, while no change in either rank of antioxidant capacity were measured in lines with no all-*trans*-lycopene. The significance of the ability to dissolve all-*trans*-lycopene is apparent when comparing the *og* ^{c} genotype with the *tt* genotype, a line with elevated prolycopene, the tetra-cis isomer of lycopene. The *tt* genotype extracts did not have visible undissolved carotenoid crystals, and had the highest antioxidant activity in the unmodified $ORAC_{FL}$ analysis. The

score for the *tt* genotype was unchanged in the modified method. Assuming that

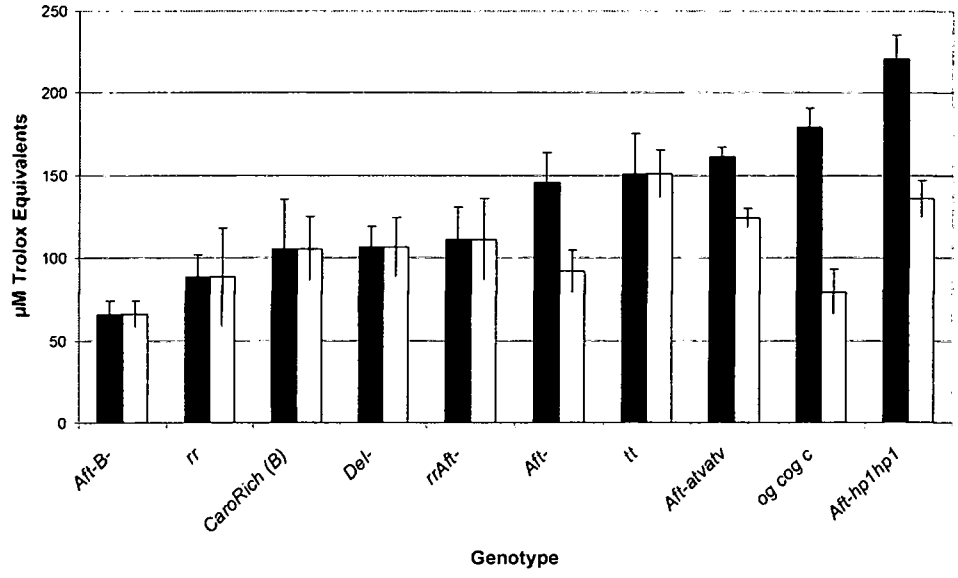


Figure 4.1. Lipophilic antioxidants determined using the unmodified $ORAC_{FL}$ method (□) and the DMSO-modified $ORAC_{FL}$ method (■).

the observed effect is not solely attributable to increased phytoene content of the *tt* genotype, this indicates that both prolycopene and all-*trans*-lycopene act as antioxidants, but that prolycopene is the more easily dissolved isomer of the two forms. The implications of this phenomenon on human bioavailability and antioxidant potential are considered in the human intervention study discussed in Chapter 5.

Aside from lycopene, the effect of the other carotenoids was difficult to assess. Total carotenoids correlated (R^2=0.64) with antioxidant activity excluding data for *Aft-atvatv, rr* and *Aft-rr* fruit, but correlated poorly when data from these lines was included (R^2=0.39). There could be several reasons for this: 1) lutein has a highly significant antioxidant contribution, 2) limited tocopherols present in the extracts may have also had a significant effect, and 3) another uncharacterized

lipophilic antioxidant may have been present. The first hypothesis is unlikely based on previous antioxidant studies of purified carotenoids. The third hypothesis is possible, as some colorless lipophilic flavonoids may not have been removed during the extraction procedure. At first, this possibility seemed unlikely. However, the carotenoid HPLC lower limit of detection was set at 250 nm, while flavonoids absorbance is highest around 220 nm. Absorbance anomalies at the lower end (below 260 nm) were initially thought to be due to solvents. These anomalies may have been due to the presence of flavonoids. However, the presence of flavonoids is not consistent with the antioxidant data. The low carotenoid genotypes *rr* and *rrAft-* had high antioxidant scores despite negligible carotenoid content, but the higher antioxidant score line *rrAft-* only had higher tocopherol content, not higher total carotenoids or total phenolics (Figure 2.14). Thus, it appears that the most likely hypothesis is the second one proposed: that tocopherol content affected antioxidant scores. Tocopherol content of the extracts is shown in Figure 2.15. The α-tocopherol content shows a moderate (R^2=0.65) correlation with the $ORAC_{FL}$ scores. Lines with high tocopherol and carotenoid contents produced high $ORAC_{FL}$ scores, while lines with low total carotenoids and/or low tocopherol content produce low $ORAC_{FL}$ scores.

Juice from *og* ^{c}, *Del*, *r*, and *B* genotype tomatoes was tested using the $ORAC_{FL}$ system, with additional analysis of ascorbic acid content, carotenoids, and tocopherols. Results of these analyses are shown in Tables 4.2 and 4.3.

Table 4.2. Tomato juice carotenoid profiles, μg g^{-1} FW [a, b].

Genotype	*r/r*	*t/t*	*og* [c]/*og* [c]	*B*	*Del*	*Aft;atv/atv*
cis/trans-Lutein	0.53	0.51	0.58	0.45	0.87	0.56
Phytoene, E/Z		26.51	11.32	6.79	14.66	9.05
Prolycopene		12.73				
Delta-carotene					10.01	
β-carotene	trace		6.03	20.48	2.83	6.20
α-carotene					trace	
13-*cis*-Lycopene		7.85	0.86	0.91	1.28	4.23
all-*trans* Lycopene		1.11	69.38	5.10	8.08	32.32
Total carotenoids	0.53	48.72	88.16	33.73	37.73	52.37

[a] mean of 8 replicate extractions

[b] trace phytofluene, cis-β-carotene and less common lycopene isomers not reported

Table 4.3. Tomato genotype juice nutrient content. Carotenoids, ascorbic acidn and tocopherols are expressed as μg g^{-1} FW, and ORAC scores as μMoles trolox equivalents/ml juice.

Genotype	γ-tocopherol	o-tocopherol	β-tocopherol	Ascorbic Acid	Total carotenoids	$ORAC_{FL}$ hydrophilic	$ORAC_{FL}$ lipophilic
og [c] *og* [c]	1.12 [b]	9.91 [a,b]	0.0131 [b]	143.19 [a]	88.16	5840.3 [a]	183.45 [a]
Aft-atvatv	1.36 [a]	10.437 [a]	0.0135 [a]	146.5 [a]	33.73	5702.3 [a]	121.12 [b]
B-	0.53 [e]	7.10 [c]	0.0134 [a]	94.39 [d]	48.72	4719.4 [b]	161.25 [a,b]
rr	1.12 [b]	9.02 [b]	0.0132 [b]	98.4 [c,d]	0.53	5751.8 [a]	130.35 [b]
tt	0.93 [c]	7.97 [c]	0.0135 [a]	130.78 [b]	33.73	- [z]	170.05 [a,b]
Del-	0.72 [d]	7.18 [c]	0.0131 [b]	102.39 [c]	52.37	- [z]	204.75 [a]

[a-e] symbols indicate significant difference within a column, ($P<0.05$) determined by Fisher's LSD

[z]: *data unavailable*

The differences in the lipophilic $ORAC_{FL}$ scores corresponded to both total carotenoids and tocopherols. Given that the carotenoids were 5 and 10 times more abundant than the tocopherols, it seems likely that of the two classes of compounds, the carotenoids have a great impact on the antioxidant activity of the juice. There were two elements within this data set that might dispute such a hypothesis. First, the *rr* juice did not have a near-zero lipophilic $ORAC_{FL}$ score, evidence that the carotenoids are not the primary antioxidants measured by this assay. Second, the *Aft-atvatv* juice has lower total carotenoids than the *og* [c]*og* [c] juice, but has a higher lipophilic $ORAC_{FL}$ score. This discrepancy can be

explained by the differences in tocopherol content. The *Aft-atvatv* juice has the highest measured levels of the α- and γ- forms of tocopherol.

The hydrophilic $ORAC_{FL}$ scores of the tomato juice indicated the presence of antioxidants other than ascorbic acid. Phenolics have already been demonstrated to contribute to hydrophilic antioxidant activity. It is likely that they were at least partially responsible for the variation among the tomato genotypes, as ascorbic acid content does not correlate well with the $ORAC_{FL}$ scores. The juices were not tested for total phenolics, preventing further interpretation. One very important conclusion to be drawn from comparisons of the lipophilic and hydrophilic $ORAC_{FL}$ scores is that the bulk of the antioxidant activity was found in the water-soluble portion. While the tocopherols and carotenoids contribute to total antioxidant activity, it was evident that their impact on antioxidant capacity of the fruit pales in comparison to the water-soluble antioxidants. Breeding a 'high-antioxidant' tomato is thus not a function of how red the tomato is (with high lycopene content), but rather on the genetic control of the water soluble antioxidants such as ascorbic acid and the phenolics.

ANTIOXIDANT CAPACITY USING THE PHOTOCHEM SYSTEM

In order to analyze the carotenoid contribution to antioxidant activity using as many radical generators and measurement methods as possible, the tomato juices were tested using the PhotoChem system from AnalytikJena AG (Konrad Zuse Straße 1, Jena, Germany). In order to make use of the full spectrum of tests available on this machine, both the water and lipid soluble antioxidants were

extracted and tested. The results of the ACW test of hydrophilic compounds, expressed as ascorbic acid equivalents, (Figure 4.2) correlate with the water

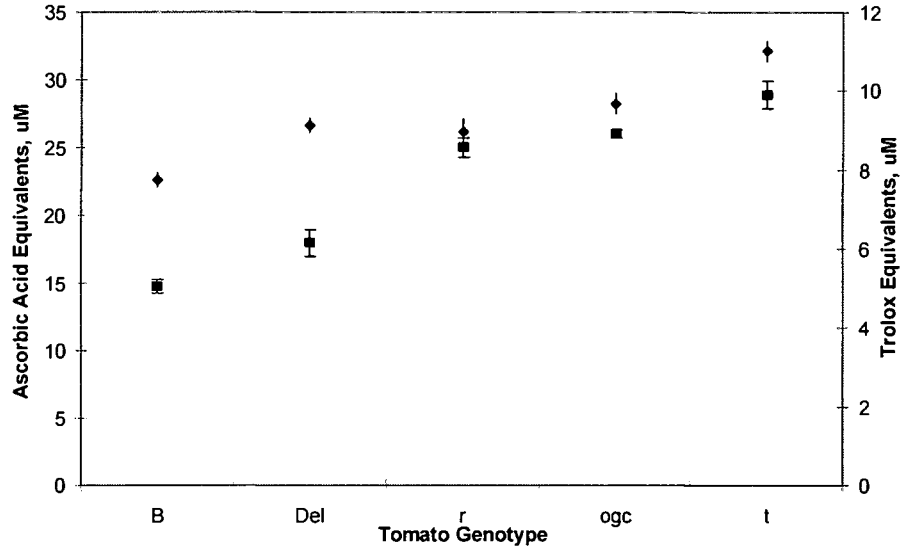


Figure 4.2. PhotoChem tomato antioxidant scores. ▪ Water-soluble antoxidants (left axis), ♦ Lipid-soluble nutrients (right axis).

s

oluble components tested by the $ORAC_{FI}$ assay (R^2=0.4). The tomato juice with the allele *t* had the highest score, with successively lower scores for *og* ^{c}, *Del*, *r*, and *B*. This corresponds well with the ascorbic acid content (Table 4.3) (R^2=0.53), with one exception. The lowest score in the PhotoChem ACW assay was observed in the high β-carotene (*B*) mutant tomato, although it had significantly higher ascorbic acid than the low carotenoid (*rr*) mutant. This indicated that compounds other than ascorbic acid were affecting the PhotoChem ACW assay. Likely candidates were the phenolics, as measured by Folin-Ciocalteau (F-C), which are present in the extract, and which have been demonstrated to correlate with antioxidant activity as measured by the ORAC assay. Differences in phenolics, as

measured by the F-C assay, may explain why the *B* mutant has a higher score than the *rr* mutant.

Results of the ACL assay (lipophilic compounds, Figure 4.2), expressed as trolox equivalents, were problematic. The scores generally did not correlate well with those of the lipophilic compounds tested by $ORAC_{FL}$. The highest scoring genotype in the PhotoChem system assay was the tangerine (*t*) mutant tomato, not the high total carotenoids crimson (og^c) mutant tomato. This was not the case in the $ORAC_{FL}$, scores, where the $og^c og^c$ genotype had the higher measured antioxidant activity of the two. The PhotoChem assay, like the unmodified $ORAC_{FL}$ assay, had the limitation that the effect of lycopene was not accurately measured: lycopene crystals were clearly visible in the sample tubes used to prepare the extracts for both assays, indicating their absence in antioxidant assay. The cause of this problem is that lycopene does not dissolve well in methanol. It is this limitation that makes the assay unsuitable for testing tomatoes, particularly those with high lycopene content.

Like the $ORAC_{FL}$ assay results, the PhotoChem analysis implicates compounds other than carotenoids as contributing to antioxidant activity in the lipophilic system. Tocopherols may certainly explain part of the variation, but unlike the situation in the $ORAC_{FL}$ extracts, the flavonoids were present and active as antioxidants in both extract types of the PhotoChem system. This was caused by water and methanol basis of the ACL solvent system, these being solvents that readily solublize flavonoids. It is highly likely that the same flavonoid antioxidant was measured in both the ACL and ACW systems, and for this reason no 'total'

antioxidant values based on the two kits was calculated.

RELEVANCE OF TOMATO ANTIOXIDANTS

When this project was initiated, we believed that lycopene was the predominant antioxidant in red tomatoes both in terms of quantity and efficacy. While the former is true in high-lycopene varieties, the latter is disputable based on the findings reported here. Lycopene did have a significant effect on the $ORAC_{FL}$ lipophilic scores. However, the tocopherols also contributed, despite the limited quantity present in tomato fruit. Whether lipophilic flavonoids contributed to antioxidant capacity was not clearly proven, although water-soluble flavonoids clearly had a significant effect. Furthermore, the water soluble antioxidants had significant impact on the total antioxidant capacity of the fruit, as observed in the PhotoChem system, having ~15-28 μM ascorbic acid equivalents compared to ~8-11 μM trolox equivalents for the lipophilic antioxidants. While the ascorbic acid and trolox equivalents are not identical scales for comparison between the lipid- and water-soluble antioxidants, the data indicates a greater portion of antioxidants were present in the aqueous fraction. Consistent with this finding, the $ORAC_{FL}$ aqueous scores of several tomatoes (Table 4.4) were several fold higher than the $ORAC_{FL}$ lipophilic scores. Thus it is not requisite for a 'high antioxidant' tomato to be red from high lycopene content. Instead, a tomato with elevated phenolics, ascorbic acid, tocopherols, and carotenoids (in relative order of contributution to total antioxidant capacity of fruit) is the 'best' in terms of total and partitioned antioxidant capacity.

Chapter 5.

TOMATO CAROTENOIDS AND *EX VIVO* ANTIOXIDANT ACTIVITY

INTRODUCTION

The primary carotenoid in red tomatoes is lycopene, a known antioxidant. Other carotenoids found in tomatoes also act as antioxidants. To determine the contribution, if any, of these carotenoids to increased antioxidant activity following tomato consumption, a nutritional pilot study was designed to document the effects of specific carotenoids on antioxidant capacity in human plasma. This project was initiated to identify nutritionally significant and bioavailable carotenoids that can be exploited to produce tomatoes with elevated antioxidant benefits. Understanding the antioxidant activity of specific carotenoids will allow for selection of tomatoes with improved antioxidant capacity. Such improvements would benefit consumers of tomato products. By choosing the appropriate mutants, it is possible to compare the antioxidant and health effects of carotenoids that are typically masked by lycopene and beta-carotene. Research efforts have been focused primarily on lycopene and β-carotene, leaving most of the other carotenoids essentially unstudied in terms of *in vivo* and *ex vivo* antioxidant activity. This research will begin to bridge the gap in our understanding of these "minor" tomato carotenoids.

Various colored tomatoes are commercially available for consumption, but only red tomatoes and their processed products have been tested for beneficial effects following human consumption. Red tomatoes have been associated with

several health benefits, including decreased prostate cancer and reduction of oxidative stress in blood plasma LDLs. In many studies, this reduction in oxidation is measured *ex vivo* by copper induced oxidation of plasma LDLs or through area-under-curve measurements following introduction of a radical generator. It would be ideal to measure a reduction in *in vivo* oxidation of plasma LDLs. However, this would require a long term dietary intervention well over six months for all the carotenoids of interest. Such a time frame was considered well beyond the scope of this project, so the decision was made to design a 10 week dietary intervention with measurement of oxidation using *ex vivo* methods.

MATERIALS AND METHODS

Tomato juice was made from tomatoes with the genes *r*, *t*, *og* ^{c}, *B*, or *Del* (Figure 5.1). The tomatoes were grown during the summer of 2003 at the Oregon

Figure 5.1. Heat processed tomato juice. Genotypes from left: *rr, tt, $og^{c}og^{c}$, B-, Del-*.

State University Vegetable Research Farm, Corvallis, OR. Eighteen kg (40 lbs) of ripe fruit were harvested in a once over picking of each genotype, and were immediately processed into juice at the OSU Food Science and Technology Pilot

Plant using the method described in chapter 4.

HUMAN DIETARY INTERVENTION STUDY

Two volunteers were recruited from the OSU student population for the dietary intervention study. Both students had normal dietary habits which were not entirely dependent on vegetables nor taking any dietary supplements, were in good health, and non-smokers. One subject was placed on a carotenoid-restricted diet throughout the study to deplete the carotenoids from the subject's plasma, with the exception of those carotenoids supplied by the tomato juice (Appendix 6). The other subject maintained a normal diet throughout the study, supplementing it with tomato juice.

Table 5.1. Dietary Intervention Juice Consumption Schedule.

Day	Plasma Sample	Dietary Intervention
-14	t -14	washout
1	t 0	*r* gene juice
4		
5	t 5	washout
14		
15	t 15	*t* gene juice
18		
19	t 19	washout
28		
29	t 29	*B* gene juice
32		
33	t 33	washout
42		
43	t 43	*og c* gene juice
46		
47	t 47	washout
56		
57	t 57	*Del* gene juice
60		
61	t 61	

After a two week carotenoid-washout period for subject 1, both subjects supplemented their diets with tomato juice. Juice was consumed during lunch and dinner meals daily during alternate weeks (Table 5.1). Juice servings were 8 fl oz (~240ml), the equivalent of one serving of raw tomato. Thus, subjects consumed ten tomato servings during each week of intervention. After a week of drinking juice, the subjects observed a tomato juice-free week as a carotenoid washout

period. Carotenoids have a half-life of 14-21 days in the human body (Allen et al., 2003), and seven days of fasting results in a 35% reduction in plasma lycopene (Hadley et al., 2003). This study allowed 10 days for carotenoid washout; far less than the ideal 21 days, as well as less than the low end of 14 days for a 50% reduction. It was known from the outset that this period of time would not be sufficient to restore initial conditions. However, the study was time-restricted in the availability of both volunteers and phlebotomist, and so the compromise of a shorter-than-ideal washout period was made. A trained phlebotomist drew blood from the volunteers into ethylene-diamine-tetra-acetic acid (EDTA)-treated vacutainers to prevent oxidation, as described by Yeum et al. (1996). The initial draws occurred prior to lunch at the start of a week of tomato juice consumption, and again five days later, 16 hours after the last tomato juice

Table 5.2. Juice intervention schedule.

Time	Monday	Tuesday	Wednesday	Thursday	Friday
10:00 AM	Blood draw				Blood draw
12:00 PM	8fl oz juice with lunch	8fl oz juice with lunch	8fl oz juice with lunch	16fl oz juice with lunch	
6:00 PM	8fl oz juice with dinner	8fl oz juice with dinner	8fl oz juice with dinner	16 fl oz juice with dinner	

was consumed (Table 5.2). Immediately after the blood was drawn, the samples from each subject were centrifuged at 800x g at 4°C for 20 minutes to extract the plasma. Plasma was then aliquoted and stored at -80°C until analyzed.

PLASMA CAROTENOID EXTRACTION

Carotenoids were extracted from the plasma samples using a method based

on that of Porrini et al. (1998). Aliquots of plasma were thawed from -80°C and used immediately for extraction. One-hundred fifty µl of plasma was extracted with 150µl ethanol and 300µl hexane in a glass microtube. The mixture was vortexed for 1 minute, followed by centrifugation for 5 minutes at 1000 xg. Two-hundred fifty µl supernatant was drawn off and evaporated under nitrogen. The extract was immediately dissolved in 100% methylene chloride for injection into the HPLC system.

JUICE CAROTENOID EXTRACTION

Carotenoids were extracted from tomato juice using a modified method based on that of Ferruzzi et al. (2001). Briefly, six replicates of 10 g tomato juice were added to 1 g $CaCO_3$ and 25 ml methanol in a glass vial with a teflon-lined cap. Samples were vortexed for 2 minutes, followed by centrifugation at 710 xg for 5 minutes. The supernatant was collected in a separate sealed flask, and the juice solids were re-extracted with 25ml 1:1 acetone-hexane solution. The vials were vortexed and centrifuged, and supernatant added to the sealed flask. The tomato juice was extracted again with 25 ml 1:1 acetone-hexane, but not centrifuged after the final extraction. The collected supernatant was vacuum filtered through Whatman #1 qualitative paper, and the remaining solid material in the vial was also vacuum filtered into the same flask. The vial and supernatanat flask were both triple rinsed with ~1-2ml 1:1 acetone-hexane, pouring the solutions into the vacuum flask. Filtrate was added to a separatory funnel together with 2% NaCl solution, and allowed to stand sealed for 20 minutes. Next, the

lower aqueous layer was decanted, and the upper hexane layer saved. The hexane layer was placed in a 50 ml flask with one g $NaSO_4$ to remove residual water. This solution was then pipetted into a 50 ml glass vial with a Teflon-line cap. The $NaSO_4$ solution was triple rinsed with 1:1 acetone:hexane, the wash solution added to the glass vial. The solution was then dried under nitrogen in a water bath at 35°C, and the contents sealed under nitrogen when dry. Ten ml methylene chloride was added to the vial to dissolve the carotenoids as well as any other lipophilic compounds present.

CAROTENOID ANALYSIS BY HPLC

Prepared carotenoid extracts in methylene chloride were filtered through a Waters 0.45μm filter PTFM membrane Acrodisc into an amber vial for carotenoid analysis by HPLC. Conditions for HPLC carotenoid analysis were based on the conditions described by Lin and Chen (2003), as described in chapter 2. Juice extractions were preformed in triplicate, with two injections into the HPLC system per sample.

DETERMINATION OF LIPOPHILIC ANTIOXIDANT ACTIVITY

Measurement of *ex vivo* lipophilic antioxidant activity in the plasma samples and tomato extracts was made using the Aldini et al. method (2001) now called the TAP assay (Yeum, pers. comm.). In collaboration with Yeum, frozen aliquots of plasma and nitrogen-dried and sealed carotenoid extract vials were sent to Tufts University for analysis. The reason for using the TAP assay rather than

any other antioxidant assay such as the $ORAC_{FL}$ is that it has a proven selectivity and sensitivity to the lipophilic antioxidants.

The ferric reduction assay of plasma (FRAP) was used to test the plasma of subject 1 for non-lipid specific changes in plasma antioxidant capacity. The method (Benzie and Strain, 1996) used a ThermoMax microplate spectrophotometer (Molecular Devices, Foster City, CA) to measure change in absorption λ_{max}=595 nm. This change results from the formation of ferrous-TPTZ complex as a result of reduction of ferric iron to ferrous iron. Plasma aliquots were prepared with TPTZ and sodium acetate at pH 3.6, and absorption measured after 20 minutes.

RESULTS AND DISCUSSION

Tomatoes with primary carotenoids other than lycopene may have beneficial effects similar to red tomatoes. However, if the antioxidant benefits of tomatoes are primarily due to lycopene, such benefits would not be observed in products of non-red tomatoes. To test this hypothesis, a human dietary intervention study was devised to compare the effects of different carotenoid-profile products on human plasma. Instead of testing fresh tomatoes, heat-processed tomato juice was used. This was done to enhance the bioavailability of the carotenoids over raw fruit, as indicated by Stahl and Sies (1992). This may have introduced or increased carotenoid isomers in the tomato juice (Shi and Le Maguer, 2001; Nguyen et al, 2001), though it is now believed that this phenomenon in heat treated tomato products may be limited (Re et al., 2002).

Averaged carotenoid profiles of the tomato juices are shown in Table 5.3. Two replicates of plasma extractions were also injected twice per sample, with results shown in Figures 5.2 and 5.3 for subjects 1 and 2, respectively. Analysis of

Table 5.3. Carotenoid profiles of processed tomato juices derived from tomato lines with different mutations affecting carotenoid and flavonoid biosynthetic pathways.

Genotype	*r/r*	*t/t*	*og* [c] */og* [c]	*B*	*Del*	*Aft;atv/atv*
cis/trans-Lutein	0.53	0.51	0.58	0.45	0.87	0.56
Phytoene, E/Z		26.51	11.32	6.79	14.66	9.05
Prolycopene		12.73				
Delta-carotene					10.01	
β-carotene	trace		6.03	20.48	2.83	6.20
α-carotene					trace	
13-*cis*-Lycopene		7.85	0.86	0.91	1.28	4.23
all-*trans* Lycopene		1.11	69.38	5.10	8.08	32.32
Total carotenoids	0.53	48.72	88.16	33.73	37.73	52.37

[a] mean of 8 replicate extractions

[b] trace phytofluene, cis-β-carotene and less common lycopene isomers not reported

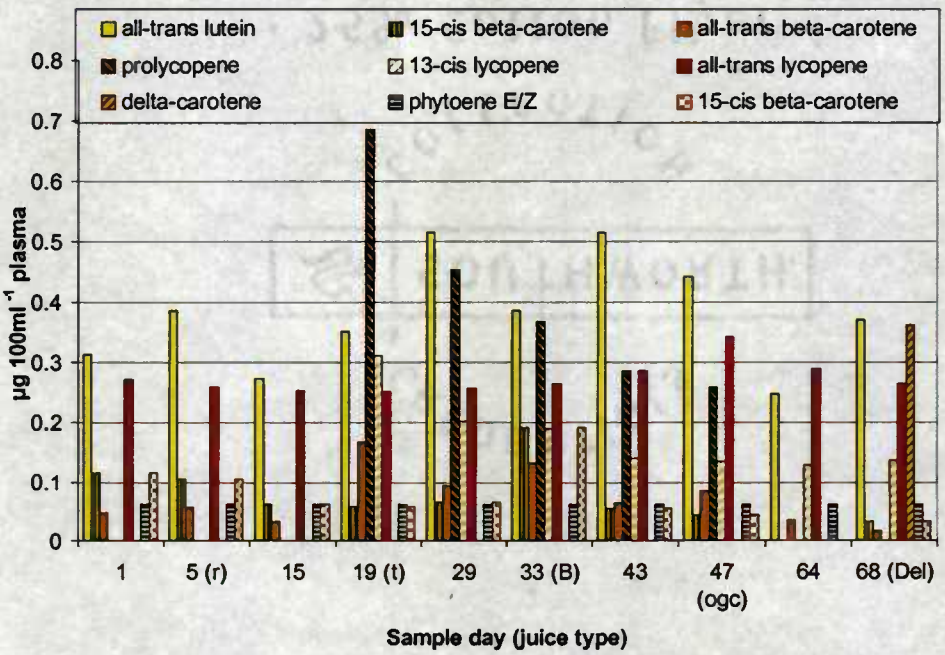


Figure 5.2. Plasma carotenoids of Subject 1. Following a 14 day washout period, tomato juice with specific carotenoid profiles was consumed at 10 day intervals.

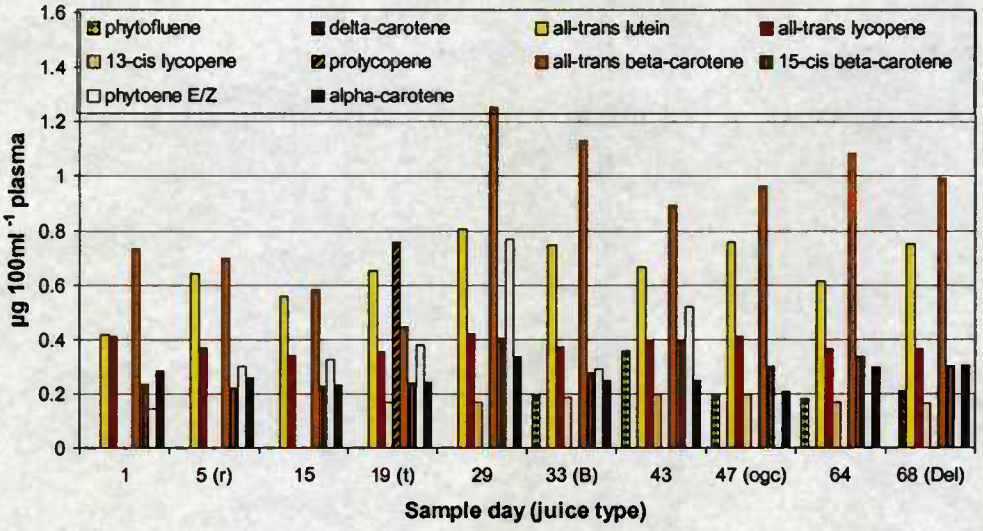


Figure 5.3. Plasma carotenoids of Subject 2. Tomato juice with specific carotenoid profiles was consumed at 10 day intervals.

the plasma samples revealed a significant difference between the two subjects. There was clearly an effect of diet because the carotenoid-restricted subject 1 had fewer types of carotenoids detected as well as less of them relative to subject 2. Apart from this, both subjects responded in a similar manner to the consumed juices. This is most evident with the unique carotenoids prolycopene and δ-carotene, which are not detected in either subject until the consumption of juices containing the respective carotenoids. Prolycopene in particular is interesting as both subjects, despite their different diets, responded similarly to the *tt* genotype juice with ~0.7 µg prolycopene per 100ml plasma. This demonstrates the rapid absorption of prolycopene relative to the other carotenoids, none of which had such a dramatic response following juice consumption. The other novel carotenoid introduced by the tomato juice was δ-carotene, which was absorbed at approximately ½ the rate of prolycopene, taking into account the differences in

carotenoid content of the two juices containing these carotenoids. Under a passive absorption model, this is best explained by the tetra-*cis* nature of prolycopene that increases the bioavailability of the carotenoid compared with other di-*cis* or all-*trans* carotenoids.

Results of the restricted diet (subject 1) and the unrestricted diet (subject 2) on antioxidant capacity are shown in Figure 5.4. Subject 1 exhibited the greatest changes in antioxidant activity. There was a surprising effect of fasting on this subject, as the TAP score increases by almost 20% after two weeks on a zero carotenoid diet. In retrospect, this effect was likely due to the dramatic change in diet for this subject, who reported increasing red meat intake from 3 servings per week to 7 servings per week. Other dietary changes included exchange of apple

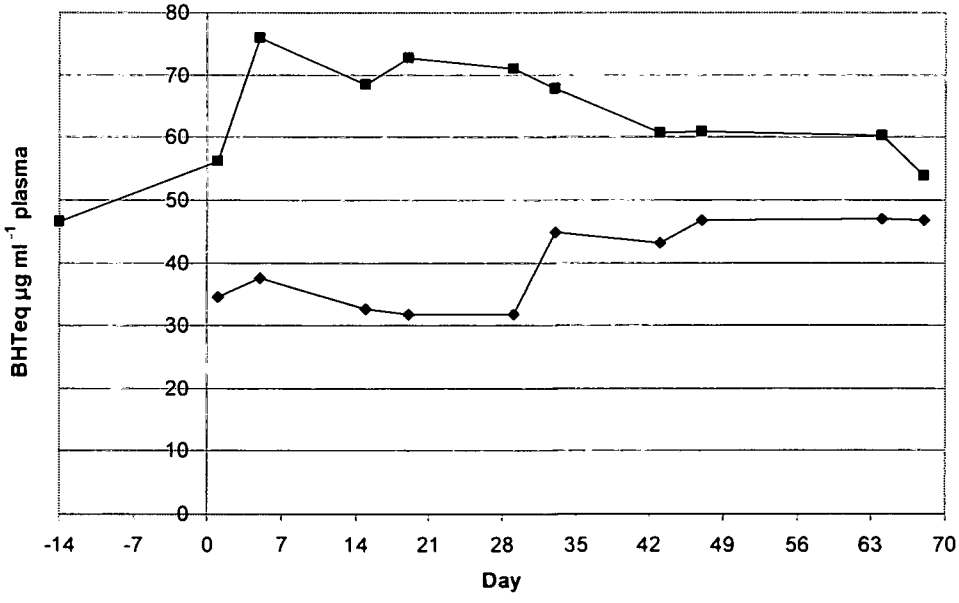


Figure 5.4. TAP plasma antioxidant scores of human subjects. Sampling order: day 5 followed *r* juice, day 19 followed *t* juice, day 33 followed *B* juice, day 47 followed *og* ^{c} juice, and day 68 followed *Del* juice. ■: subject 1, ♦: subject 2.

juice for orange juice, nightly rice or dry pasta instead of vegetables and meats,

and bread and cheese lunch instead of peanut-butter and jelly sandwiches. It is unclear which of these dietary changes were responsible for the observed change in antioxidant activity during the carotenoid washout period. However, the dietary effect for the duration of the juice intervention period were judged minimal, as the subject's meals were consistent throughout this period.

Comparing the effects of the juices on subject 1, either some of the juices had a minimal effect on antioxidant capacity while others had large effects, as measured by the TAP assay, or all of the juices had a limited effect and the changes observed are due to factors unrelated to the juice carotenoids. Most unexpected was the dramatic changes following low-carotenoid (*rr*) juice consumption. This juice had the 2nd lowest ascorbic acid content, average α-tocopherol content, and limited lutein to contribute to lipid antioxidant capacity. Despite the relatively low antioxidant nutrient content of the juice, the *rr* genotype appears to have had the most dramatic effect on the antioxidant status of subject 1. However, the carotenoid profile changed very little as a result of consuming the *rr* juice, with lutein increasing slightly and lycopene decreasing slightly. Thus it appears that there was variation in the lipophilic antioxidant content of subject 1 plasma, other that the carotenoids. In light of this finding, explaining the absence of any subsequent dramatic shifts in subject 1 plasma antioxidant capacity despite the change in carotenoid intake and plasma carotenoid profile becomes simplistic: the carotenoids are not causing a highly significant effect, and thus observed differences are probably caused by other plasma antioxidants. The best case scenario would require the assumption that the observed changes were the

exclusive result of differences in carotenoids. If this were true, then the increases in antioxidant capacity after consuming the juices carrying the *t* and og^c genes were the result of increases in prolycopene and all-*trans*-lycopene, respectively. Juices with the *B* and *Del* genes that resulted in decreased antioxidant capacity could be explained by decreases in total carotenoids in the case of *B*, and by a lack of effect of δ-carotene in *Del*. The *r* gene juice data is difficult to interpret as due to the pattern of increasing antioxidant activity continuing from the carotenoid-washout period. It is likely that variation in lipophilic antioxidants not examined in this study would certainly violate this assumption, and so the conclusions based on this assumption are very likely unsound. To improve the interpretation of this type of experiment would require analysis of all lipophilic antioxidants found in the plasma extracts as well as using a larger number of test subjects.

The FRAP assay has little information pertinent to carotenoid antioxidant activity. However, plasma samples of the fasting volunteer were analyzed to determine if there were any major changes in non-lipid antioxidant activity.

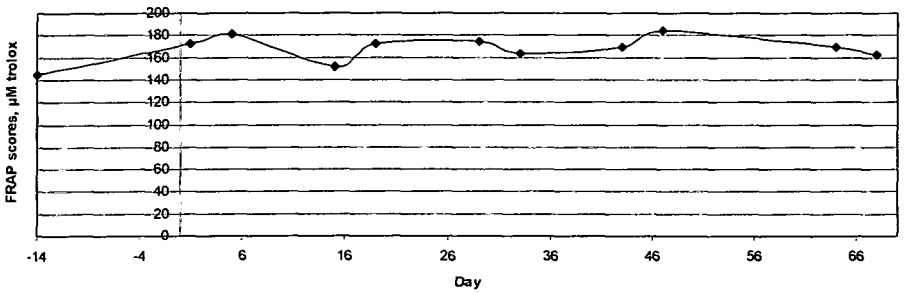


Figure 5.5. FRAP plasma antioxidant scores of human subjects. Sampling order: day 5 followed *r* juice, day 19 followed *t* juice, day 33 followed *B* juice, day 47 followed og^c juice, and day 68 followed *Del* juice.

Similar to the TAP assay, there was a trend of increased antioxidant capacity

during the fasting period through the first week of juice consumption. After that period it is difficult to explain the pattern of changes. Though not specific to the carotenoids, this assay does show that gross changes to plasma antioxidant status did not vary widely over the course of the study. However, the pattern of changes mimics the TAP assay measurements in the observed changes during juice consumption. This indicates that the tomato juices with the *t* and og^c and possibly *r* genes do result in increased antioxidant capacity of plasma. When all of the juice nutrients are considered, this may make sense, as the *r*, *t*, and og^c, juices have higher tocopherols compared to the *B* and *Del* juices, and have higher ascorbic acid content compared to *B*.

In conclusion, this research demonstrates the complexity of studying antioxidants *ex vivo* following tomato consumption. Despite the relative abundance of lycopene in the og^cog^c genotype tomato juice and absence of the same from the *rr* genotype tomato juice, there appears to be no significant effect of all-*trans*-lycopene on plasma antioxidant status as measured by the TAP assay. The statistical significance of this study based on results from two volunteers is of course extremely limited. However, this work demonstrates the need for a shift in focus away from lycopene as an antioxidant. While there were changes in antioxidant status over the course of the dietary intervention study, these changes were not clearly attributable to changes in any of the carotenoids. Since the carotenoids are known antioxidants it seems that their effects in plasma are relatively limited. This is not to say that the carotenoids are not beneficial to human health. Instead, their primary benefits reported elsewere (anti-cancer, anti-

inflammatory, enhanced gap junction communication, etc) appear to be due to a characteristic other than antioxidant activity, or at least not due to antioxidant activity in plasma as measured by the TAP assay, and it is my opinion that these benefits and mechanisms of influence of lycopene need to be studied further. The interaction of carotenoids with other compounds such as α-tocopherol also bears further exploration because some of the benefits of tomato consumption may be caused by synergistic activity between the two.

CHAPTER 6. CONCLUSIONS

While purple tomatoes are not likely to hit the market in the immediate future, the continued marketing of 'high antioxidant' fruits and vegetables will very likely prepare a niche market for such a novelty crop. The anthocyanidins responsible for the purple color have been reported to have antioxidant activity in previous reports. Testing of the various genetic combinations of tomato fruit anthocyanin-expression genes revealed a trend of increased antioxidant capacity with increasing purple color intensity. The increase in anthocyanins was found to correlate to an increase in total phenolic content. Phenolics, known for their antioxidant activity, may be beneficial for human consumption, and also have antioxidant activity. The trend of increased phenolics and anthocyanins may be exploited by breeders by using the anthocyanin-expression-enhancing genes and other genes influencing the flavonoid pathway to produce tomatoes with elevated phenolic content, resulting in increased water soluble antioxidant content in tomato fruit skin. The combination of the flavonoid altering genes *Aft*, *atv*, and *hp-1* together with the high lycopene gene og ^{c} will further increase the antioxidant content of the tomato, elevating ascorbic acid, α-tocopherol, carotenoid, and phenolic content. Such a tomato with be a truly 'high antioxidant' fruit, boasting high levels of both water and lipid soluble antioxidants.

Tomatoes and tomato products are currently marketed using phrases like; "contains lycopene, a known antioxidant". While true, this may be misinterpreted to mean that tomatoes without high lycopene content are not as beneficial in terms

of antioxidant activity. Such reasoning is flawed because all-*trans*-lycopene is not the primary antioxidant in tomatoes, nor has its antioxidant activity been proven to have significant beneficial effect *in vivo*. Indeed, human tomato and tomato product consumption studies show that the tomato has beneficial effects on *ex vivo* antioxidant activity, but this is not proof that lycopene is the cause of the observed increase in antioxidant activity. Instead, lycopene may produce beneficial effects following human consumption due to its proposed ability to interact with human nuclear genes such as the connexins. Future studies of health benefits and antioxidant effects should include both red (lycopene-containing) tomatoes and yellow (lycopene free) tomatoes to increase our understanding of the effects of the antioxidant, lycopene. The human dietary intervention pilot study reported here is an attempt to achieve this, with the results indicating that tomato benefits to antioxidant activity are not necessarily the result of lycopene. Rather, the tomato appears to increase antioxidant activity as measured by the TAP assay without the presence of all-*trans*-lycopene. This suggests that lycopene, a known antioxidant, may compliment the already health-beneficial compounds found in tomato. Thus, to produce a 'high antioxidant' tomato will require focus not only on lycopene, but on all the contributing antioxidants found in the fruit.

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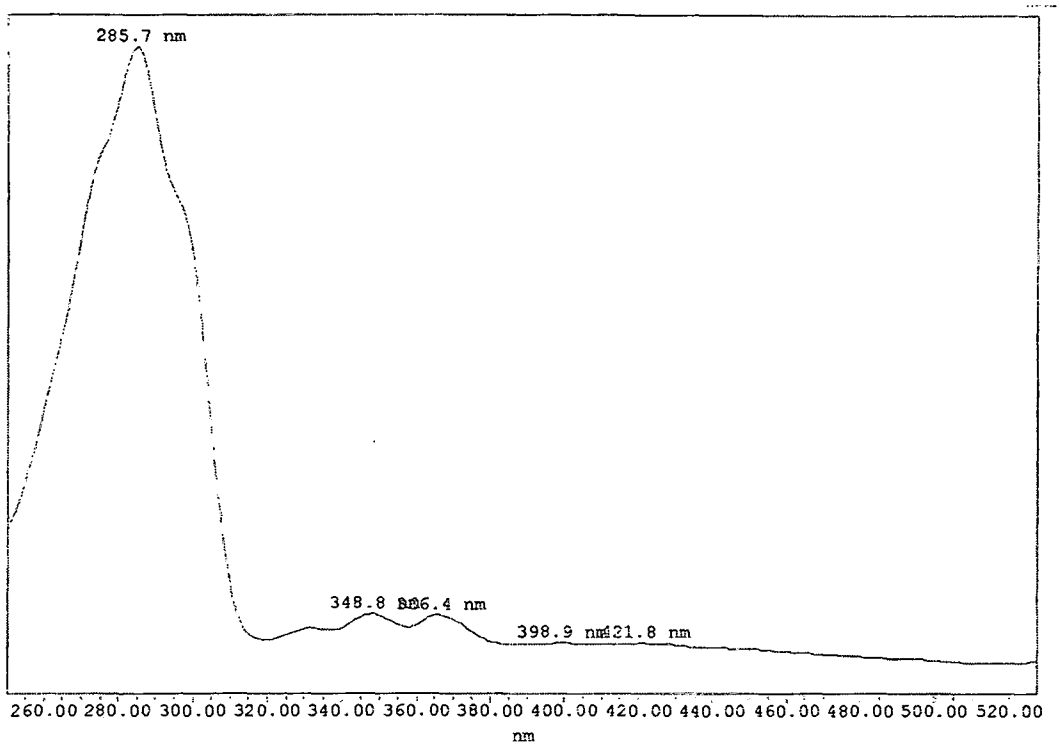
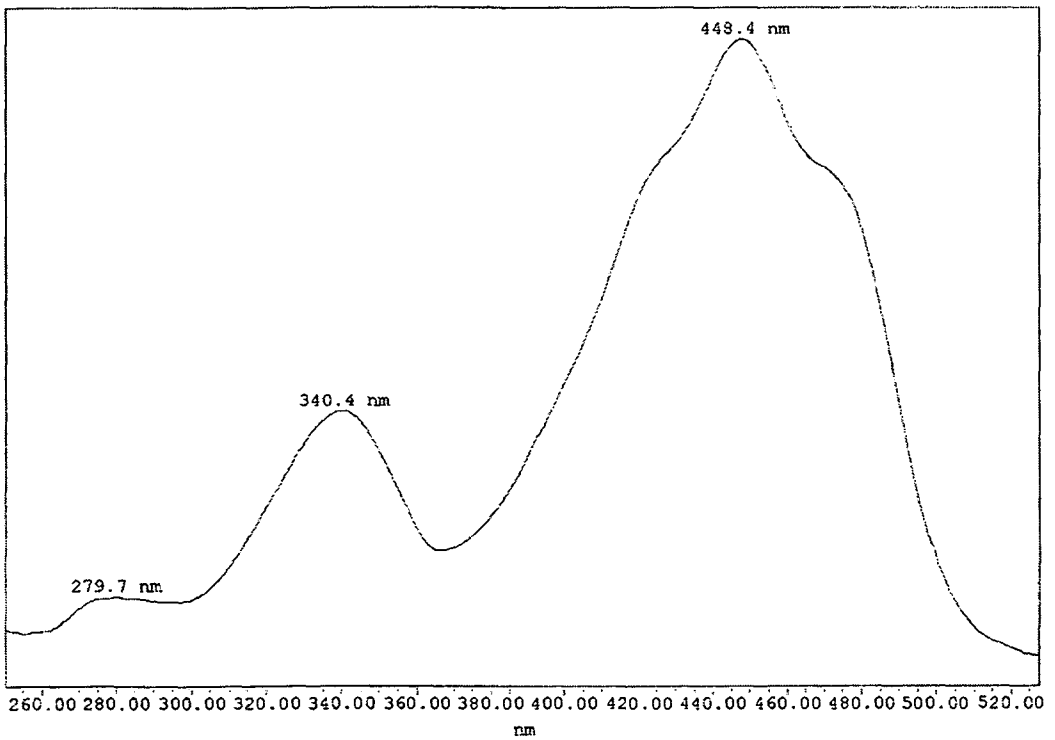
APPENDICES

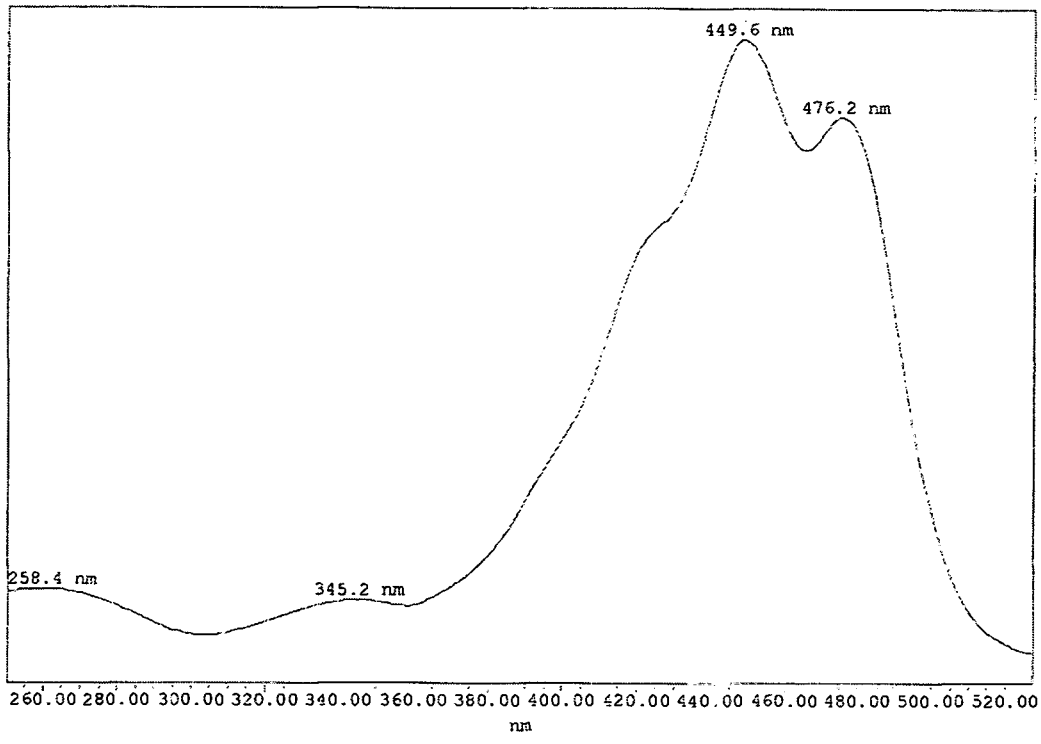
APPENDIX 1. CAROTENOID SPECTRA



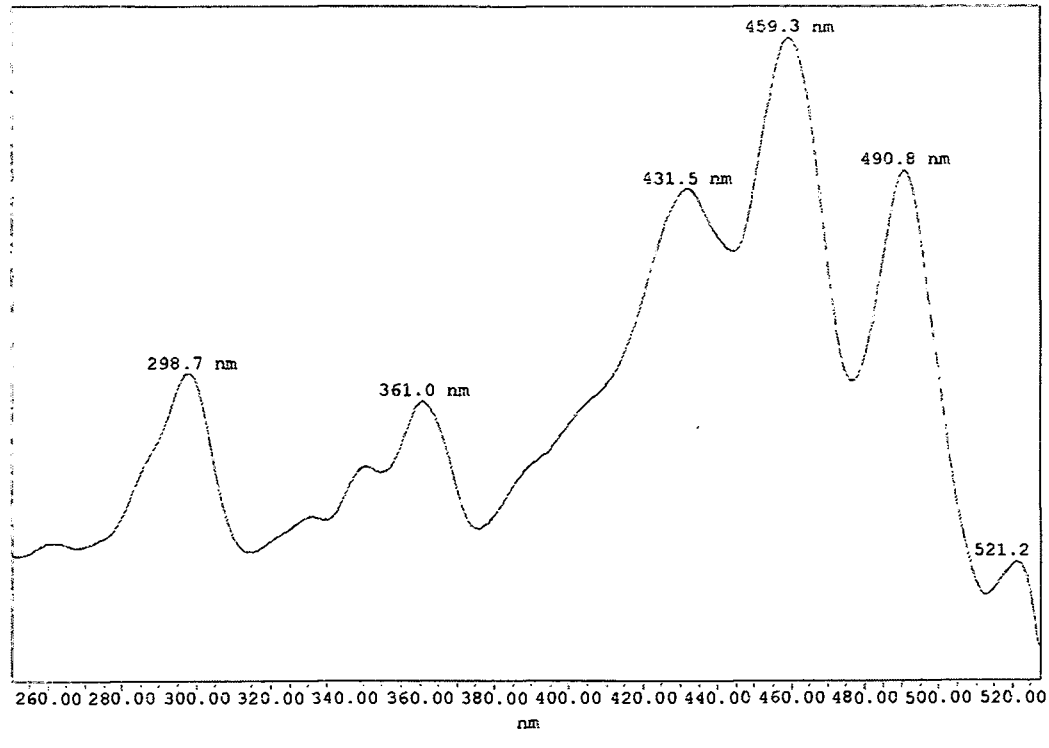
cis-phytoene



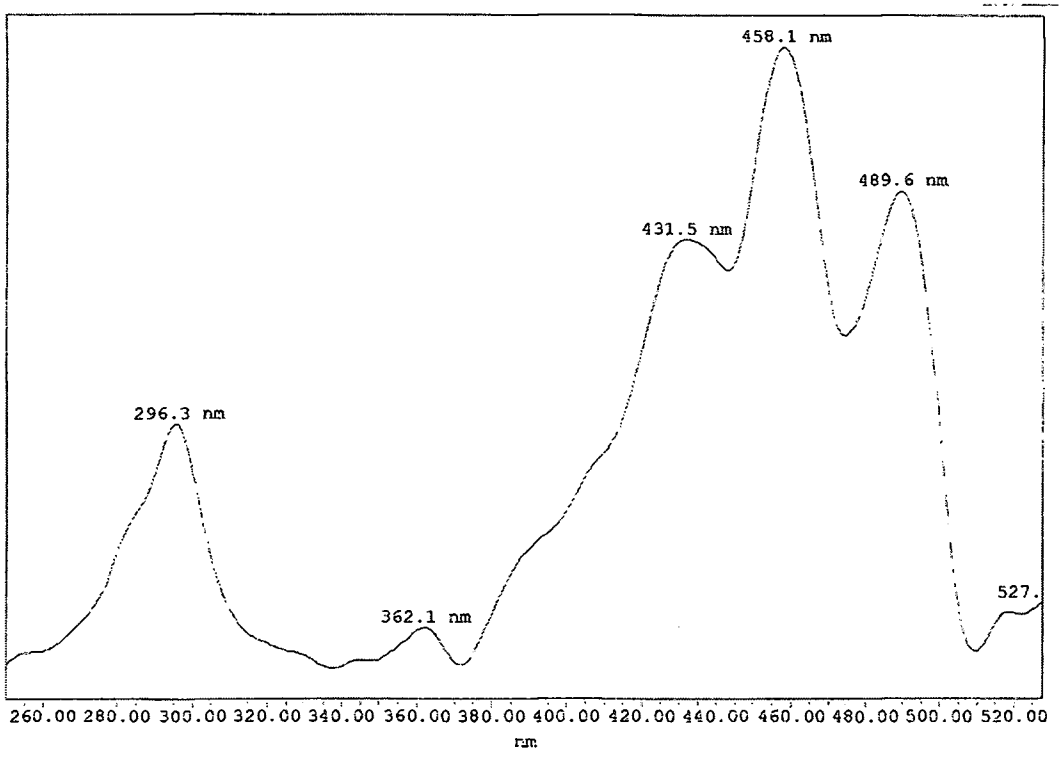
15-cis β-carotene, 7.9 minutes retention



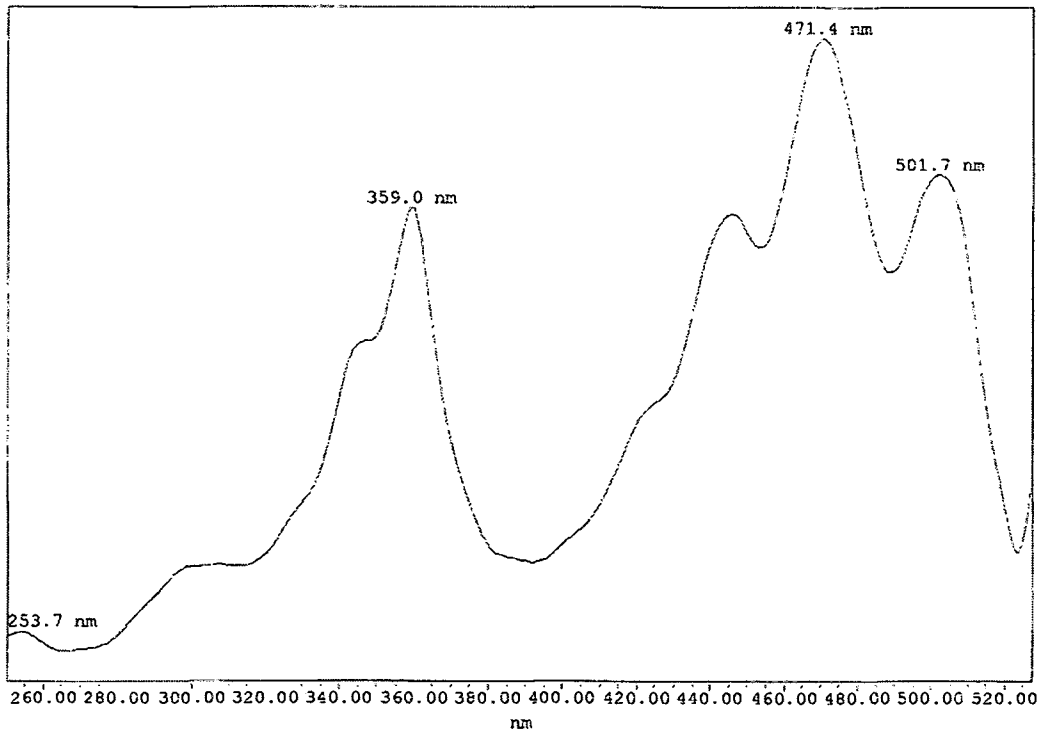
Unknown 1. 12.1 minute retention time (CaroRich)



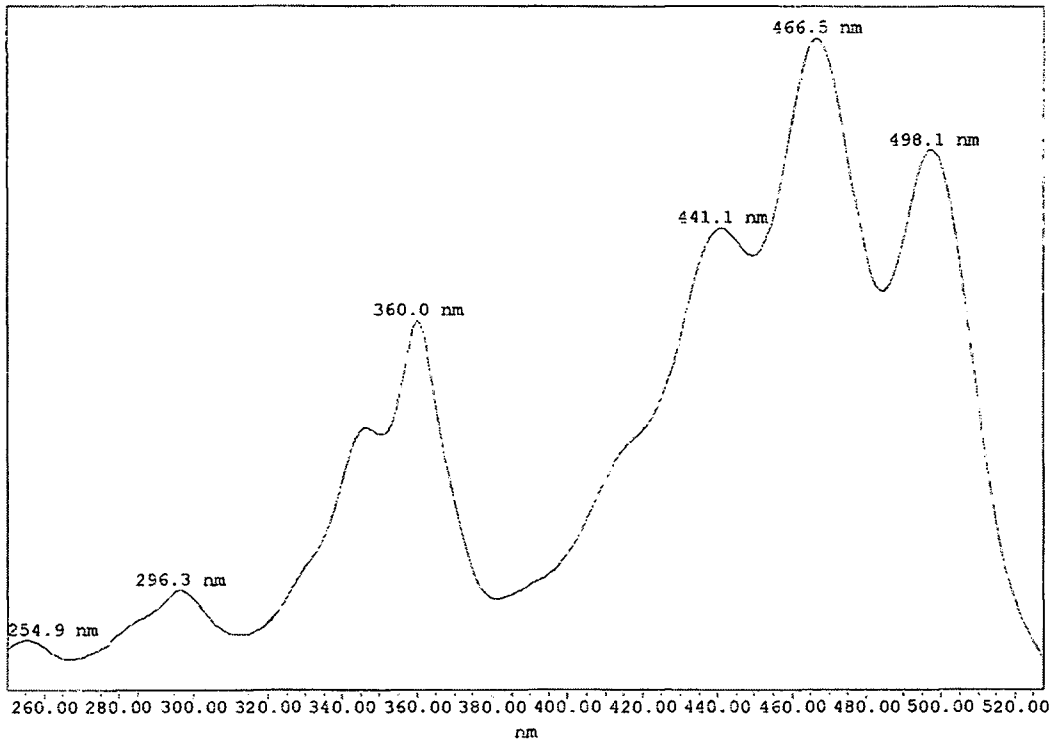
Cis-δ-carotene, 15.8 minutes retention time



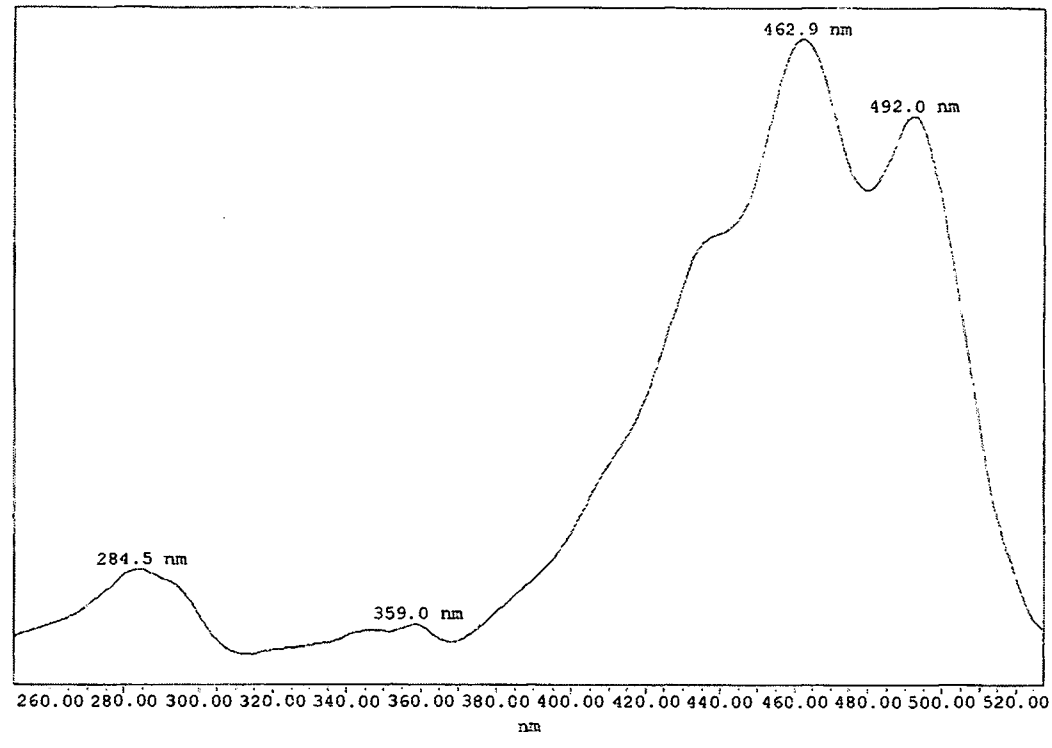
Cis δ-carotene, 19.8 minutes retention



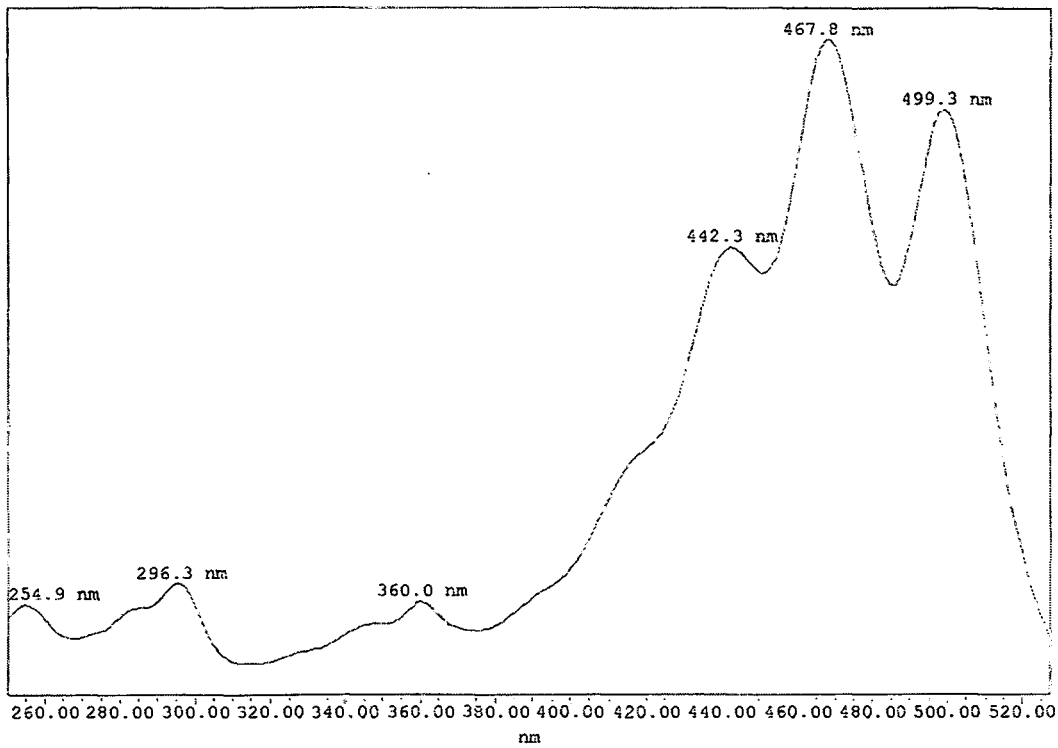
15-*cis*-lycopene, 17.8 minutes retention time



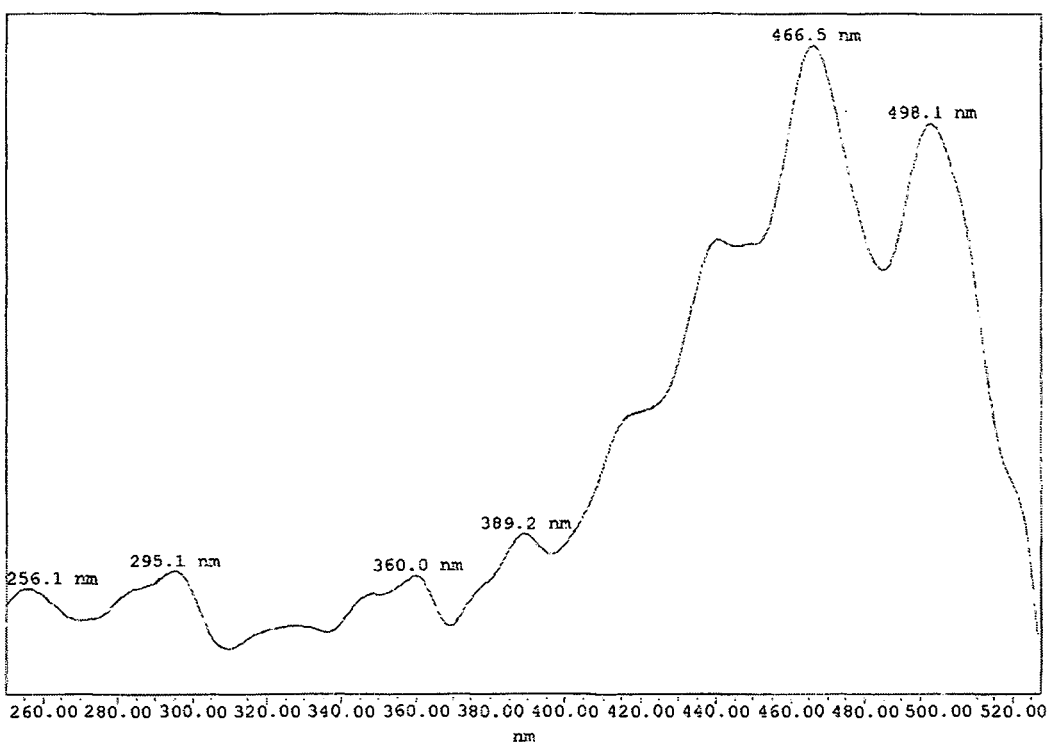
9,13-cis-lycopene, 20.9 minutes retention



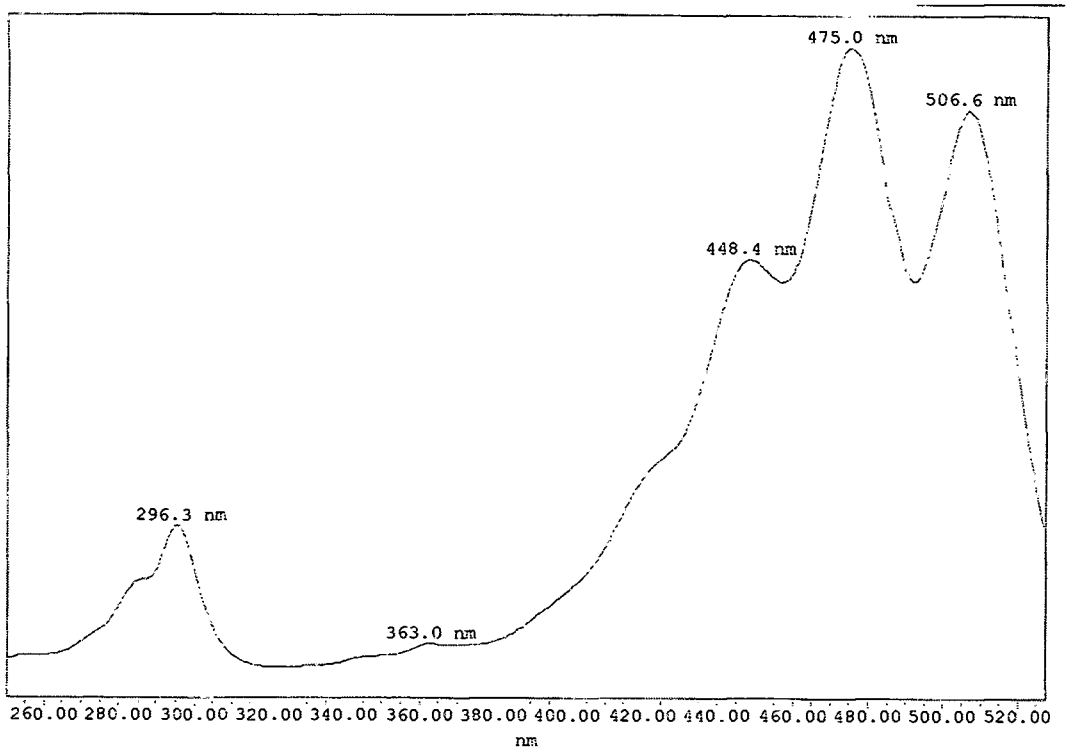
γ-carotene, 24.3 minutes retention



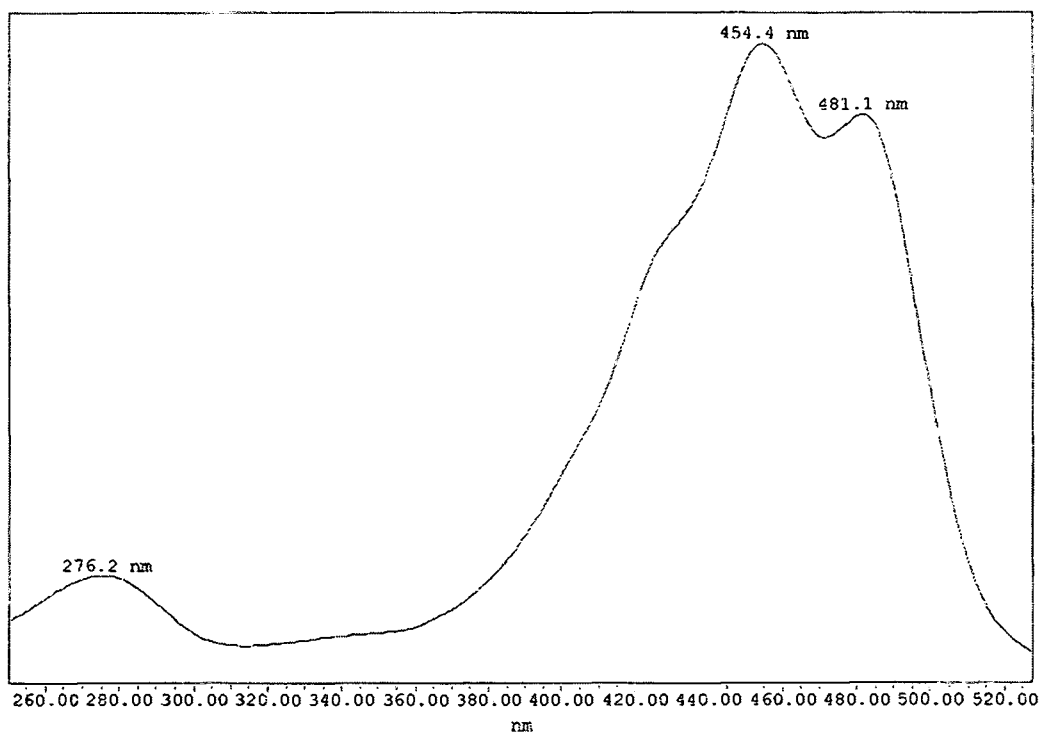
9,13-di-cis-lycopene?, 31.5 minutes retention



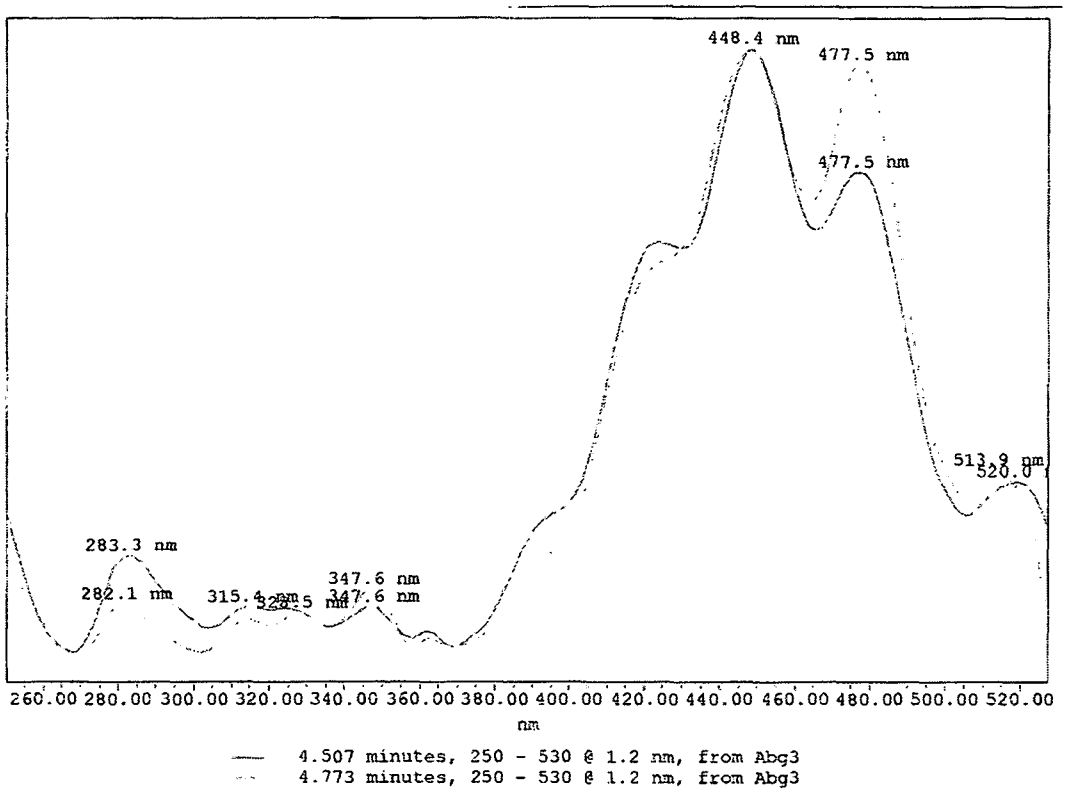
9,13-di-cis-lycopene again?, 34.5 minutes retention



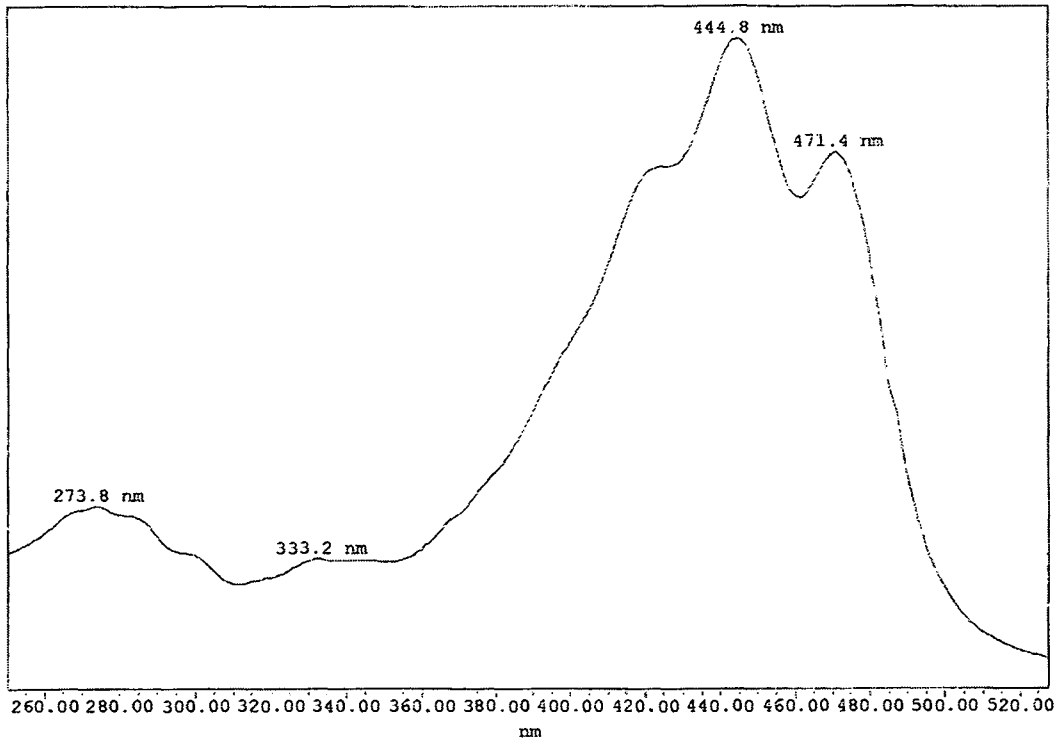
All-trans-lycopene, 49 minutes retention



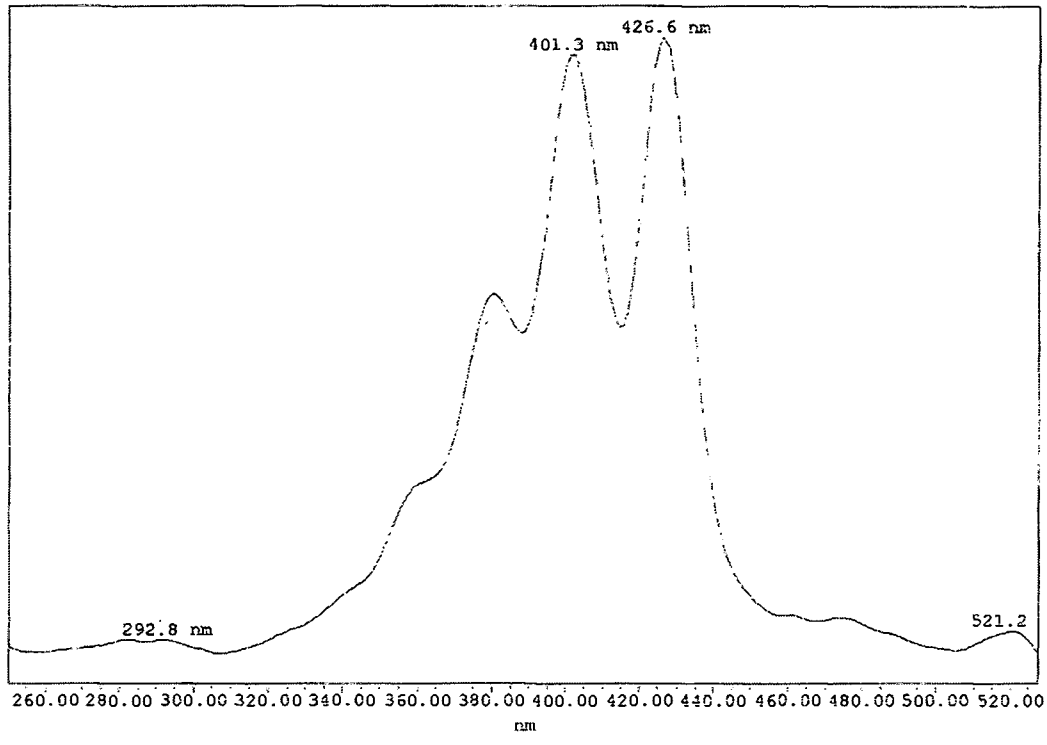
All-trans-β-carotene, 11.3 minutes retention



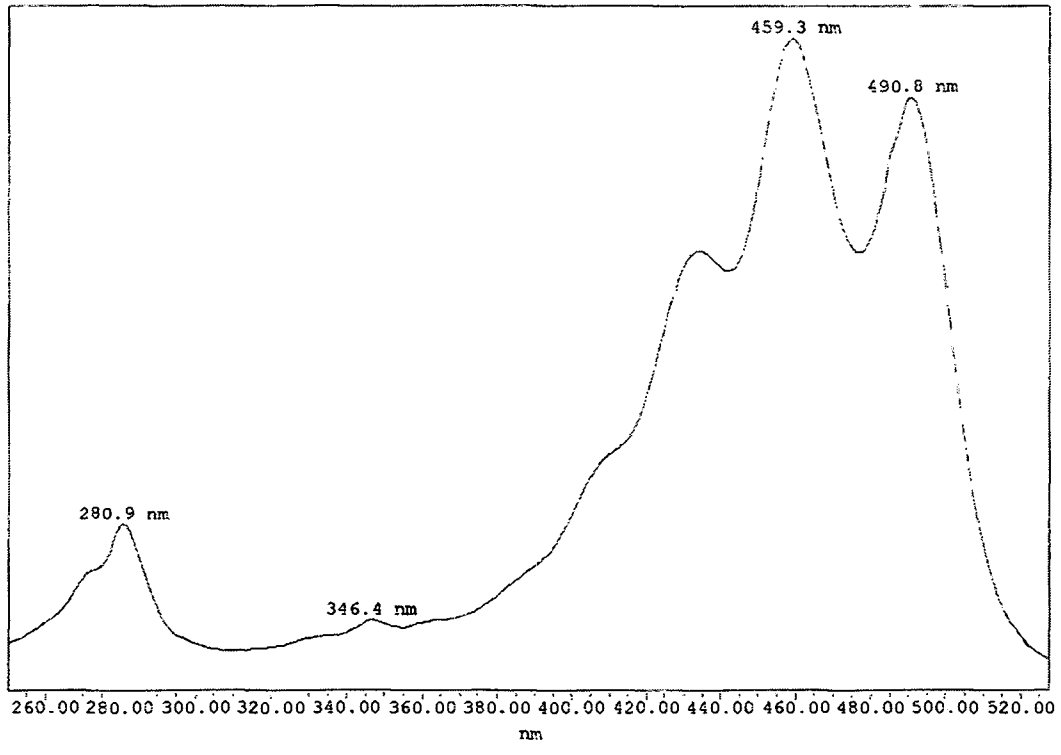
Lutein isomers, 4.5 and 4.7 minutes retention



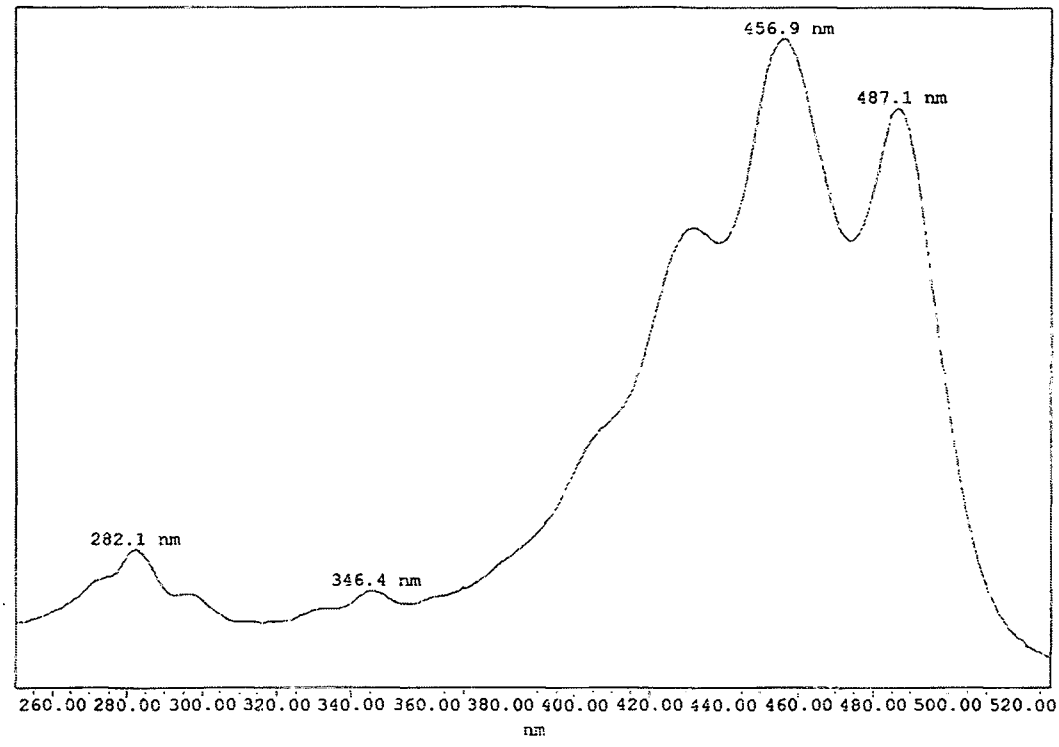
α-carotene, 4.9 minutes retention



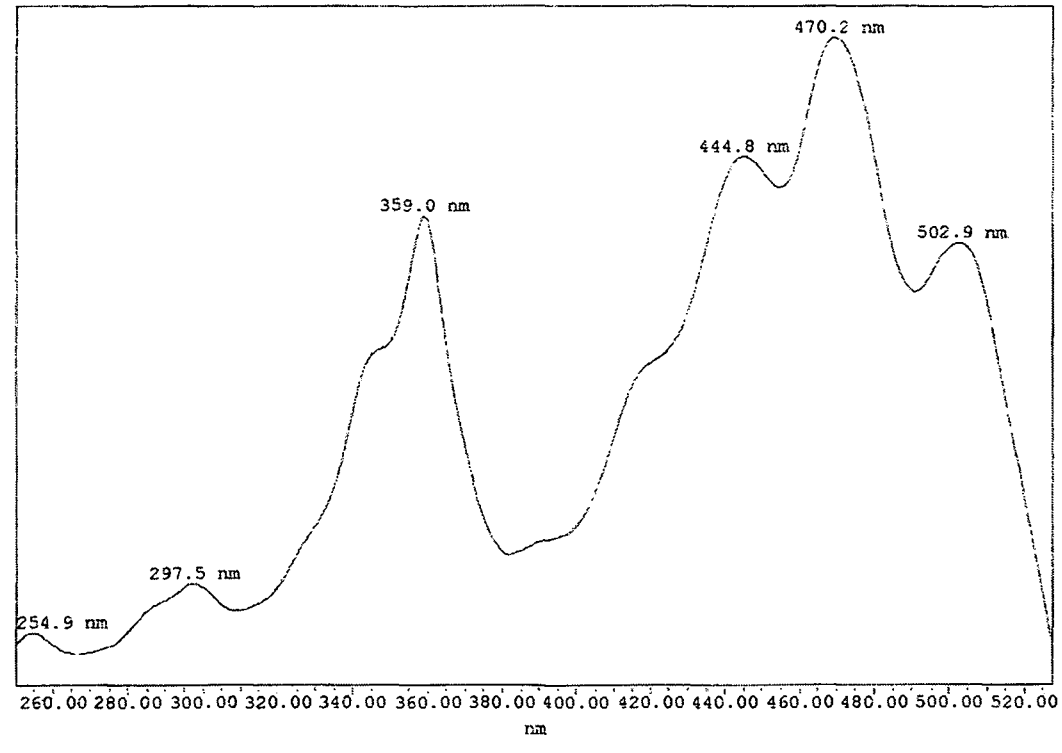
ζ-carotene, 9.7 minutes retention



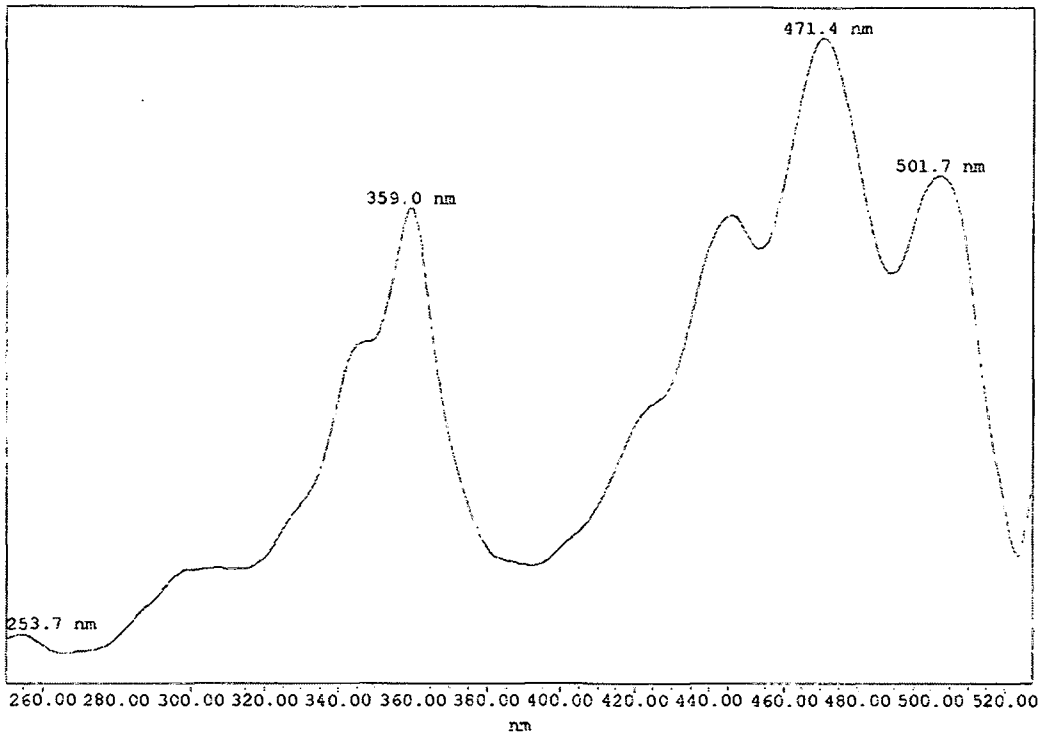
δ-carotene, 16.2 minutes retention



?cis δ-carotene, 17.7 minutes retention



Unknown, 17.2 minutes retention



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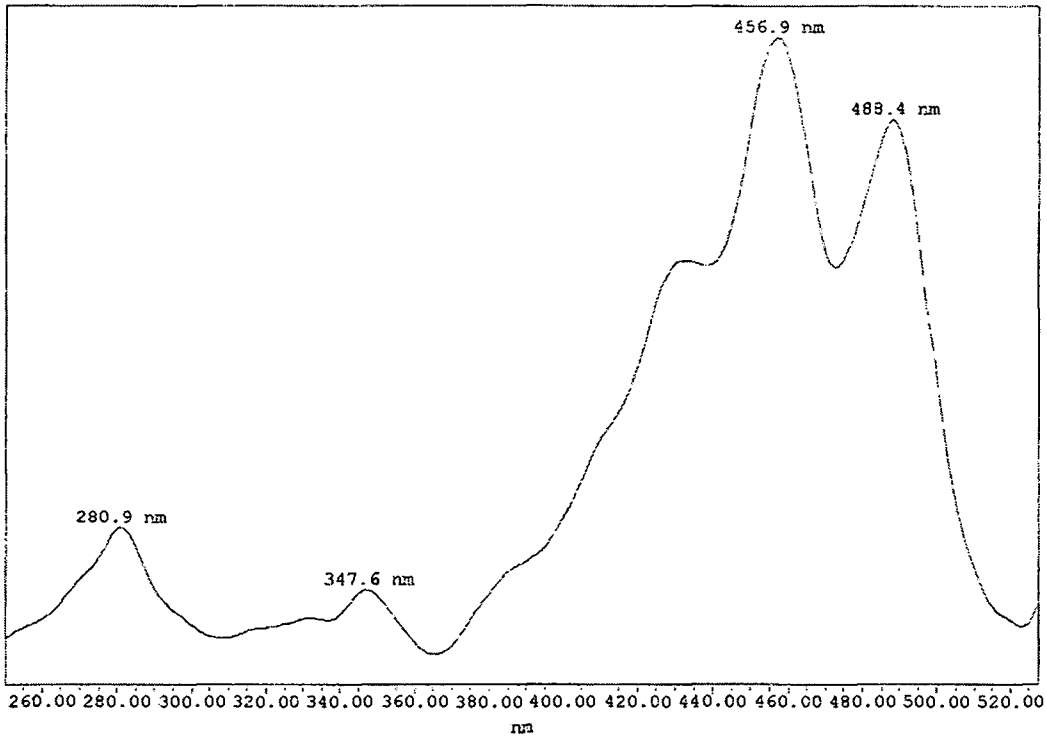
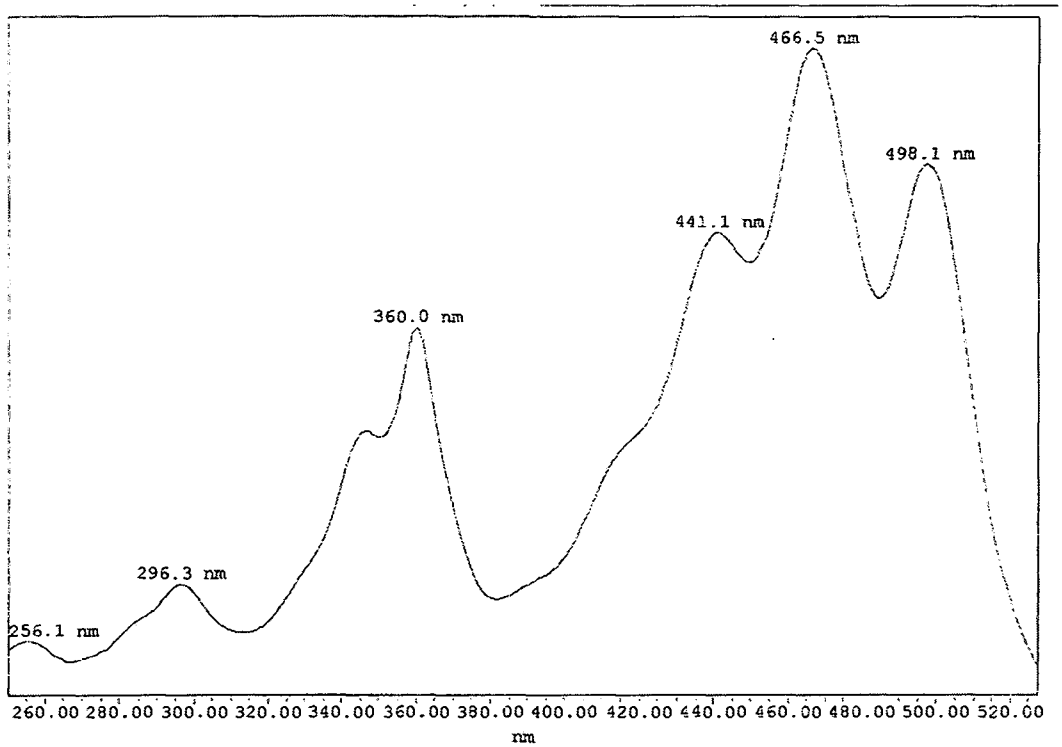
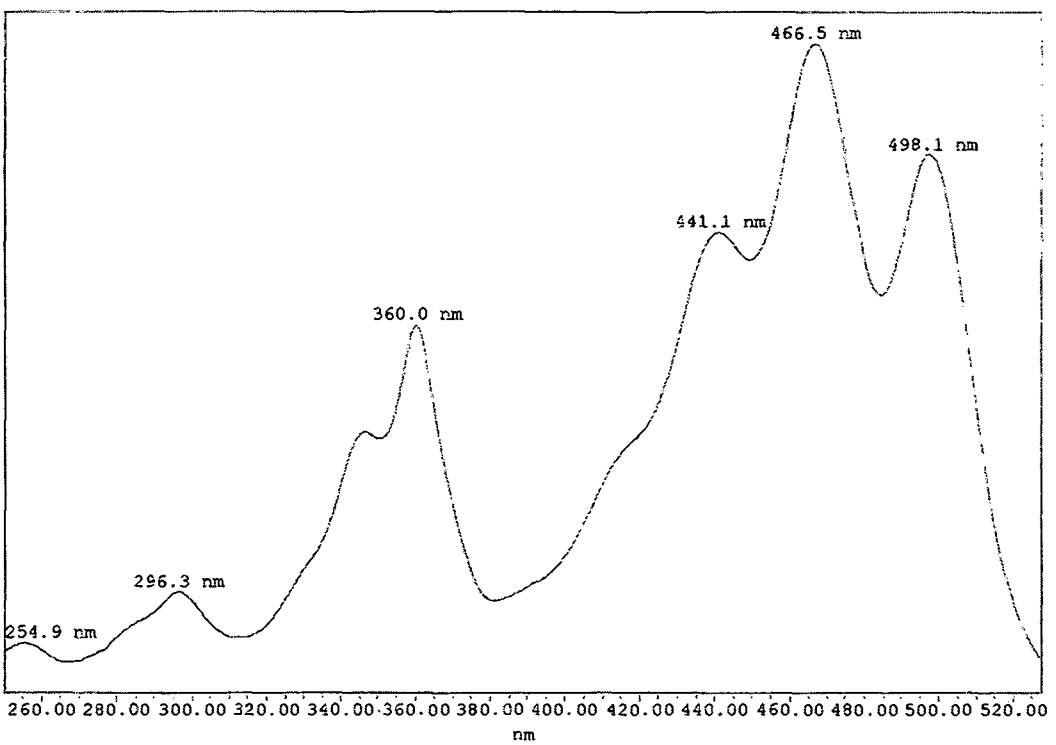


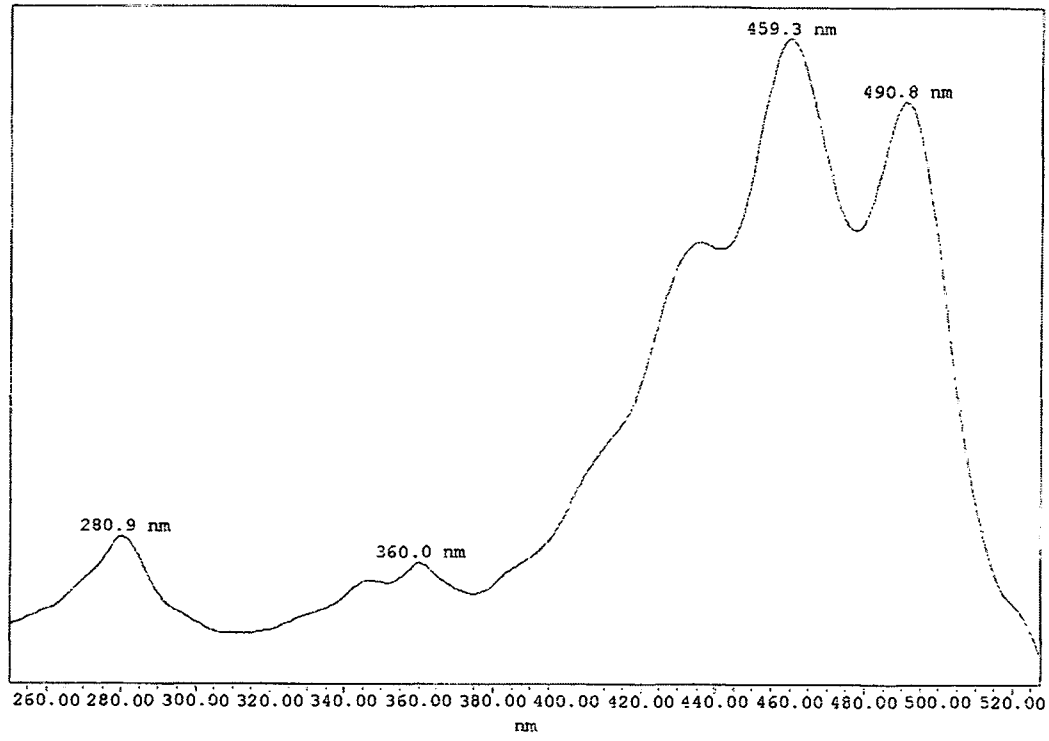
Unknown, 18.9 minutes retention



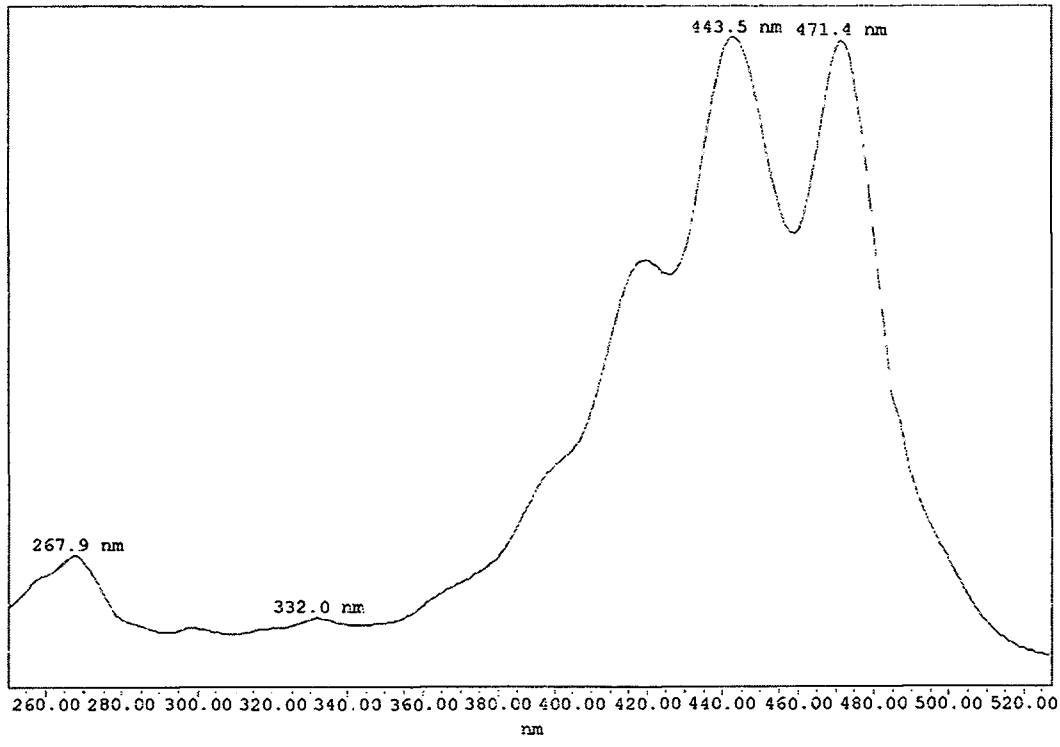
Unknown, 20.3 minutes retention



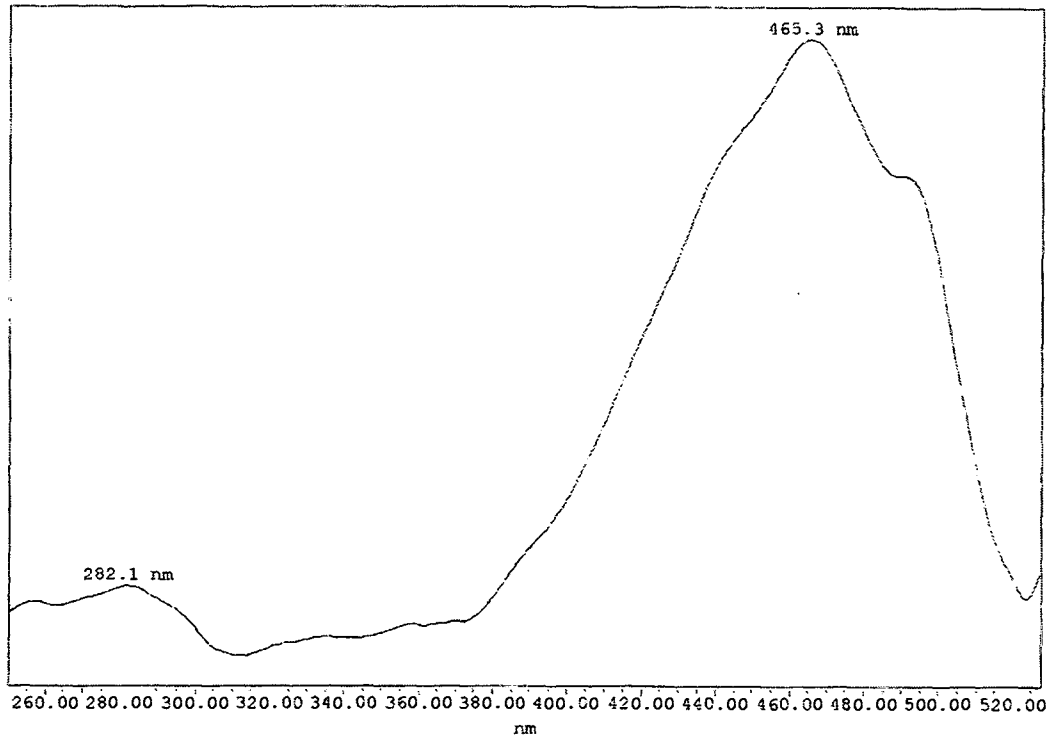
13-cis-lycopene, 20.9 minutes retention



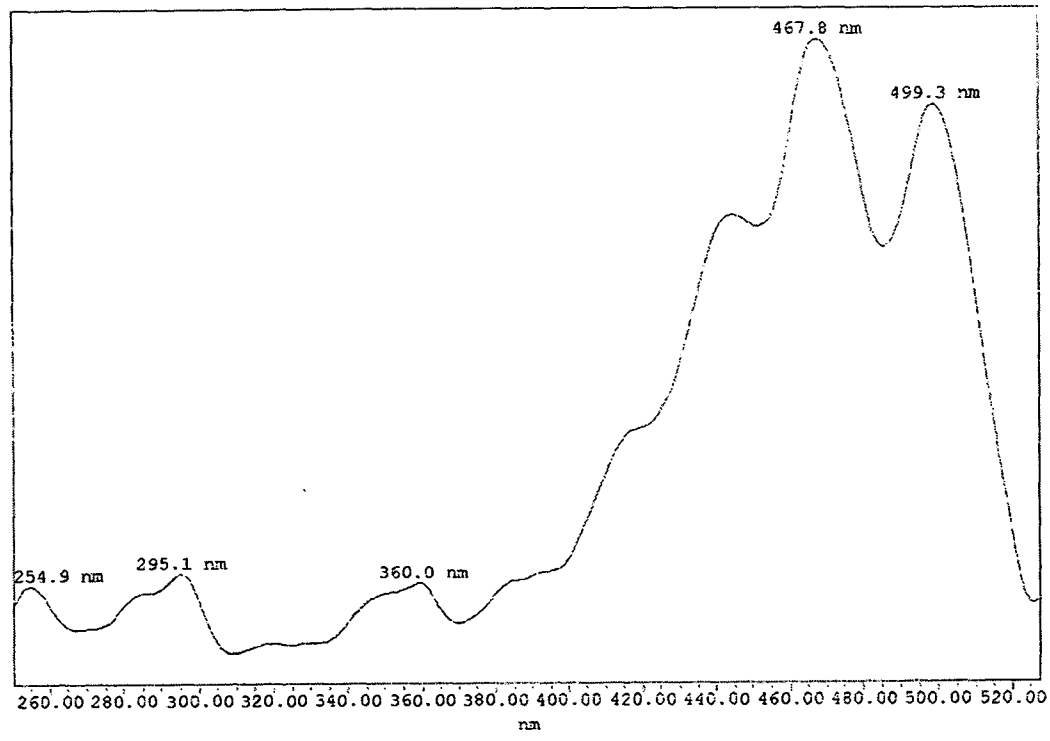
Unknown, 21.5 minutes retention



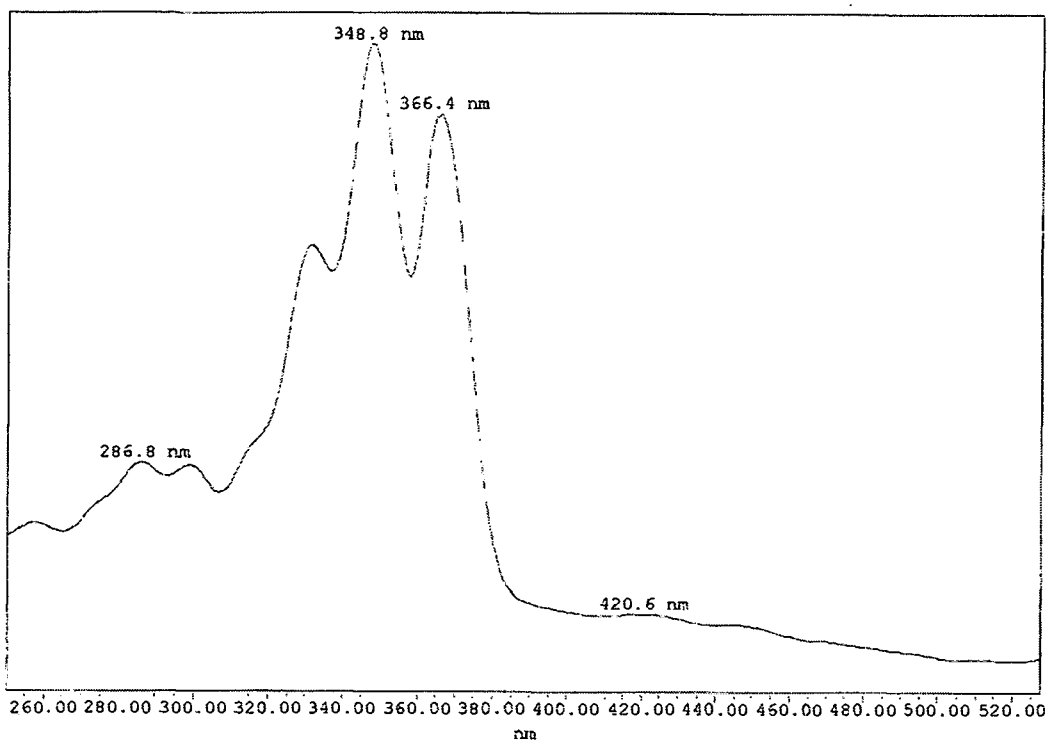
Neurosporene, 22.1 minutes retention (from Carotenature standard)



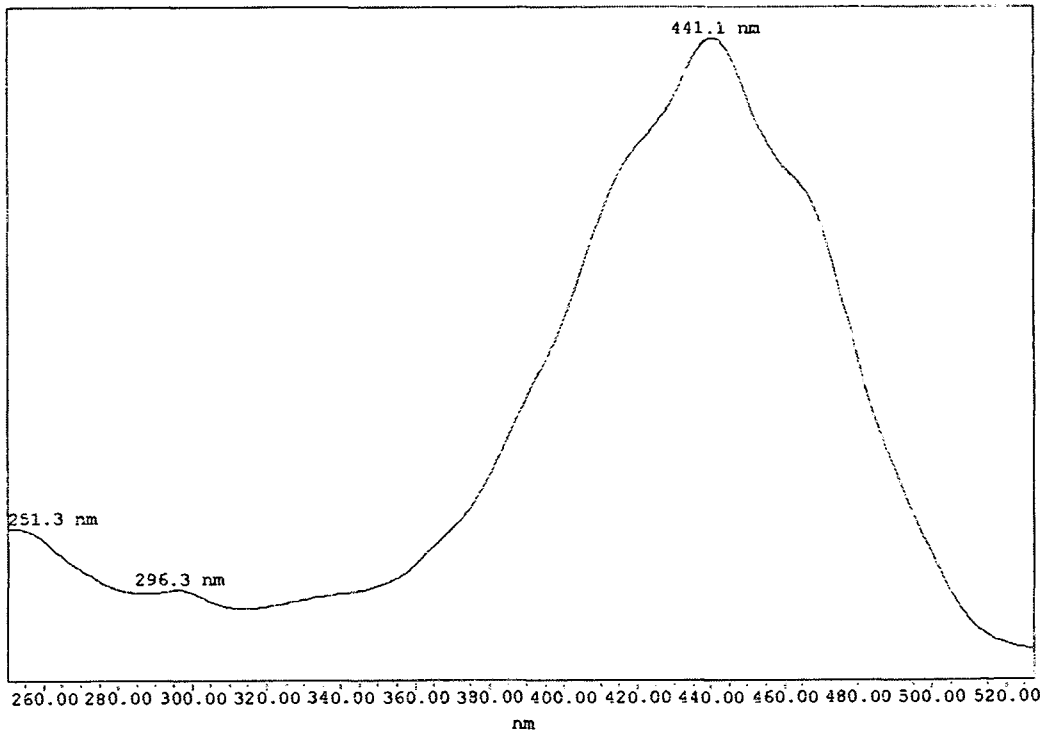
Unknown, 24.2 minutes retention



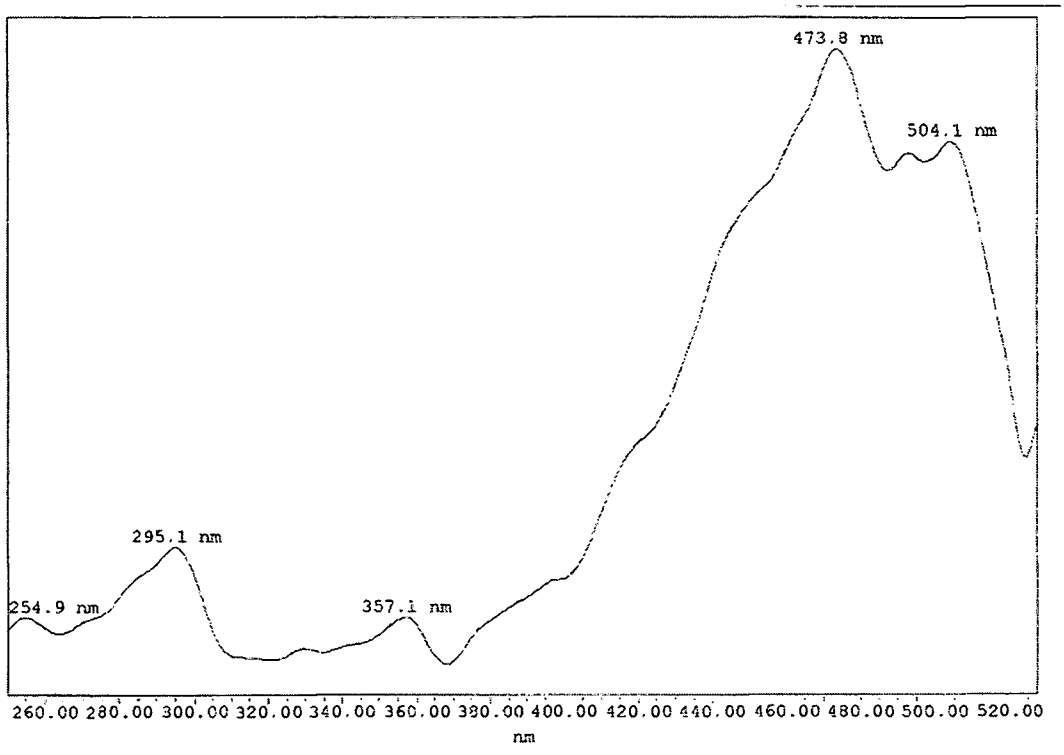
Unknown, 30.3 minutes retention



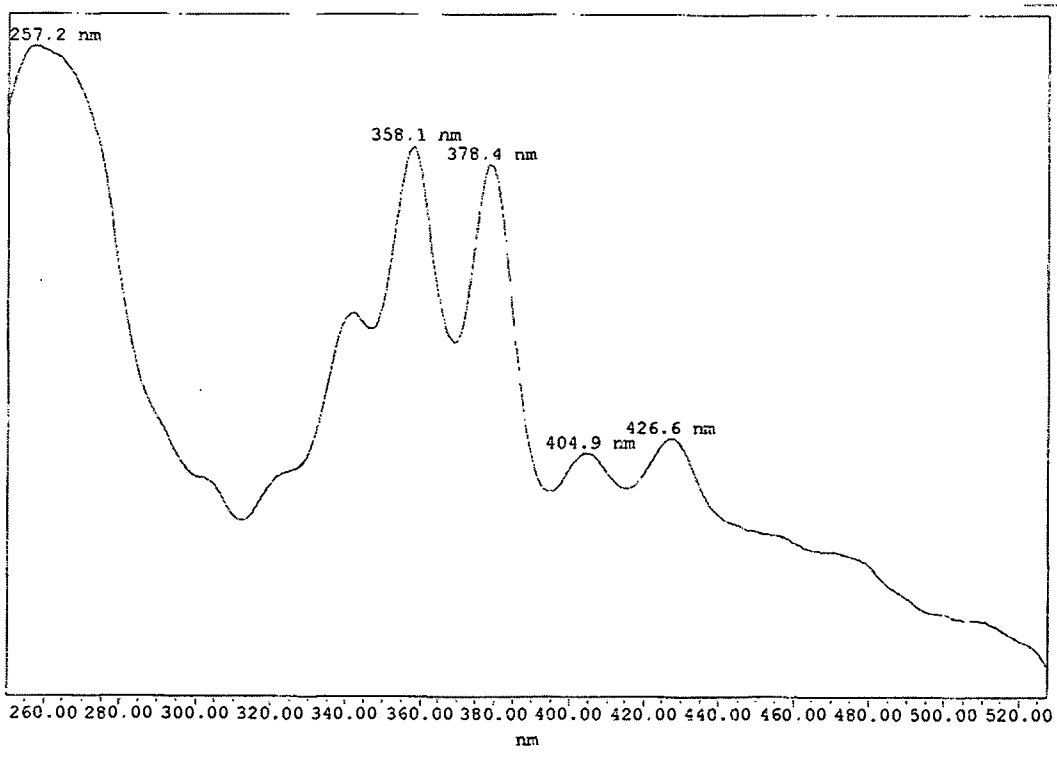
Phytofluene, 5.8 minutes retention



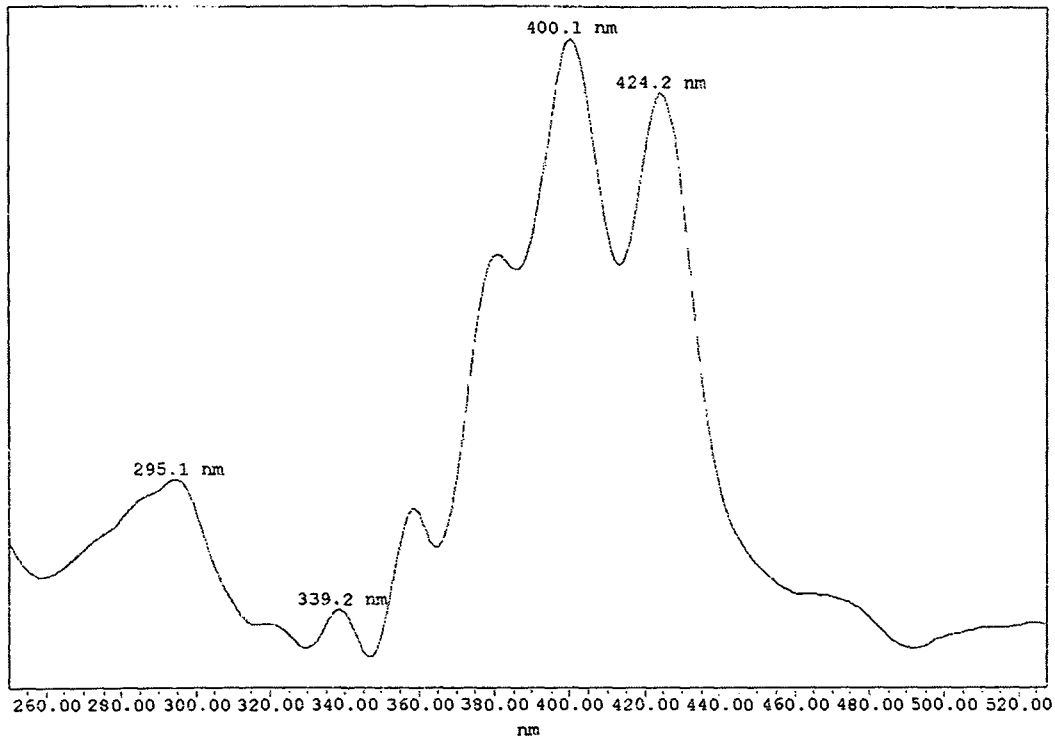
Prolycopene, 10.3 minutes retention



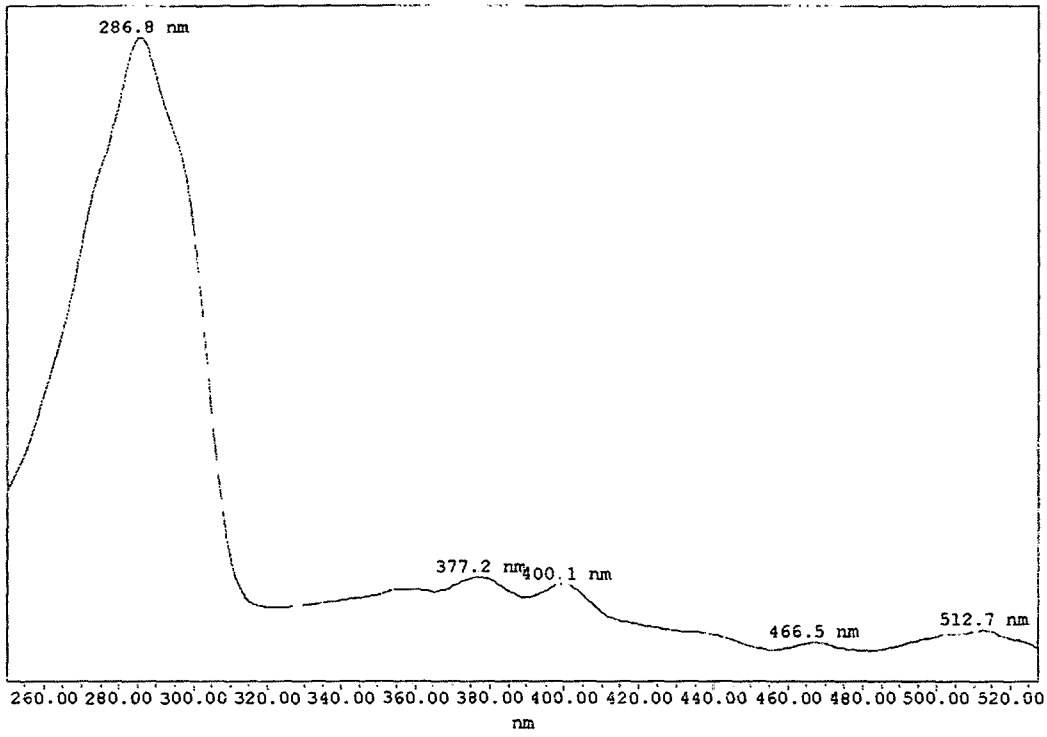
Unknown, 42.5 minutes retention



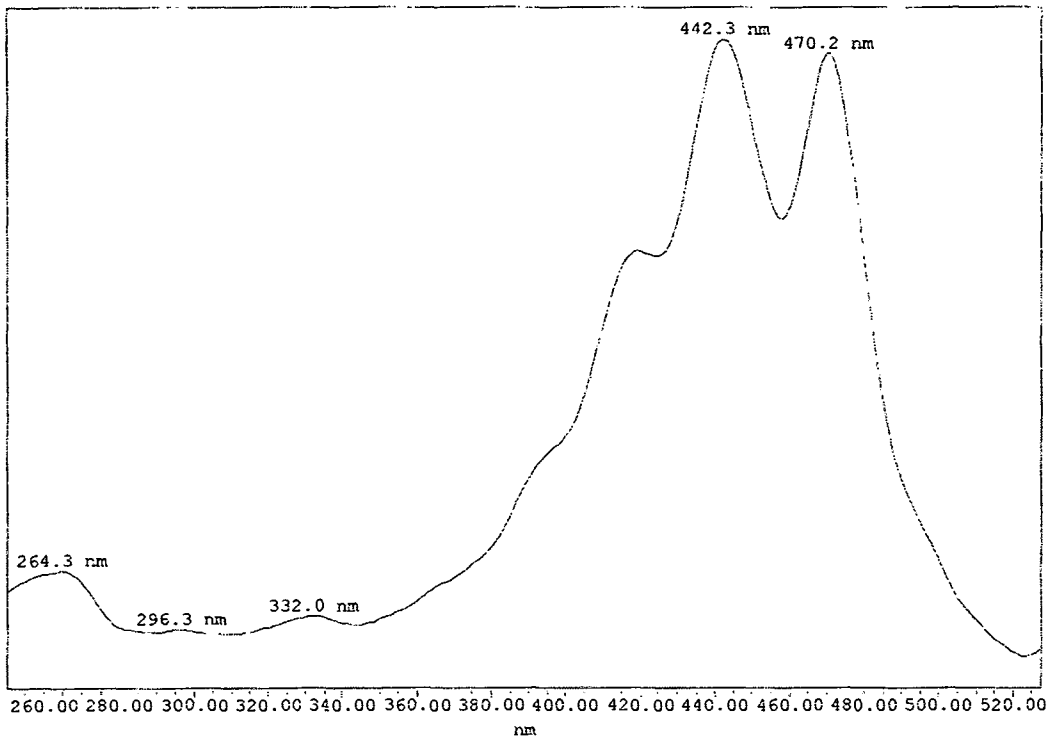
Coelution of two unknowns, 257 an unknown, 358.1 likely ? from *t*, 2.7 minutes retention



ζ-carotene isomer in *t*, 7.2 minutes retention

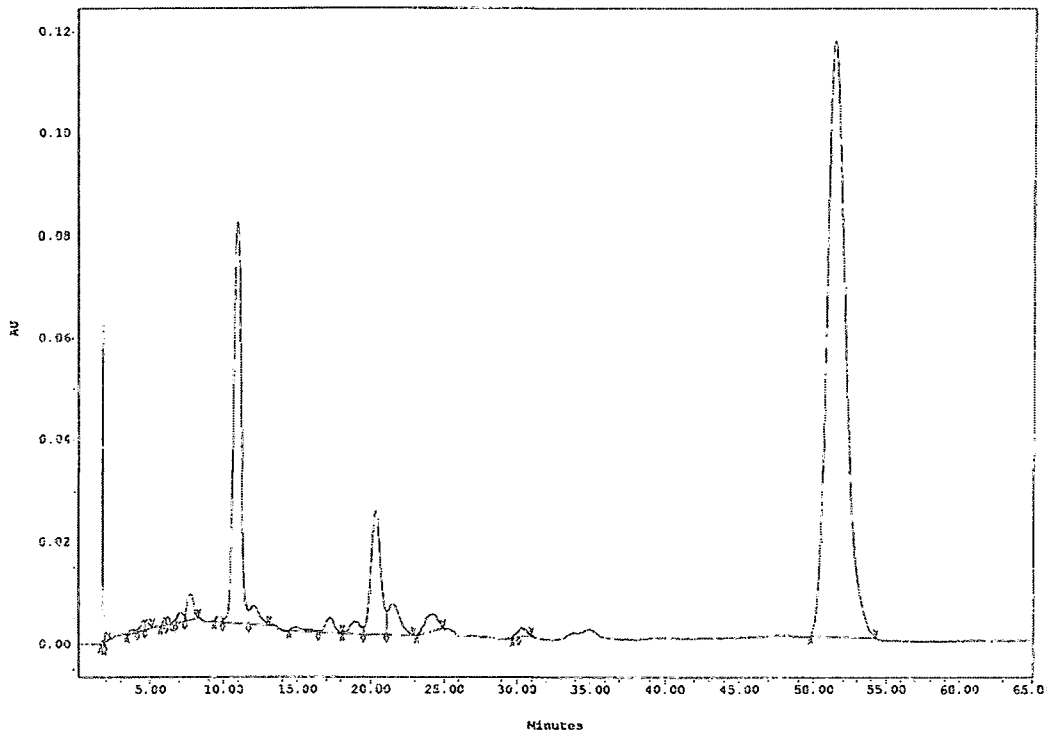


All-*trans*-Phytoene, 5.2 minutes retention

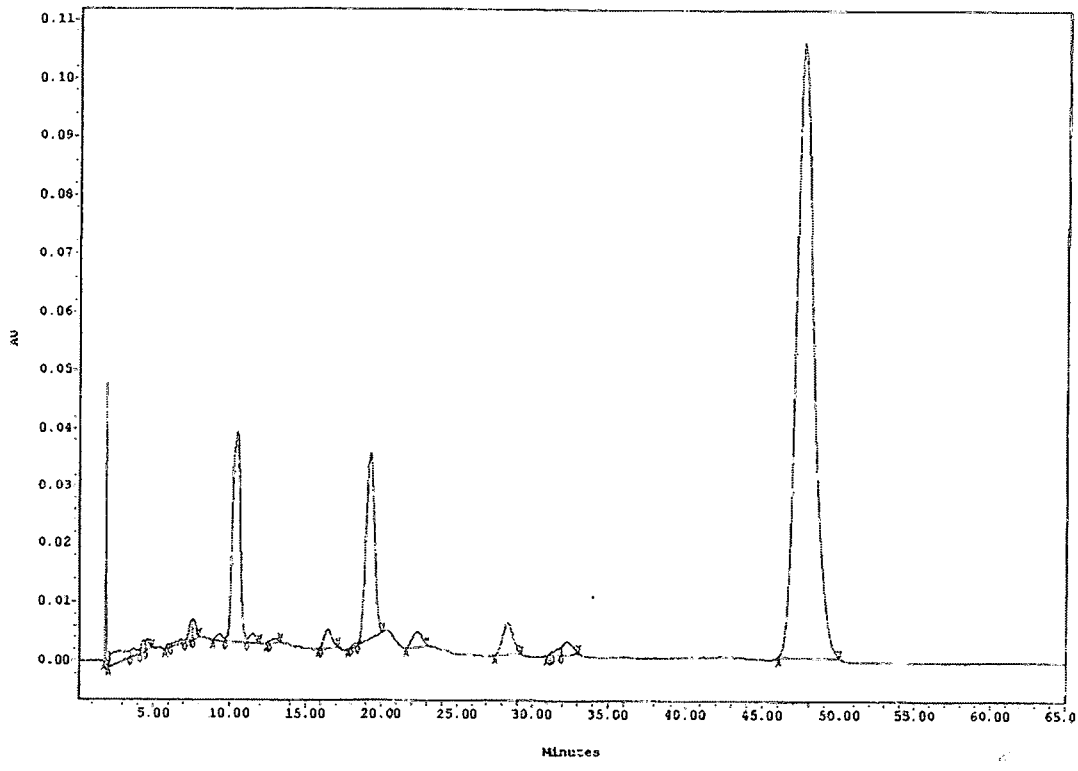


Neurosporene isomer in *t*, 19.9 minutes retention

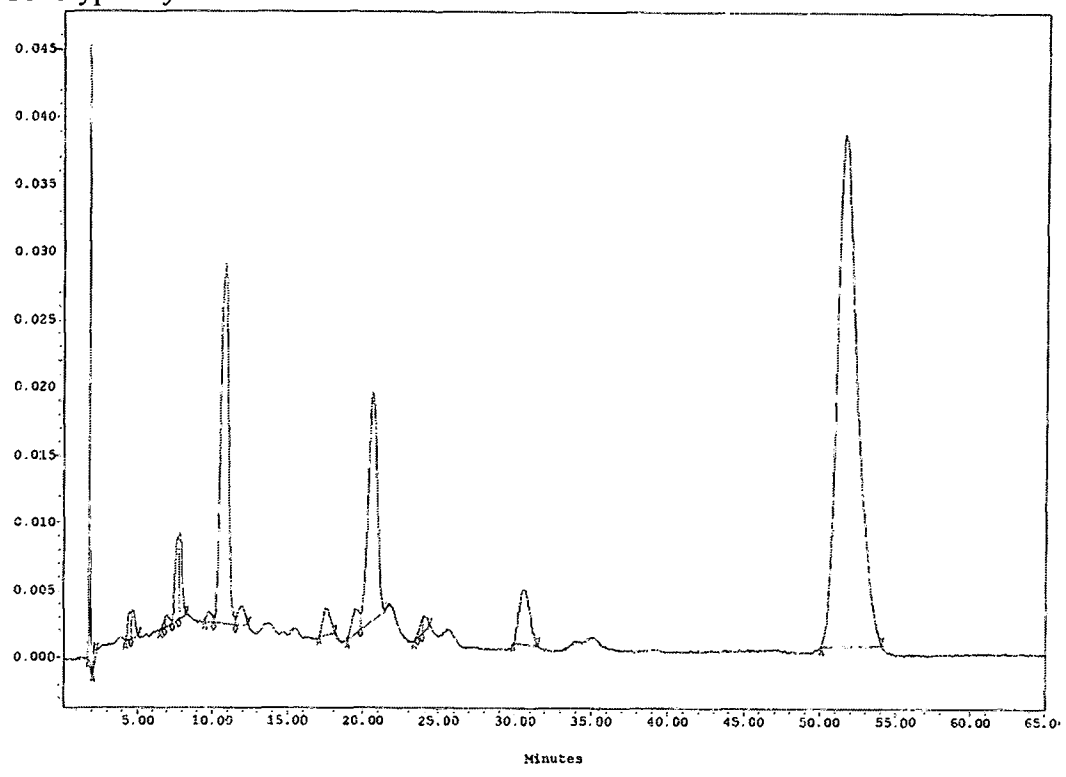
APPENDIX 2. TOMATO CAROTENOID CHROMATOGRAMS @ 450nm



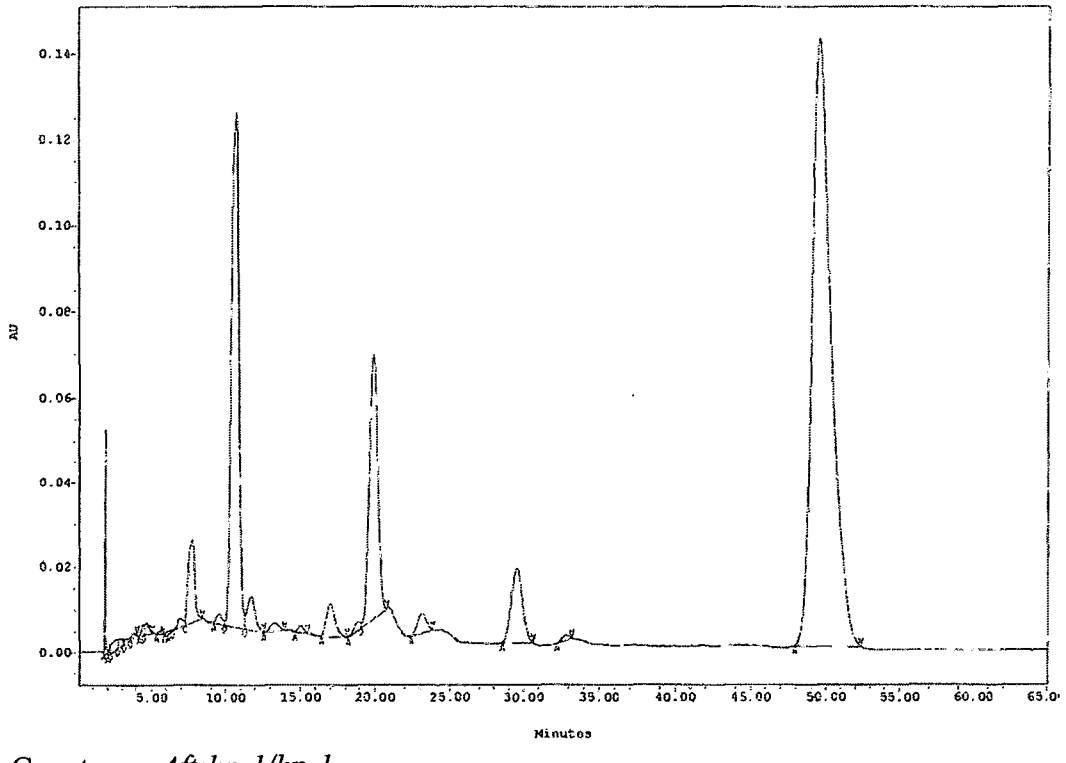
Genotype: *Abg*



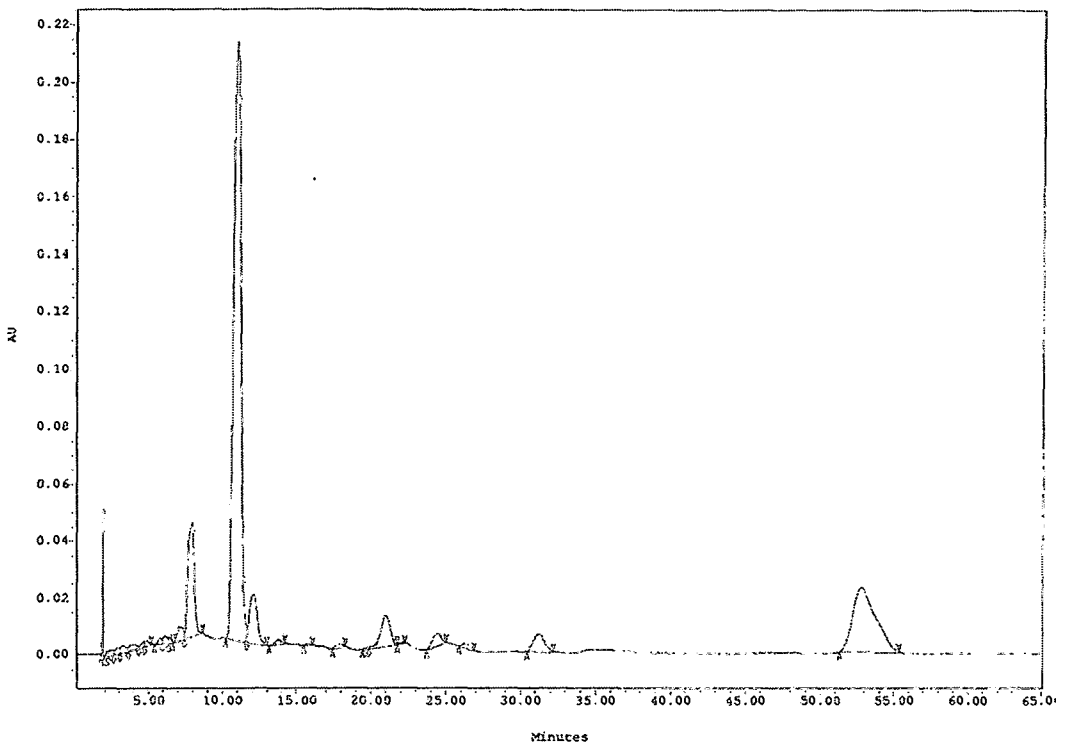
Genotype: *Aft*



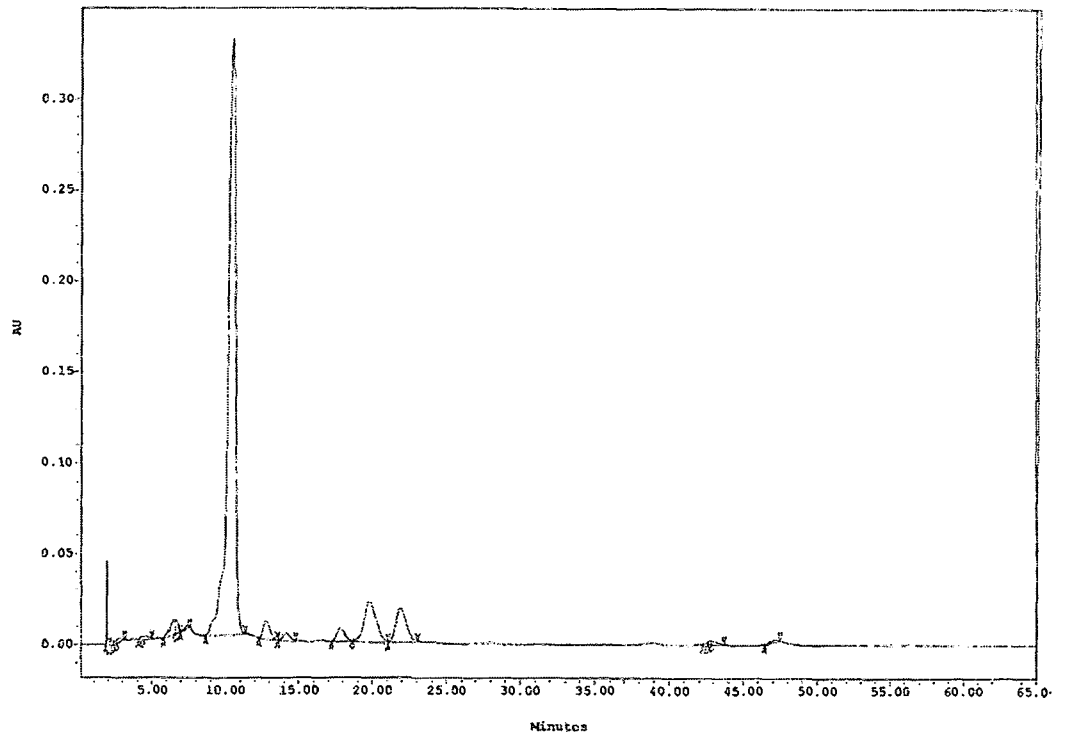
Genotype: *Aft-atvatv*



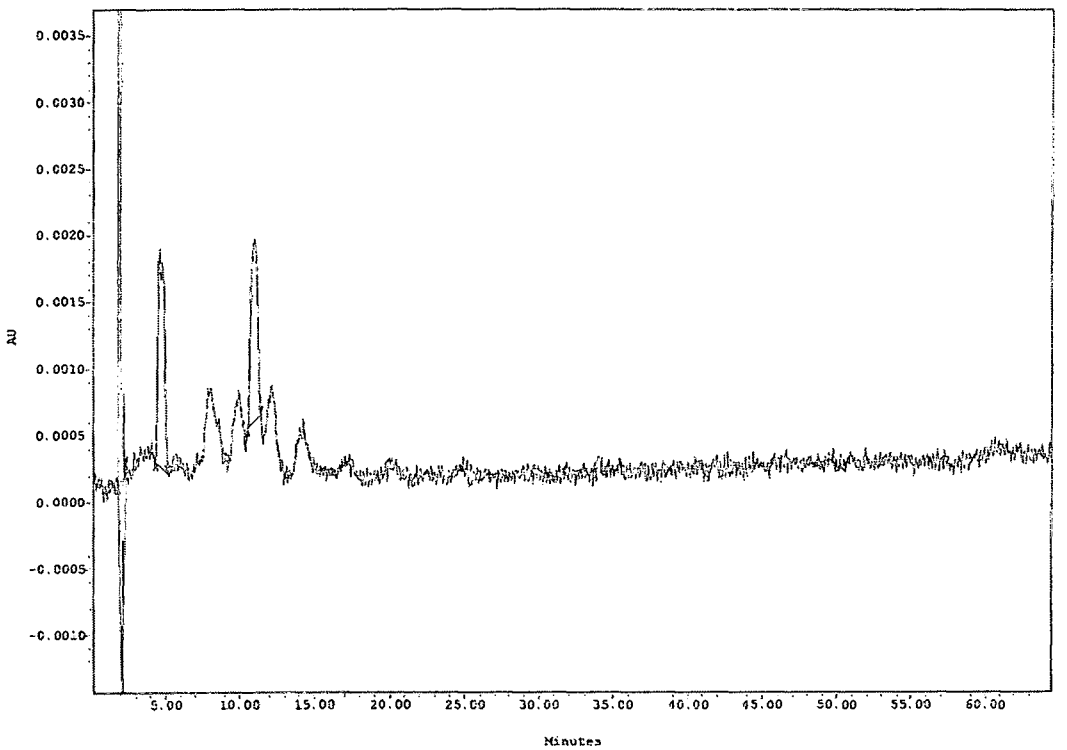
Genotype: *Aft;hp-1/hp-1*



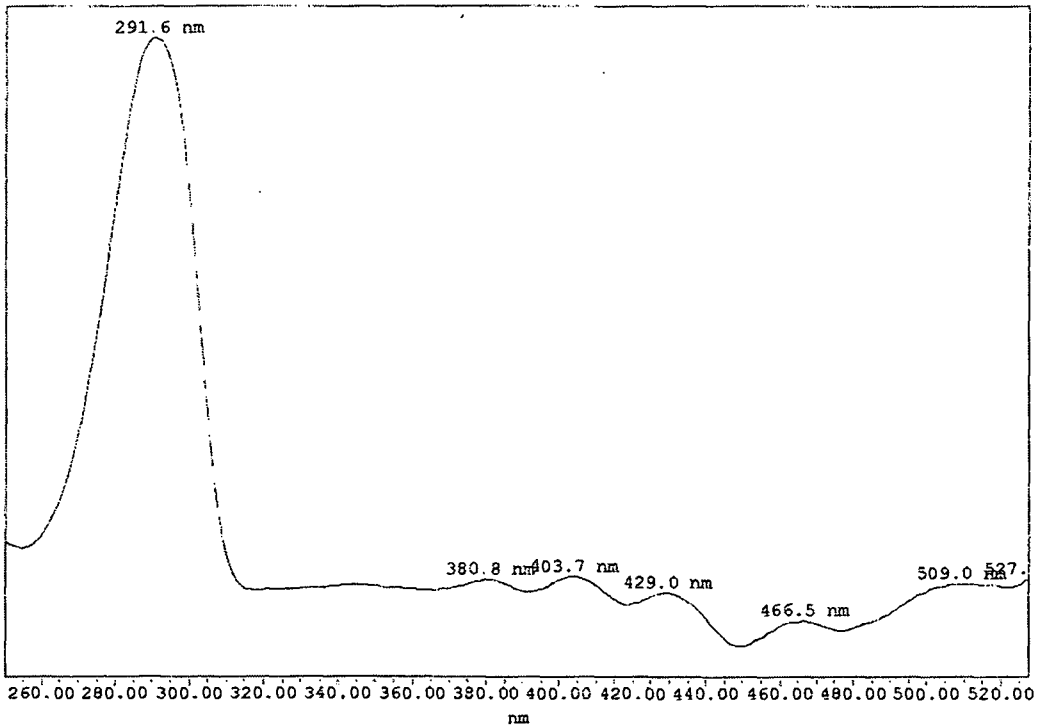
Genotype: *B*



Genotype: *t*

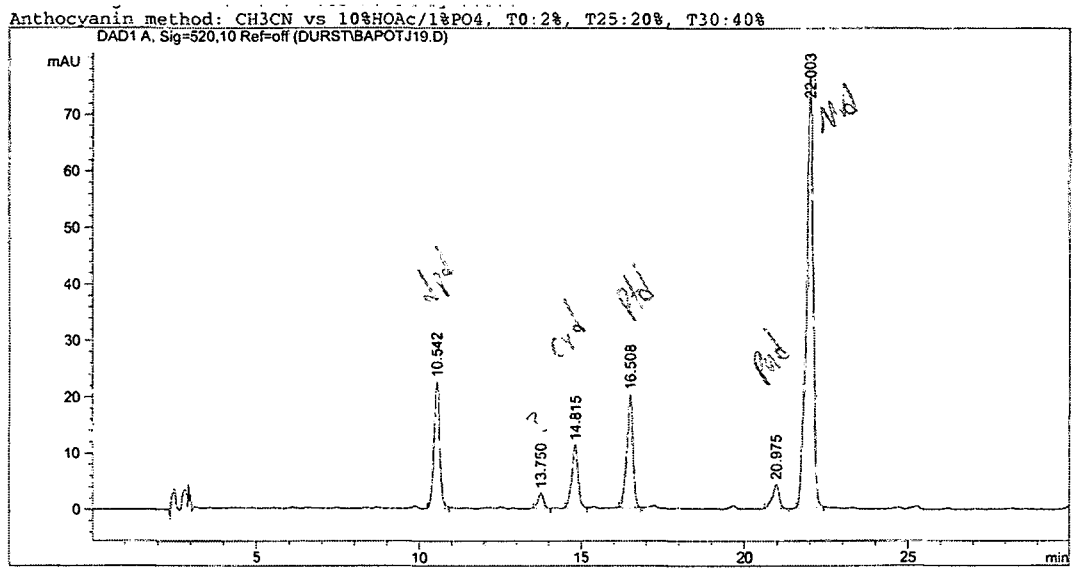


Genotype: *Aft;rr*

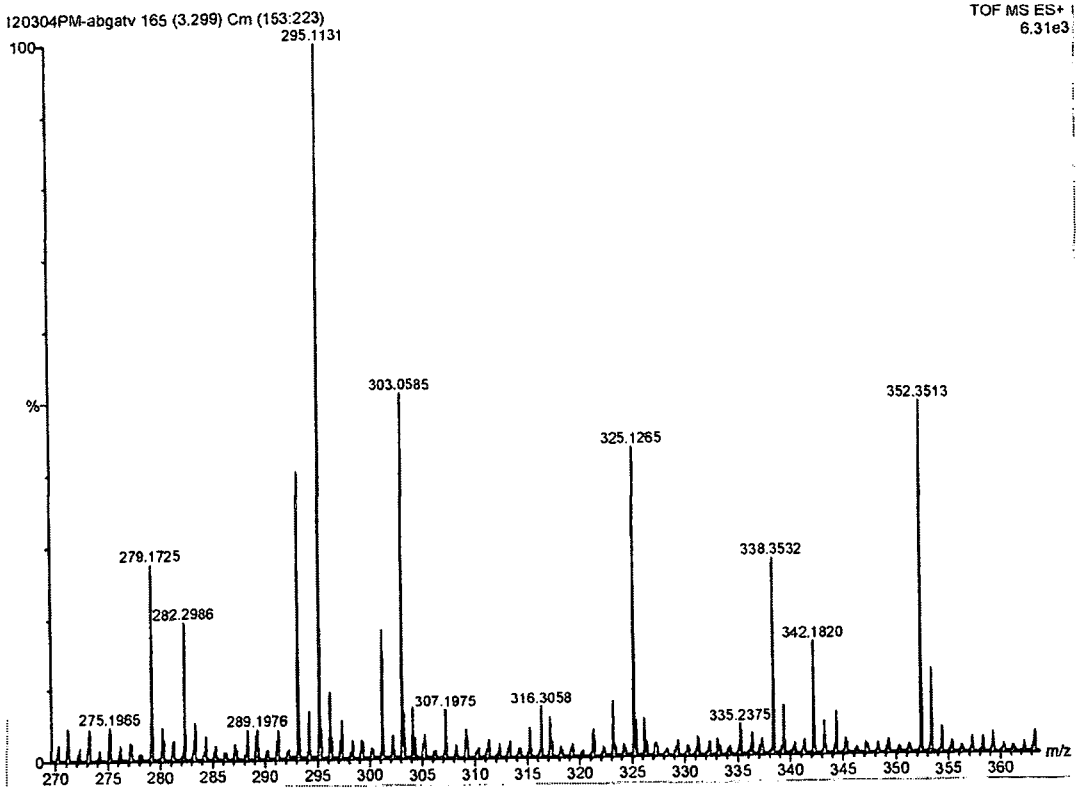


Tocopherol, 3.9 minutes retention

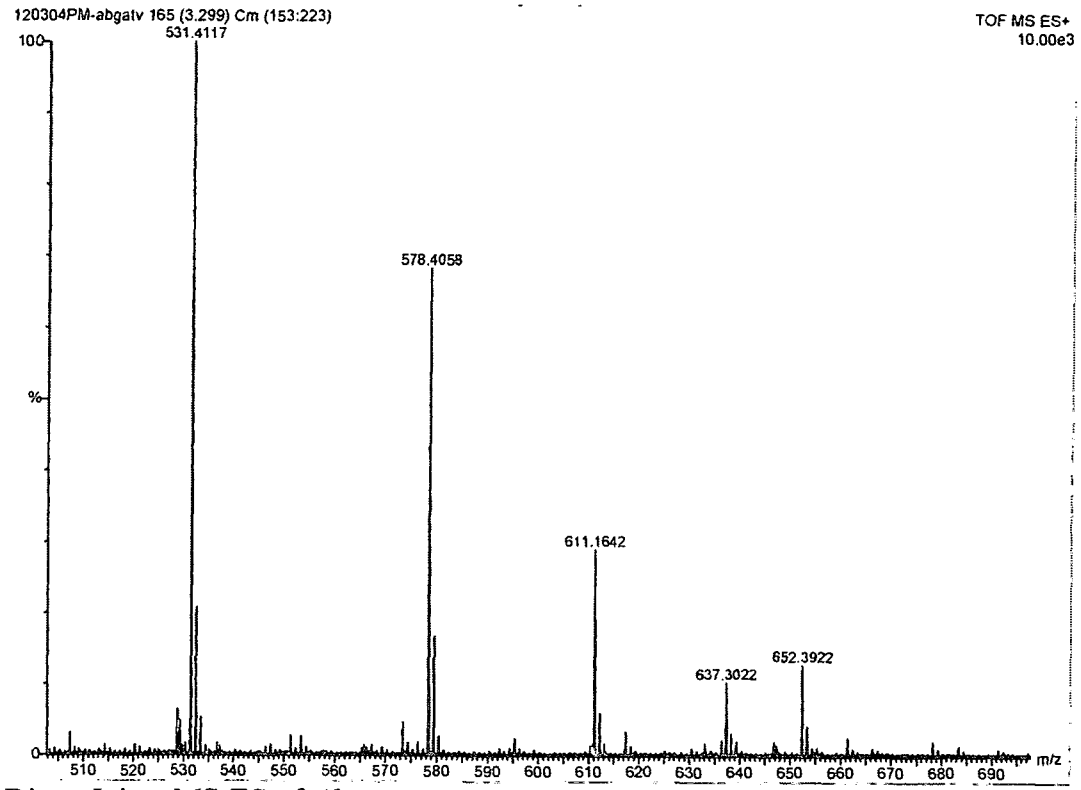
APPENDIX 3. ANTHOCYANIN HPLC/MS CHROMATOGRAMS



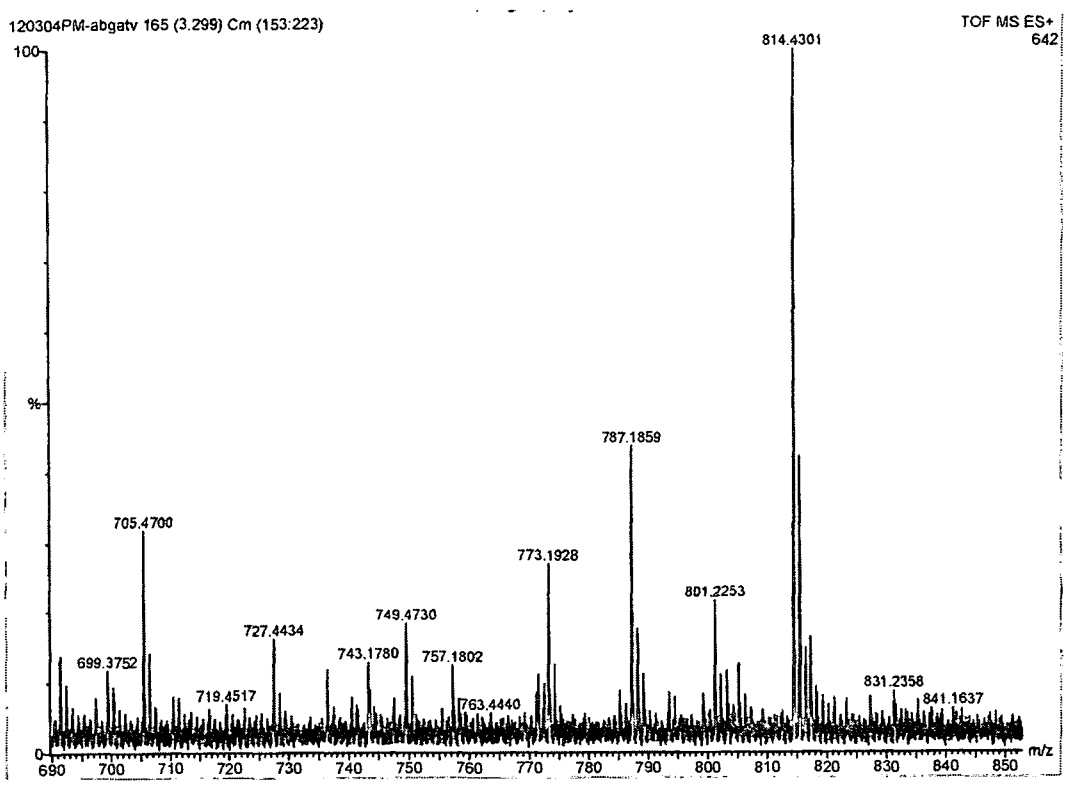
Acid-hydrolyzed Welch's Grape Juice anthocyanidins standards



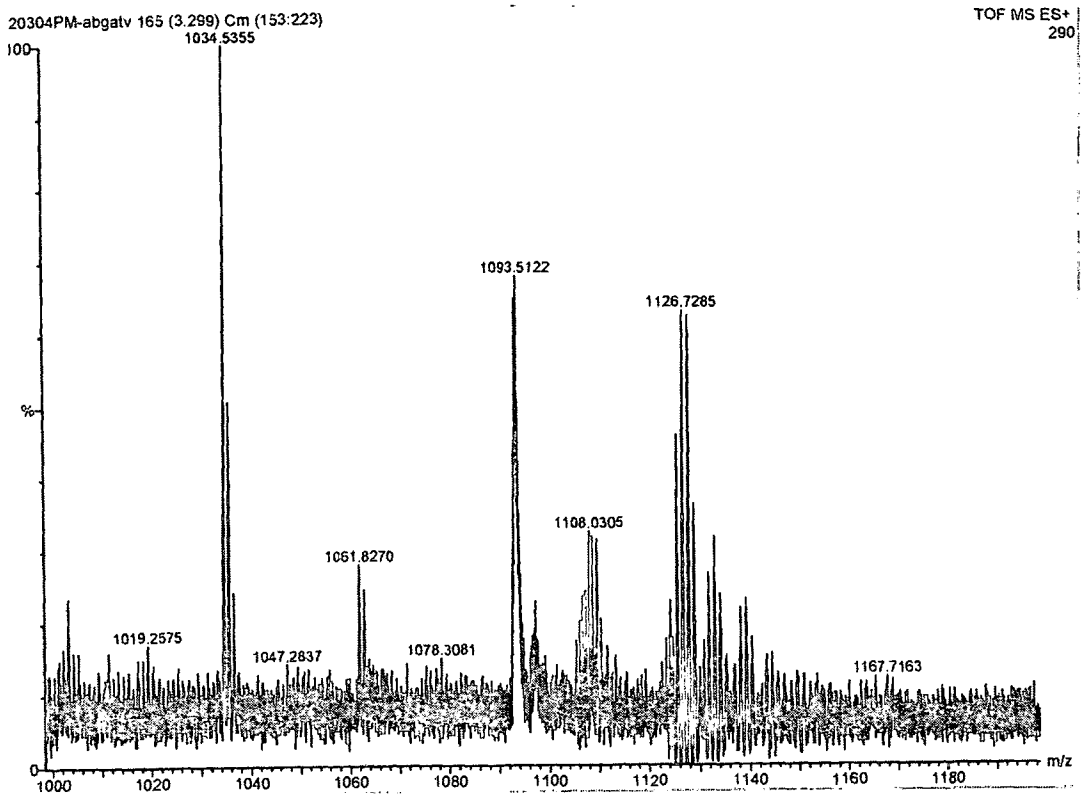
Direct Inject MS-ES of *Abg-atvatv*



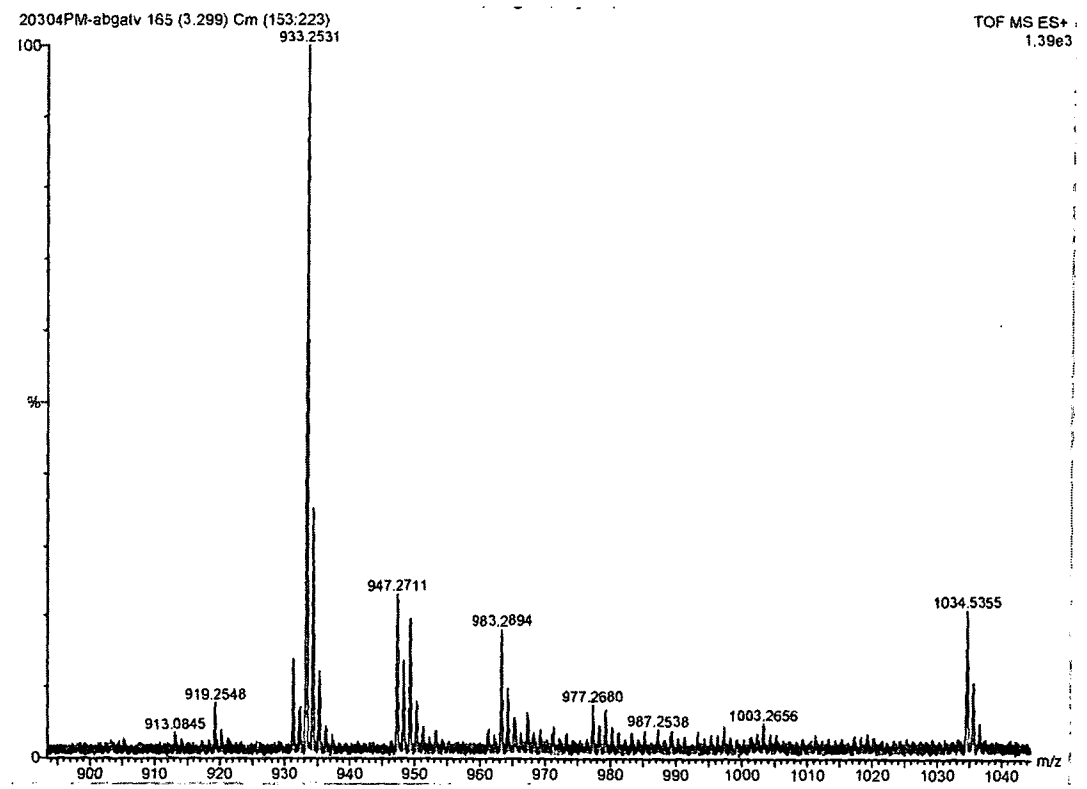
Direct Inject MS-ES of *Abg-atvatv*



Direct Inject MS-ES of *Abg-atvatv*



Direct Inject MS-ES of *Abg-atvatv*



Direct Inject MS-ES of *Abg-atvatv*

APPENDIX 4. TOMATO PHENOLIC EXTRACT EFFECTS ON *BOTRYTIS CINAREA* SPORE GERMINATION AND MYCELIAL GROWTH.

INTRODUCTION

Qualitative observations of field-grown anthocyanin-expressing tomato fruit indicated a possible antimicrobial effect compared to non-purple tomatoes. The primary difference between these types of tomatoes is the increased phenolics and anthocyanins in the purple tomatoes. In an effort to determine if the anthocyanins or increased phenolics are the cause of this observed difference, a pilot study was initiated to measure the effects of these compounds on a common tomato pathogen, *Botrytis cinarea.*

MATERIALS AND METHODS

PURIFICATION OF ANTHOCYANINS AND PHENOLICS

Anthocyanins and other flavonoids expressed in tomatoes were extracted from fruit skin using the basic protocol 1 of Rodriguez-Saona and Wrolstad (2001). Pooled skins of fruit carrying the *Abg-atvatv* gene combination and weighed skins of *rr* and *Aft-atvatv* genotype fruit were cryogenically milled for extraction. The anthocyanins were separated from complex phenolics and water soluble sugars and acids by passage through a C_{18} solid phase column, using basic protocol 2 of Rodriguez-Saona and Wrolstad (2001), collecting both the ethyl acetate fraction (complex phenolics) and the methanol fraction (anthocyanins and flavonoids). Total monomeric anthocyanins in the anthocyanin fraction

(methanol) were quantified by the pH differential method. The concentrated extract of *Abg-atvatv* contained 3.36 mg ml^{-1} anthocyanin. Serial dilutions were prepared from it at concentrations of 1.68, 0.84, and 0.42mg ml^{-1}monomeric anthocyanin (50, 25, and 12.5%, respectively), and identical dilutions were made of the ethyl acetate extract, although anthocyanins were not detected in this fraction. Both the anthocyanin (methanol) and phenolic (ethyl acetate) fractions were tested for total phenolics using the F-C method. The *rr* and *Aft-atvatv* genotype fruit extracts were also analyzed for total phenolics, and the *Aft-atvatv* extracts tested for total monomeric anthocyanins. These extracts were diluted to 50% and 25% original concentrations.

DETERMINATION OF SPORE GERMINATION INHIBITION

Tomato extract antimicrobial activity was measured against *Botrytis cinerea*, the causal agent of grey mold. Potato dextrose broth (PDB) was inoculated with *B. cinerea* spores diluted to $2x10^3$ ml^{-1}. Fifty µl inoculated PDB and 50µl of ethyl acetate or methanol extract fractions taken from a concentrated skin extraction of *Abg-atvatv* fruit were added to microplate wells and incubated in the dark for 24 hours, with control wells containing 50 µl water in place of extract. Spore germination counts were made at 6 and 23 hr. A similar experiment was conducted using the methanol and ethyl acetate extracts of the purple fruit (*Aft-atvatv*) and yellow fruit (*rr*), this time incubated for 48 hours. Spore germination was counted as any spore exhibiting a protrusion of hyphal growth beyond the

circumference of the original spore wall. This method of counting spore germination does not take into account growth cessation following germination, as was observed in many samples. Effects of the extracts on hyphal growth were assessed by visual inspection, and are discussed in qualitative terms.

RESULTS AND DISCUSSION

The inhibition of spore germination in the methanol extracts of both the *Aft-atvatv* and *rr* skins was greatest at the highest concentration of extract, and decreased dramatically upon dilution (Figure A4.1). Immediately evident was that both *Aft-atvatv* and *rr* skin extracts inhibited spore germination at the highest tested concentration, with both extracts having 100% inhibition for the entire 48 hour incubation. This effect may be partially pH driven. However, given that plant cells partition the anthocyanins and flavonoids into the vacuole, where pH is very low, the result is likely relevant with respect to physiological conditions. The diluted extracts exhibited a dosage response: increasing dilution resulted in

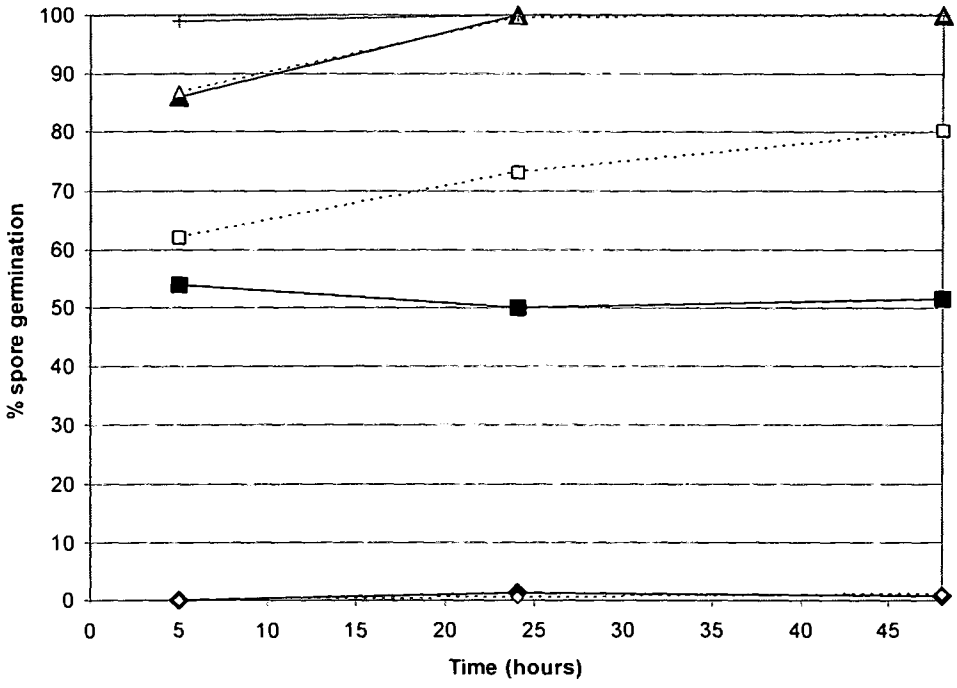


Figure A4.1. Methanol extracts of *Aft-atvatv* (♦:1011GAE, ■:505.5GAE,▲:253 GAE), *rr* skins (◊ :253 GAE, □: 127 GAE,Δ: 64 GAE), and water control (+)

increased spore germination. Particularly peculiar is that the purple fruit extract at 505 gallic acid equivalents had approximately 50% germination inhibition, while the undiluted yellow fruit extract at 253 gallic acid equivalents had 100% germination inhibition. There are three interpretations of this: 1) there is a pH threshold which is crossed after the first dilution; 2) gallic acid equivalents is a poor measure for extract contents as correlating to antimicrobial activity; or 3) there are compounds present in yellow fruit extract with higher inhibitory effects than are found in the purple fruit extract, though their effects are not observed under field conditions. As other publications have indicated a high correlation of phenolic content to antimicrobial activity, the use of GAE is not likely the cause of this data anomaly. The ORAC scores of these extracts were substituted for GAE

but relative ranking remained the same: the undiluted *rr* extract exhibited greater inhibition at lower ORAC score than the *Aft-atvatv* material. It is unlikely that the yellow fruited plants have a genetic effect that may cause differential phenolic or flavonoid pathway regulation and expression. Unfortunately neither red, yellow, nor purple colored tomatoes were analyzed for complete flavonoid profiles, and so evidence to support this hypothesis is not provided here. Because expression within the flavonoid pathway is probably similar (apart from the anthocyanins and complex phenolics) between *rr* and *Aft-atvatv* genotypes, this suggests that pH may affect germination inhibition. Further complicating matters, flavonoid and anthocyanin conformations are pH sensitive, which may affect the ability to inhibit microbial growth. pH may act as a confounding factor depending on the concentration of these compounds. It is clear that pH does not explain all of the inhibition: comparing the methanol extracts with the ethyl acetate extracts (Figure A4.2) shows a difference in the undiluted extracts: 0% germination versus 6% and 8% germination. Assuming the pH is the same in these extracts due to identical dilution, the difference can only be explained by the presence of the compounds found in the methanol extracts.

Because the methanol extract contains compounds other than anthocyanins it was not possible to attribute the observed inhibition to anthocyanins alone. As further evidence of the activity of other compounds, the ethyl acetate extracts (Figure A4.2) also inhibit spore germination. However, the inhibition in the ethyl

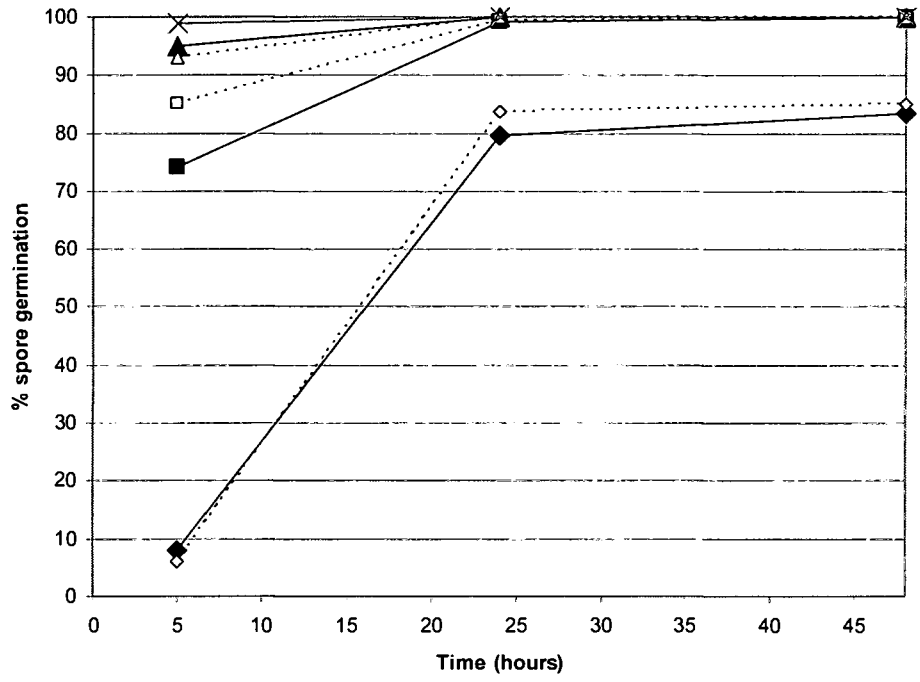


Figure A4.2. Ethyl acetate extracts of *Aft-atvatv* (♦:466 GAE, ■:233 GAE, ▲:116 GAE), *rr* skins (□:266 GAE, ◊:133 GAE, Δ:67 GAE), and water control (x)

acetate extracts of both the *Aft-atvatv* and *rr* skin extracts is ineffective in preventing *B. cinarea* spores germination at all dosages. This is unlike the situation for the methanol extracts, where the two most concentrated extracts from *Aft-atvatv* and from *rr* allow spore germination at rates of 0% and 50%, and 0% and 60-80% germination, respectively. This suggests that the compounds present in the methanol extract were the most potent inhibitors of germination. It also indicated that the anthocyanins had a limited, if any, effect on spore germination: despite the higher extract concentration, the *Aft-atvatv* methanol extract did not exhibit a significant increase in inhibition when compared with the *rr* extract. Not only do anthocyanins not appear to inhibit spore germination, the anthocyanin tomato extracts have less of the actual inhibiting compound(s) compared to *rr*

tomatoes, since it takes ¼ as much yellow tomato extract to achieve the same level of inhibition as the purple tomato extract.

Inhibition of spore germination followed a dose-response curve in concentrated purple fruit extracts from *Abg-atvatv* for both the ethyl acetate and methanol fractions (Table A4.1). The inhibitory effects broke down at an earlier

Table A4.1. Inhibition of *B. cinarea* spore germination by ethyl acetate and methanol extracts from purple tomato fruit (genotype *Abg-atvatv*).

Genotype	Extract	anthocyanin concentration, mg/ml $^{-1}$	Gallic acid equivalents, PPM	% spore germination at 6 hours	% spore germination at 23 hours
Blank	Water	0.00	0.00	74	100
Abgatv	Ethyl acetate	0.00	1085.00	0	0
Abgatv	Ethyl acetate	0.00	542.50	0	0
Abgatv	Ethyl acetate	0.00	271.25	15.4	91.4
Abgatv	Ethyl acetate	0.00	135.63	74.6	100
Abgatv	Methanol	1.68	7890.50	0	0
Abgatv	Methanol	0.84	3945.25	0	0
Abgatv	Methanol	0.42	1972.63	0	0
Abgatv	Methanol	0.21	986.31	3.6	0

dilution in the ethyl acetate extract compared to the methanol extract. Indeed, this experiment provides the best evidence that even after accounting for pH effects, the compounds present in the both extracts are having an inhibitory effect, with the methanol extract having the stronger inhibitory effect of the two extracts. That the methanol extract dilution did not exhibit spore germination at any concentration after 24 hours supports the hypothesis that the flavonoids and/or anthocyanins are inhibiting *B. cinarea* spore germination. Of particular interest is that 3.6% germinating spores were observed in the four-fold dilution (986 GAE, 0.21mg ACY ml^{-1}) at six hours incubation, but no germinated spores were observed after

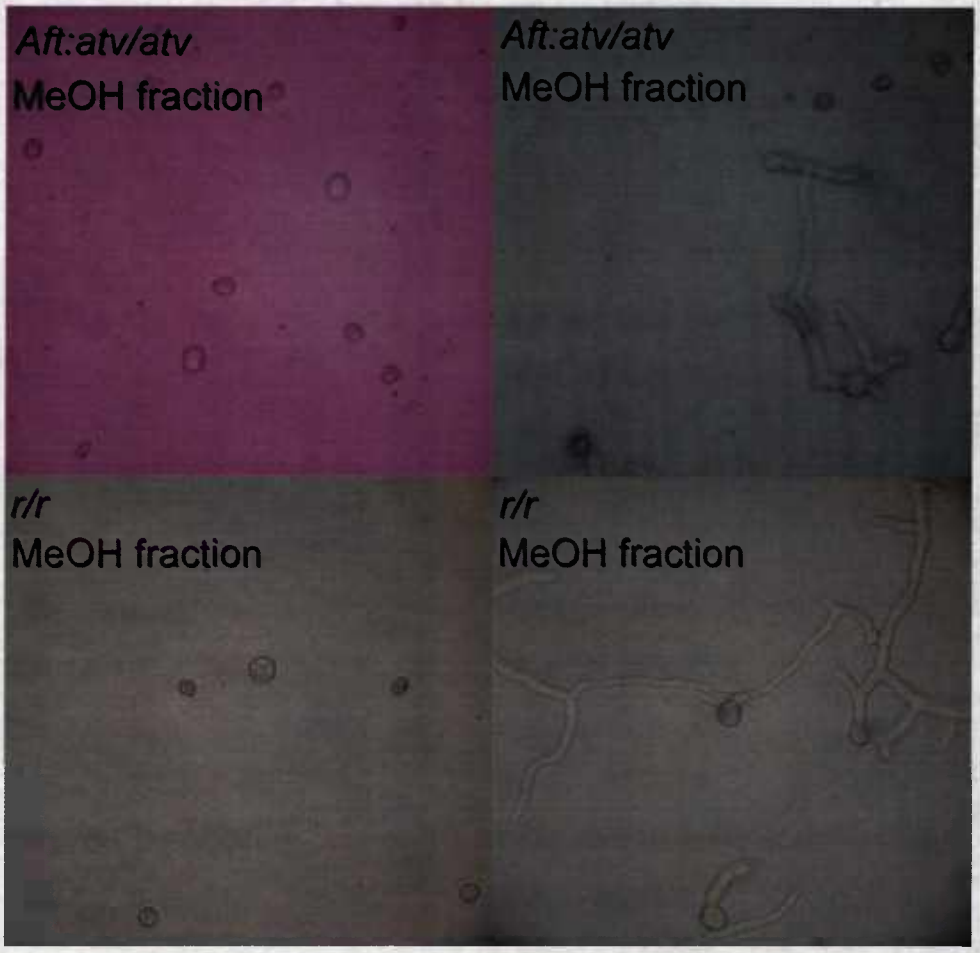


Figure A4.3. *Botrytis cinarea* mycelial growth at 23 hours in tomato methanol extracts. Clockwise from top left: dilutions at 1011 GAE, 505 GAE, 127 GAE, and 254 GAE.

24 hours. The percentage counts were based on counts of 100 spores at 6 hours. At 24 hours, all microplate wells (5 replicates) were searched for germinating spores, but none were found. This seems to indicate that at this concentration, germinating spores die and lyse. Further testing of this effect is warranted.

Mycelial growth was also reduced by the extracts. Both the methanol and ethyl acetate fractions from yellow and purple tomatoes inhibited mycelial growth (Figures A4.3, A4.4, and A4.5). Gallic acid equivalents values for each

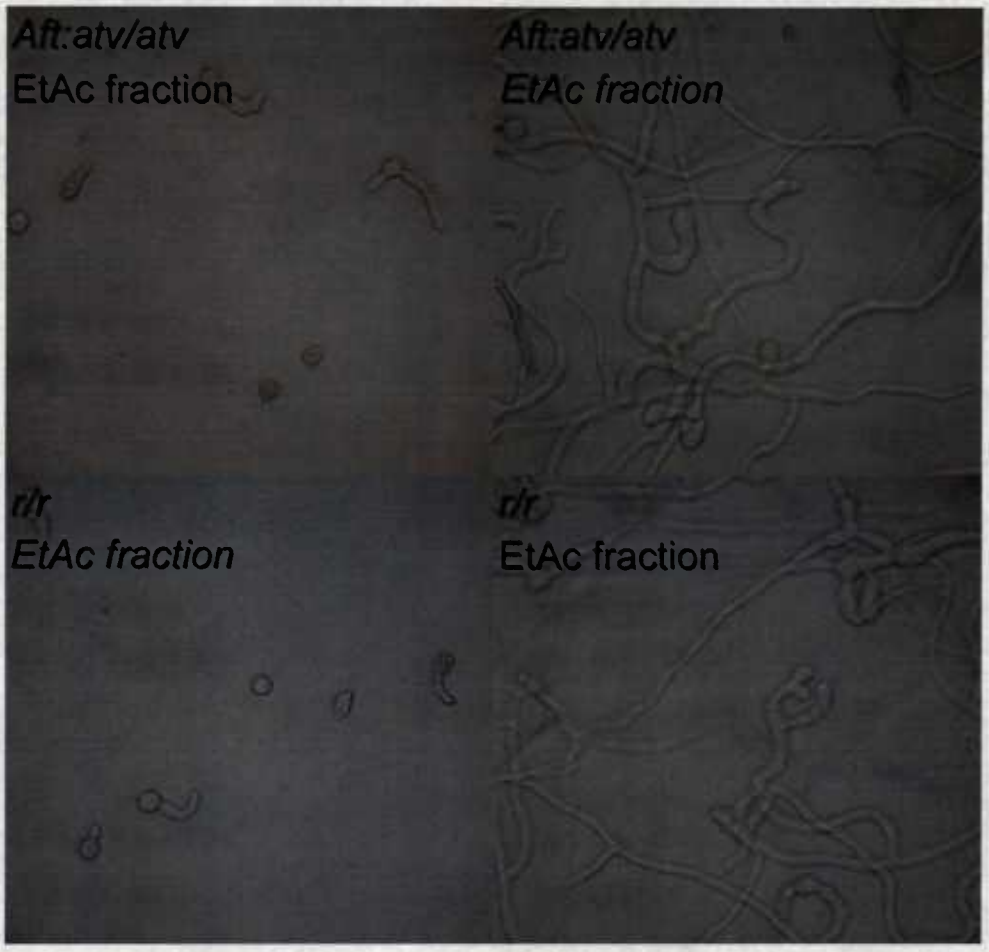


Figure A4.4. *Botrytis cinarea* mycelial growth at 23 hours in tomato ethyl acetate extracts. Clockwise from top left: dilutions at 466 GAE, 233 GAE, 133 GAE, and 266 GAE.

fraction are given for comparison. Due to differences in skin mass used in the initial extractions, the *rr* extracts are 57% strength compared to the *Aft-atvatv* extracts in terms of their respective skin concentrations.

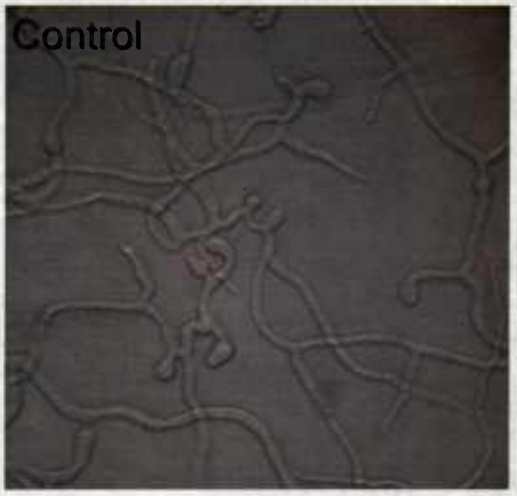


Figure A4.5. *Botrytis cinarea* growth at 23 hours in water.

APPENDIX 5. IRB DOCUMENTATION

INSTITUTIONAL REVIEW BOARD

OREGON STATE UNIVERSITY
312 Kerr Administration Building · Corvallis, Oregon · 97331-2140
E-MAIL: IRB@oregonstate.edu · PHONE: (541) 737-3437 · FAX: (541) 737-3093

REPORT OF REVIEW

TO: James Myers,
Horticulture

RE: Carotenoid Antioxidant Capacity: Determining the Effects of Different Tomato Genotypes in Humans (Student Researcher: Peter Mes)

Protocol No. 2107

The referenced project was reviewed under the guidelines of Oregon State University's Institutional Review Board (IRB). The IRB has **approved** the application. This approval will expire on **2/12/2004**. This modification request was reviewed at the Expedited level. A copy of this information will be provided to the full IRB committee.

- Any proposed change to the approved protocol, informed consent form(s), or testing instrument(s) must be submitted using the MODIFICATION REQUEST FORM. Allow sufficient time for review and approval by the committee before any changes are implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB.
- In the event that a human participant in this study experiences an outcome that is not expected and routine and that results in bodily injury and/or psychological, emotional, or physical harm or stress, it must be reported to the IRB Human Protections Administrator within three days of the occurrence using the ADVERSE EVENT FORM.
- If a complaint from a participant is received, you will be contacted for further information.
- Please go to the IRB web site at:
  http://osu.orst.edu/research/RegulatoryCompliance/HumanSubjects.html to access the MODIFICATION REQUEST FORM and the ADVERSE EVENT FORM as needed.

Before the expiration date noted above, a Status Report will be sent to either close or renew this project. It is imperative that the Status Report is completed and submitted by the due date indicated or the project must be suspended to be compliant with federal policies.

If you have any questions, please contact the IRB Human Protections Administrator at IRB@oregonstate.edu or by phone at (541) 737-3437.

for Dr. Anthony Wilcox
Institutional Review Board Chair

Date: 10/15/03

NSTITUTIONAL REVIEW BOARD

OREGON STATE UNIVERSITY

Office of Sponsored Programs and Research Compliance
312 Kerr Administration Bldg.
Corvallis, Oregon
97331-2140

Telephone
541-737-3437

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541-737-3093

Email
IRB@oregonstate.edu

TO: James Myers,
Horticulture

RE: Carotenoid Antioxidant Capacity: Determining the Effects of Different Tomato Genotypes in Humans (Student Researcher: Peter Mes)

IRB Protocol No. 2107

The referenced project was reviewed under the guidelines of Oregon State University's Institutional Review Board (IRB). The IRB has approved the application. This approval will expire on 2/4/2005. This continuation request was reviewed at the Full Board level. A copy of this information will be provided to the full IRB committee.

Enclosed with this letter please find the approved informed consent document for this project, which has received the IRB stamp. This information has been stamped to ensure that only current, approved informed consent forms are used to enroll participants in this study. All participants must receive the IRB-stamped informed consent document.

- Any proposed change to the approved protocol, informed consent form(s), or testing instrument(s) must be submitted using the MODIFICATION REQUEST FORM. Allow sufficient time for review and approval by the committee before any changes are implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB.
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If you have any questions, please contact the IRB Human Protections Administrator at IRB@oregonstate.edu or by phone at (541) 737-3437.

Laura K. Kimden Date: 2/5/04

for Drs. Courtney Campbell and Wayne Kradjan
Institutional Review Board Co-Chairs

pc: 2107 file

APPENDIX 6. SUBJECT 1 LOW CAROTENOID DIET PROFILE DURING JUICE SUPPLEMENTATION

Low Carotenoid September 9, 2003

Total Weight: 1857.30 g (65.51 oz-wt.)
Serving Size: 1857.30 g (65.51 oz-wt.)
Serves: 1.00
Cost: --

Sources & Amounts for Calories

Amount	Food Item	Calories		0 13 25 38 50
2 each	Grilled Cheese Sandwich-Whole Wheat	863.15	49%	
16 fl oz	Apple Juice-Frozen Conc + Water,Unsweet.	224.66	13%	
2 cup	General Mills Cheerios Cereal GML	219.00	13%	
32 oz-wt	Campbell's Tomato Juice CAM	186.67	11%	
1 each	EskimoPie VanillaIceCream Bar-DrkChocCtd	165.50	9%	
0.5 cup	Long Grain White Rice-Instant-Enr-Cooked	80.85	5%	
1 tbs	Soy Sauce-Low Sodium	8.48	0%	
	Total	1748.31	100%	

% comparison to: US Male (19-30 years)

Basic Components			
Calories	1748.31		61%
Calories from Fat	627.95		73%
Calories from Saturated Fat	321.24		125%
Protein	59.49	g	99%
Carbohydrates	225.74	g	54%
Dietary Fiber	18.79	g	57%
Soluble Fiber	5.89	g	
InSoluble Fiber	9.04	g	
Sugar - Total	98.02	g	
Monosaccharides	--	g	
Galactose	--	g	
Glucose	--	g	
Fructose	--	g	
Disaccharides	--	g	
Lactose	0	g	
Sucrose	--	g	
Maltose	--	g	
Other Carbs	104.59	g	
Fat - Total	69.77	g	73%
Saturated Fat	35.69	g	125%
Mono Fat	1.34	g	4%
Poly Fat	0.62	g	2%
Trans Fatty Acids	1.55	g	
Cholesterol	134.18	mg	45%
Water	1475.44	g	
Ash	26.80	g	
KiloJoules	7314.86		
Vitamins			
Vitamin A IU	8150.31	IU	181%
Vitamin A RAE	937.27	RAE	104%
Vitamin A RE	1507.30	RE	167%
A - Carotenoid	373.33	RE	
A - Retinol	750.60	RE	
A - Beta Carotene	2188.48	mcg	
Thiamin-B1	1.31	mg	109%
Riboflavin-B2	1.69	mg	130%
Niacin-B3	16.36	mg	102%
Niacin Equiv.	18.24	mg	114%
Vitamin-B6	1.51	mg	116%
Vitamin-B12	0.91	mcg	38%
Biotin	15.10	mcg	50%
Vitamin C	122.49	mg	136%
Vitamin D IU	105.53	IU	53%
Vitamin D mcg	2.64	mcg	53%
Vit E Alpha-Tocopherol	3.37	AToco	22%
Vit E-Alpha Equiv.	3.39	mg	23%
Vitamin E IU	5.05	IU	
Vitamin E mg	3.39	mg	
Folate	508.83	mcg	127%
Folate DFE	726.67	DFE	182%
Vitamin K	--	mcg	
Pantothenic Acid	1.81	mg	36%