Lycopene and its oxidation products as
potential biological antioxidants

by

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I would like to dedicate this Thesis to my parents,

Samuel and Elizabeth Liken.

Without their help, both as an undergraduate student

at the University of Glasgow, and also as a graduate

student here at Iowa State, I would never have been

able to do as well as I have. Thanks, folks !

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To date approximately 600 carotenoids have been characterized in nature, from both plant and animal kingdoms, the majority being C-40 compounds. The evolution of such a vast range of carotenoids has certainly not been without purpose. Survival of photosynthetic species has required competitive adaptation, and two primary mechanisms have been identified with respect to carotenoid diversity. First, carotenoids extend the wavelengths of light that can be used in photosynthesis. While chlorophylls typically absorb light at wavelengths greater than 600 nm, the carotenoids tend to absorb in the range 400 - 600 nm, and transduce this energy to the photosynthetic reaction center. Secondly, the carotenoids are able to greatly reduce photooxidative damage induced by light sensitization of chlorophyll, first observed in studies on carotenoid deficient mutants of the photosynthetic bacterium, *Rhodopseudomonas spheroides* (Sistrom et al., 1956). Consequently it appears that the high degree of structural diversity seen in the carotenoids has facilitated enhancement of both functions, so providing a competitive edge in species survival.

In nature, carotenoids have been found free, or conjugated with proteins, sugars and other molecules. In either form, they can be divided into two main categories: the *carotenes*

and the *xanthophylls*. The carotenes are purely hydrocarbon in structure, and comprise only about 10% by number of naturally occurring carotenoids. The xanthophylls, on the other hand, are much more abundant, being formed from the carotenes by various oxidative reactions. Figure 1 provides the structures of a number of commonly occurring carotenoids.
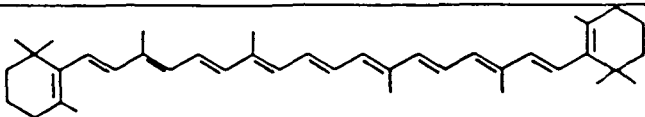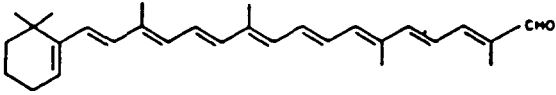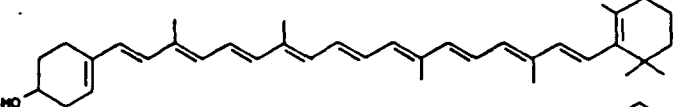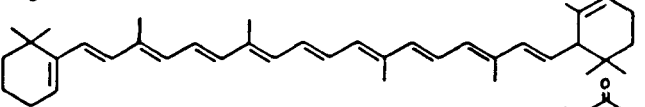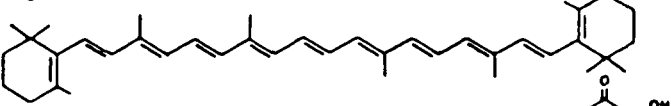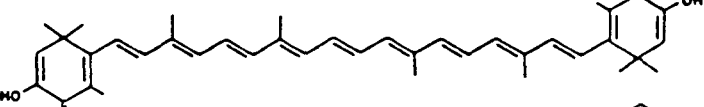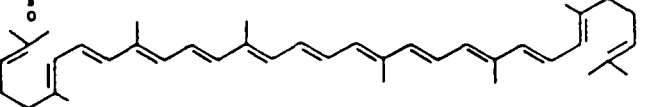
Compound	Structure
β-Carotene (widely distributed)	
β-Apo-8'-carotenal	
Cryptoxanthin (orange)	
α-Carotene (widely distributed)	
Echinenone (sea urchin)	
Astacene (crustacean)	
Lycopene (tomato)	

Figure 1. The structure of some commonly occurring carotenoids

The *de novo* synthesis of phytoene, and its subsequent conversion to other acyclic

and cyclic carotenoids is illustrated in Figure 2. The *de novo* synthesis of carotenoids occurs

only in photosynthetic microorganisms and plants, and not in animals.
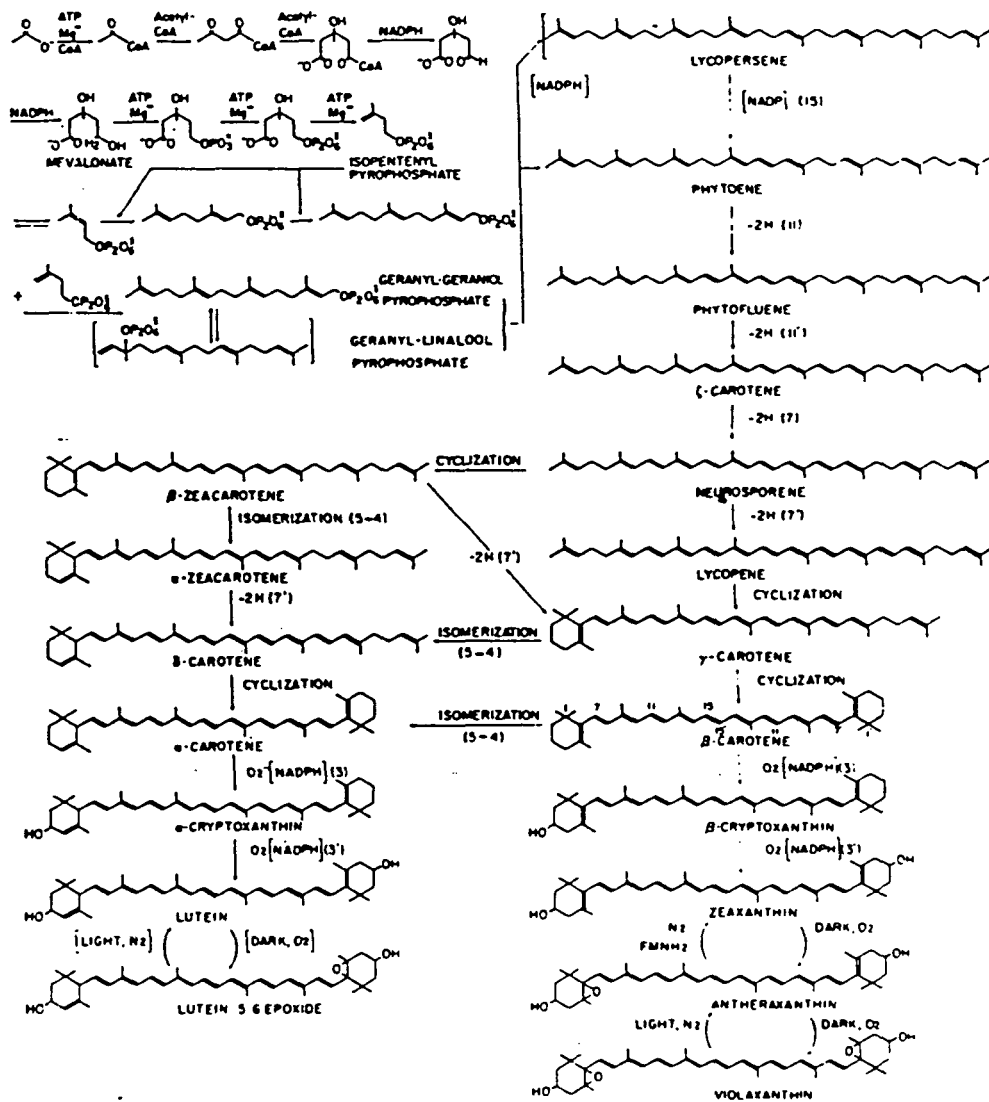
Figure 2. Synthesis of the major carotenoids from acetyl CoA

Structural properties

The carotenoids are characterized by long conjugated double-bond systems, ranging from 5 to 15 conjugated double bonds in many common carotenoids. In general, as the level of conjugation increases, the wavelength maxima increase (bathochromic shift), and the absorption range broadens. Cyclization in the system results in a decrease in wavelength maxima (hypsochromic shift), with a slight drop in absorption range. The introduction of carbonyl groups at the 4 and 4' carbons increases the degree of conjugation in the carotenoid, causing both a bathochromic shift and increase in absorption range. Similarly, a bathochromic shift follows from hydroxylation at C-3 and C-3'. Table 1 illustrates wavelength phenomena for a number of carotenoids.

The data in Table 1 applies to all-trans isomers of these carotenoids (more properly, all-E isomers). Additionally cis (Z) isomers exist in nature. Indeed, ß-carotene is known to have at least 3 cis isomers, 7-cis, 9-cis and 13-cis. At least four cis isomers of lycopene have been spectrophotometrically detected, but not structurally identified, in carrot oil (Saleh and Tan, 1991). The inability as yet to identify cis-isomers by position has led to the adoption of such nomenclature as "neo-A" and "neo-B" lycopene, as well as "nor" lycopene. The position of the cis double bonds affects the wavelength properties of the carotenoid, generally causing a hypsochromic shift of up to 10 nm, with a smaller loss in absorption range.

Table 1.   Wavelength phenomena of carotenoids as affected by structure

Carotenoid name [a]	conjugated bonds	structure and functionality	wavelength range (nm)[b]
phytofluene	5	acyclic	331-367 [36]
ζ-carotene	7	acyclic	376-422 [46]
neurosporene	9	acyclic	414-468 [54]
lycopene	11	acyclic	444-502 [58]
γ-carotene	11	monocyclic	414-468 [54]
ß-carotene	11	bicyclic	417-471 [54]
ß-cryptoxanthin	11	bicyclic, 3-OH	425-480 [55]
zeaxanthin	11	bicyclic, 3-OH and 3'-OH	425-480 [55]

[a] structures are provided in Figure 2.

[b] spectral data from De Ritter and Purcell, 1981. Wavelength data indicate maxima of first and third peaks, most carotenoids having three distinctive wavelength maxima. The distance between the first and third peaks (the absorption range) is indicated in parenthesis. All spectral data are for carotenoids in petroleum ether solvent.

Lycopene

Lycopene is a particularly interesting carotenoid for many reasons. It is the most highly conjugated natural acyclic C-40 carotenoid, with a broad wavelength maxima range over 58 nm. This, as well as its symmetrical planarity, makes it a powerful light absorbing pigment in photosynthetic organisms. Unlike ß-carotene, lycopene is not metabolized to vitamin A when ingested, because of its acyclic nature. Other carotenoids, such as canthaxanthin, have no provitamin A function for different structural reasons. Lycopene is present in many widely consumed pink-red fruits and vegetables, most noticeably in tomatoes, at approximately 40 mg/kg, as well as carrots, pink grapefruit and pumpkin. In ripe commercial tomatoes, lycopene constitutes 78 - 90% of total carotenoids, in contrast to only 5 - 10% for ß-carotene. The observations that tomatoes and tomato products are rich in lycopene, and that tomatoes are the most widely consumed vegetable in the United States (Rick, 1978), have been parallelled by studies on carotenoid distribution in human plasma. At least six studies in the last ten years have looked at the human serum levels of three carotenoids, α-carotene, ß-carotene, and lycopene (Miller et al., 1984; Bieri et al., 1985; Stacewicz-Sapuntzakis et al., 1987; Barua et al., 1989; Krinsky et al., 1990a; Khachik et al., 1992b). The ranges in these studies have been quoted between 54-260 nmol/l plasma for α-carotene, 265-630 nmol/l plasma for ß-carotene, and 350-1168 nmol/l plasma for lycopene.

Obviously a number of factors have affected these values, including sample preparation, method of analysis, dietary consumption, and inter-individual physiological

variations. These factors have been reviewed (Krinsky et al., 1990a; Stahl and Sies, 1992; Parker, 1989). The main points to note here are that ß-carotene is not the only major carotenoid found in human plasma, and that lycopene is at least equally abundant.

As has been shown by Krinsky et al. (1958), lycopene is transported in blood by low- and high- density lipoproteins (LDLs and HDLs). HDLs transport about 17% of serum lycopene, Sf 10-1000 LDLs about 5%, and Sf 3-9 LDLs about 75%. Any ß-carotene not converted to vitamin A during absorption follows the same distribution pattern. Several studies have indicated some specificity in absorption of carotenoids. Stahl and Sies (1992) have reported that lycopene absorption in humans was greater from heat-processed than from unprocessed tomato juice. They have also reported that the two common cis isomers of lycopene, 9-cis- and 13-cis- lycopene, were either absorbed better than all-trans lycopene, or were metabolized to a lesser extent. The latter observation has also been made by Krinsky et al. (1990a). It has been suggested that absorption, metabolism and isomerization varies between carotenoids (Parker, 1989) and between different species (Krinsky et al. ,1990a). In general the best animal models for simulating human carotenoid metabolism have been reported as the ferret (Ribayada-Mercado et al., 1989), the female rhesus monkey (Mathews-Roth et al., 1990; Krinsky et al., 1990b), and the baboon (Leo et al., 1992). For the ferret model, this has been accounted for by similarities to human gastrointestinal tract anatomy and physiology.

Little information exists on the tissue distribution of lycopene. Mathews-Roth et al. (1990) have shown that ^{14}C lycopene resided predominantly in the liver of monkeys, with

significant amounts in adipose tissue, intestine, and colon. However, they have pointed out that the interindividual variation in the five monkeys used was high.

The subcellular distribution of lycopene has not been studied to date. Canthaxanthin has been isolated from the hepatic membranes of chicks fed canthaxanthin (Mayne and Parker, 1988a). ß-carotene has been shown to reside preferentially in the mitochondrial fraction of cow mammary tissue (Patton and Kelly, 1980) and chick liver (Mayne and Parker, 1986). O'Fallon and Chew (1984) have also indicated the presence of ß-carotene in bovine corpus luteum microsomal membranes. Mathews-Roth (1975) has also shown ß-carotene to be present in erythrocytes of carotenemic and noncarotenemic individuals. The lack of studies concerning the subcellular distribution of lycopene is unusual, bearing in mind that lycopene is at least as equally abundant as ß-carotene in human plasma.

This is an important observation in the context of this thesis; in the last fifteen years, carotenoids have been investigated primarily on the basis of their hypothesized benefits to human health, most noticeably in cancer prevention. Until recently, ß-carotene alone has been the focus of much of this research. Investigations into the health promoting effects of other carotenoids have come about with the realization that many other carotenoids are present in human plasma. To date, ß-carotene and other carotenoids have been associated with three main areas of health improvement:-

a) Improvement of general cardiovascular health;

b) Enhancement of immune function;

c) Reduced risk of cancers.

While reference to ß-carotene as the primary carotenoid investigated in each of these fields is necessary, emphasis has been placed on non-provitamin A carotenoids in the rest of this review.

Health Promoting Effects of Carotenoids

Cardiovascular health

Preliminary studies have observed inverse correlations between dietary carotenoids and heart disease and stroke (Anon, 1990), as well as plasma ß-carotene concentrations and angina risk (Riemersma et al., 1991). It has also been proposed that ß-carotene, in combination with other antioxidants, protects against lipoprotein oxidation (Estebauer, 1989), and so plays an important role in retarding the progression of atherosclerosis. Oxidized low density lipoproteins (LDLs) have been associated with atherosclerosis occurrence (Lue and Fruchart, 1991) and increased platelet aggregation (Ardlie et al, 1990). To date no studies have been performed on non-provitamin A carotenoids. The above observations have been made only within the last three years, and must be substantiated with further research. They are not discussed further here.

Immunoenhancement

The enhancement of immune function by carotenoids was first observed in the 1930s. First, infections of the ear, gut, bladder and kidney in vitamin A deficient rats were prevented when the rats were fed ß-carotene (Green and Mellanby, 1930). Similarly, severe ear infections in children were observed to decline with increased intake of ß-carotene (Clausen, 1931). These two early reports do not indicate that ß-carotene specifically was responsible; it must be remembered that ß-carotene, a provitamin A carotenoid, is rapidly converted to vitamin A in the intestinal mucosa . Hence the immunoenhancement observed may have been partly or fully due to vitamin A, which indeed plays an important role in the maintenance of immune function (Bendich, 1989a).

Two strategies have been adopted in eliminating possible provitamin A effects. First, non-provitamin A carotenoids (such as canthaxanthin and lycopene) have been used in controlled immunoenhancement studies. However, even comparing the results between a canthaxanthin fed group and a ß-carotene fed group does not necessarily eliminate vitamin A effects. Alternatively, carotenoids can be introduced into the blood plasma by intravenous or intraperitoneal injection, bypassing the major site of vitamin A biosynthesis, the intestinal mucosa. It should be noted, though, that the liver and kidneys, among others, can also convert provitamin A carotenoids to vitamin A (Bauernfeind, 1981). Using non-provitamin A carotenoids eliminates these problems. In a study performed by Lingen et al. (1959), lycopene in crystalline suspension was introduced into white hybrid mice intravenously or

intraperitoneally. Fourteen to twenty-four hours later the mice were challenged with various infectious agents, and resistance to infection was assessed by prolongation of survival time against control groups. The mice were challenged either by infection with strains of *Klebsiella pneumoniae* bacteria, by total-body X-ray irradiation at 1250r, or by injection of Ehrlich ascites tumors from pre-infected mice. The researchers concluded that lycopene induced a markedly enhanced non-specific resistance in all cases.

Not until 20 years later was further work undertaken. Since then the effects of carotenoids on many aspects of immune function have been investigated. Recent review articles have summarized these investigations (Bendich, 1989a; Krinsky, 1991; Canfield et al., 1992). Table 2 summarizes some of the main effects of carotenoids on the immune system.

The mechanisms by which carotenoids enhance immune function have not yet been elucidated, but at least one line of research is being pursued. Bendich (1989a, 1989b) has suggested that carotenoids may quench reactive oxygen species and free radicals generated in the initial response to infections. White blood cells and polymorphonuclear leukocytes (PMNs) produce these species to destroy invading bacteria, but overproduction can result in self-injury, as well as injury to neighboring cells and tissues. Bendich has also speculated that by preventing membrane lipid peroxidation, carotenoids help to maintain the membrane receptors essential for immune function, e.g. macrophage receptors for antigen recognition. Furthermore, by preventing lipid peroxidation, carotenoids may decrease the formation of immunosuppressive peroxides. This observation has been echoed by one other study on

Table 2.   Observed immunoenhancing effects of carotenoids

Carotenoid	Model	Effect
- ß-carotene	mouse	Reduction of radiation-induced thymus involution and lymphopenia
- ß-carotene	mouse	Increased graft vs. host response
- ß-carotene/ bixin[a]	mouse	Enhanced regression of virally-induced tumors
- ß-carotene	human	Increased human helper T-lymphocyte cells
- ß-carotene	mouse	Slowed growth of implanted syngenic tumors
- ß-carotene/ canthaxanthin[a] astaxanthin[a]	hamster/ mouse	Increased cytotoxic macrophages and T-cell activity in tumor models, elevated levels of tumor necrosis factor (TNF)
- ß-carotene/ canthaxanthin[a]	human	Maintained macrophage receptors for antigens
- ß-carotene/ α-carotene	mouse	Increased natural killer (NK) cell lysis of tumor cells
- ß-carotene/ α-carotene	human	Enhanced cytokine production
- canthaxanthin[a]	mouse	Reduction of immunosuppression in ultraviolet irradiated animals
- ß-carotene	human	Increased NK activity in HIV+ patients[b]

[a] Indicates non-provitamin A carotenoid.

[b] Garenal et al., 1992.

modulation of immunosuppression in UV-irradiated mice by the carotenoid canthaxanthin (Gensler, 1989). Gensler has suggested that canthaxanthin may prevent the oxidation of sterols, so reducing immunosuppression.

As yet, the immunomodulating effects of carotenoids at the molecular level have only been investigated in terms of general antioxidative capacity. Further research is merited.

Cancer prevention

The concept that carotenoids may be able to prevent cancer appeared in 1981. Peto et al. (1981) have performed meta analysis on various data, both from human epidemiological studies and from animal studies, and have hypothesized that ß-carotene has a specific preventitive action against human cancer, independent of any provitamin A mechanism (Peto et al., 1981). Since then a number of studies have been undertaken to establish a relationship between cancer risk and intake of dietary carotenoids, primarily ß-carotene. The scope of these studies has ranged from the induction of carcinogenic or pre-carcinogenic states in cell cultures, organ cultures, and whole animals, to the evaluation of human epidemiological data.

Induction of mutagenesis and carcinogenesis in cell cultures, organ cultures, and whole animal systems

In a wide range of studies, mutagenesis and carcinogenesis have been experimentally initiated under controlled conditions, both chemically and by exposure to X-ray and ultraviolet (UV) irradiation.

In bacterial, animal and human cell cultures, carotenoids have been shown to prevent mutagenesis. In these studies, UV-A light has been used to initiate mutagenesis, as have a whole range of known mutagenic and genotoxic agents, including 8-methoxypsoralen (8-MOP), dimethylsulfoxide (DMSO), cyclophosphamide, phorbol myristyl acetate (PMA), methylmethanesulfonate (MMS), and 4-nitroquinoline-1-oxide (4-NQO). Although strains of *S. typhimurium* have been used in such studies, the most common cell culture models have utilized mouse mammary cells or Chinese hamster ovary (CHO) cells (Krinsky 1989, 1991). Mutagenesis has been assessed in two general ways: either by increased numbers of histidine revertants after exposure to the mutagen (the Ames test), or by the appearance of sister chromatid exchanges (SCE), indicative of nuclear damage and development of pre-malignant states. Translocations, general chromosomal aberrations, and the appearance of micronuclei have also been shown to be indicative of mutagenesis. To date only ß-carotene has been used in cell culture mutagenesis studies; it has been concluded that ß-carotene does prevent mutagenesis, in a dose-dependent manner. However, ß-carotene has not been observed to protect against all mutagenic or genotoxic compounds, including tannic acid, gallic acid, and hydrogen peroxide (Stich and Dunn, 1986). In addition, the concentrations of ß-carotene in

the common solvent vehicles used, methanol and ethanol, have come under scrutiny (Krinsky, 1991). In some cases, solubility may have been exceeded, causing carotenoid precipitation, and thus confounding the study results.

Although mutagenesis has also been assessed in organ cultures (Manoharan et al., 1985), most researchers have tended to look at malignant transformation and tumorigenesis. Manoharan et al. (1985) have observed that ß-carotene in hexane at concentrations of 10^{-6}M prevented malignant transformations in mouse mammary cell organ cultures exposed to various carcinogens such as dimethylbenzanthracene (DMBA), N-methylnitrosurea, and N-nitrosodiethylamine, noting that ß-carotene acted at both initiation and promotion. Pung et al. (1988) have observed that ß-carotene and canthaxanthin in CH_3OH at 10^{-5} M inhibited neoplastic transformation in C3H 10T1/2 cell organ cultures. Using both methylcholanthrene (MCA) and X-ray treatment, they have concluded that the carotenoids are effective at promotion, but not during initiation.

In whole animal models the chemopreventitive effects of carotenoids have normally been assessed by tumor count relative to controls. It is difficult to assess the anticarcinogenic effects of carotenoids in animal models, as has been discussed by Krinsky (1991) and Moon (1989). Although a large volume of studies have been performed, there is a lack of unity in experimental design. This includes test animal, tumor site evaluated, carcinogen(s) used, and the dose level, dose rate and study duration. Even when the same experimental design has been used, the results have often contrasted.

Using mainly mice, rats and hamsters, the majority of studies have indicated a significant anticarcinogenic effect of carotenoids, primarily in skin, colon and pancreas (Krinsky, 1991). However, the number of studies indicating lack of effect of carotenoids and the lack of systematical experimental design, leaves little room for meaningful conclusions.

Human epidemiological data

The interpretation of data from studies on dietary carotenoids and cancer risk in humans has been even more difficult than in animal models. Two forms of study have been used, and both suffer from problems of data interpretation. Retrospective studies match known cancer patients with selected controls, and compare predisease dietary data, or blood samples. Prospective studies follow a population of healthy individuals over a period of time, with frequent monitoring of diet and blood. After a certain time, individuals who develop cancer (or die from cancer) are compared to the "non-cases" in the population with respect to diet and blood carotenoid composition.

Before looking at some of these studies, it is useful to first put them in context by examining some of the sources of data interpretation problems. The issue has been more fully discussed by others (Moon, 1989, Temple and Basu, 1988, Ziegler, 1989, and Thurnham, 1990).

In retrospective studies the matching of cases to controls becomes increasingly difficult with the elimination of uncommon factors; it is difficult to eliminate all confounding

factors, and so it is dubious whether cancer can be attributed to one component in the diet. Furthermore the onset of cancer may have affected either the patient's diet, or memory and, hence, dietary recall ability.

The form of exposure to carotenoids confounds interpretation further. Ziegler (1989) has quoted five representative studies where subjects consumed carotenoids in the form of fruits, berries, soups, and various vegetables. As Temple and Basu (1988) have noted, the true protective factor in such studies may have been any one of a number of factors associated with intake of these foods: dietary fiber, low fat, phenols, glucoronidase inhibitors, low meat intake, etc.

When quantifying blood plasma carotenoids, two problems exist. First, often ß-carotene alone is measured. Secondly, carotenoids are often analyzed by dietary total vitamin A (DTVA) assays (i.e. retinol + ß-carotene). Retinol and its analogs have some anti-carcinogenic properties (Moon,1989), thus the effects of carotenoids are confounded.

As with animal models, there is a real and immediate requirement for improved experimental design, including the standardization of dietary carotenoid form and feeding regime, control selection in statistically significant numbers, and uniformity in sampling and analysis of blood plasma carotenoids. Table 3 provides a summary of cancer studies to date, with respect to carotenoids, based on epidemiological data obtained in the last decade.

In summary, a strong inverse relationship has been shown to exist between dietary ß-carotene and the risk of lung or stomach cancer, while other cancer sites require further investigation. In addition, investigation of other specific carotenoids is warranted.

Table 3. Correlation between human cancer risk and carotenoid intake[a]

Site	Conclusions
Lung	Strong inverse relationship with ß-carotene[b]
Stomach	Fairly strong inverse relationship with ß-carotene
Colon/ rectum	No evidence of any relationship
Breast	Possible inverse relationship[c]
Ovary/ cervix	Possible inverse relationship[d]
Prostrate	Weak inverse relationship[e]
Bladder	Limited evidence for conclusion

[a] Carotenoids measured by dietary intake, generally by consumption of green and yellow vegetables, total carotene and dietary total vitamin A (DTVA), or by blood analysis of plasma ß-carotene or total plasma carotene. Data summarized from Temple and Basu, 1988, Moon, 1989, Ziegler, 1989, Stählein et al., 1991, Canfield et al., 1992.

[b] For squamous cell- and small cell-carcinoma, but not adenocarcinoma.

[c] Especially in post-menopausal women.

[d] Especially for ovarian cancer risk in younger women.

[e] Inconsistent data.

Mechanism of Cancer Prevention

As Krinsky (1989) has observed, carotenoids may operate by prolonging the promotional phase, indicated by studies in which the latency period of carcinogenesis increased without overall decrease in the number of animals with tumors. Other studies have reported tumor regression after exposure to carotenoids, thus carotenoids apparently have an effect at the progression stage. In addition, while carotenoids have been observed to inhibit the initiation of carcinogenesis in many studies, it has been suggested that effects at promotion and progression are independent of initiation (Pung et al., 1988; Temple and Basu, 1988). The main focus of recent research has been how carotenoids operate at the cellular and molecular levels. To date four possible mechanisms have been proposed.

Conversion of ß-carotene to retinoids and apocarotenoids

ß-carotene and other provitamin A carotenoids are metabolized *in vivo* to both retinoids and apocarotenoids. These may either enhance or inhibit the effects of retinoic acid (Canfield et al., 1992). Retinoic acid is known to block promotion, modulate enzyme action, and repress oncogene expression. There is no evidence that provitamin A carotenoids prevent cancer in this manner, and non provitamin A carotenoids are not able to be metabolized to

retinoids in the first place. However, this does not rule out the mechanism; the carotenoids may well function in a number of structure-dependent ways.

Modification of xenobiotic metabolizing pathways

Temple and Basu (1988) have observed that the levels of some hepatic drug-metabolizing enzymes were reduced in mice fed supplemental ß-carotene. Specifically, the levels of cytochrome P-450 oxidases and biphenyl-4-hydroxylase decreased, with no effect on p-nitroanisole-O-demethylase or antipyrine-N-demethylase. These researchers have speculated that this may cause carcinogens to be routed along a detoxification pathway rather than an activation pathway, presupposing that ß-carotene is active during initiation. This would likely be very dependent on the nature of the carcinogen. Edes et al. (1989) have reported that supplemental ß-carotene in rat diets caused a significant increase in the activity of the carcinogen metabolizing enzyme aryl hydrocarbon hydroxylase (AHH) in the intestine after benzo(a)pyrene challenge. These two studies may indicate some modulation of xenobiotic metabolism by carotenoids.

Destruction of tumor cells

Carotenoids have been shown to beneficially stimulate the immune response in a number of ways. In relation to cancer prevention, carotenoids have increased macrophage

cytotoxicity towards hamster tumor cells, enhanced lymphocyte production, influenced human interferon action, enhanced cytokine production, and elevated levels of tumor necrosis factor (Temple and Basu, 1988; Bendich, 1989a). Other carotenoids have been observed to cause some of the same effects, and so it has been concluded that carotenoid-enhanced immune response to tumors may play an important role in cancer prevention.

Antioxidant activity of carotenoids

A great deal of evidence has been presented for a role of prooxidant states in carcinogenesis. Cerutti (1985) has concluded that prooxidant states can promote initiated cells to neoplastic growth. Prooxidant states can be defined as an increase in concentration of active oxygen species, such as hydrogen peroxide, hydroxyl radical, and singlet oxygen (O_2^{1*}), or organic peroxides and organic free radical species. Cerutti has specifically suggested a role for lipid peroxidation in carcinogenesis, and additionally the oxidation of other cellular macromolecules.

Palozza and Krinsky (1992b) have defined biological antioxidants [bioantioxidants] as compounds capable of protecting biological systems against the potentially harmful effects of processes or reactions that can lead to excessive oxidations. The carotenoids have been implicated as bioantioxidants in a number of studies, and three mechanisms have been postulated whereby carotenoids may function in this capacity: via interactions with vitamin E, as deactivators of active oxygen species, and as inhibitors of lipid peroxidation.

Carotenoid-vitamin E interactions

Vitamin E is the best studied and most abundant antioxidant *in vivo*. Levels in human plasma range between 11-27 μmol/l, of which about 80-90% is α-tocopherol, the rest being composed of ß-, γ- and δ-tocopherols, depending on dietary consumption. These levels are between 20 to 50 times greater than ß-carotene in plasma; some researchers have put the ratio closer to 150:1 (Khachik et al., 1992b). Mayne and Parker (1986,1988b) have observed that chicks fed ß-carotene showed a 4-fold increase in liver total vitamin E, with a 50% decrease in plasma vitamin E. In addition, they have shown that chicks fed canthaxanthin exhibited 2 x hepatic α-tocopherol levels. Palozza and Krinsky (1992a) have demonstrated that ß-carotene and α-tocopherol are synergistic antioxidants in model membrane systems, noting that α-tocopherol prolongs the antioxidative effectiveness of ß-carotene by some sacrificial mechanism. These studies seem to indicate that vitamin E has been mobilized to peroxidation sites to provide synergistic support of ß-carotene in its antioxidative capacity. Further studies are required to clarify the mechanism of synergism.

Deactivation of active oxygen species

Active oxygen species have been implicated in cellular lipid peroxidation. A number of studies have shown the carotenoids to be capable of deactivating these species (Krinsky, 1979; Packer et al., 1981; Krinsky and Deneke, 1982; Krinsky, 1989; Palozza and Krinsky,

1992b). Furthermore, the mechanism by which carotenoids deactivate singlet oxygen has been elucidated (Foote, 1968; Foote and Denny, 1968), and lycopene has been demonstrated to be the most effective biological singlet oxygen deactivator (Di Mascio et al., 1989).

Inhibition of lipid peroxidation

A number of studies have indicated that ß-carotene and other carotenoids function as inhibitors of *in vitro* lipid peroxidation, specifically concerning linoleic acid (Monaghan and Scmitt, 1932; Terao, 1989) and arachidonic acid (Halevy and Sklan, 1986). Burton and Ingold (1984) have presented a mechanism for the antioxidant activity of ß-carotene: in essence, the hypothesis states that ß-carotene functions as a chain-breaking antioxidant at low partial pressures of oxygen (pO_2) by `quenching' lipid peroxy radicals, as shown in Figure 3. This mechanism has been widely accepted, although none of the potential intermediates proposed have yet been isolated. The effectiveness of ß-carotene as an antioxidant has been shown to be greatly dependent on pO_2. Reaction [5] in Figure 3 illustrates the effect of oxygen: at high partial pressures, equilibrium shifts to the right, favoring formation of a chain-carrying radical and stimulating propagation. At low partial pressures, equilibrium shifts to the left, preventing chain propagation by the carotenoid-carrying radical. Burton and Ingold have shown that at normal pO_2 of 760 torr, ß-carotene takes on prooxidant activity after a brief period of antioxidant activity; at reduced pO_2, for example at 150 torr, ß-carotene remains a highly effective inhibitor of lipid peroxidation. These observations have

a) Lipid autoxidation

initiation ► RH → $R\bullet$ + $H\bullet$ [1]
                lipid   lipid
                              radical

propagation ┌► $R\bullet$ + O_2 → $ROO\bullet$ [2]
            │   peroxy radical
            │
            │ $ROO\bullet$ + RH → $ROOH$ + $R\bullet$ [3]
            └─────────────────────────────────┘
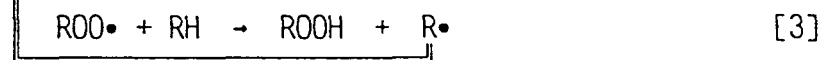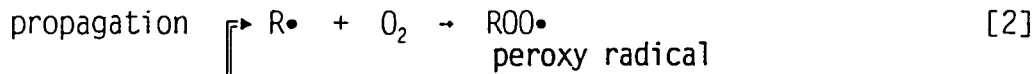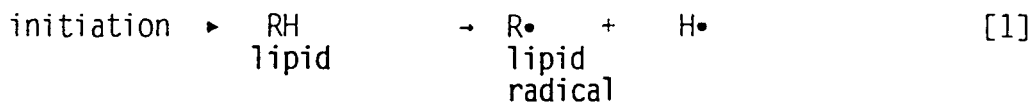
b) peroxy radical quenching

$ROO\bullet$ + carotenoid   → $\bullet$carotenoid-O-O-R   [4]
                                     carbon-centered resonance
                                     stabilized radical

$\bullet$carotenoid-O-O-R + O_2 → $\bullet$O-O-carotenoid-O-O-R   [5]
stable radical   new reactive chain-
                                     carrying radical

$\bullet$carotenoid-O-O-R + $X\bullet$ →   stable products   [6]
            organic
            radical
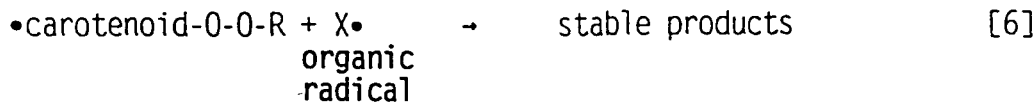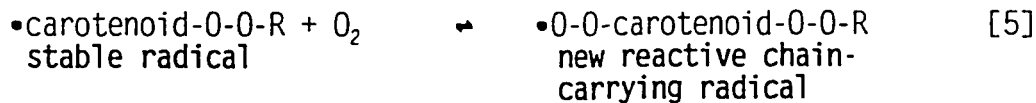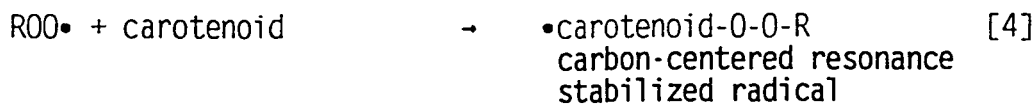
Figure 3. Inhibition of lipid peroxidation by ß-carotene (Burton, 1989)

been reproduced in a number of laboratories (Palozza and Krinsky, 1992b). Since pO_2 found

in mammalian tissues are low, beta carotene and other carotenoids have the potential to act

as lipid soluble chain-breaking antioxidants *in vivo*.

Various membrane models, commonly rat liver microsomes, have been used to

monitor the inhibition of lipid-peroxidation by carotenoids. Most studies have reported

modest to high inhibition, and one study has reported that lycopene is superior in antioxidant capacity to both α- and ß-carotene (Kim, 1990). Zamora et al. (1991) have investigated the effects of ß-carotene on spontaneous lipid peroxidation in rat erythrocytes and plasma, and have concluded that ß-carotene may work as an antioxidant *in vivo*. Leibovitz et al. (1990), however, have observed little protection by ß-carotene against lipid peroxidation in rat tissue slices, and Mayne and Parker (1989) have noted only marginal antioxidant effects of canthaxanthin in chick liver homogenates. Most investigations have used the thiobarbituric-acid reactive substances (TBARS) assay for lipid peroxidation, an assay which measures the secondary products of lipid peroxidiation. But in at least one study (Petyayev et al., 1975), free radical concentrations have been measured directly by electroparamagnetic methods. Petyayev et al. have reported that lycopene induced a decrease in the concentration of free radicals in experimentally induced rat Guerin's tumor.

The lack of consistent data concerning carotenoids as antioxidants in animal tissue models has led Palozza and Krinsky (1992b) to conclude that as yet there can be no direct claim that carotenoids function as antioxidants in human disease prevention. However, this concept has been the main area of carotenoid research in the last decade.

Toxicity of Carotenoids

The complete lack of toxicity of ß-carotene in humans has been demonstrated (Mathews-Roth, 1986; Bendich, 1988). ß-carotene has been used for over twenty years in the treatment of patients with erythropoietic protoporphyria (EPP), and no toxicity has been observed.

Excessive consumption of carotene or lycopene in the diet , or as dietary supplements, has been observed to cause carotenemia or lycopenemia respectively. Both conditions are characterized by yellow-orange coloration of the skin, caused by the deposition of excess pigment in epithelial tissue. Coloration disappears over time as the ingestion of excess carotenoid is discontinued (Reich et al, 1960). For provitamin A carotenoids, excessive intake has not been shown to induce hypervitaminosis A, nor have liver stores of the carotenoid or vitamin A greatly increased. In general, carotenoid absorption decreases as intake increases, with proportional fecal excretion of the intact carotenoid (Klaui and Bauernfeind, 1981).

There has been at least one report of fatal carotenoid toxicity, associated with canthaxanthin. This diketocarotenoid is a GRAS (Generally Recognized As Safe) food colorant approved by the FDA. It is found naturally in some fish and mushrooms, but is approved for use in many food products, including poultry, eggs, cheese, meat products and tomato products. Due to its colorant properties, canthaxanthin has been used in Europe in tanning pills, although it has not received FDA approval for this purpose in the US. The
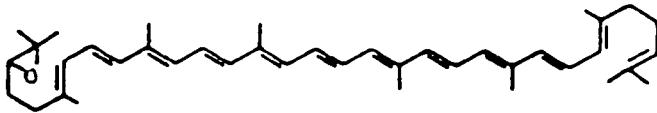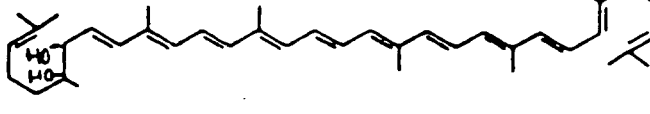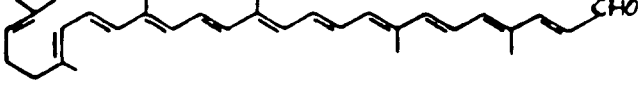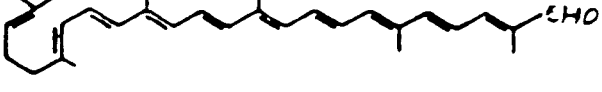
toxicity report has indicated death by aplastic anemia in a patient using tanning pills (Bluhm et al., 1990). This case has been debated in two letters (Mathews-Roth, 1991; Herbert, 1991). Herbert has claimed that canthaxanthin can reside in the body for several months, and has linked the carotenoid to retinopathy, hepatitis, and urticaria. Mathews-Roth has argued that canthaxanthin has been used in conjunction with ß-carotene in the treatment of EPP patients in Europe without any observed toxicity, and that retinopathy caused by canthaxanthin is reversible with time. She has also pointed out that the Bluhm report did not indicate composition of the tanning pill, nor dose. In summary, carotenoid toxicity does not seem to be an issue as yet, but further investigation will put recent studies in context.

Derivatives of Lycopene: Occurrence and Significance

In investigating the benefits of carotenoids to human health, or any other food or drug component for that matter, a fundamental question arises: are the effects attributable to the intact molecule or to one or more derivatives? This question has been asked of ß-carotene, with specific reference to its provitamin A function, the conclusion being that the effects observed are independent of any provitamin A function. However, little research has been conducted on the possibility that oxygenated derivatives of lycopene and other carotenoids may be partly or fully responsible for the beneficial health effects reported. Oxygenated

derivatives of lycopene have been detected in foods, and processing may stimulate formation or enhance levels of these compounds. At least one oxygenated derivative of lycopene has been found in human plasma; this product may represent a natural food component, or a product of general metabolism of lycopene and its oxygenated derivatives in food. To date five oxygenated derivatives of lycopene have been identified in foods (specifically tomatoes) and human plasma. These products are indicated in Table 4.

Table 4.   Oxygenated derivatives of lycopene in food and human plasma

Lycopene	
Lycopene-1,2-epoxide	
Lycopene-5,6-epoxide	
Lycopene-5,6-diol	
6'-apolycopenal	
8'-apolycopenal	

Ben-Aziz et al. (1973) have isolated a number of lycopene derivatives from tomatoes, including lycopene-1,2-epoxide, lycopene-5,6-epoxide, 6'-apolycopenal and 8'-apolycopenal. Lycopene-5,6-epoxide has been measured in raw and stewed tomatoes by Khachik et al. (1992a) at 5 mg/kg fresh weight, with no quantification of lycopene-1,2-epoxide. A number of studies have been conducted to determine the nature of the products formed by the processing of ß-carotene, with the identification of a number of ß-carotene aldehydes, ketones, epoxides and diols (Marty and Berset, 1986, 1988; Chandler et al., 1988). Khachik et al. (1992a) have identified lycopene-5,6-epoxide at levels of 12.5-21 mg/kg in tomato paste, and lycopene-5,6-diol at levels of 2.3-10 mg/kg.

At least one study has reported the presence of oxygenated lycopene derivatives in human plasma (Khachik et al., 1992b). They have detected only lycopene-5,6-diol at levels of 72.5 nmol/l plasma, a significant quantity, being about 28% relative to ß-carotene, and 6% relative to intact lycopene. The absence in plasma of other oxygenated products found in tomatoes may indicate differences in absorption or metabolism (Khachik et al., 1992a, 1992b), possibly caused by destruction or rearrangement of these compounds in the acid conditions of the stomach (Parker, 1989).

It is fully possible that lycopene and any oxidation products undergo further oxidative metabolism at target tissue sites. It is known that epoxidation/de-epoxidation reactions involving ß-carotene and other carotenoids occur in some plants, caused by photosynthetic stimulation of membrane electron transport systems (Ashikawa et al., 1987). ß-carotene epoxidation is also known to result during cell injury in plants (Ben-Aziz et al., 1973).

Tyczkowski et al. (1987) have identified various metabolites of canthaxanthin in chickens, and have proposed a pathway for canthaxanthin metabolism. They did not observe further oxygenation of canthaxanthin, rather metabolism of the carbonyl functions. No oxidized forms of lycopene have been reported in human cells, but the possibility should not be ruled out, bearing in mind the high degree of unsaturation seen in carotenoids. In addition, if the carotenoids function by way of some free-radical mediated antioxidant mechanism, the carotenoids would be expected to be modified in the process (Handelman et al., 1991). It would be expected that naturally- or metabolically-oxygenated lycopene products would be more soluble in aqueous systems than lycopene itself. If the oxygenated product is not bound to membranes, it may be more effective than the parent molecule in its antioxidant capacity, assuming that antioxidative function is still present. If the product is bound to membranes, or an integral part of membranes, increased solubility may lead to increased antioxidant function at the membrane/fluid interface, assuming the oxygenated product is orientated perpendicular to the plane of the membrane. This assumption may not be valid: the symmetrical planarity of lycopene may cause it to be oriented within the membrane bilayer, and hence lycopene would be more effective at preventing oxidation within the membrane. Orientation may change to perpendicular as a result of oxidation, but this is merely speculation. The orientation of carotenoids in membranes must be better understood to allow an assessment of their function.

Preparation and Analysis of Lycopene

A number of factors must be taken into consideration when extracting lycopene from foodstuffs. The highly unsaturated carotenoids are susceptible to the oxidizing and isomerizing effects of heat, light, oxygen, acids, metals and free halogens among others (De Ritter and Purcell, 1981; Krinsky and Welankiwar, 1984). Lycopene must be extracted and stored rapidly with minimum exposure to these factors. Storage temperatures should be low, and as Craft and Soares, Jr (1992) have indicated, the choice of storage solvent is crucial: on examining eighteen solvents for solubility and ten-day storage stability of ß-carotene and lutein, they have shown butylated hydroxytoluene (BHT)-stabilized tetrahydrofuran (THF) to be the most appropriate solvent. Halogenated solvents are not recommended for use as carotenoid storage solvents.

Extraction of carotenoids from foodstuffs

A large number of methods exist for carotenoid extraction from foods. De Ritter and Purcell (1981) have listed extraction techniques for over forty carotenoid sources, including animal tissue, fruits and vegetables, and microorganisms. Two methods have recently been published for the extraction of tomato paste carotenoids (Tan, 1988; Sadler et al., 1990). Tan (1988) has extracted tomato paste carotenoids into EtOH, preparing concentrates after refluxing with petroleum ether (PE) at 48°C. Sadler et al. (1990) have developed a more

rapid technique without using elevated temperatures: extraction of the tomato paste carotenoids by agitation with 2:1:1 hexane:acetone:ethanol, and concentration of the lycopene-bearing hexane phase.

Analysis of tomato paste carotenoids

Three classes of chromatographic methods have been employed for carotenoid analysis: open column chromatography, thin layer chromatography (TLC), and high performance liquid chromatography (HPLC). Open column- and thin layer- chromatography have been outperformed by HPLC in terms of rapidity of analysis and resolving power, and thus are used fairly infrequently (Krinsky and Welankiwar, 1984) or in conjunction with HPLC methods (Tan et al., 1986).

Normal-phase HPLC has long since been supplanted by reversed-phase (RP) HPLC, commonly using C-18 columns and non-aqueous mobile phases. RP-HPLC techniques have allowed the rapid separation of carotenoids of varying polarities with the option of using isocratic, step-gradient or continuous gradient mobile phases. A number of different RP columns have been utilized, with a correspondingly wide range of mobile phase systems (Miller et al.,1984; Quackenbush, 1987; Khachik and Beecher, 1986; Ng and Tan, 1988). Bushway (1986) has used a Vydac C-18 218TP54 column with isocratic mobile phase of 40:56:4 MeCN:MeOH:tetrahydrofuran for the separation of polar and non-polar carotenoids. Vydac C-18 and Zorbax ODS C-18 columns are now most widely used in carotenoid RP-

HPLC (Craft et al., 1990; O`Neil et al., 1992). Saleh and Tan (1991) have optimized mobile phases for both of these columns with reference to carotenoid resolution, recommending 80:18:2 MeCN:MeOH:CH$_2$Cl$_2$. The use of MeCN and MeOH in RP-HPLC carotenoid separations has become common, often with the use of a non-polar modifier such as dichloromethane or chloroform to adjust solvent strength (Nells and De Leenheer, 1983). Khachik and Beecher (1987) have used a Brownlee RP-18 column fitted with guard cartridges and a 22:55:23 MeOH:MeCN:CH$_2$Cl$_2$ mobile phase, with continuous UV-photodiode array (PDA) detection of carotenoids eluted from the column. Khachik et al. (1992a, 1992b) have further developed gradient mobile phases for the separation of carotenoids in tomatoes and green vegetables, and in human plasma. The separations used a 4-solvent system set-up of acetonitrile:methanol:hexane:dichloromethane at 85:10:2½:2½ for the first ten min, followed by a 30 min gradient to a final composition of 45:10:22½:22½ acetonitrile:methanol:hexane: dichloromethane. This method has allowed the best resolution of polar and non-polar carotenoids in various isomeric forms to date, enhanced by the use of a compatible injection solvent, 4:2:2:2 acetonitrile:methanol:hexane: dichloromethane. The researchers have been able to identify twenty carotenoids present in human plasma, of which nine have been resolved as two or more geometrical isomers. The same gradient elution system has been used for semi-preparative chromatography of carotenoids, employing a Rainin Microsorb C-18 column.

The use of PDA detection methods has now become common in carotenoid analysis. The system allows detection over a range of wavelengths, with the added advantage that

spectral data can be stored, analyzed, and compared to library spectra. Consequently the collection of eluate and subsequently UV-spectrophotometric analysis to determine wavelength maxima is no longer necessary, making analysis a lot simpler and a great deal faster.

Identification of lycopene

As stated, PDA detection has allowed the identification of carotenoids by wavelength absorption maxima. These data can be compared to tabulated values in appropriate solvent (Foppen, 1971; De Ritter and Purcell, 1981). However, wavelength maxima alone are not sufficient to identify carotenoids. Commonly the carotenoids are subjected to mass spectrometry, using either desorption chemical ionization (DCI) or electron capture negative ion (ECNI) methods. Khachik et al. (1992a), for example, have identified lycopene by its characteristic molecular anion at m/z 536 in methane, as well as other key fragment ions, by DCI- and ECNI-MS.

Infra-red (IR), Raman, and resonance Raman (RR) spectroscopic methods have been used to a lesser extent to identify lycopene and other carotenoids (Rimai et al., 1973; Koyami et al., 1982). Parker (1971) has used RR methods to identify ß-carotene in carrots, and lycopene in tomatoes and tomato sauce, by comparison of characteristic vibration fundamental wavenumbers with known carotenoid RR spectra.

Yield and purity analysis

Lycopene in crude form or after purification has often been analyzed for yield and purity. Purity has been evaluated by calculation of lycopene peak area as a % of total peak area on an HPLC chromatogram. This is generally adequate, but does not allow for the possibility of co-eluting compounds within the peak. A distinct advantage of PDA detection is its ability to execute spectral scans continuously; the peak can be broken down by spectral scan to search for any non-lycopene components.

Yield analysis can also be performed using PDA methods if standard curve data is present in the PDA files (Rouseff et al., 1992). Most commonly though, UV-spectrophotometric methods have been used, based on the Beer-Lambert equation. This technique has the disadvantage that in crude extracts other carotenoids with similar wavelength maxima can elevate absorbance above the real value. This has been observed by Lime et al. (1957); when compared to cleaned-up samples, crude extracts of lycopene showed 10% higher concentrations. However, the method is common. Sadler et al. (1990) have used the method recently in quantifying lycopene in crude tomato paste extracts.

Preparation and Analysis of Lycopene Oxidation Products

Preparation methods

A variety of techniques have been employed for the preparation of lycopene oxidation products such as those found in Table 4. The most obvious method of preparation has been by isolation from vegetables. However, these compounds were present in very small amounts; ripe commercial tomatoes, for example, yield only about 5 mg/kg lycopene-5,6-epoxide, and other products were below the limits of detection in conventional analytical techniques (Khachik et al., 1992a). Preparation from tomato paste has been more effective, the oxidation products being present in a more concentrated form. While these methods have been used to isolate small quantities of product for assessment of chemical, spectroscopic, and chromatographic properties (Ben-Aziz et al., 1973), they are not practical for routine preparation of larger quantities of product.

For ß-carotene at least, oxidation products resulting from autoxidation or co-oxidation reactions in its capacity as an antioxidant have been characterized. Handelman et al. (1991) have subjected ß-carotene to spontaneous oxidation, peroxy-radical induced autoxidation, and chemical attack by NaOCl. They have observed a number of ß-apo-carotenals and at least one epoxide, as well as a number of unidentified products. Liebler and Kennedy (1992) have autoxidized ß-carotene by thermally initiated azo-radical formation from azobis(2,4-

dimethylvaleronitrile) (AMVN) and have characterized at least seven epoxides. To date no oxidation products of lycopene have been characterized by these methods of oxidation.

A number of oxidized derivatives of lycopene have been prepared by various chemical treatments. Bush and Zechmeister (1957) have prepared lycopene-5,6-epoxide by treatment with perphthalic acid, and lycopene-5,6-diol by treatment with BF_3. Further treatment of this diol with BF_3 has been shown to yield 5,6,5',6'-tetrahydroxylycopene. The researchers have postulated a mechanism for the formation of lycopene-5,6-diol from lycopene, and by way of methylation reactions have concluded that the two hydroxyl groups were unequal in nature. A simpler method for the preparation of oxidation products has been proposed by Rodriguez et al. (1976) and Ritacco et al. (1984a, 1984b). These investigators have observed that lycopene readily undergoes oxidation in the presence of Micro-Cel C, a commercial synthetic calcium silicate powder, and a suitable solvent. Lycopene exposed to water-reactivated Micro-Cel C (MCC) in petroleum ether solvent for one hour has been observed to yield a number of products, at least twelve being resolved by TLC of the product mixture. 6'-Apolycopenal and lycopene-5,6-epoxide have been characterized by mass spectrometry, and lycopene-5,6-diol has been tentatively identified. A reaction scheme for the hydroxylation mechanism has been proposed, similar to the BF_3 reaction described by Bush and Zechmeister; it has been suggested that Lewis acid sites on the silicate surface may function in a similar way to the BF_3 mechanism. An oxidation reaction scheme has been presented as shown (Ritacco et al., 1984b) :

lycopene → lycopene-5,6-epoxide → lycopene-5,6-diol → 6'-apolycopenal
+ 6-methyl-5-hepten-2-one
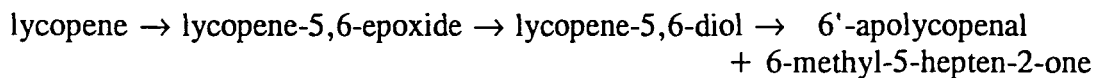
Sub-milligram quantities of each product have been isolated from the reaction of 5 mg lycopene with 150 g MCC. The advantage of the MCC method is its ability to form a number of oxidation products rapidly and with only one treatment. Extraction of the products is simple, involving the use of acetone to wash the products from hydroxylating surfaces, partitioning into petroleum ether, and concentration of the extract.

Analysis of products

Similar to lycopene, RP-HPLC has been most commonly used for the resolution and identification of its oxidation products. PDA detection allows calculation of wavelength absorbance maxima, for comparison with tabulated data. Fractions can be collected from semi-preparative chromatography of extracts and characterized.

Raman spectroscopy has been used to a very small degree in the characterization of carotenoid oxidation products (Ashikawa et al., 1982), as has IR spectroscopy (Tsukida and Zechmeister, 1958). In general, however, mass spectrometry remains the preferred method of characterization (Baldas et al., 1966; Ben-Aziz et al., 1973; Foppen, 1971). Ritacco et al. (1984a) and Ben-Aziz et al.(1973) have provided excellent mass spectral data for each of the lycopene oxidation products listed in Table 4, giving both molecular ion and key fragment ion m/z data.

Quantification of lycopene oxidation products has been achieved by the use of UV-spectrophotometric analysis, using published molar extinction coefficients (ε) for the appropriate wavelength maxima (Khachik et al., 1992a, 1992b).

Assessment of the Antioxidant Capacity of Carotenoids

As has previously been discussed, the antioxidant capacity of carotenoids may operate in one or more ways: via interactions with vitamin E, as deactivators of active oxygen species, or as inhibitors of lipid peroxidation. Carotenoid-vitamin E interactions have begun only recently to attract interest, and are not discussed here.

Research on the quenching of active oxygen species has focused primarily on singlet oxygen (O_2^{1*}), although this has not been proven to be the primary active oxygen species *in vivo* (Canfield et al.,1992). Direct singlet oxygen quenching by lycopene has been observed *in vitro* by Di Mascio et al. (1989), through observing the disappearance of 3,3'-(1,4-naphthyl-idene) dipropionate (NDPO$_2$) generated singlet oxygen via IR emission spectroscopy. Anderson et al. (1974) have observed inhibition of liposomal lipid peroxidation by ß-carotene in the presence of singlet oxygen generated by radiofrequency discharge and photosensitization. However, this study provided no direct evidence that lipid peroxidation inhibition was caused by carotenoid quenching of singlet oxygen, a point which

has been mentioned by Packer et al. (1981). The significance of singlet oxygen *in vivo* must be ascertained prior to further work.

The majority of investigations on carotenoid antioxidative capacity have centered on their radical quenching properties in lipid peroxidation, as shown in Fig. 3. These properties have been assessed by a number of methods, in controlled studies with carotenoid and non-carotenoid groups. Studies can be divided into three main areas: chemical studies, liposome studies, and tissue/whole animal studies.

Chemical studies

Monagahan and Schmitt (1932) have reported carotene inhibition of linoleic acid oxidation, quoting 50% inhibition in the first hour. In this study, linoleic acid was present in a non-polar lipid homogenous environment, common in the majority of chemical studies performed to date. The researchers initiated oxidation spontaneously by exposure of the reaction mixture to air, and measured oxidation by oxygen uptake in Warburg respirometers. Oxygen uptake methods have been assessed as versatile and quantitative tools (Yamamoto et al., 1982), and have been used a great deal. Wayner and Burton (1992) have measured oxygen uptake by lipids during peroxidation by way of both Clark-type oxygen electrodes and the measurement of pressure change in sample headspaces by way of pressure transducers (Mahoney et al., 1964). The latter more sensitive method has been used by Burton and Ingold (1984) and Niki et al. (1985) among others. Both groups have controlled

the rate of initiation of peroxidation by using azo initiators such as azoisobisbutyro-nitrile

(AIBN), azobisamidinopropane hydrochloride (AAPH), and azobis-dimethylvaleronitrile

(AMVN). These compounds dissociate thermally at 37°C and above at a uniform

reproducible rate to yield carbon centered radicals capable of initiating lipid peroxidation,

as shown in Figure 4.

$$A-N=N=A \qquad \rightarrow N_2 + 2e\ A\bullet + (1\text{-}e)\ A\text{-}A \qquad [1]$$
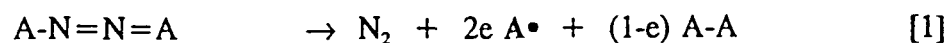
where A is an organic group, and e is the efficiency of
radical generation.

$$A\bullet + O_2 \rightarrow AOO\bullet \qquad [2]$$

$$AOO\bullet + RH \rightarrow AOOH + R\bullet \qquad [3]$$
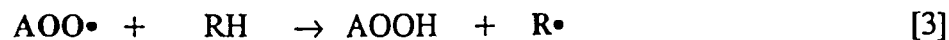
where RH is a lipid

Figure 4. Initiation of lipid oxidation by azo initiators

The use of these initiators is now common. Yamamoto et al. (1982) have used

AMVN to initiate oxidation of methyl linoleate and methyl linolenate, and have used a

number of procedures to measure oxidation, including the determination of peroxides.

Peroxides have been measured by Hamm et al. (1965) in a sensitive spectrophotometric

assay, but hydroperoxide assays are more common. Hydroperoxides have been measure directly by Terao (1989) and Terao et al. (1992) using HPLC methods, and similarly by Terao et al. (1986) after first reducing the hydroperoxides using $NaBH_4$. These workers have initiated methyl linoleate oxidation by AMVN, and have assessed the effects of various carotenoids on the oxidation.

Liposome studies

Liposome systems have been used for a number of years in investigating carotenoid antioxidant effects. They have the advantages that they act as a model membrane system, and not just a simple lipid homogenous solution, and allow the manipulation of pH, temperature, and liposome lipid composition. Carotenoids in the formulation are readily incorporated into the liposome. A number of mixture protocols have been published for liposomes in carotenoid and other investigations. Kinsky et al (1968) has used egg lecithin (phosphatidylcholine), dicetylphosphate, and cholesterol, while Barclay and Ingold (1981) and Barclay et al. (1986) have formed multilamellar liposomes from dilinoleoyl-phosphatidylcholine (DLPC). These two methods have been used extensively in carotenoid research (Anderson and Krinsky, 1973, 1974; Krinsky and Deneke, 1982; Doba et al., 1985). Liposomal lipid peroxidation has been initiated in a number of ways, for example by UV light, ferrous ions, K_3CrO_8, spontaneously, and by azo-initiators (Palozza and Krinsky, 1992b). The oxidations have been performed in various solvents. Organic phases

such as chloroform have been used, with the use of lipid-soluble initiators such as AMVN.
Lipsomes in aqueous suspension have been subjected to initiation by water-soluble initiators
such as AIBN or lipid-soluble initiators. In general, lipid-soluble initiators are more effective
at initiating oxidation within the liposome, while water-soluble initiators tend to perform
better at the lipid-water interface. Lipid peroxidation has been measured by oxygen uptake
methods (Doba et al., 1985), by enzymatic determination of glucose released from
peroxidized liposome aqueous compartments (Anderson and Krinsky, 1973), and by the
TBARS assay for malondialdehyde (MDA). This assay has been widely used, and involves
the reaction of MDA, a secondary product of lipid peroxidation, with thiobarbituric acid
(TBA), and colorometric determination of the product at 535-548 nm.

Tissue/whole animal studies

Zamora et al. (1991) have studied the effects on lipid peroxidation of dietary ß-
carotene incorporated into rat erythrocyte membranes, lipid peroxidation being initiated by
$BrCl_3C$, and measured by the TBARS assay. These researchers have observed no antioxidant
effects of ß-carotene. Mayne and Parker (1989) have performed a similar study on chicks
fed canthaxanthin. Liver homogenates and hepatic membrane suspensions from the chicks
were subjected to ferrous sulfate initiated oxidation, and TBARS assays of lipid peroxidation
indicated only marginal effects of dietary canthaxanthin.

Palozza and Krinsky (1992a) have assessed the AAPH-initiated lipid peroxidation of isolated rat liver microsomes. Halevy and Sklan (1987) have subjected bovine seminal- and kidney-vesicles to heat-induced lipid oxidation. Arachidonic acid oxidation was monitored by formation of the natural products prostaglandin and hydroxyicosatetraenoic acid (HETE).

In a number of studies, Tappel's group has assessed lipid peroxidation in rat tissue slices initiated by various halogenated hydrocarbons ($BrCl_3C$, dichloromethane, CBR_4) and *tert*-butyl hydroperoxide (t-BOOH) (Sano et al., 1986; Fraga et al., 1987, 1988). In addition to TBARS, these researchers have used total aldehydes measured as cyclohexanedione-reactive substances (CHDRS) to estimate lipid peroxidation. Fraga et al. (1988) have initiated peroxidation by the above methods in rat tissue slices, liver homogenates, and liver microsomes, with TBARS assessment of lipid oxidation, and have observed that TBARS methods are adequate indices of lipid peroxidation in all three systems. Gavino et al. (1984) have also assessed rat tissue slice lipid peroxidation using total ethane and pentane (TEP) release from the slices, concluding that this method was far more sensitive than the TBARS assay. Kunert and Tappel (1983) have used similar methods for live guinea pigs, measuring TEP in expired air, and have identified TEP methods as useful indices of *in vivo* lipid peroxidation.

Research Objectives

The last fifteen years have witnessed considerable research into the health-beneficial effects of carotenoids. While the anticarcinogenic effects of carotenoids have been well investigated, the more recent reports of improved cardiovascular health and immunoenhancement resulting from ingestion of carotenoids have stimulated further research in both of these areas. At this stage, however, it is necessary to offer an explanation for the mechanism of action of these compounds. Indeed, more than one mechanism may be responsible. A great deal of evidence has pointed towards antioxidant mechanisms, most noticeably the ability of carotenoids to inhibit lipid peroxidation both *in vitro* and *in vivo*.

While earlier research on carotenoids and human health benefits has been directed primarily towards ß-carotene, a major carotenoid found in many green and yellow vegetables, recent investigations have begun to look at other carotenoids, including those with no provitamin A activity. These investigations have used two carotenoids of interest, canthaxanthin (Palozza and Krinsky, 1992c) and lycopene. Canthaxanthin is a widely used GRAS food colorant used in fish, red meat, poultry, eggs, tomato products, and candies, to name but a few. Lycopene is the dominant carotenoid in commercial tomatoes and tomato products, and has been shown to be the most abundant carotenoid in human plasma (Khachik et al., 1992b). In addition, other carotenoids including lutein, ß-cryptoxanthin and phytofluene, have been shown to be present at levels comparable to ß-carotene. Lycopene

has been identified as having greater antioxidant potential than ß-carotene in at least one *in vitro* study (Kim, 1990).

Interest in the antioxidant potential of carotenoid oxidation products, however, is virtually non-existent. Of the twenty carotenoids in human plasma identified by Khachik et al., four are oxygenated derivatives of the parent carotenoids, lycopene and ß-carotene. These compounds may have been present as natural components in the diet, or may have been oxidized in the stomach, intestine or other tissues; being present at around 5-8% of the parent carotenoid, their levels are significant. These oxygenated products, whether they be naturally occurring or products of metabolism, may exhibit greater antioxidant capacities than the parent carotenoid. All four of the oxidation products would be expected to show increased water solubility, in turn leading to increased antioxidant activity, either as free cellular components or as membrane-associated molecules. In addition, three of the four oxidation products were more conjugated than the parent molecule: increased conjugation has been shown to elevate the antioxidant capacity of carotenoids by increasing the stability of carotenoid-lipid radical intermediates (see Figure 3). Furthermore, oxidation products of polyunsaturated fatty acids (PUFAs) have recently been shown to destroy certain cancerous cells (Haumann, 1993). The mechanism is not yet understood, but the structural similarities between PUFAs and carotenoids may infer that carotenoid oxidation products may also be capable of killing cancerous cells.

Consequently it would be interesting to see whether lycopene and some of its oxidation products have any antioxidant capacity in lipid peroxidation studies. The volume

of *in vitro* studies performed on ß-carotene as an antioxidant makes this carotenoid a suitable reference in such studies.

The requirement for controlled experimental conditions in such studies can be met by using *in vitro* methods. Lipid peroxidation studies in this project have been carried out in purely chemical environments. The antioxidant capacities of lycopene and some of its oxidation products have been evaluated in this study, with ß-carotene as a reference compound.

EXPERIMENTAL INVESTIGATION

Preparation and Analysis of Lycopene

Preparation of crude lycopene extracts from tomato paste

Canned tomato paste (Hunt, Fullerton, CA) was purchased locally and stored at -30°C. Prior to extraction the paste was allowed to stand for 45 min in a 30°C water bath to reduce the thickness of the paste. Tomato paste lycopene extracts were prepared by two methods. All work was performed in subdued yellow light.

The method of Tan, 1988 (modified)

A 150g pre-weighed sample of tomato paste was transferred to a 2L beaker containing 750 mL of 100% EtOH. The mixture was homogenized for 20 min using a Polytron homogenizer and vacuum filtered. After decanting the filtrate, the residue was homogenized with 250 mL EtOH, filtered, and the filtrate added to the first filtrate. The pooled extract was evaporated to about 40 mL and added to a 100 mL round-bottomed (RB) flask containing 40 mL of crude petroleum ether (PE). The mixture was refluxed for 15 min at 50°C, and the PE phase was decanted; the reflux was repeated after adding a further 40 mL PE. The PE phases were pooled and transferred to a separatory funnel. After addition of 10

mL saturated $NaCl_{(aq)}$ and sodium sulfate to partition EtOH and remove traces of water, the extract was evaporated to dryness and redissolved in 2-3 mL of CH_2Cl_2. A sample of crude lycopene extract was immediately analyzed by HPLC.

This procedure was later modified. Instead of evaporating the EtOH filtrate, 500 mL EtOH was used in the initial extraction, and volumes of 500 mL PE were used in the refluxing steps.

The method of Sadler et al., 1990 (modified)

An $18 \pm 0.2g$ sample of tomato paste was weighed into each of 12 500mL screw-top Erlenmeyer flasks. Three hundred milliliters of 2:1:1 hexane:acetone:ethanol (all reagent grade) extractant was added to each flask. The flasks were shaken for 20 min on a wrist-arm shaker. Fifty milliliters water was then added to the flasks to separate polar and non-polar phases, and the flasks were shaken a further 5 min at half speed.

The mixture was vacuum filtered and transferred to a separatory funnel, to which was added 100 mL saturated $NaCl_{(aq)}$ to ensure complete phase separation. The filtrate was treated as shown in Figure 5. In initial experiments, hexane phases from both re-extracted pulp and re-extracted polar phase were analyzed by HPLC to determine how much lycopene could be recovered. The crude lycopene extract was either stored at -70°C or analyzed by HPLC methods.

Analysis of crude lycopene extracts

Crude extracts were analyzed by reversed-phase (RP) HPLC, using non-aqueous mobile phases in an isocratic system. Samples were injected on column via a Rheodyne 7010 injector equipped with loop filler port and $20\mu L$ sample loop (Rainin, Woburn, MA). Detection was achieved using a Waters 991 photodiode array (PDA) detector in series with an NEC Power Mate SX Plus computer (NEC, Boxborough, MA) and a Waters 5200 printer/plotter (Waters, Milford, MA).

Two RP-HPLC C-18 analytical columns were employed separately in different runs: a Vydac 218TP54 column (Rainin, Woburn, MA) and a Zorbax ODS column fitted with compatible guard cartridge (MacMod, Chadds Ford, PA). Three HPLC mobile phases were used, all employing methanol (MeOH) and acetonitrile (MeCN) with a solvent strength modifier: 40:56:4 MeCN:MeOH:tetrahydrofuran (Quackenbush, 1987), 63:20:15:2 MeCN:MeOH: tetrahydrofuran:CH_2Cl_2, and 80:18:2 MeCN:MeOH:CH_2Cl_2 (Saleh and Tan, 1991). Samples were run at 1.0 mL/min for 30 min.

Identification of lycopene

Two methods were used to identify lycopene in crude extracts. First, the PDA Spectrum Analysis function was used to calculate wavelength maxima (λ_{max}) for each peak on the analytical HPLC chromatogram. These λ_{max} values were compared to tabulated values
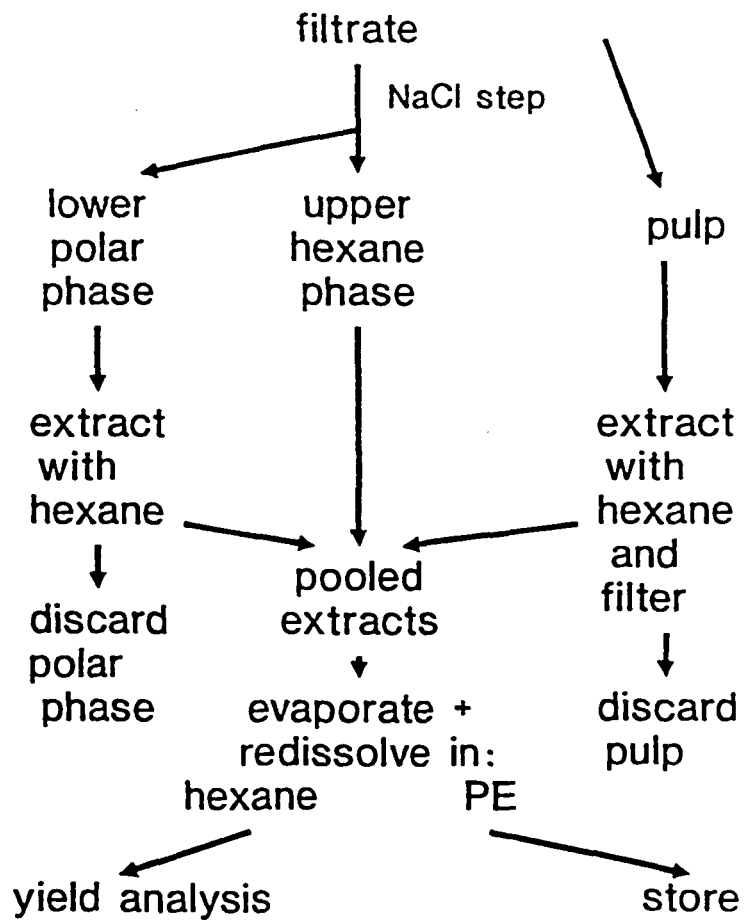
Figure 5. Preparation of crude lycopene extracts using the method of Sadler et al. (1990): treatment of the primary filtrate

for tentative identification of each carotenoid.

For absolute identification of the suspected lycopene peak, desorption chemical ionization (DCI) mass spectrometry was employed. This required a purified lycopene extract, which was obtained using semi-preparative HPLC methods. A 5 mL sample of crude extract was injected onto a Whatman Partisil M9 10/25 ODS-3 semi-preparative column (Whatman, Hillsboro, OR) via a Rheodyne 7010 injector equipped with loop filler port and 5 mL sample loop (Rainin, Woburn, MA). Eluate was detected by a Beckman UV detector (Beckman, Fullerton, CA) fitted with semi-preparative flow cell and 436 nm UV filter, with output being recorded on a Fisher Recordall 5000 chart recorder (Fisher, Pittsburgh, PA). Samples were run for 30 min with an 80:18:2 MeCN:MeOH:CH_2Cl_2 mobile phase isocratic at 2 mL/min using a Beckman 110A pump (Beckman, Fullerton, CA). "Heart cutting" techniques were used to collect the central 1 min portion of a 3 min peak elution volume. Four samples were run, and the eluates were pooled, evaporated to dryness at 35°C, and redissolved in HPLC grade hexane. The purified lycopene extract was stored at -29°C prior to use.

Purified extracts were analyzed in the same way as crude extracts, using a Vydac C-18 column with 80:18:2 MeCN:MeOH:CH_2Cl_2 mobile phase at 1 mL/min. Using the PDA Spectrum Analysis function, the lycopene peak was analyzed at 0.1 min intervals to assess peak integrity, i.e., the absence of any co-eluting compounds.

Desorption chemical ionization (DCI) mass spectrometry was performed on a Finnigan Model 4000 mass spectrometer (Finnigan, Cincinnati, OH), using NH_3 as the

reagent gas, at a source block temperature of 200°C. Both M^+ and M^- mass spectra were obtained.

Purity and yield analysis of crude lycopene extracts

The integrity of the lycopene peak in crude extracts was assessed in the same way as in purified extracts, by way of the PDA Spectrum Analysis function. Percentage purity of lycopene in the crude extract was calculated as the peak area of lycopene relative to total chromatogram peak area.

Yield was calculated by way of the Beer-Lambert equation:

$$A = \varepsilon c l$$

where A = absorbance
ε = molar extinction coefficient, M^{-1} cm^{-1}
c = concentration, mol/L
l = path length, cm

Concentration was calculated and converted to g/L using the molecular weight of lycopene, 536 amu. From this value, the total mass of extracted lycopene and the yield per 100 g tomato paste were calculated. Samples of crude extract were diluted 1:1000 in hexane prior to measuring absorbance with a Milford 250 spectrophotometer, path length 1 cm. Absorbance was measured at 472 nm, the λ_{max} for lycopene in hexane, after calibration with a hexane blank. Concentrations were calculated using an ε of 1.85 x 10^5 M^{-1} cm^{-1} (Sadler et al., 1990).

Preparation and Analysis of Lycopene Oxidation Products

Oxidation of crude lycopene extracts

The preparation of lycopene oxidation products was required for determination of their antioxidant capacities later in this study. Four methods for the formation of lycopene oxidation products were assessed. All work was performed in subdued yellow light.

Oxygen electrode chamber methods

In these methods, lycopene was oxidized in oxygen electrode chamber apparatus, as described by Wayner and Burton (1992). A schematic of the apparatus used is provided in Figure 6.

The reaction mixture and stir-bar occupied approximately 11 mL in a 20 mL volume flat-bottomed cylindrical glass reaction chamber. The oxygen electrode, connected to a YSI Biological Oxygen Monitor (YSI, Yellow Springs, OH) in series with a Fisher Recordall 5000 chart recorder, screwed into a plastic housing which then sat tightly within the reaction chamber, with the electrode tip just protruding into the reaction mixture. A small channel running the length of the housing allowed both expulsion of air when the housing was pushed into the chamber, and the introduction of small volumes of reagent into the reaction mixture.

OXYGEN ELECTRODE
IN REACTION TUBE

SECONDARY
WATER CELL

PRIMARY WATER
BATH, AT 37°C   STIR PLATE   OXYGEN   CHART
                                MONITOR   RECORDER
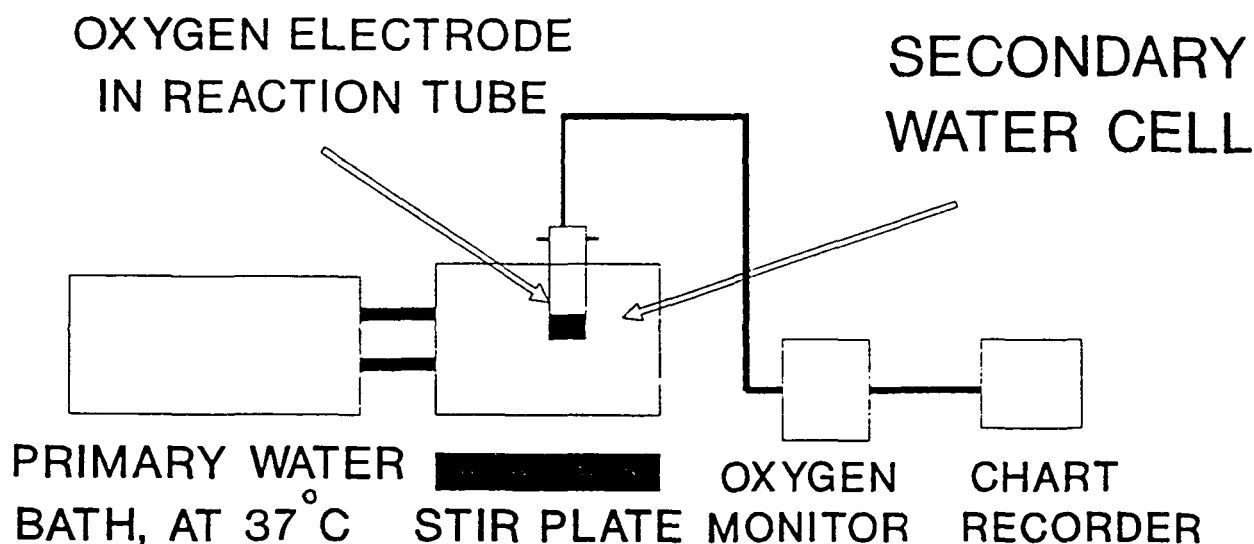
Figure 6. Oxygen electrode chamber apparatus for lycopene oxidation

The electrode itself was a YSI 1331 Clark-type oxygen electrode (YSI, Yellow Springs, OH). Reaction temperatures were controlled by using a primary thermostated water bath connected to a smaller secondary closed water cell, in which the reaction tube was bathed.

Oxidations were performed in two organic solvents, methanol and hexane. Both solvents were air-saturated 15 min prior to use to give final dissolved oxygen concentrations in the μM range. Two forms of oxidation were attempted: direct oxidation of lycopene, and co-oxidation of lycopene in the presence of methyl linoleate. The thermally dissociable initiator azoisobisbutyro-nitrile (AIBN) was used to initiate oxidation in these experiments. AIBN (Eastman Kodak, Rochester, NY) was prepared at a concentration of 5×10^{-2}M, 95%

pure methyl linoleate (Sigma, St Louis, MO) at $5x10^{-3}M$, and 95% pure all-trans lycopene (Sigma) at $5x10^{-5}$ to $5x10^{-1}M$ concentrations.

Before oxidations were initiated, baseline drift on the oxygen monitor was followed over a 10 min period, using 10 mL air-saturated solvent in the reaction chamber. The % baseline drift/min was calculated for use as a correction factor.

All oxidations used a total reaction volume of 10 mL. For the direct oxidation of lycopene, 9 mL of air-saturated solvent was equilibrated in the reaction chamber for 10 min at 37°C with the oxygen electrode in place. Using a 500 μL long-needled Hamilton syringe (Rainin, Woburn, MA), 0.5 mL of lycopene solution was introduced into the reaction volume, followed by 0.5 mL AIBN reagent. For the lycopene/methyl linoleate co-oxidation, 7 mL air-saturated solvent was equilibrated, 2 mL of methyl linoleate reagent was added, and the oxygen electrode was placed in the chamber. Five hundred μL AIBN reagent was introduced after 1 min, and a few seconds later 0.5 mL lycopene solution was added. Oxidations ran for 25-30 min, with continuous monitoring of dissolved oxygen status on the chart recorder.

Since earlier investigations showed that the plastic electrode housing melted slowly in hexane, customized identical Teflon housings were manufactured by the Iowa State University Engineering Research Institute Machine Shop.

Following the oxidations the reaction volume was evaporated and redissolved in 4-5 mL of 80:18:2 MeCN:MeOH:CH_2Cl_2 mobile phase, and filtered through a 0.45 μm nylon

filter. The filtrate was immediately analyzed on a Vydac analytical C-18 HPLC column, with the mobile phase above isocratic at 1 mL/min for 30 min.

Air/oxygen saturation methods

These methods essentially involved the forced oxidation of lycopene in crude extract form by continuously bubbling air or pure oxygen through the extract.

In the air saturation experiments, crude lycopene extracts were prepared by the modified method of Sadler et al. (1990), evaporated, and redissolved in an adequate volume of MeOH. The volume was ultrasonicated in a water bath to ensure complete solubilization of lycopene, and transferred to a 100 mL side-arm Erlenmeyer flask. The flask was sealed with a rubber stopper fitted with a glass tube feeding into the reaction mixture. Air was bubbled into the lycopene solution at medium flow-rate for 2 hours, with sampling at 30 min intervals. Sample aliquots were run on an analytical Vydac C-18 HPLC column using a gradient mobile phase developed by Handelman et al. (1991) for autoxidation products of ß-carotene. At a flow rate of 0.7 mL/min, an 8 min isocratic run of 85:15 MeCN:MeOH was followed by a switch to 30% isopropanol over 12 min.

Later air-saturation experiments used petroleum ether (PE) as a solvent, and employed similar methods to those used in oxygen electrode chamber methods, namely both the direct oxidation of lycopene, and co-oxidation of lycopene with methyl linoleate. AIBN was used to initiate oxidations. AIBN was prepared in PE at $10^{-2}M$, methyl linoleate as a 5%

solution, and a 60 mL volume of lycopene in PE prepared by the modified method of Sadler et al. (1990) described earlier in this chapter.

Five milliliters AIBN reagent was transferred to a 250 mL side-arm Erlenmeyer flask, and the temperature was raised to 45-50°C using a heating plate. Twenty milliliters methyl linoleate reagent was added to the flask, and air was bubbled in for 30s. After adding the 60 mL volume of lycopene reagent, air was bubbled through for 90 min, with sampling at 15 min intervals. Five hundred μL 5×10^{-3}M butylated hydroxytoluene (BHT) was immediately added to sample aliquots to prevent degradation of any oxidation products formed. The aliquots were evaporated and redissolved in a vehicle solvent, filtered through a 0.45μm nylon filter, and analyzed on a Vydac C-18 column as previously described. A number of vehicle solvents were used to improve the solubility of lycopene oxidation products: 85:15 MeCN:MeOH; 100% MeOH; 1:1 MeOH:THF; 1:3 MeOH:THF; 1:1 MeOH:toluene; 10% acetone in hexane; and 1:3 toluene:(85:15 MeCN:MeOH).

Oxygen saturation methods were performed in a similar manner. The apparatus used is shown in Figure 7.

In these experiments toluene was used as a solvent. AIBN was prepared at 10^{-2}M in toluene, methyl linoleate as a 5% solution, and crude lycopene extracts at a total volume of 20-25 mL in toluene. Reactions were performed at 58-60°C in a 500 mL RB flask. In the direct oxidation of lycopene, 20-25 mL lycopene solution, 60 mL toluene, and 10 mL AIBN reagent were added to the flask. Heat was applied and pure oxygen bubbled through at 7-10 psi. The reaction was allowed to proceed for 2 hours, with sampling at 20 min intervals. In

the lycopene/methyl linoleate co-oxidations, 10 mL methyl linoleate reagent and 10 mL

AIBN reagent were first added, and oxygen flow was commenced. Then the 20-25 mL

lycopene solution was added to the flask along with 60 mL toluene. Sample aliquots were

redissolved in vehicle solvents similar to those used in the air-saturation experiments, and

analyzed by the same HPLC methods.

In some cases, heating was complemented by the application of UV light from a high

power UV lamp for the first 20 min of the oxidation. This was used to assist both in the

dissociation of AIBN and in the oxidation of lycopene itself.
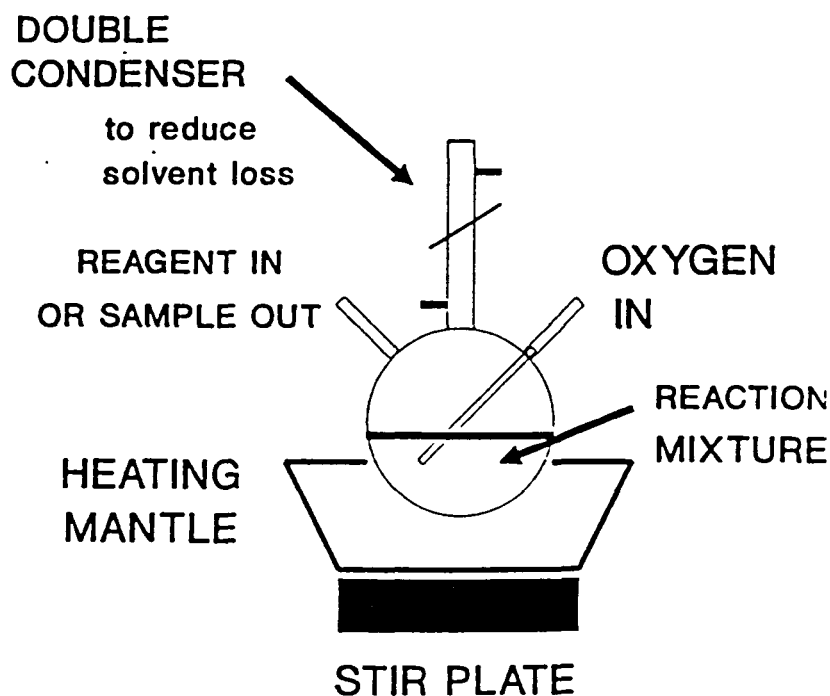
Figure 7. Oxygen saturation apparatus used in lycopene oxidation

Peroxyphthalic acid oxidation of lycopene

Bush and Zechmeister (1951) prepared lycopene-5,6-epoxide by reacting lycopene with monoperphthalic acid in absolute ether, and allowing the reaction mixture to stand for a week in darkness. Similarly, Tsukida and Zechmeister (1958) prepared ß-carotene mono- and di-epoxide from ß-carotene. In this study, magnesium peroxyphthalate was used in place of monoperphthalic acid, being a stronger oxidizing agent. It was thought that a stronger oxidizing agent could improve the very low yield of lycopene-5,6-epoxide (1.5%) obtained by Bush and Zechmeister. The reaction mixture was filtered, and the filtrate was washed with saturated sodium bicarbonate solution, evaporated, and redissolved in 40:20:20:20 MeCN:MeOH:CH_2Cl_2:hexane. The residue was also dissolved in this solvent. Both samples were analyzed on the Vydac 218TP54 analytical HPLC column, using an isocratic mobile phase of 85:10:2½:2½ MeCN:MeOH:CH_2Cl_2:hexane at 0.7 mL/min.

The Micro Cel C method

The oxidation of carotenoids on Micro Cel C (MCC), a synthetic hydrous calcium silicate adsorbent, was first reported by Rodriguez et al. (1976). Ritacco et al. (1984a) have prepared a number of lycopene oxidation products using the MCC method. This study used the methods employed by Ritacco et al. (1984a) to oxidize lycopene in crude extract form, with minor modifications.

Approximately 300g MCC (Manville Sales Corporation, Denver, CO) was dried in a vacuum oven at 100°C for 24 hours, transferred to a screw-top glass bottle, and stored in an upright desiccator. Fresh MCC was prepared weekly. The MCC (30 $\pm$ 1 g) was weighed into a 500 mL screw-top Erlenmeyer flask, to which was added exactly 3 mL distilled water (10% v/w) to partially reactivate the MCC. Ritacco et al. (1984a, 1984b) have chosen to use the term reactivation, since the addition of a certain amount of water to MCC tended to make it more reactive, unlike silica gel compounds. The flask was shaken for 30 min at quarter speed on a wrist-arm shaker to equilibrate the MCC. A 25 mL extract of crude lycopene in PE, prepared by the modified method of Sadler et al. (1990), and corresponding to about 35 mg lycopene as determined spectrophotometrically, was added to the flask. This translated to about 1 mg lycopene/g MCC, whereas Ritacco et al. (1984a) used ratios closer to 1mg/30g. Crude PE was added to the flask with shaking, to just cover the MCC. The volume of PE added was important: some lycopene tended to diffuse into excess PE, and thus would not be in contact with the MCC hydroxylating surfaces. Initial experiments showed that the thickness of the slurry did not affect the course of the reaction, nor the amount of each product formed. The reaction mixture was allowed to stand for 60 min at room temperature, and was then transferred with rinsings to Büchner vacuum filtration apparatus with #4 Whatman filter paper. Volumes of acetone were added to the Büchner funnel and the slurry was agitated with a spoon to remove products from reaction surfaces. This procedure was repeated until the residue was yellow-pale orange. The filtrate was transferred to a 2L separatory funnel. Knowing the approximate volume of filtrate, a volume

of PE equivalent to 1¼ x this volume was added to the separatory funnel, along with 100 mL saturated $NaCl_{(aq)}$ for phase separation. The mixture was shaken and settled for 2 min to allow partitioning of oxidation products into the PE phase. After discarding the lower polar phase, 25 mL distilled water was added to the flask, which was shaken and allowed to settle a further min. The polar phase was again discarded, and the PE phase was shaken with a small amount of anhydrous sodium sulfate to remove traces of water. The PE phase was decanted into a 3L RB flask, evaporated, and redissolved in 25 mL 4:2:2:2 $MeCN:MeOH:CH_2Cl_2$:hexane. This solvent was developed by Khachik et al. (1992a, 1992b) as a compatible injection solvent for the analysis of carotenoid oxidation products. The final preparation was stored at -70°C prior to use. Analyses were performed within 1 hour of preparation.

Some experimental modifications were attempted to improve the oxidation procedure. The % water in MCC reactivation was used at 5%, 10% and 20%. The mass of MCC used was varied from 30g to 100g to 150g. Oxidations were performed both at room temperature, and at 40°C in a water bath. Finally, oxidations were assessed at 15, 30, 45 and 60 min duration. Additionally, the effects on MCC of drying and reactivating with water were assessed. MCC treated in this manner was compared to untreated MCC, in terms of lycopene-oxidizing capacity. Also, to assess how much oxidation product was left on the MCC residue during filtration, the residue was soaked for 30 min in acetone, filtered through Whatman #42 paper, evaporated, redissolved in injection solvent, and subjected to analytical HPLC.

Apart from the single modifications, oxidation procedures and HPLC analyses were standardized. The effects of modifications upon the oxidation were determined using the peak area of oxidation products relative to the peak representing unoxidized lycopene. When this peak yielded too low an area, absolute peak areas of oxidation products were used.

Oxidation products were analyzed on a Vydac C-18 analytical HPLC column using the method of Khachik et al (1992a). An isocratic mixture of 85:10:2½:2½ MeCN:MeOH:CH$_2$Cl$_2$:hexane (solvent A) at time 0 was followed by a linear gradient from time 10-40 min, ending at 100% 45:10:22½:22½ MeCN:MeOH:CH$_2$Cl$_2$:hexane (solvent B). Flow rate was set at 0.5 mL/min. Injection solvent and mobile phases were prepared fresh weekly.

Analysis of oxidation products

Chromatograms from the analytical HPLC of oxidation products were analyzed using PDA Spectrum Analysis and Spectrum Index Plot functions. Wavelength maxima were calculated for each peak, and peaks were dissected and assessed for peak integrity. Chromatograms were compared to those of crude lycopene extracts analyzed by the same HPLC methods, to observe the formation of new peaks and increases in area of existing peaks. Wavelength maxima data were compared to tabulated values (De Ritter and Purcell, 1981) for tentative identification of the peaks.

Isolation and identification of oxidation products of lycopene

After oxidizing crude lycopene extracts using the Micro Cel-C method, and tentatively identifying the products by analytical HPLC, specific oxidation products were then isolated, for absolute identification by mass spectrometry (MS), and for studies on their antioxidant capacities. Product isolation was attempted using semi-preparative RP-HPLC on a Vydac 218TP510 C-18 semi-preparative column. Apart from the column, the equipment used was identical to that employed in the preparation of lycopene from crude extracts for MS analysis, with the addition of a fraction collector. For effective isolation of oxidation products, a mobile phase had to be optimized, with the aim of improving resolution of the oxidation products. Mobile phase optimization was performed on the Vydac 218TP54 analytical column, using a number of mobile phases, in both isocratic and gradient elution regimes, as shown in Table 5.

Selection of appropriate mobile phases was performed as detailed in the Results section. These mobile phase systems were used both for semi-preparative and analytical HPLC. A 5 mL sample of oxidized lycopene was injected onto the semi-preparative column, and fractions were collected at appropriate intervals, with flow rate set at 2 mL/min. Initially fractions were collected into 15 mL test tubes already containing 4 or 5 drops of 1% BHT in Khachik's injection solvent (40:20:20:20 $CH_3CN:MeOH:CH_2Cl_2$:hexane), to prevent degradation of the oxidation products prior to analytical HPLC. Analytical HPLC of the fractions was performed on the Vydac 218TP54 column, with flow rate set at 0.7 mL/min,

Table 5.   Mobile phases assessed for the isolation of lycopene
oxidation products

85.00 : 10.00 : 2.50 : 2.50   A:M:D:H

88.00 : 10.00 : 1.00 : 1.00   A:M:D:H

86.25 : 9.88 : 1.90 : 1.90   A:M:D:H

84.50 : 9.75 : 2.80 : 2.80   A:M:D:H

82.75 : 9.67 : 3.70 : 3.70   A:M:D:H

81.00 : 9.50 : 4.50 : 4.50   A:M:D:H

85 : 15 A:H

85 : 10 A:M

75 : 20 : 0.25 H:D:M

100% A^b → 100% M^b, over 25 min

100% M^b, isocratic for 25 min

100% M^b → 100% A^b, over 25 min

100% M^b → 50:50 A^b:M^b, over 25 min

100% M^b from 0-10 min, to 50:50 A^b:M^b from 10-35 min

75:25 M^b:A^b → 100% A^b, over 25 min

100% M^b → 100% EA, over 25 min

90:10 M^b:THF → 100% EA, over 25 min

100% M^b → 45 : 10 : 22.5 : 22.5 A:M:D:H

100% M^b → 100% H

100% M^b → 75:25 H:M^b, over 25 min

100% M^b → 100% H, over 12 min, 100% H 12-25 min

100% M^b, from 0-5 min, to 100% H over 25 min

90:10 M^b:H_2O → 100% H, over 25 min

[a] A = acetonitrile, M = methanol, D = dichloromethane, H = hexane
EA = ethyl acetate, THF = tetrahydrofuran

[b] with 2.5 : 2.5 H:D added

using a Beckman Model 502 Autosampler (Arlington Heights, IL). The visible purity of the fractions was assessed from analytical HPLC chromatograms. Oxidized lycopene samples were also rechromatographed at 1:5, 1:10, 1:100 and 1:1000 dilutions, to assess the effects of sample concentration on resolution of the oxidation products.

Later, resolution of the oxidation products was attempted using another RP-HPLC column. The system used was identical to that employed in the purification of lycopene, as previously described. A 5 mL sample of oxidized lycopene sample was injected onto a Whatman Partisil M9 10/25 ODS-3 semi-preparative column, and was eluted isocratically with hexane at 2 mL/min.

Initial experiments with other mobile phases were unsuccessful: only the highly apolar hexane provided adequate resolution of the oxidation products. Fractions of eluate were collected and analyzed by RP-HPLC, using the Vydac 218TP54 analytical column with an isocratic mobile phase of 85:10:2½:2½ MeCN:MeOH:CH_2Cl_2:hexane at 0.7 mL/min.

Pooled fractions from a number of semi-preparative runs were rechromatographed with the aim of increasing purity of the oxidation products. Purity was assessed by analytical HPLC, based on peak area as a % of total peak area. Peak integrity was assessed using the PDA Spectrum Analysis function.

Prior to antioxidant studies, all purified and non-purified fractions were stored at -4°C prior to use. HPLC studies showed that the oxidation products were stable for up to two days at this temperature. After two to five days, however, the oxidation products would start

to disappear, possibly being further converted to aldehydes and ketones, though these conversions were not followed.

Products isolated by the above methods were identified by DCI mass spectrometry.

Assessment of Antioxidant Capacities of Lycopene, the Oxidation Products of Lycopene, and ß-carotene

The final aim of this research investigation was to evaluate the antioxidant effects of lycopene and some of its oxidation products, with ß-carotene as a well-studied reference carotenoid. Experiments were carried out in purely chemical, lipid homogenous systems, with antioxidant capacities of the above compounds assessed by their ability to hinder the spontaneous oxidation of a lipid substrate.

Experimental Design

Studies on antioxidant capacities were designed to be carried out in lipid homogenous chemical environments, as used by Burton and Ingold (1984) and Terao (1989). Experimental conditions were selected with reference to these papers, particularly Terao's work, on account of his experimental scale. Those conditions are outlined below.

The lipid substrate used in these experiments was squalene, a highly unsaturated C-30 hydrocarbon with six unconjugated double bonds. Squalene was chosen on account of its high oxidizability.

Both Terao, and Burton and Ingold, used azo-initiators to initiate lipid oxidations. No initiators were used in this study, first because it was felt that the presence of initiators would complicate the experiments, and second, because the high oxidizability of squalene removed the need for an initiator. It was realized that lack of an initiator would make the kinetics of peroxidation initiation less reproducible and reliable, but not to a major extent.

As has previously been observed, oxygen partial pressures affect the antioxidant capacities of carotenoids (Burton and Ingold, 1984; Burton, 1989). To observe the effects of oxygen partial pressures on carotenoid antioxidant capacities, a range of pO_2 values were selected: 2% oxygen, 10% oxygen, and 20% oxygen, with the balance as nitrogen gas. These concentrations are not the same as those used by Burton and Ingold (15, 150, and 760 torr oxygen), but corrspond to 15, 75 and 150 torr oxygen, respectively. While Burton and Ingold (1984) employed a pressure transducer system to monitor and control pO_2 , no such apparatus was available for these experiments. To compensate for this, the headspace: reaction volume ratio was designed to be high, to ensure that the overall pO_2 did not change significantly during the course of the experiment. The ratio of headspace volume to squalene volume was set at about 550 (0.2g squalene = 0.22 mL squalene, and headspace volume = 125 mL). Additionally, headspaces were periodically replaced with fresh gas mixture.

Burton and Ingold (1984) also observed that the concentrations of carotenoids affected their antioxidant capacities. They showed that the antioxidant capacity of ß-carotene increased significantly from 0.05mM to 0.5mM in lipid systems, with a smaller increase from 0.5mM to 5mM. In this study, carotenoid concentrations were calculated relative to squalene. Carotenoids in hexane were added directly to reaction vials containing squalene, and the hexane was evaporated in a 40°C oven until constant weight was reached (after approximately 4 hours). Consequently, the amount of carotenoid per gram squalene was calculated. A number of exponential concentrations were assessed, and from these preliminary data suitable concentrations were chosen.

Two methods were used to assess the degree of oxidation of squalene. First, the oxygen weight-gain method was employed (Olcott and Einset, 1958a, 1958b). This method was based on direct measurement of the lipid sample for weight changes caused by oxygen uptake. The weight increased after an initial induction period, and then began to decrease as volatile secondary products of lipid peroxidation were formed. By following weight changes over time, the degree of oxygen uptake could be assessed. Second, peroxide values (PV) were measured. The peroxide value (PV) by titration assay (AOAC, 1990) involved the oxidation of I^- to I_2 by lipid peroxides, and the subsequent titration of I_2 with sodium thiosulfate. Other methods were investigated, but were determined to be unsuitable. The conjugated diene method, which spectrophotometrically determined the formation of conjugated diene in the early rearrangement stages of unsaturated-lipid oxidation, was not used on account of high solvent costs, and inadequate sensitivity. The Stamm test, a more

sensitive spectrophotometric assay for peroxides (Hamm et al., 1958; Duve and White, 1991; Liu and White, 1992), was not used on account of the complexity of the method, as well as long set-up times. The TBARS assay (Pikul and Kummerow, 1991; Ramanathan and Das, 1992) was assessed, but was not sensitive enough to detect TBARS in the small lipid sample sizes used. The p-anisidine assay (AOAC, 1990), which spectrophoto- metrically determined secondary products of oxidation, was not used, since the absorption wavelength of 350 nm coincided with carotenoid spectra.

Materials and Methods

All reagents used in this section were prepared as soon as possible prior to use, to reduce the risk of degradation.

Lycopene-5,6-epoxide and lycopene-5,6-diol samples were obtained as previously described. Lycopene was prepared from crude lycopene extracts, by previously described purification steps. All-trans ß-carotene (Grade IV) was obtained commercially from Sigma Chemicals (Sigma, St. Louis, MO). Squalene was obtained commercially (Sigma, St Louis, MO) and stored at 0°C prior to use. All carotenoids were prepared at appropriate concentrations in HPLC-grade hexane and were stored at -4°C prior to use. In preparing each carotenoid, concentrations were determined spectrophotometrically, using the appropriate molar extinction coefficients (ε) and wavelength maxima: for ß-carotene, 11.0×10^4 M^{-1} cm^{-1} at 450 nm (Sadler, 1990), for lycopene, 18.5×10^4 M^{-1} cm^{-1} at 472 nm

(Sadler, 1990), for lycopene-5,6-diol, 16.1×10^4 M^{-1} cm^{-1} at 455 nm (Bush and Zechmeister, 1956) and for lycopene-5,6-epoxide 16.1×10^4 M^{-1} cm^{-1} at 454 nm (Khachik, personal communication). The ε for lycopene-5,6-epoxide is not available as yet, due to the difficulty of preparing it in sufficient quantities for calculation of the value. Therefore, the ε used was identical to that of lycopene-5,6-diol. This could only serve as a reasonable approximation, but the structural similarities between the 5,6-epoxide and the 5,6-diol meant that using the given ε for lycopene-5,6-epoxide resulted in less than 10% error in calculations (Khachik, personal communication).

Gases used in the experiments, oxygen and nitrogen, were prepared in the laboratory using commercially obtained cylinders of pure oxygen and pure nitrogen, and mixing the gases at determined flow rates and pressures to achieve the desired oxygen:nitrogen ratios. Gases were mixed as required.

Treatments were randomized using complete randomized design to minimize non-treatment effects.

Oxygen weight-gain methods

Oxygen weight-gain methods were based on the weight change of a lipid sample associated with the uptake of oxygen during the oxidation process. Initial experiments assessed suitable concentrations for carotenoids, and suitable time periods for measurement. Oxidations were performed in 125 ml screw-top amber vials, to which was added accurately about 200 mg squalene, and carotenoid in hexane. Carotenoids were added at $1 \mu g$

carotenoid/g squalene, 10 μg/g squalene, and 100μg/g squalene. Controls consisted of squalene alone. The hexane solvent was evaporated under a stream of nitrogen gas until the sample reached constant weight. The vials were then flushed gently with appropriate gas mix for 60 seconds, enough to fill the vial, capped and incubated at 70°C in a convection oven. At periodic intervals, the vials were removed from the oven, cooled to room temperature for 20 min in an upright desiccator, opened, and weighed to constant mass. The vials were again flushed with appropriate gas mix, capped, and returned to the oven. After 72 hours, weight/time plots were drawn, and by comparing the plots suitable concentrations and time periods were calculated. These initial plots showed that regardless of concentration, a short decrease in weight was followed by a weight increase from 8 to 25 hours. In terms of concentrations, the 1μg/g samples showed little difference from controls, while the 10μg/g and 100μg/g samples differed significantly in terms of rate of oxygen uptake after the induction period. Having selected experimental parameters, an experimental design was constructed. The methodology used was as described above. Four carotenoids, lycopene, lycopene-5,6-epoxide, lycopene-5,6-diol, and ß-carotene, were used, as well as a control consisting of squalene alone. Two concentrations were used for each carotenoid: 10μg carotenoid/g squalene and 100μg carotenoid/g squalene. Oxidations were performed at three pO_2 values for each carotenoid at each concentration: 2% oxygen, 10% oxygen, and 20% oxygen. Two replicates were used in each of two replicate experiments.

Peroxide value (PV) measurements

Peroxide value was assessed using the AOAC Official Method (AOAC, 1990). All volumetric glassware was calibrated. To 125 mL reaction vials was added accurately about 1g squalene, enough to allow PV calculations on four 200 mg aliquots. The vials were treated as in the oxygen weight-gain methods. At selected intervals, 200 mg aliquots were removed from the vial to a 150 ml beaker, to which was added 30 mL acetic $CH_3COOH:CHCl_3$ (3:1 v/v). After shaking to dissolve the squalene, 1 mL saturated aqueous KI solution was pipetted into the beaker, which was agitated for 30 sec and set in the dark for 5 min. Following this 30 mL distilled water was added to the beaker. The iodine produced by the peroxide-induced oxidation of iodide was titrated with standardized 0.0005N sodium thiosulfate solution, using a 1% starch solution as the endpoint indicator. Titration volumes were converted to PV, after calibration against a blank.

Statistical Analysis of Data

Analysis of the data collected in this study was performed by using the Statistical Analysis System (SAS) produced by SAS (Cary, NC). Data from the antioxidant studies were analyzed for differences among the compounds tested, effects of concentration, oxygen pressure effects, and time effects. All data were analyzed by using analysis of variance

(ANOVA) techniques, and treatments were compared using Student's t-tests. Peroxide value data was analyzed on the basis of the slope from 0 hours to 25 hours; data at 52 hours was not incorporated into the analysis, since values at 52 hourss indicated that oxidations were complete after 25 hrs, and after 25 hours secondary products of oxidation were beginning to form. Oxygen weight-gain data was not analyzed on a slope basis, since oxygen uptake patterns were observed to vary with pO_2. For this reason, data was analyzed as maximum oxygen uptake; the greatest value of oxygen uptake seen over 52 hours was used as the maximum oxygen uptake value. These values were compared to each other, for effects of compound, concentration and partial pressure of oxygen.

RESULTS AND DISCUSSION

Preparation and Analysis of Lycopene

Two methods were used to prepare crude extracts of lycopene from tomato paste, the modified methods of Tan (1988) and Sadler et al. (1990). HPLC analyses of extracts were performed on Vydac and Zorbax C-18 reversed-phase columns, using three mobile phases: 40:56:4 acetonitrile (MeCN):methanol (MeOH):tetrahydrofuran (THF), 63:20:15:2 MeCN:MeOH:THF:CH_2Cl_2, and 80:18:2 MeCN:MeOH:CH_2Cl_2.

Extraction methods were compared in terms of simplicity and speed, reproducibility as assessed by analytical HPLC, and the quantity of lycopene prepared relative to other tomato paste carotenoids. The modified method of Tan (1988) suffered in all aspects. The method was time intensive, laborious, and irreproducible on occasion. Lycopene yield was good, but other tomato paste carotenoids (phytoene, phytofluene, and ß-carotene) were present at significant levels. Analyses were performed on the Vydac C-18 column initially using the 40:56:4 MeCN:MeOH:THF mobile phase, but changing to the 80:18:2 MeCN:MeOH:CH_2Cl_2 phase later. The reason for the change becomes apparent later. The method of Sadler et al. (1990) yielded better results. The method required less time and labor effort, was highly reproducible, and yielded high levels of lycopene with smaller quantities of other carotenoids. HPLC analyses of re-extracted pulp and re-extracted polar

phase in the procedure indicated only very small quantities of lycopene. In the interests of time, these steps were omitted from the procedure in routine extractions.

The Vydac C-18 column continually performed markedly better than the Zorbax ODS column, in terms of carotenoid resolution, for all three mobile phases. This observation was in agreement with Saleh and Tan (1988), who concluded that Vydac columns performed better than Zorbax columns in the separation of more lipophilic carotenoids.

The best mobile phase of the three used was determined to be 80:18:2 MeCN:MeOH:CH_2Cl_2, which gave very good resolution of lycopene and its isomers, as well as other carotenoids. The 40:56:4 MeCN:MeOH:THF phase also performed well, but suffered from poorer carotenoid resolution. The 63:20:15:2 MeCN:MeOH:THF: CH_2Cl_2 phase gave poor resolution of carotenoids in early runs, and was not used further.

In summary, the optimum method for the preparation and analysis of crude lycopene extracts involved the modified extraction method of Sadler et al. (1990) followed by HPLC analysis of extracts on a Vydac C-18 column, using an 80:18:2 MeCN:MeOH:CH_2Cl_2 mobile phase isocratic at 1 mL/min for 30 min. Figure 8 illustrates a chromatogram of crude lycopene extract prepared and analyzed by this optimum method.

Identification of lycopene and other carotenoids

Chromatographic peaks were tentatively identified by their wavelength maxima (λ_{max}).
The upper box in Figure 8 provides spectra for each peak on the chromatogram. As can be seen, carotenoid spectra were distinguished by the presence oee λ_{max} points. The sharpness of these points decreased at low degrees of conjugation
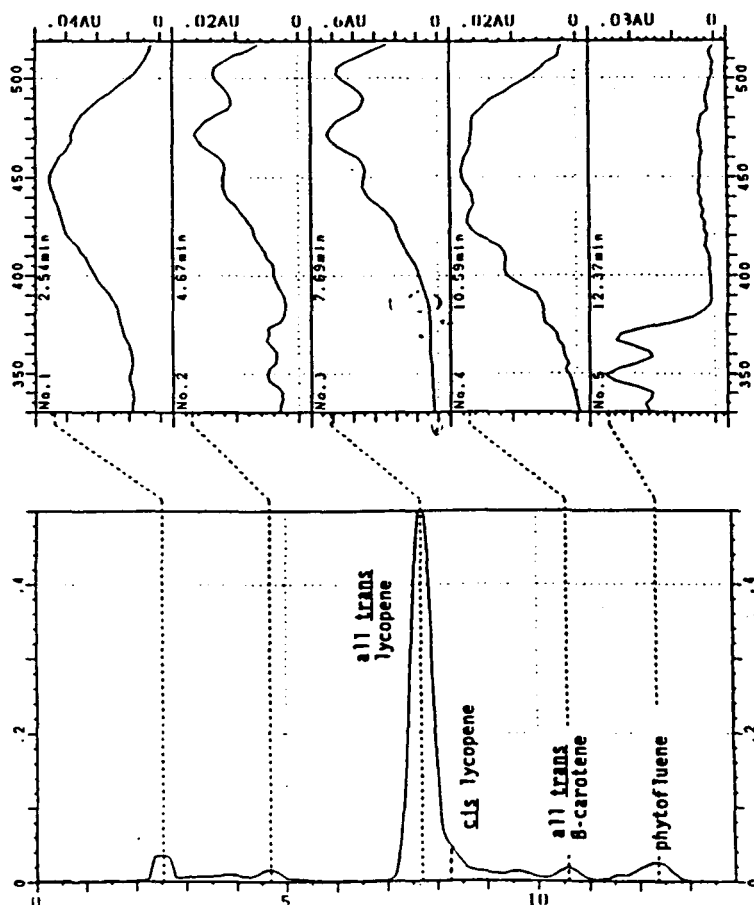
Figure 8. HPLC chromatogram of crude lycopene prepared and analyzed by the optimized method, in PDA Spectrum Index Plot mode

(as in phytoene) and with cyclization in the carotenoid (compare ß-carotene to lycopene). Cis-lycopene was characterized by increased absorbance at 364nm compared to trans-lycopene.

Using the Spectrum Analysis function to accurately determine the λ_{max} values of each peak, these values were then compared to tabulated values (De Ritter and Purcell, 1981; Tan, 1988; Rouseff et al., 1992). Table 6 provides experimental and tabulated λ_{max} values for each peak in Figure 8, and tentative identification of each peak.

Table 6.   Tentative identification of carotenoids in crude lycopene extracts by wavelength maxima comparison

Peak #	RT[a] (min)	exptl. λ_{max}	tabulated λ_{max}	carotenoid
1	2.54	432 448 476	---	unidentified[b]
2	4.67	448 472 502	---	unidentified[b]
3a	7.69	450 474 504	449 473 502	all-trans lycopene
3b	8.29	442 469 493	440 466 493	cis (neo B) lycopene
4	10.29	427 454 478	425 452 478	all-trans ß-carotene
5	12.37	334 349 367	334 350 368	phytofluene

[a]   RT = retention time.
[b]   Possibly oxidation products.

Although identification by λ_{max} alone was adequate for most of the peaks, the identity

of the suspected lycopene peak had to be confirmed by DCI mass spectrometry. Mass spectra

for a purified lycopene extract were obtained as described previously. The DCI-NH$_3$(+)

mass spectrum is shown in Figure 9.

The major molecular anion at m/z 536.3 was indicative of lycopene (Khachik et al.,

1992a). On the M⁻ mass spectrum (not shown), key fragment ions at 431.5, 372.3, and 348.3

were also indicative of lycopene (Foppen, 1971). By using both λ_{max} data and mass spectral

data, the identity of peak 3 in Figure 8 was confirmed as being lycopene.
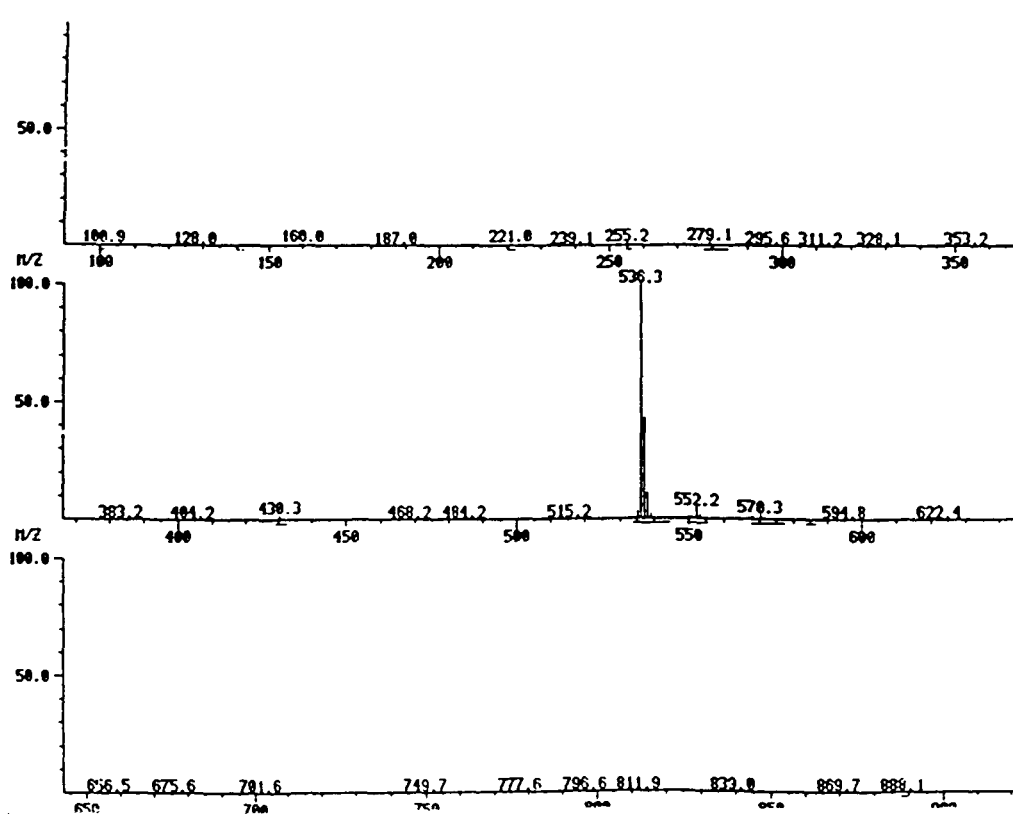
Figure 9. DCI-NH$_3$(+) mass spectrum of a purified lycopene extract

Purity and yield analysis of lycopene

The integrity of lycopene in crude extracts was ascertained by spectral scans of the lycopene peak at 0.1 min intervals. The lycopene peaks were pure i.e. they contained no co-eluting compounds. The purity of lycopene in crude extracts was assessed as the area of lycopene peaks as a percentage of total chromatogram area, and was determined to be greater than 90%. However, later analyses using the HPLC gradient elution system of Khachik et al. (1992a) proved more sensitive, resolving some very small peaks suspected to be natural oxidation products of lycopene. Using this mobile phase system, purity was determined to be only 80-85%.

Yield of lycopene in crude lycopene extracts was determined spectrophotometrically, by application of the Beer-Lambert equation. Total masses of lycopene in the crude extract lay in the range 16.1-17.5 mg lycopene/100 g tomato paste. These values were close to those calculated by Sadler et al. (1990), 15.5-16.1 mg lycopene/100 g tomato paste. The slight increase was most probably due to increased paste:extractant ratios and longer extraction times. However, the proportionally small increases in yield indicates that Sadler et al. (1990) were probably correct in calculating optimal paste:extractant ratios of 4g:100mL. Lime et al. (1957) reported that carotenoid extracts analyzed spectrophotometrically yielded artificially increased concentrations, of about 10%. This would translate to a 1.5-2.0 mg lycopene/100 g paste lower yield. Even accounting for this factor, final yield was determined to be very acceptable.

Preparation and Analysis of Lycopene Oxidation Products

Oxidation of crude lycopene extracts

Four methods were assessed for the oxidation of lycopene: oxygen electrode chamber methods, air/oxygen saturation methods, peroxyphthalic acid oxidation of lycopene, and the Micro Cel-C method.

Oxidations in oxygen electrode chamber apparatus were assessed both by monitoring dissolved oxygen status, and by analytical HPLC of oxidation products. Dissolved oxygen concentrations would be expected to decrease as oxidation progressed, and the % change recorded (ΔO_2) would reflect this change. However, ΔO_2 data was highly irreproducible. All data was calibrated for baseline drift, and compared to control oxidations (no AIBN initiator present). In some experiments, the ΔO_2 was negative, as expected (i.e., dissolved oxygen concentrations decreased during the oxidation). However, the magnitude of ΔO_2 varied greatly between runs. In other experiments, oxygen concentrations changed only minimally, and in others the ΔO_2 was in fact positive, indicating increased dissolved oxygen concentrations. No air bubbles were observed in the reaction mixture, the electrode was noted to be tightly screwed into the housing, and the housing was firmly in contact with the reaction chamber. The only possible route of entry for oxygen from the atmosphere into the reaction chamber was via the channel running the length of the housing, normally used for

introduction or removal of reagents by syringe. Leaving the syringe in the channel during oxidations did not improve results. Fresh AIBN and methyl linoleate reagents were purchased and prepared, again with no effect on reproducibility. Squalene, a highly unsaturated C-30 hydrocarbon (6 C=C double bonds), was prepared in hexane at $5x10^{-3}M$, and substituted for methyl linoleate reagent; squalene typically oxidizes more readily than methyl linoleate, but in these experiments, ΔO_2 values did not indicate any such increase. The most obvious explanation for irreproducibility in ΔO_2 is the poor solubility of oxygen in the non-aqueous solvents used, particularly in hexane. No data exists for the solubility of oxygen in hexane and methanol, and so it can only be assumed that inadequate dissolved oxygen was present for oxidations to proceed in a reproducible manner. Analytical HPLC of the reaction products yielded no evidence of lycopene oxidation products, only a large parent lycopene peak.

Air and oxygen saturation methods were similar to those used in oxygen electrode chamber oxidations, but with continuous bubbling or air or pure oxygen into the reaction volume. This would be expected to at least partially overcome low oxygen solubility in the organic solvents used. In these systems, a large headspace was present, and so dissolved oxygen concentrations were not monitored. Analytical HPLC of the reaction products yielded a small poorly resolved peak-cluster eluting before lycopene. Investigation of this cluster using the Spectrum Analysis function indicated the occasional presence of two compounds, giving λ_{max} at 441, 467, and 497 nm and 431, 455, and 483 nm respectively. Using completely different methodology, Ritacco et al (1984a) obtained spectral data for an

oxidation product of lycopene, giving λ_{max}, at 439, 467 and 495 nm, similar to those of the former compound. The compound was not identified by these researchers, nor was it identified in this study. Based on λ_{max} values, the latter compound could represent either lycopene-5,6-epoxide or lycopene-5,6-diol. The reaction product mixture also contained a large lycopene peak, indicating poor oxidation of lycopene. These results are surprising in that no oxidation products were formed in quantity, particularly since air or pure oxygen was continuously bubbled into the reaction mixture. Again, very low oxygen solubilities in hexane and methanol may have been responsible for the low degrees of oxidation. It is unlikely that the oxidation products formed proceeded towards formation of volatile secondary products of oxidation, based on the lack of visible decolorization of the reaction mixture, and the amount of residual parent lycopene in the product mixture. According to the studies performed by Burton and Ingold (1984a), carotenoids tend to act as pro-oxidants at higher pO_2, after a short period of antioxidant activity, but the carotenoid itself would be partially oxidized by that stage, and these oxygenated products would be detectable. In summary, the air/oxygen saturation methods were disappointing with respect to the formation of lycopene oxidation products.

The attempted oxidation of lycopene using monoperoxyphthalic acid was also unsuccessful. No oxidation products could be detected either in the filtrate or in the residue, although a large lycopene peak was observed.

In contrast, the Micro Cel-C (MCC) method for the formation of lycopene oxidation products was extremely successful. The method itself was simple, requiring little time- and

labor-effort, and yielded a number of well-defined oxidation products. Initial experiments focused on optimizing the experimental conditions used. In brief, a 1 hour oxidation at room temperature using oven-dried, 10% water-reactivated MCC was adequate for the successful oxidation of lycopene. Fresh MCC performed just as well as oven-dried, reactivated MCC, but the latter form was selected to maintain reproducibility. The extremely low density of MCC made the use of more than 30g very difficult at the acetone-wash stage, on account of ineffective agitation of the slurry to remove oxidation products from hydroxylating surfaces. However, analytical HPLC of the final oxidation products indicated near-complete oxidation of lycopene using 30g MCC with approximately 30 mg lycopene extract, as shown in Figure 10. In testing the MCC residue for residual oxidation product, it was observed that the acetone-treated MCC residue contained only the carotenoid phytoene in significant proportions (approximately 0.15 AU), with negligible quantities of oxidation product (most abundant was one compound at 0.03 AU), and so re-extraction of the residue was not incorporated into the procedure.

A number of oxidation products can be seen on the chromatogram between 5 and 16 min retention time. Table 7 provides spectral data for each of the peaks shown in Figure 10. Three main groups of oxidation products existed. Peaks 1 to 4 have identical spectra, and can be considered as isomers of one compound [group (A) compound]. Peaks 5 to 7 also represent isomers of one compound [group (B) compound], but in this case the hypsochromic shift typical of cis isomers is noticeable; peak 5 would be expected to be the trans isomer, while peaks 6 and 7 represent cis isomers. Peak 10 can be considered to be the third
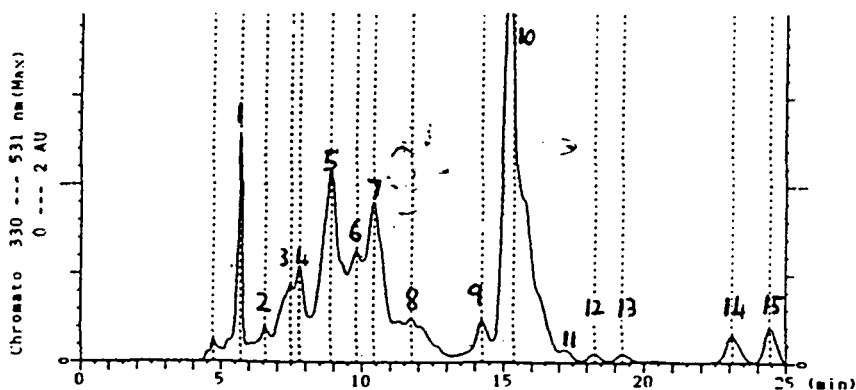
Figure 10. Analytical HPLC chromatogram of MCC-oxidized lycopene

Table 7. Spectral data for MCC-oxidation products of lycopene shown in Figure 10

Peak number	Retention time (min)	λ_{max} (nm)
1	5.72	413 435 462
2	6.58	413 435 462
3	7.49	413 435 462
4	7.79	413 435 462
5	8.89	433 458 488
6	9.81	431 456 483
7	10.41	431 456 483
8	11.75	— 471 —
9	14.26	— 453 478
10	15.35	433 459 489
11	17.20	445 468 498
12	18.29	417 441 468
13	19.25	417 441 468
14	23.10	380 402 426
15	23.44	378 399 423

[a] --- denotes wavelength maxima not calculable.

main group [group (C) compound]. Peaks 8 and 9 are too small for consideration. Peak 11 represents lycopene itself, based on retention time and spectral data. Peaks 12 and 13 represent ε-carotene, and peaks 14 and 15 represent isomers of ξ-carotene.

Isolation and identification of lycopene oxidation products

The isolation of oxidation products of Micro Cel-C-treated lycopene was initially attempted using semi-preparative RP-HPLC on the Vydac 218TP10 column. Mobile phase optimization indicated that a gradient from 100% methanol with 2.5:2.5 hexane: dichloromethane (solvent A) to 100% hexane (solvent B) over 25 min provided the best resolution of the oxidation product groups. While all the other mobile phases assessed gave a maximum time separation of 1.25 min between the (B) and (C) groups, the above system gave a separation of slightly more than 2 min. Other promising mobile phase systems assessed on the semi-preparative column included 85:10:2½:2½ acetonitrile: methanol:dichloromethane: hexane isocratic, 100% methanol to 100% ethyl acetate over 25 min, and 100% hexane to 75:25 hexane:methanol over 25 min. Extensive investigations indicated that it was not possible to resolve any one of the three oxidation groups on the Vydac column. Even rechromatographing pooled fractions of the unresolved mixture yielded no improvement in resolution.

Instead of continuing with semi-preparative HPLC on the Vydac column, a switch was made to the Whatman Partisil M9 ODS-3 column. Initial experiments with this column

were not encouraging, since again the three oxidation groups were not completely resolved. An earlier fraction block was observed to be completely free of group (C) compound, while the later fraction block was unresolved. However, rechromatographing this latter unresolved block, after evaporating and redissolving in 40:20:20:20 acetonitrile:methanol: dichloromethane:hexane, led to the resolution of group (C) away from groups (A) and (B); this was not reproducible unless the second fraction block was diluted 1:2 prior to reinjection, probably due to column overloading. Testing fractions of the rechromatographed second block by analytical HPLC indicated that group (C) compound eluted over a small number of fractions, and that the elution time was reproducible.

In the first fraction block, however, it was not possible to resolve groups (A) and (B). They appeared to be inseparable under any conditions. By testing fractions of eluate using analytical HPLC, it was observed that the peak area ratios of group (A) to group (B) changed gradually between fractions; this observation was utilized in selecting fractions in such a way that levels of group (A) compound were limited, and levels of group (B) compound were maximized.

In the final analysis, two fairly pure fractions were obtained: a group (C) fraction with only about 12% contamination, and a fraction comprised of groups (A) and (B), with no contamination by group (C) product.

By looking at the % total of peak area of purified group (C) compound over a number of wavelengths, it was ascertained that the compound was present at between 88-90% purity. Using the PDA Spectrum Analysis function, spectral scans executed across the peak did

reveal the presence of a co-eluting compound in very small amounts. This co-eluting compound was found in all fractions of compound (C). Its wavelength maxima, 450, 474, and 510 nm suggested that this compound could possibly be 6'-apolycopenal, but this was not confirmed. Looking at the fraction comprised of groups (A) and (B), the same investigations showed no co-eluting compounds to be present, and by collecting an appropriate range of fractions, group (A) compound was shown to make up less than 1% of total peak area, while group (B) compound made up between 92 and 94% of total peak area. Purity at this level would suggest that continued efforts to separate group (A) and (B) compounds would be pointless.

Figures 11a) and 11b) provide typical chromatograms of both the fraction containing group (A) and (B) compounds, and the fraction containing group (C) compound, respectively. In preparing the fractions for both MS identification and antioxidant studies, both fractions were pooled from a number of chromatographic runs. Since the 60:20:20:20 acetonitrile:methanol:dichloromethane:hexane injection solvent used to solubilize the oxidation products eluted from the column at about the same time as the first fraction block, a phase separation occurred between the injection solvent and the hexane mobile phase. This was also the case with the second fraction block, but to a very minor extent. For the first fraction block, the pooled volume was evaporated just to dryness, and redissolved in HPLC-grade hexane to a known volume. For the second block, which was rechromatographed for purification, the volume was diluted 1:2 with 40:20:20:20 acetonitrile:methanol :hexane:dichloromethane prior to injection; no attempt was made to remove the small
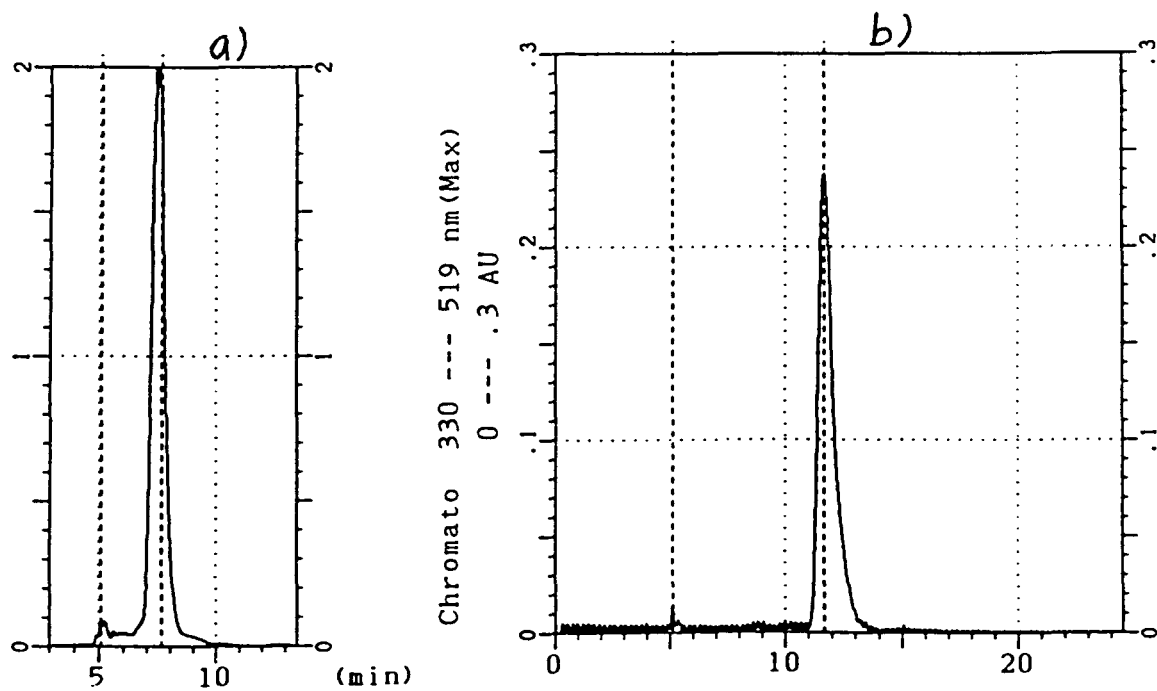
Figure 11. Analytical HPLC chromatograms of a) fraction containing
group (A) and (B) compounds, and b) fraction containing
group (C) compound
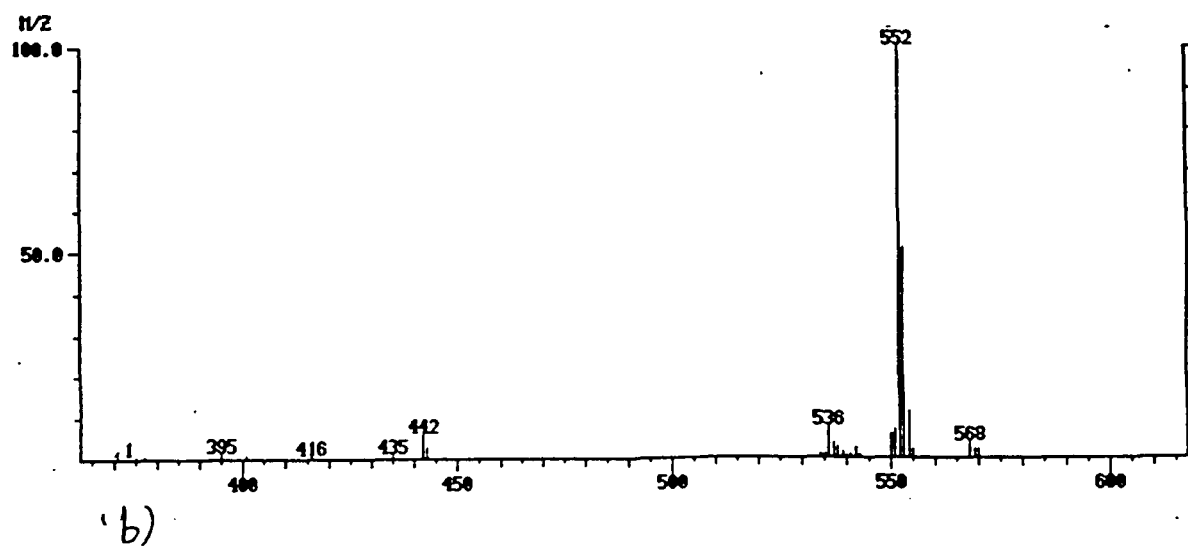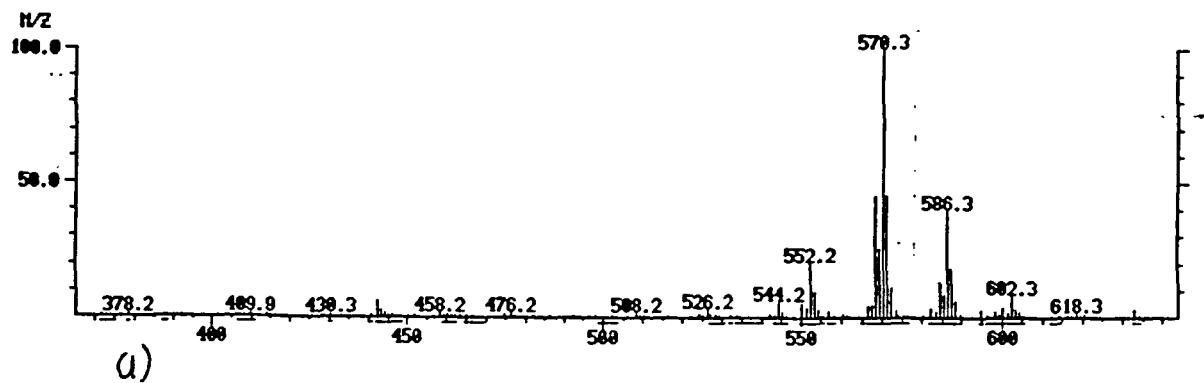
Figure 12. DCI mass spectra for a) lycopene-5,6-diol
and b) lycopene-5,6-epoxide

polar phase.

Mass spectrometry, in conjunction with wavelength maxima data, succeeded in identifying compound (B) as lycopene-5,6-diol, and compound (C) as lycopene-5,6-epoxide. Compound (A) was not identified. DCI NH_3(-) mass spectra for both the epoxide and the diol are provided in Figure 12. Both the epoxide and the diol were identified by comparison with published MS data (Ben-Aziz et al., 1973; Ritacco et al., 1984a; Khachik et al., 1992a, 1992b). The 5,6-diol was characterized by the major molecular anion at m/z 570 (100%), and key fragment ions at m/z 586 (40%, M+NH_4), m/z 552 (20%, M-H_2O), and m/z 602 (8%). The 5,6-epoxide was identified by the major molecular anion at m/z 552 (100%), and key fragment ions at m/z 536 (8%, M-O), m/z 568 (4%, M+NH_4) and m/z 442.

In oxidizing 5 mg lycopene with MCC, Ritacco et al (1984a) observed that three main compounds were formed: 6`-apolycopenal at 12.5% of final products, lycopene-5,6-epoxide at 23%, and lycopene-5,6-diol at 33%. By calculation the diol:epoxide ratio was 1.4. In this study, the same ratio was calculated as 1.4, but the relative percentages of diol and epoxide were higher; lycopene-5,6-epoxide at 33%, and lycopene-5,6-diol at 47%. Only a trace of 6'-apolycopenal was detected in this study, and it was calculated that the unidentified compound (A) was present at around 13%. Based on wavelength maxima data, compound (A) was not detected by Ritacco et al. (1984a).

Extrapolating from the yields calculated by Ritacco et al., the MCC-oxidation of about 35 mg lycopene (the amount routinely used in this study) would yield approximately 2.5 mg of lycopene-5,6-diol, and 1.7 mg of lycopene-5,6-epoxide. By spectrophotometric

calculations, a final yield of 2.7 -3.1 mg lycopene-5,6-diol was determined. This is within

range of values obtained by Ritacco's group, differences being attributed to longer oxidation

times. Lycopene-5,6-epoxide was determined to be present at about 0.8 mg. This yield is

smaller than calculated by Ritacco's group, probably due to differences in methodology

used.

Assessment of Antioxidant Capacities of Lycopene, the Oxidation
Products of Lycopene, and ß-carotene

The antioxidant capacities of lycopene, lycopene-5,6-epoxide, lycopene-5,6-diol, and

ß-carotene were assessed in small volume, high headspace lipid systems, antioxidant

capacities being determined by their effects on squalene oxidation. The test compounds were

used at two concentrations, and under three headspace concentrations of varying pO_2. The

oxidation of squalene was assessed both by oxygen weight-gain and peroxide value.

Oxidations were allowed to run over 72 hours, with sampling at selected intervals. The

selection of time intervals was performed by removal of squalene samples from the oven

every 2 hours, weighing to constant mass, and introducing fresh gas mixture into the reaction

vial. These preliminary experiments indicated induction periods for squalene, rate of oxygen

uptake after the induction period, total oxygen uptake, the maximum for oxygen uptake, and

optimum time intervals for introducing fresh gas mixture into the vials to ensure that oxygen

was not a limiting factor in the oxidations. For all partial pressures of oxygen, fresh gas at 4 hr intervals was determined to be sufficient. Preliminary studies indicated that the oxygen weight-gain method was very reproducible, even although the weight gain observed in a 200 mg sample amounted to only 7-8 mg. Concerns over the effects of water-, carbon dioxide- and aldehyde-losses on sample weight gain have previously been dispelled by Olcott and Einset (1958a, 1958b), and so were not viewed as a major source of error in this study.

Oxygen weight-gain experiments

Figures 13-16 illustrates oxygen uptake of squalene over a 52 hr period as affected by ß-carotene, lycopene, lycopene-5,6-epoxide and lycopene-5,6-diol, respectively. Figure 17 illustrates oxygen uptake for the controls (no carotenoid). Each figure shows uptake patterns for the compound at the various pressure/concentration combinations. Tables A1-A5 provide the data used in the construction of Figures 13-17. Figure 18 indicates the relative effects on oxidation of all four compounds at 2% O_2 and 10μg carotenoid/g squalene. In terms of general trends, this pressure/concentration combination was representative of all other combinations, apart from the 2% O_2 and 100μg carotenoid/g squalene combination, which is discussed later.

For all compounds and all pressure/concentration combinations, a rapid oxygen uptake phase between 0 and 12 hours was characteristic. Figure A1 in the Appendix indicates that this uptake phase was linear after an initial induction period of approximately 5 hours.

Figure 13.   Oxygen weight-gain of squalene as influenced by lycopene[a,b]

[a]   10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b]   All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
Lycopene

Legend:
- ○ 2% O2, 10ug/g
- ⊙ 10% O2, 10ug/g
- ▨ 20% O2, 10ug/g
- □ 2% O2,100ug/g
- ◀ 10% O2,100ug/g
- ▶ 20% O2,100ug/g

X-axis: time (hrs)
Y-axis: mgs O2/g lipid

.

Figure 14.   Oxygen weight-gain of squalene as influenced by ß-carotene[ab]

[a]   10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b]   All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
Beta carotene

2% O2, 10ug/g
10% O2, 10ug/g
20% O2, 10ug/g
2% O2,100ug/g
10% O2,100ug/g
20% O2,100ug/g

time (hrs)

mgs O2/g lipid

Figure 15. Oxygen weight-gain of squalene as influenced by lycopene-5,6-epoxide[a,b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b] All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
Lycopene-5,6-epoxide

○	2% O2, 10ug/g
⊙	10% O2, 10ug/g
▦	20% O2, 10ug/g
□	2% O2,100ug/g
◀	10% O2,100ug/g
▶	20% O2,100ug/g

mgs O2/g lipid

time (hrs)

Figure 16. Oxygen weight-gain of squalene as influenced by lycopene-5,6-diol[a][b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b] All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
Lycopene-5,6-diol

Legend:
- ○ 2% O2, 10ug/g
- ⊙ 10% O2, 10ug/g
- ▨ 20% O2, 10ug/g
- □ 2% O2,100ug/g
- ◄ 10% O2,100ug/g
- ▶ 20% O2,100ug/g

x-axis: time (hrs)
y-axis: mgs O2/g lipid

Figure 17. Oxygen weight-gain of squalene by no-carotenoid control[a]

[a] All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
Control

2% O2,
10% O2,
20% O2,

time (hrs)

mgs O2/g lipid
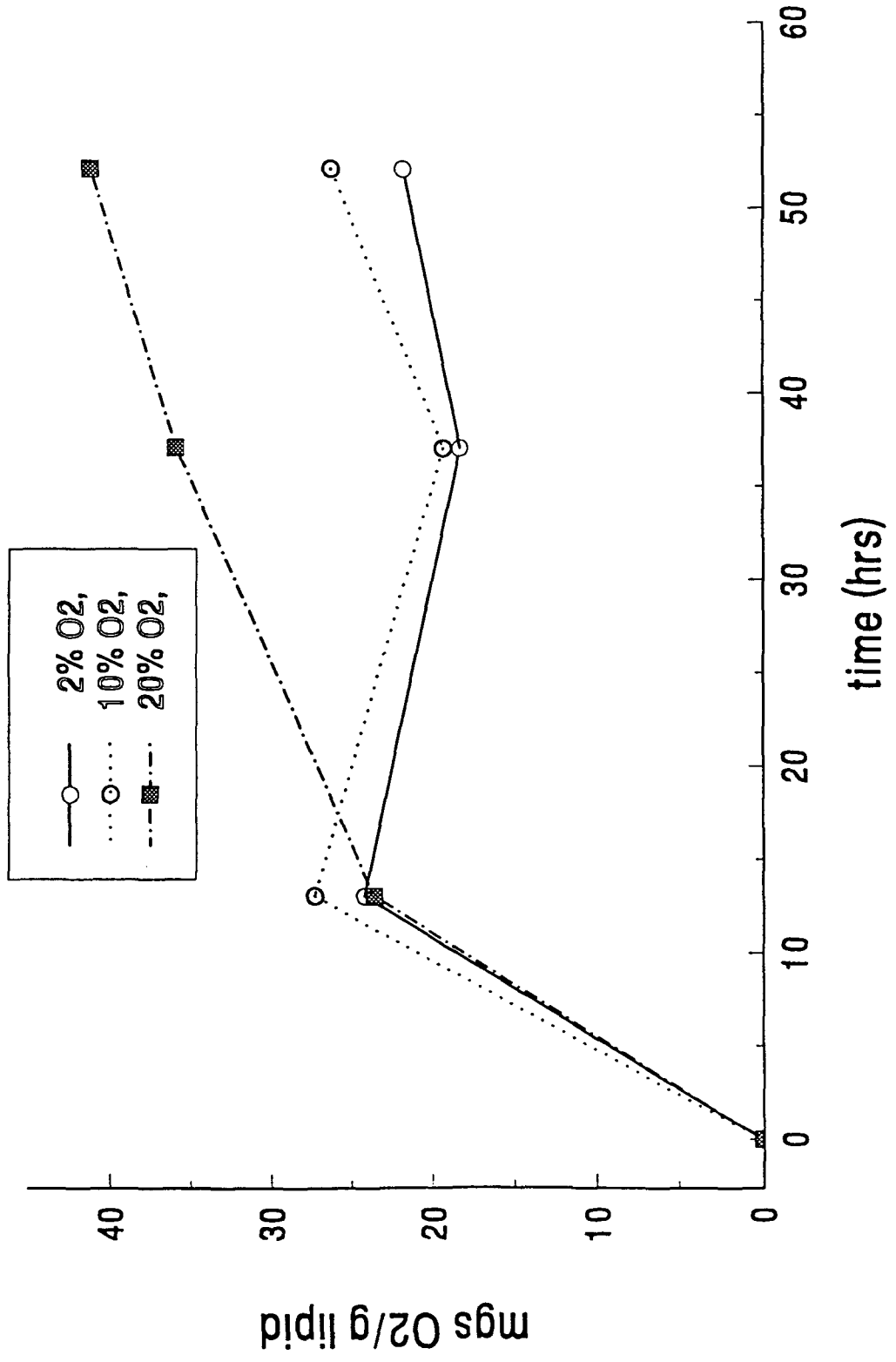
Figure 18. Relative oxygen weight-gain of squalene as affected by carotenoids and no-carotenoid control, at 2% O_2 and 10μg carotenoid/g squalene[a][b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

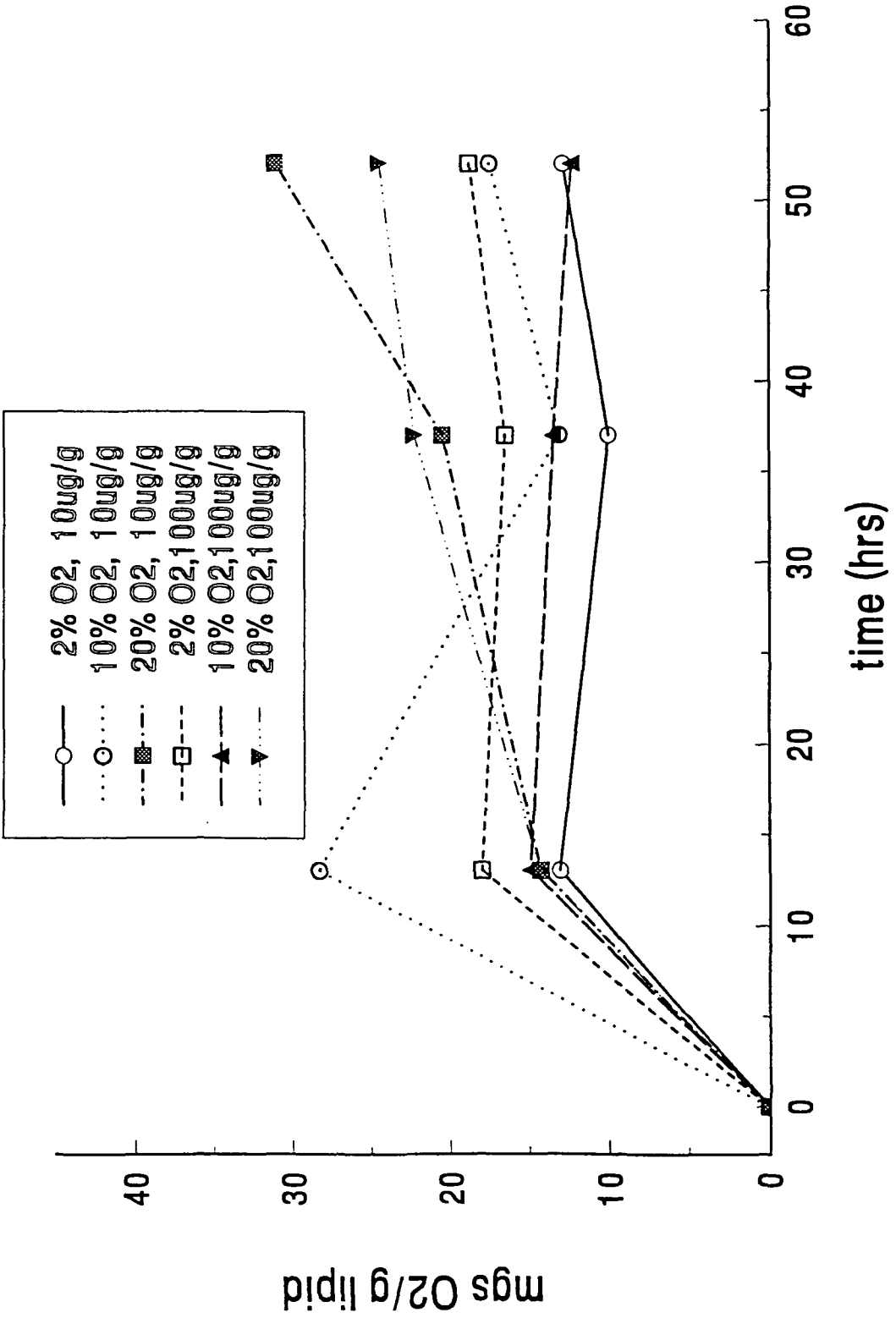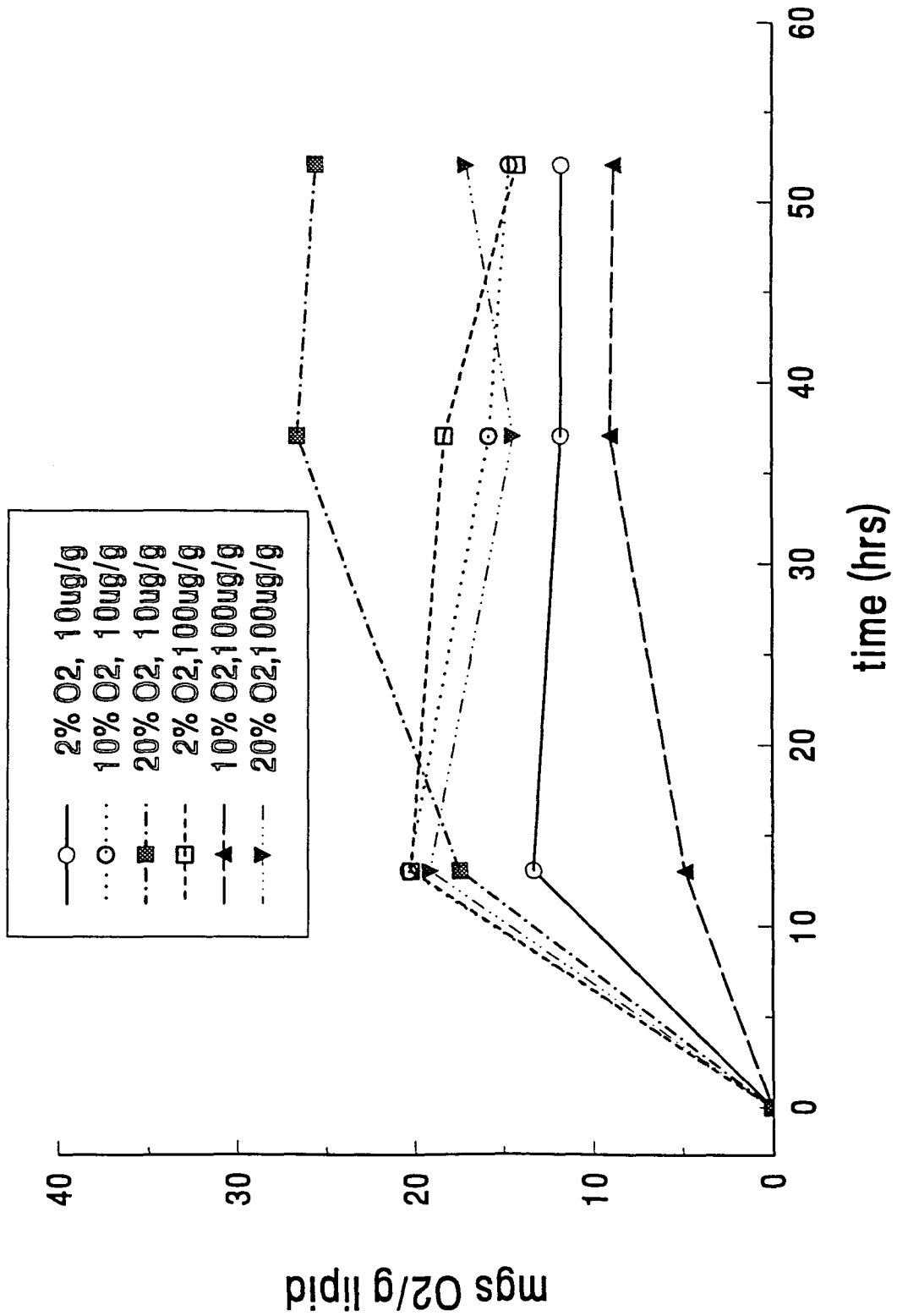[b] All values are averages of two replicates of two replicates.

OXYGEN UPTAKE
at 2 % O2, 10ug/g

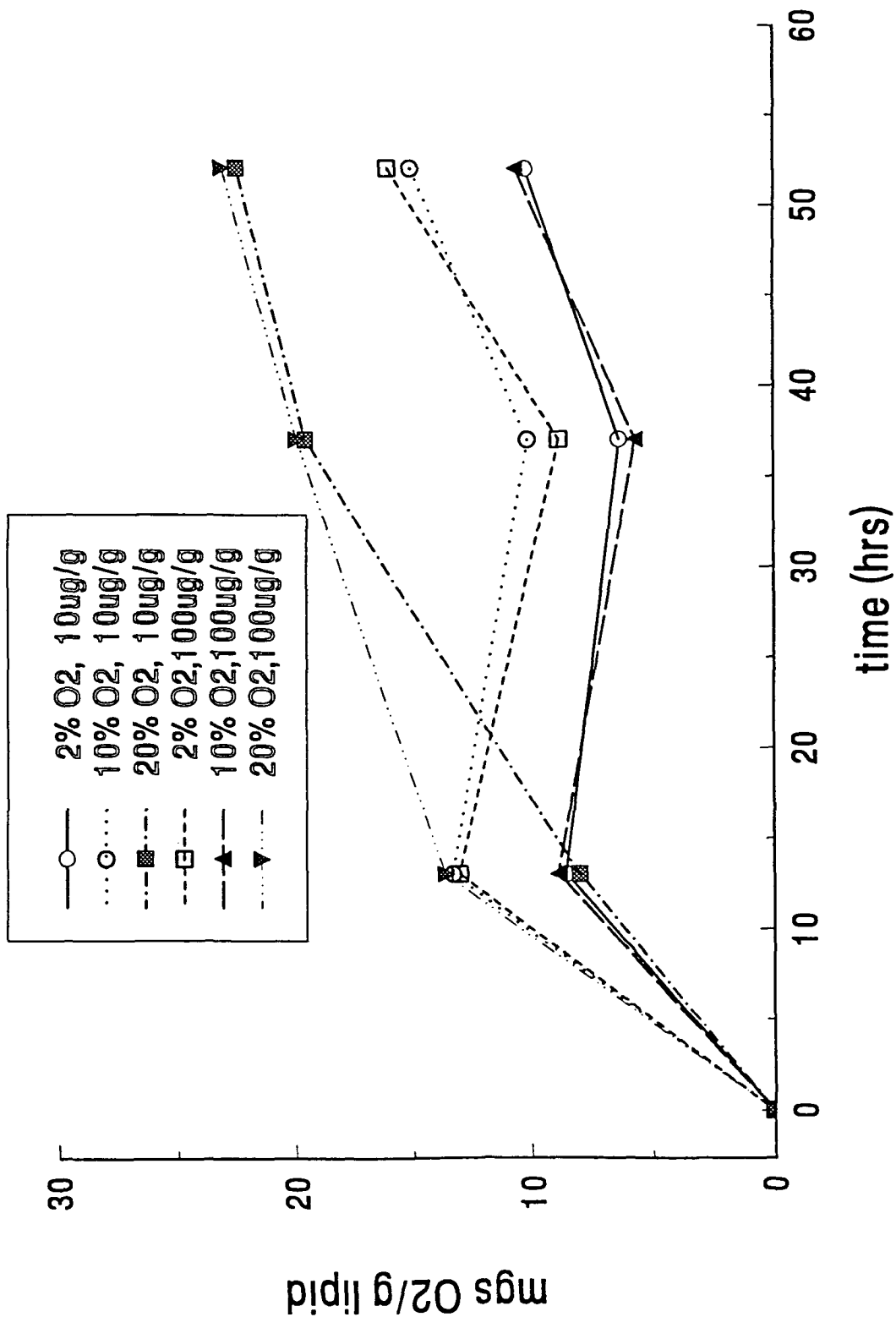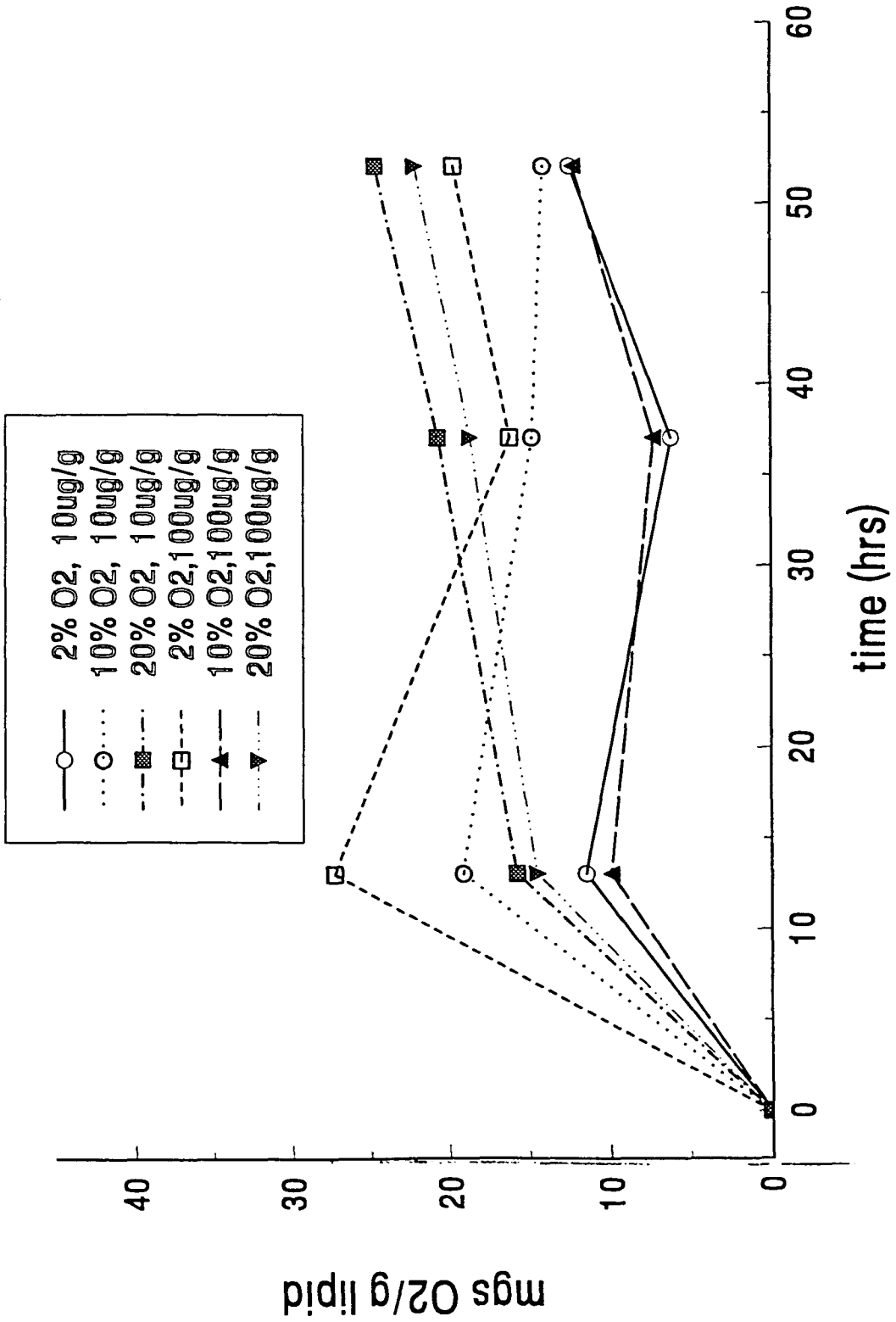Legend:
- Lycopene
- 5,6-diol
- Beta carotene
- 5,6-epoxide
- Control

mgs O2/g lipid

time (hrs)
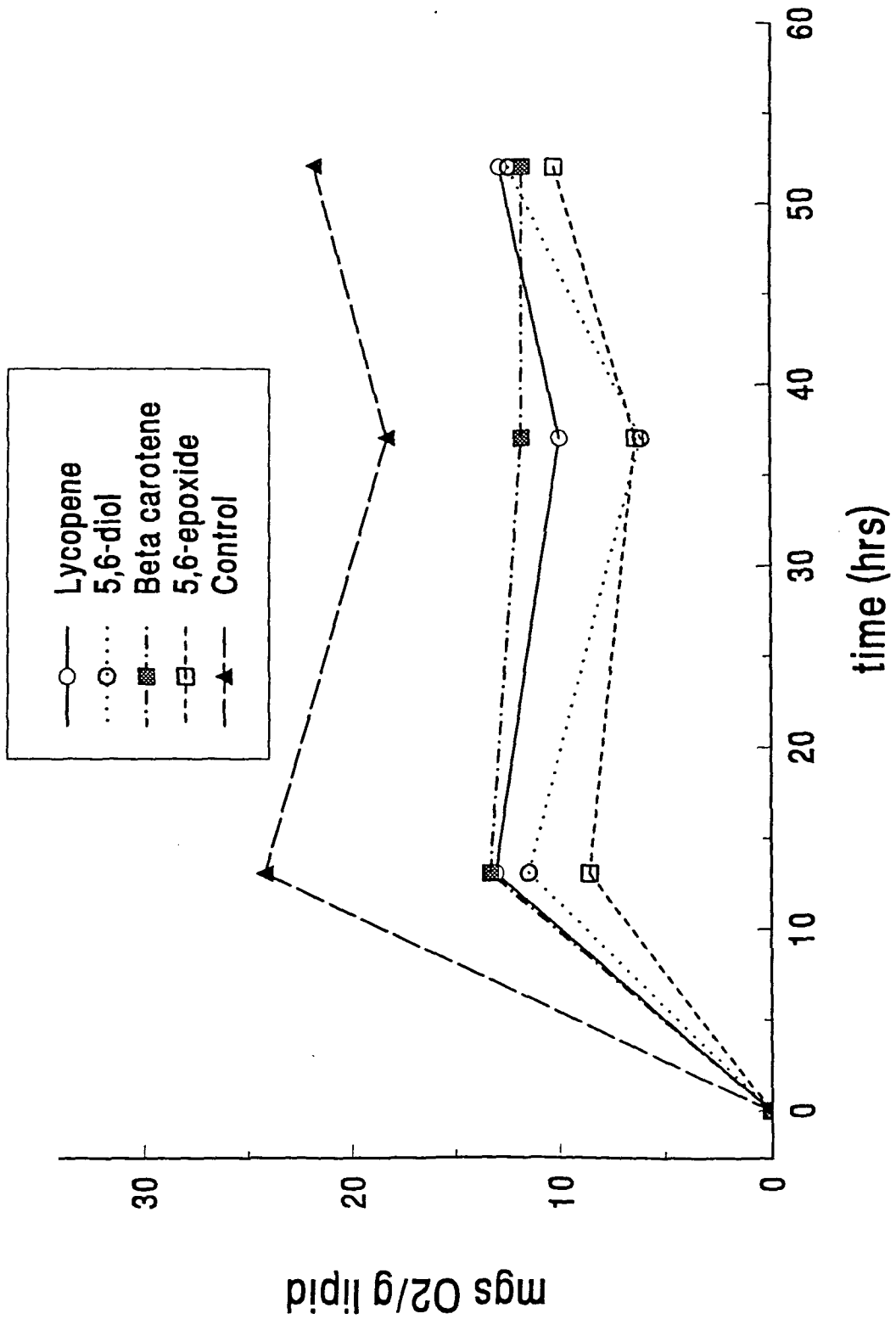
Figure A1 utilizes data from preliminary work, on lycopene-5,6-diol, since no data was collected between 0 and 12 hours in later studies. Beyond 12 hours, the patterns varied with pressure. In general, all combinations involving pressures of 2% O_2 and 10% O_2 tended to exhibit either a tailing-off effect in oxygen uptake, or more often a slight decrease in oxygen weight-gain, from 12 to 52 hours, often decreasing from 25 to 37 hours and increasing again from 37 to 52 hours. However, for combinations involving pressures of 20% O_2 the rapid uptake phase was followed by a second slower uptake phase which was fairly linear from 12 to 52 hours. This would seem to indicate that the rate of oxygen uptake was influenced by the partial pressure of oxygen. The same effect can be seen in Figure 17; the fact that the control followed the same pattern suggests that this effect was not mediated in any way by carotenoid antioxidant mechanisms, rather indicating that the rate and kinetics of lipid oxidation were different at partial pressures of oxygen below 20% O_2.

Statistical analysis of oxygen weight-gain data averaged over pressure and concentration treatments indicated significant differences among compounds tested. At $\alpha=0.05$, all compounds were significantly different from controls, at every pressure/concentration combination. Table A11 provides a complete breakdown of antioxidant capacities for all compounds at all possible pressure/concentration combinations. Lycopene-5,6-epoxide had a significantly higher antioxidant capacity than either lycopene or lycopene-5,6-diol; lycopene-5,6-epoxide was also slightly more effective at preventing lipid oxidation that ß-carotene, but this was not statistically significant at $\alpha=0.05$. Lycopene-5,6-diol was less effective than ß-carotene, and more effective than lycopene, but differences

were not statistically significant. According to these general observations, it appears that lycopene-5,6-epoxide exhibited greater antioxidant capacity than did the parent lycopene, while lycopene-5,6-diol showed no such difference in antioxidant capacity.

The effects of concentration and pressure on antioxidant capacity of carotenoids can be seen in Figures 13-16. By breaking down the statistical analysis to look for differences between compounds at specific pressure/concentration combinations, it was observed that concentrations affected the relative antioxidant capacity of the carotenoids. At $10\mu g$ carotenoid/g squalene, antioxidant capacity increased from lycopene -> ß-carotene -> lycopene-5,6-diol -> lycopene-5,6-epoxide; the differences between lycopene and lycopene-5,6-diol were statistically significant at $\alpha=0.05$, and more interestingly the differences between lycopene-5,6-diol and lycopene-5,6-epoxide were not statistically different. At a concentration of $100\mu g$ carotenoid/g squalene, a different order of antioxidant activity was observed : lycopene -> lycopene-5,6-diol -> lycopene-5,6-epoxide -> ß-carotene. In this case, differences between lycopene-5,6-diol and lycopene were not significant, nor were differences between lycopene-5,6-diol and lycopene-5,6-epoxide. However, at 100 μg carotenoid/g squalene and 2% O_2, an unusual phenomenon was observed. The order of antioxidant activity at this combination increased from lycopene-5,6-diol > ß-carotene > lycopene > lycopene-5,6-epoxide. In all other cases, lycopene was noted to exhibit the lowest antioxidant capacity of the four compounds tested, but at this particular pressure/concentration combination it had significantly more antioxidant capacity than lycopene-5,6-diol. The reason for this anomaly was not understood.

Statistical analysis of pressure and concentration effects also revealed unusual trends

for all compounds at 100 μg carotenoid/g squalene and 2% O_2. According to Burton and

Ingold (1984), the antioxidant capacity of carotenoids increased as oxygen pressure decreased

from 20% to 10% to 2% O_2. For example, refer to Figure 14. The effects of pressure can

be observed. At 2% O_2 uptake reached its maximum at 52 hours, while at 10% and 2% O_2

the maximum was reached in only 12 hours. Comparing these maximum values, it can be

seen that less oxygen uptake (i.e. less oxidation) occured as oxygen pressure decreased. This

was the case for plots of pressure at 10 μg carotenoid/g squalene. In addition, Burton and

Ingold (1984) observed that antioxidant capacity of carotenoids increased as the concentration

increased from 0.05mM in organic solvent to 0.5mM. In this study, the concentrations used

translated to about 0.035mM (10μg carotenoid/g squalene) to 0.35mM (100μg carotenoid/g

squalene), and so oxygen uptake would be expected to decrease from 10μg carotenoid/g

squalene to 100μg carotenoid/g squalene treatments. Again, this can be observed in Figure

9, by comparing oxygen uptake between the two concentrations. However, the trend did not

apply to concentrations at 2% O_2. Going back to pressure trends, the same effect was

observed. Rather than seeing an increase in antioxidant activity from 20% -> 10% -> 2%

O_2, only the 20% -> 10% O_2 increase can be observed. Antioxidant capacity was, in fact,

less at 2% O_2 than at 20% O_2. Hence it appears that the 2% O_2/(100μg carotenoid/g

squalene) combination did not adhere to the general trends discussed. The reason for this

anomaly was not understood. The anomaly has not been observed to date, and was not

reported by Burton and Ingold (1984). The most reasonable explanation at present would be

to suggest some sort of negative synergism between the higher concentration and low oxygen pressure. From pressure data, it is obvious that the initial rate of oxygen uptake at 2% O_2 is significantly greater than at 20% O_2. Since the effect is not observed at the lower carotenoid concentration, it would appear that the combination of increased concentration and reduced pressure encouraged some form of prooxidant activity, rather than antioxidant activity.

In general, however, the trends in pressure and concentration reported by Burton and Ingold (1984) have been reproduced in this study. For all carotenoids tested, differences in oxygen uptake were statistically significant at the $\alpha=0.05$ level between 2, 10 and 20% O_2, at $10\mu g$ carotenoid/g squalene. At $100\mu g$ carotenoid/g squalene, oxygen uptake at 10% O_2 was significantly different from uptake at 2% and 20% O_2, but there was no significant difference in uptake between 2% and 20% O_2. The effects of concentration on oxygen uptake varied between carotenoids. At 2% O_2, oxygen uptake increased with concentration, and this was significant at $\alpha=0.05$ for both lycopene-5,6-diol and ß-carotene. At 10% O_2, oxygen uptake decreased with concentration, but was significant only for ß-carotene. At 20% O_2, oxygen uptake again decreased with concentration, but was significant for only lycopene and ß-carotene. Lycopene-5,6-epoxide treatments were not influenced by concentration. In summary, lycopene-5,6-epoxide exhibited a significantly higher antioxidant capacity than did lycopene, whereas lycopene-5,6-diol did not. Overall, there was no significant difference between ß-carotene and lycopene-5,6-epoxide, but ß-carotene displayed significantly more antioxidant capacity than did lycopene-5,6-diol.

Peroxide Value measurements

Figures 19-22 illustrates peroxide values (PV) of squalene over a 52 hr period as affected by ß-carotene, lycopene, lycopene-5,6-epoxide and lycopene-5,6-diol, respectively. Figure 23 illustrates PV for the controls (no carotenoid). Each figure shows PV patterns for the compound at the various pressure/concentration combinations. Tables A6-A10 provide the data used in the construction of these figures. Figure 24 indicates the relative effects on oxidation of all four compounds at 2% O_2 and 10μg carotenoid/g squalene. This pressure/concentration combination is representative of all other combinations. The PV plots are remarkably similar in their pattern; an initial rapid peroxide-forming phase from 0 to 12 hours is followed by another phase from 12 to 25 hours in which the slope of the curve increases slightly, levels off, or decreases slightly, depending on the carotenoid and the pressure/concentration combination. The pressure/concentration plots follow the same general pattern, with the sole exception of lycopene, which has no unifying pattern.

Statistical analysis of Peroxide Value data over pressure/concentration combination indicated some significant differences between carotenoids tested. Table A12 provides a complete breakdown of antioxidant capacities for all compounds at all possible pressure/concentration combinations. At $\alpha=0.05$, lycopene exhibited the greatest antioxidant capacity, whereas ß-carotene displayed least antioxidant capacity. Lycopene-5,6-epoxide was not significantly different from lycopene, whereas lycopene-5,6-diol was.

Figure 19.   Peroxide Value of squalene as influenced by lycopene[a][b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b] All values are averages of three replicates.

PEROXIDE VALUE
Lycopene

meq/kg lipid

time (hrs)

2% O2, 10ug/g
10% O2, 10ug/g
20% O2, 10ug/g
2% O2, 100ug/g
10% O2, 100ug/g
20% O2, 100ug/g

Figure 20.   Peroxide Value of squalene as
            influenced by ß-carotene[a,b]

[a] 10 μg/g and 100 μg/g indicate concentrations
     of carotenoid relative to squalene.

[b] All values are averages of three replicates.

PEROXIDE VALUE
Beta carotene

	2% O2, 10ug/g
	10% O2, 10ug/g
	20% O2, 10ug/g
	2% O2, 100ug/g
	10% O2, 100ug/g
	20% O2, 100ug/g

meq/kg lipid

time (hrs)

Figure 21. Peroxide Value of squalene as influenced by lycopene-5,6-epoxide[a,b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b] All values are averages of three replicates.

PEROXIDE VALUE
Lycopene-5,6-epoxide

2% O2, 10ug/g
10% O2, 10ug/g
20% O2, 10ug/g
2% O2, 100ug/g
10% O2, 100ug/g
20% O2, 100ug/g

time (hrs)

meq/kg lipid

Figure 22. Peroxide Value of squalene as influenced by lycopene-5,6-diol[a,b]

[a] 10 μg/g and 100 μg/g indicate concentrations of carotenoid relative to squalene.

[b] All values are averages of three replicates.

PEROXIDE VALUE
Lycopene-5,6-diol

Legend:
○ — 2% O2, 10ug/g
⊙ ····· 10% O2, 10ug/g
▨ —·— 20% O2, 10ug/g
□ — — 2% O2, 100ug/g
▲ —— 10% O2, 100ug/g
▶ ····· 20% O2, 100ug/g

x-axis: time (hrs)
y-axis: meq/kg lipid

Figure 23.   Peroxide Value of squalene by
no-carotenoid control[a]

[a]   All values are averages of three replicates.

PEROXIDE VALUE
Control

Legend:
- 2% O2, ○ (solid line)
- 10% O2, ⊙ (dotted line)
- 20% O2, ■ (dash-dot line)

x-axis: time (hrs)
y-axis: meq/kg lipid

Figure 24. Relative Peroxide Value of squalene as affected
by carotenoids and no-carotenoid control, at 2% O_2
and 10μg carotenoid/g squalene[a,b]

[a] 10 μg/g and 100 μg/g indicate concentrations
of carotenoid relative to squalene.

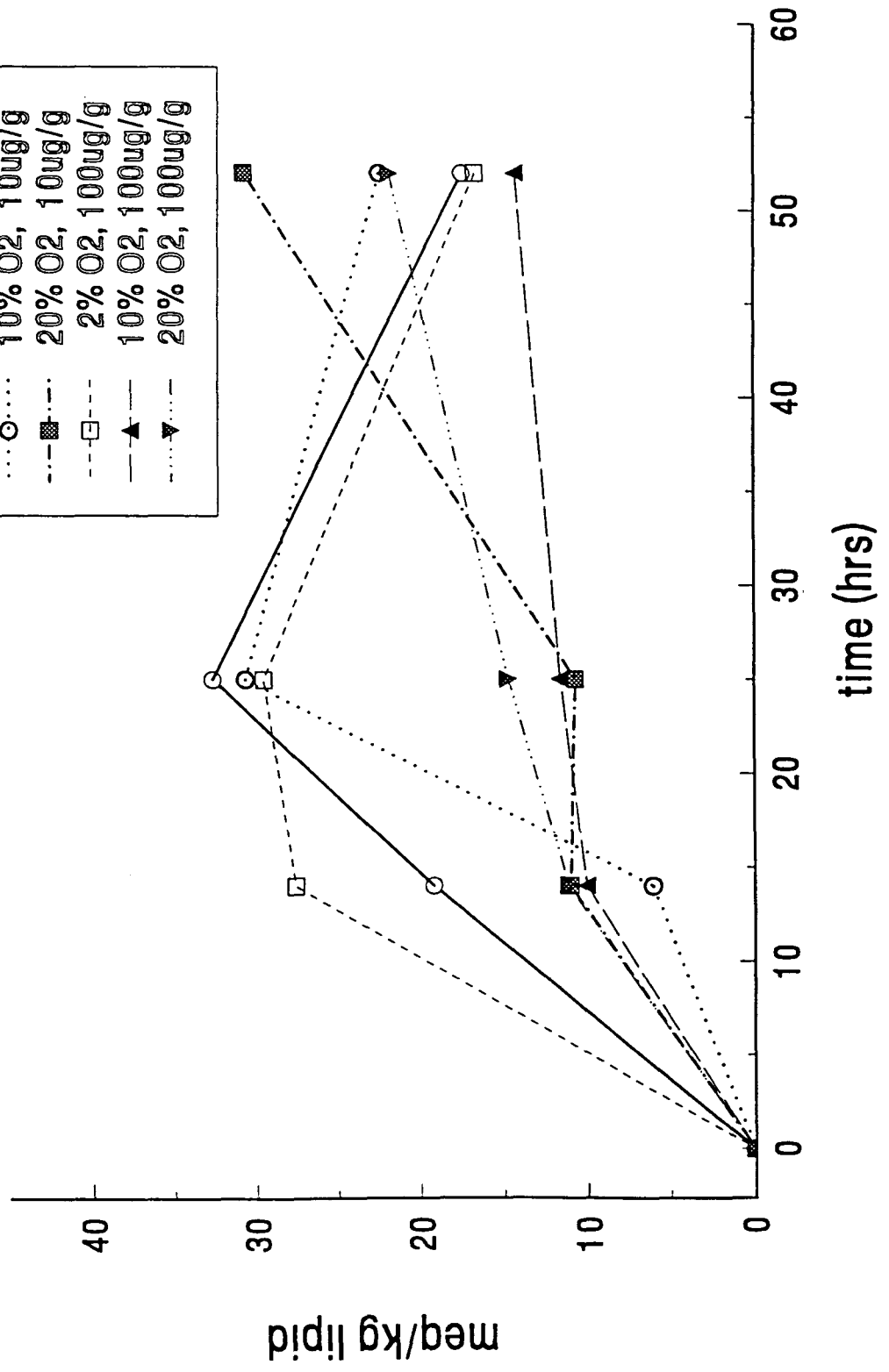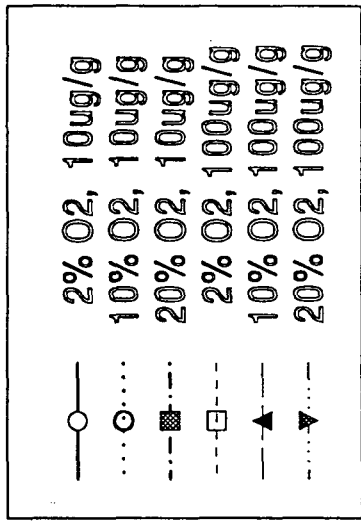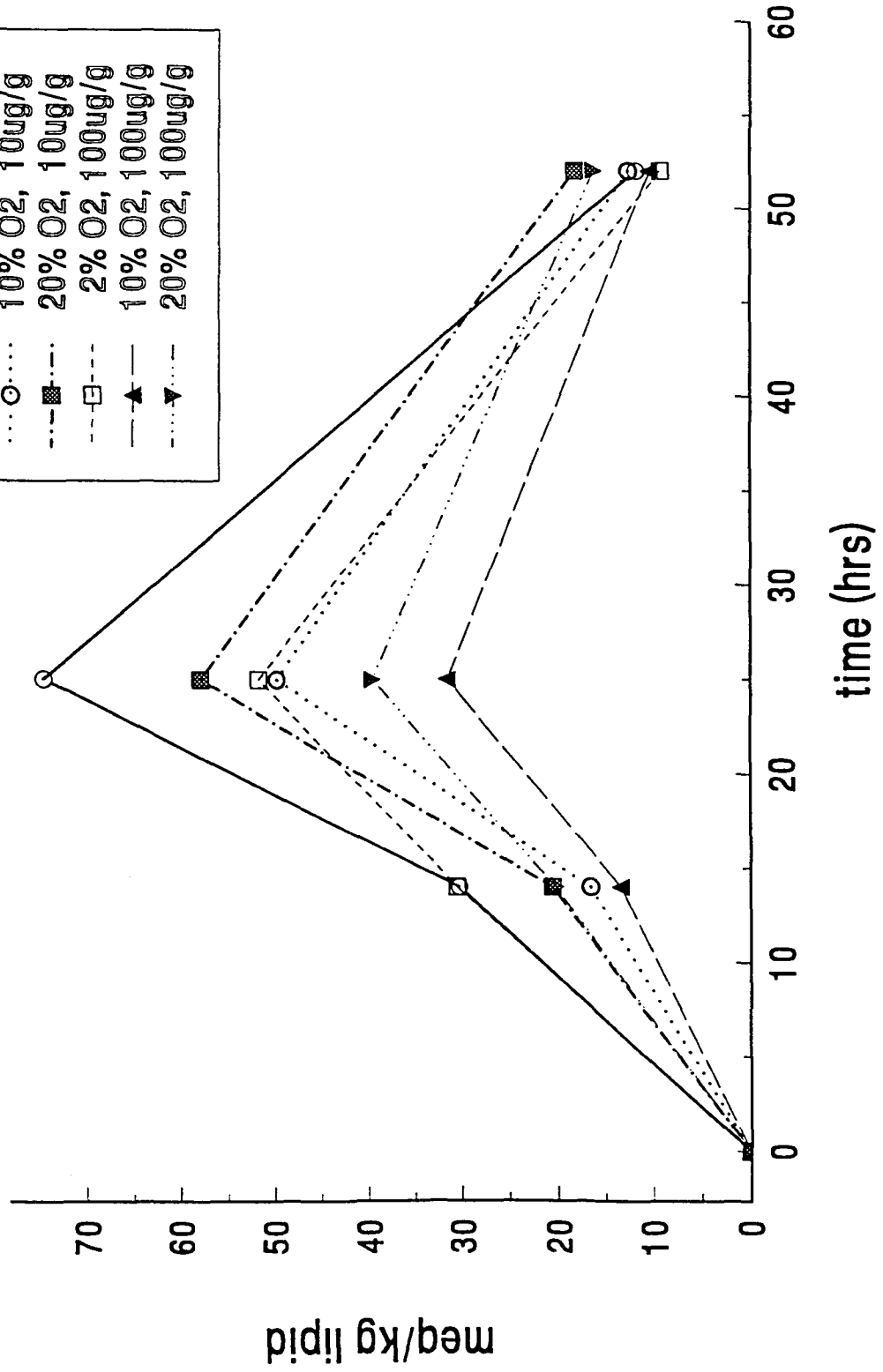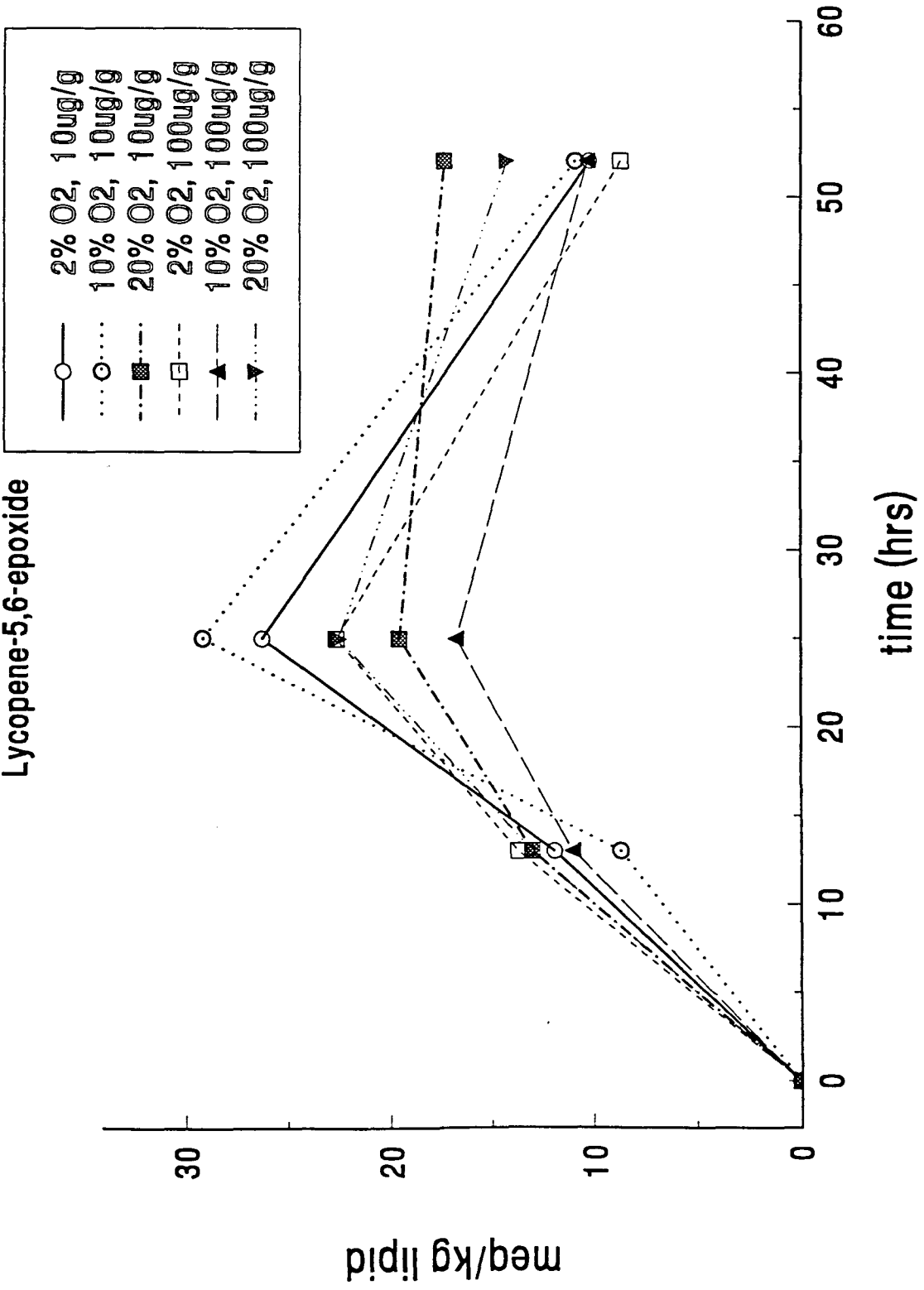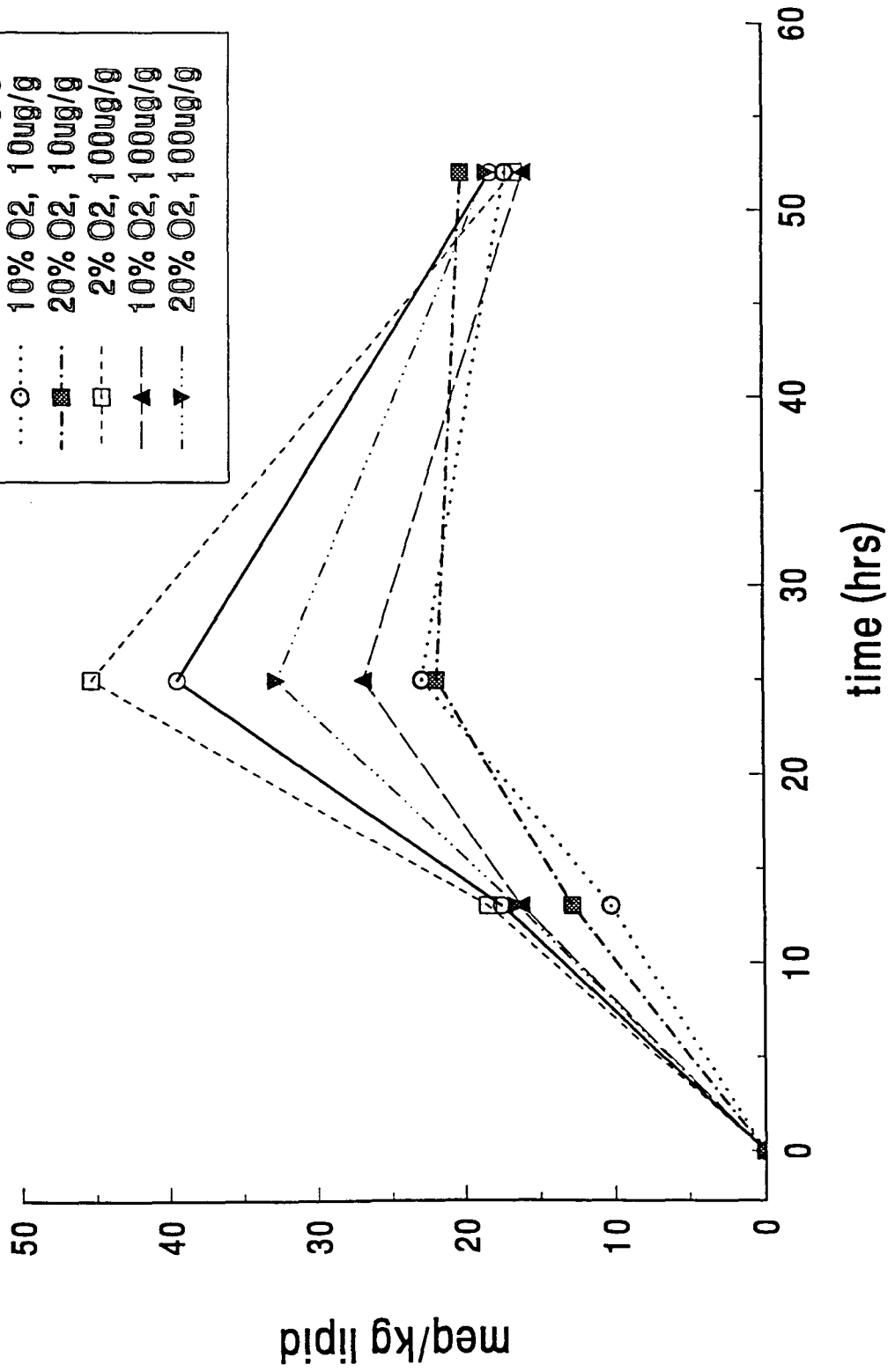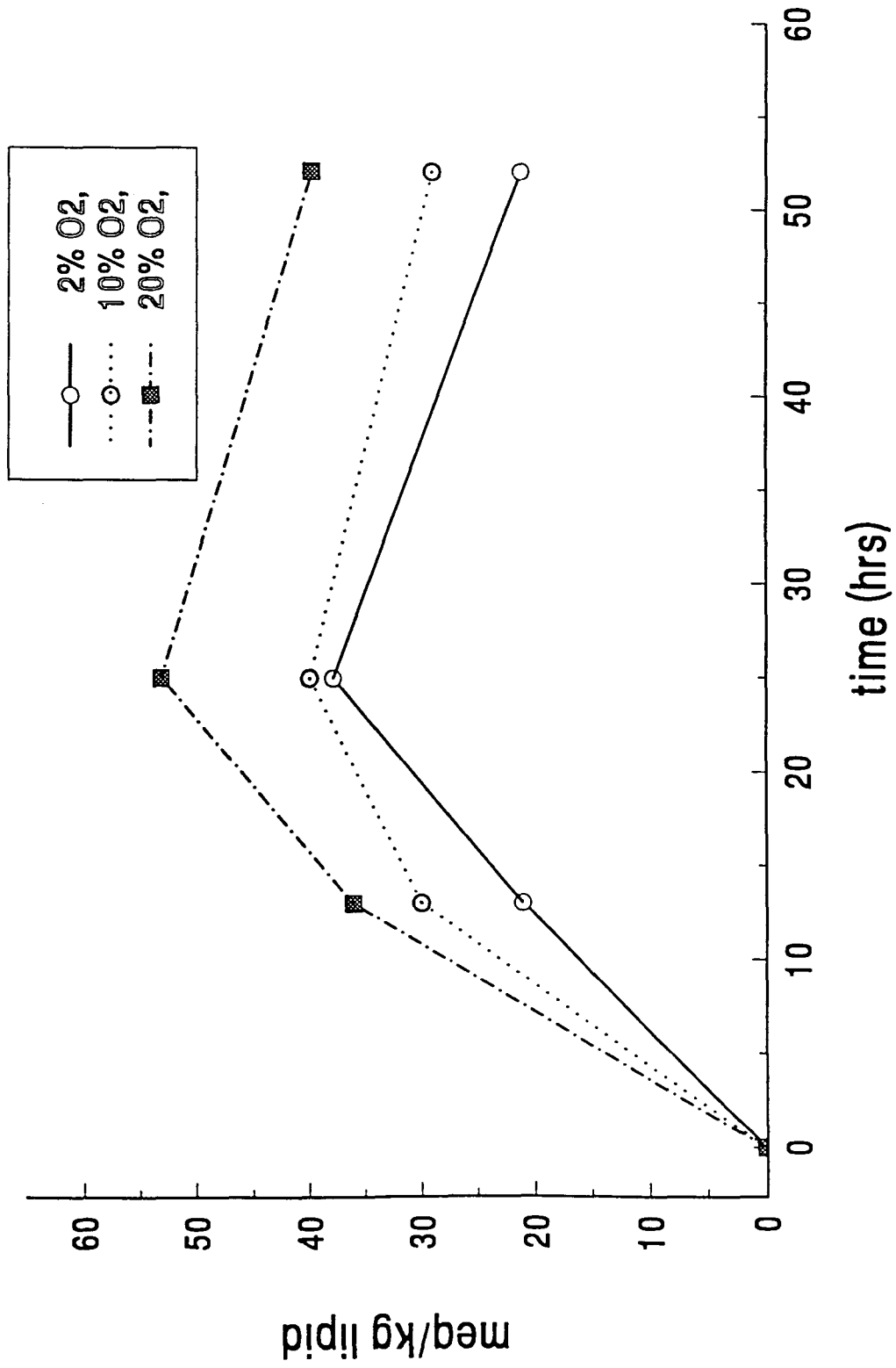[b] All values are averages of three replicates.

PEROXIDE VALUE
at 2 % O2, 10ug/g

Lycopene
5,6-diol
Beta carotene
5,6-epoxide
Control

time (hrs)

meq/kg lipid
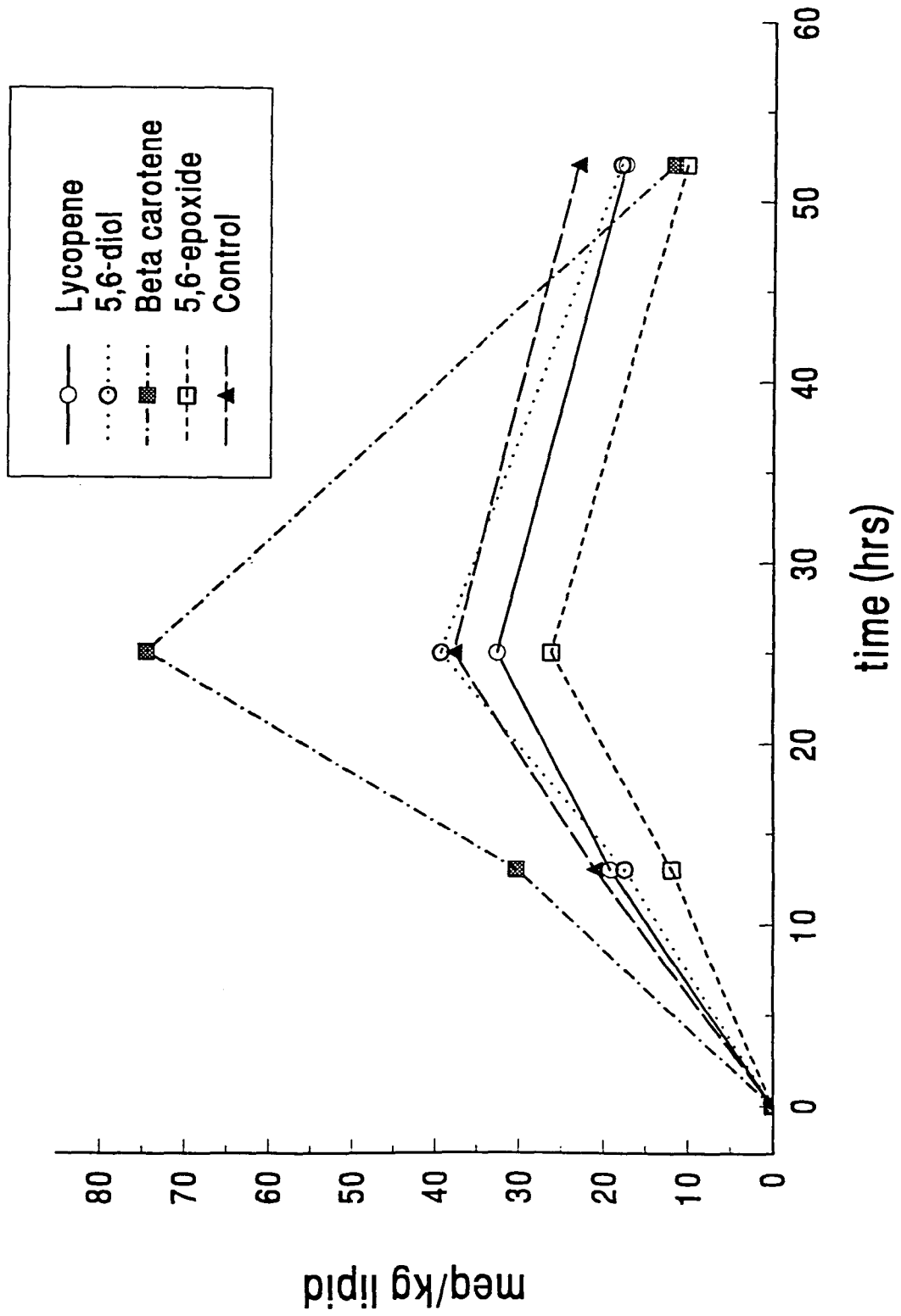
The influence of concentration and pressure upon the antioxidant capacity of the carotenoids can be seen in Figures 19-22. Concentration effects were significant at $\alpha = 0.05$. At concentrations of 10μg carotenoid/g squalene and 100 μg carotenoid/g squalene, the same general order of antioxidant capacity was observed, increasing from ß-carotene -> lycopene-5,6-diol -> lycopene-5,6-epoxide -> lycopene. At the lower concentration of carotenoid, all lycopene compounds were significantly more effective as antioxidants than was ß-carotene at $\alpha = 0.05$, but there was no significant difference among the three lycopene compounds. At the higher concentration, though, both lycopene and lycopene-5,6-epoxide were significantly more effective at preventing lipid peroxidation than was lycopene-5,6-diol, or ß-carotene. Pressure effects were also significant. At 10μg carotenoid/g squalene, antioxidant capacity of all compounds decreased as oxygen pressure decreased from 20% to 10% to 2% O_2. This is in direct disagreement with Burton and Ingold (1984), who observed exactly the opposite. At 100μg carotenoid/g squalene, antioxidant capacity decreased in the order 10% -> 20% -> 2% O_2. All pressure differences were significant. The effects of concentration on antioxidant activity were also statistically significant, antioxidant activity increasing from 10μg carotenoid/g squalene to 100μg carotenoid/g squalene in all carotenoids except lycopene-5,6-diol. However, this increase, which would be expected according to Burton and Ingold (1984), only occured at 2% and 10% O_2. At 20% O_2, the antioxidant activity decreased from 10μg carotenoid/g squalene to 100μg carotenoid/g squalene in all compounds apart from ß-carotene.

In summary, the general observations made by Burton and Ingold (1984) with regard to pressure and concentration were not reproduced in this study; partial agreements have been nullified by completely contrary results. With regard to differences in antioxidant capacity between the carotenoids tested, lycopene-5,6-epoxide seems to be at least as good an antioxidant as lycopene, while lycopene-5,6-diol exhibited significantly less antioxidant capacity than both lycopene-5,6-diol and lycopene. In all cases, all lycopene compounds displayed greater antioxidant capacities than ß-carotene.

Comparison of oxygen weight-gain and Peroxide Value methods

Overall, the two methods for assessing squalene oxidation gave different results. While the oxygen weight-gain data gave clear trends in pressure and concentration, over all compounds tested, apart from one anomaly, the Peroxide Value data displayed no sense of trend, yielding various different trends among compounds and pressure/concentration combinations. As shown on the following page, and detailed in Tables A11 and A12, the two methods gave greatly different results regarding relative antioxidant activity of each of the carotenoids.

Pressure differences are immediately obvious. Differences between compounds were significant. Whereas the oxygen weight-gain method indicated that ß-carotene had greater antioxidant capacity than did lycopene, the peroxide value method indicated the exact

opposite. Similarly, lycopene-5,6-epoxide was by far more effective than the parent lycopene according to the oxygen weight-gain method, whereas the peroxide value method indicated

Oxygen weight-gain method:C^a > L^b > D^{bc} > β^{cd} > E^d

$$P_3^a > P_2^b > P_1^b$$

$$C_1^a > C_2^a$$

Peroxide Value method: β^a > C^b > D^c > E^d > L^d

$$P_1^a > P_3^b > P_2^b$$

$$C_1^a > C_2^b$$

[1] C=control, ß=ß-carotene, L=lycopene, E=lycopene-5,6-epoxide, D=lycopene-5,6-diol.
P_1 = 2% O_2, P_2 = 10% O_2, P_3 = 20% O_2.
C_1 = 10 μg carotenoid/g squalene, C_2 = 100 μg carotenoid/g squalene.
Highest weight-gain on the left, decreasing to the right.

[abcd] Treatments in a row having the same letter are not statistically different at α=0.05.

no significant difference between the two. Lycopene was significantly more effective than was lycopene-5,6-diol with the peroxide value method, but there was no significant difference between the two based on oxygen weight-gain data.

These extreme differences between methods make it necessary to evaluate each of the methods with regard to mechanism. Oxygen weight-gain methods are based simply on the direct uptake of oxygen by a lipid substrate. The peroxide value is based on the formation of lipid peroxides following the uptake of oxygen by an initiated lipid radical. The formation of peroxides leads to a prooxidant phase where these peroxides then stimulate lipid oxidation, increasing the number of lipid peroxides over a short period of time. The large increase in number of peroxide molecules causes an increase in the reaction of these peroxides with a number of other lipid compounds at various stages in the oxidation process, yielding a diversity of peroxide reaction pathways and thus inconstant oxidation kinetics. This is a possible explanation for the lack of any apparent trend observed in the peroxide value. In addition, the peroxide value loses sensitivity at such low levels of lipid substrate, and so possibility the reliability of data comes into question, especially when performing statistical analyses. The fact that the oxygen weight-gain data correlated so well with the results from Burton and Ingolds` laboratories leads to the conclusion that this method was more suitable in following the oxidation of small quantities of lipid substrate. The fact that Burton and Ingold also monitored oxygen uptake of lipid substrates, not by weighing but by monitoring headspace oxygen disappearance in a more sensitive pressure transducer system, adds further weight to this conclusion.

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to assess some oxidation products of lycopene for potential biological antioxidant capacity. Using ß-carotene as a reference carotenoid, lycopene, lycopene-5,6-epoxide, and lycopene-5,6-diol were incorporated into antioxidant studies. Analysis of the data from the antioxidant studies indicated that of the two methods used to assess lipid oxidation, and hence antioxidant capacity, the oxygen weight-gain method was more suitable. Data from this study indicated significant pressure and concentration effects upon the antioxidant activity of all carotenoids tested, general trends being in very close agreement with the pioneering work of Burton and Ingold (1984). This study indicated that ß-carotene exhibited greater antioxidant capacity than both lycopene and lycopene-5,6-diol, though not to a statistically significant degree. Lycopene-5,6-diol did not exhibit any significant increase in antioxidant potential over the parent lycopene. Lycopene-5,6-epoxide, however, displayed significantly greater antioxidant capacities than both lycopene and lycopene-5,6-diol, as well as ß-carotene, although the latter differences were not significantly different.

From these observations, it would seem to be worthwhile to look at some of the natural oxidation products of common dietary carotenoids in terms of antioxidant potential. As yet, lycopene-5,6-epoxide has been found in plants and food, but has not been detected in human plasma. It has been postulated that carotenoid epoxides are susceptible to degradation, possibly to hydroxycarotenoids, in the acid conditions of the stomach (Parker,

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1989). Nevertheless, it is fully possible that carotenoids may undergo oxidative metabolism at non-specific or target sites in the body, since as yet there is no evidence to suggest differently.

At the same time, the assessment of antioxidant capacities of carotenoids needs to become standardized. Oxygen weight-gain methods may tend to appear somewhat empirical, but they are surprisingly quantitative and reproducible. The major problem, however, especially when working with oxygen partial pressures below 20% O_2, is the sample turn-around time, which imposes time restrictions on any study, so compromising the design. The pressure transducer system as used by Burton and Ingold (1984) is now becoming more common in the field. The system has the great advantage of sensitivity, ease of use and, most importantly, allows real time measurements. Consequently the reliability of data is greatly increased. If research into potential biological antioxidants is to advance, and the interest caused by dietary antioxidants in relation to maintenance of health in just the last few years is a strong indicator that it will, development of more suitable methods is essential.

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APPENDIX

A) OXYGEN WEIGHT-GAIN DATA

Table A1. Oxygen weight-gain data for squalene as affected
by ß-carotene[a]

	time=13 hrs	time=37 hrs	time=52 hrs
P_1C_1[b]	13.30	11.79	11.79
P_2C_1	20.37	15.84	14.70
P_3C_1	17.46	26.69	25.68
P_1C_2	20.23	18.37	14.23
P_2C_2	4.83	9.02	8.84
P_3C_2	19.15	14.45	17.15

[a] All values are expressed in mg O_2/g squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of two lots of two replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg ß-carotene/g squalene
C_2 = 100 μg ß-carotene/g squalene

Table A2. Oxygen weight-gain data for squalene as affected
by lycopene[a]

	time=13 hrs	time=37 hrs	time=52 hrs
P_1C_1[b]	13.03	9.98	12.90
P_2C_1	18.35	13.09	17.55
P_3C_1	14.24	20.53	31.22
P_1C_2	17.97	16.50	18.85
P_2C_2	14.86	13.46	12.36
P_3C_2	14.27	22.30	24.51

[a] All values are expressed in mg O_2/g squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of two lots of two replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg lycopene/g squalene
C_2 = 100 μg lycopene/g squalene

Table A3. Oxygen weight-gain data for squalene as affected
by lycopene-5,6-epoxide[a]

	time = 13 hrs	time = 37 hrs	time = 52 hrs
P_1C_1[b]	8.52	6.34	10.12
P_2C_1	13.31	9.12	15.10
P_3C_1	8.00	19.58	22.51
P_1C_2	13.02	8.83	15.08
P_2C_2	8.88	6.68	10.66
P_3C_2	13.62	19.95	22.13

[a] All values are expressed in mg O_2/g squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of two lots of two replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg lycopene-5,6-epoxide/g squalene
C_2 = 100 μg lycopene-5,6-epoxide/g squalene

Table A4. Oxygen weight-gain data for squalene as affected
by lycopene-5,6-diol[a]

	time=13 hrs	time=37 hrs	time=52 hrs
P_1C_1[b]	11.44	6.07	12.46
P_2C_1	19.13	14.72	14.07
P_3C_1	15.17	20.68	24.68
P_1C_2	27.30	16.12	19.73
P_2C_2	9.85	7.18	12.21
P_3C_2	14.46	18.65	22.16

[a] All values are expressed in mg O_2/g squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of two lots of two replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg lycopene-5,6-diol/g squalene
C_2 = 100 μg lycopene-5,6-diol/g squalene

Table A5. Oxygen weight-gain data for squalene as affected
by control[a]

	time = 13 hrs	time = 37 hrs	time = 52 hrs
P_1^b	24.21	18.31	21.87
P_2	27.31	19.35	26.30
P_3	23.60	35.88	41.29

[a] All values are expressed in mg O_2/g squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of two lots of two replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen

B) PEROXIDE VALUE DATA

Table A6. Peroxide Value data for squalene as affected
by ß-carotene[a]

	time=14 hrs	time=25 hrs	time=52 hrs
P_1C_1[b] 30.3	74.7	11.7
P_2C_1 14.5	49.9	12.5
P_3C_1 20.5	58.1	18.2
P_1C_2 30.5	51.9	93.2
P_2C_2 13.6	31.6	10.3
P_3C_2 20.2	39.7	16.2

[a] All values are expressed in mg peroxide/kg squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of three replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 µg ß-carotene/g squalene
C_2 = 100 µg ß-carotene/g squalene

Table A7. Peroxide Value data for squalene as affected
by lycopene[a]

	time=14 hrs	time=25 hrs	time=52 hrs
P_1C_1[b]	19.2	32.7	17.5
P_2C_1	6.0	30.7	22.5
P_3C_1	10.0	10.7	30.8
P_1C_2	27.6	29.6	16.7
P_2C_2	10.0	11.6	14.3
P_3C_2	11.1	14.7	21.9

[a] All values are expressed in mg peroxide/kg squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of three replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg lycopene/g squalene
C_2 = 100 μg lycopene/g squalene

Table A8. Peroxide Value data for squalene as affected
         by lycopene-5,6-epoxide[a]

	time = 14 hrs	time = 25 hrs	time = 52 hrs
P_1C_1[b]	11.9	26.3	10.2
P_2C_1	8.7	29.2	10.9
P_3C_1	13.0	19.6	17.4
P_1C_2	13.7	22.7	8.7
P_2C_2	11.0	16.8	10.3
P_3C_2	13.0	22.6	14.3

[a] All values are expressed in mg peroxide/kg squalene, and
    are expressed relative to zero at time = 0 hrs. All
    values are averages of three replicates.

[b] Code representation :
    P_1 = 2% oxygen
    P_2 = 10% oxygen
    P_3 = 20% oxygen
    C_1 = 10 μg lycopene-5,6-epoxide/g squalene
    C_2 = 100 μg lycopene-5,6-epoxide/g squalene

Table A9. Peroxide Value data for squalene as affected
by lycopene-5,6-diol[a]

	time=14 hrs	time=25 hrs	time=52 hrs
P_1C_1[b]	17.5	39.5	18.0
P_2C_1	10.2	22.9	17.2
P_3C_1	12.8	21.9	20.2
P_1C_2	18.6	45.4	16.6
P_2C_2	16.2	26.9	16.0
P_3C_2	16.5	32.8	18.4

[a] All values are expressed in mg peroxide/kg squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of three replicates.

[b] Code representation :
P_1 = 2% oxygen
P_2 = 10% oxygen
P_3 = 20% oxygen
C_1 = 10 μg lycopene-5,6-diol/g squalene
C_2 = 100 μg lycopene-5,6-diol/g squalene

Table A10. Peroxide Value data for squalene as affected
by control[a]

	time=14 hrs	time=25 hrs	time=52 hrs
P_1^b	21.0	37.9	23.2
P_2	30.0	40.0	29.1
P_3	36.1	53.2	39.8

[a] All values are expressed in mg peroxide/kg squalene, and
are expressed relative to zero at time = 0 hrs. All
values are averages of three replicates.

[b] Code representation :

P_1 = 2% oxygen

P_2 = 10% oxygen

P_3 = 20% oxygen

Table A11. Significant trends in oxygen weight-gain experiments[1]

Compounds averaged over pressure and concentration

$$C^a \;>\; L^b \;>\; D^{bc} \;>\; ß^{cd} \;>\; E^d$$
$$P_3^a \;>\; P_2^b \;>\; P_1^b$$
$$C_1^a \;>\; C_2^a$$

Compounds assessed at C_1 and C_2 averaged over pressure

At C_1 : $C^a \;>\; L^b \;>\; ß^c \;>\; D^c \;>\; E^d$
At C_2 : $C^a \;>\; D^b \;>\; L^b \;>\; ß^c \;>\; E^c$

Compounds assessed at P_1, P_2, P_3 averaged over concentration

At P_1 : $C^a \;>\; D^b \;>\; ß^c \;>\; L^d \;>\; E^e$
At P_2 : $C^a \;>\; L^b \;>\; D^c \;>\; ß^d \;>\; E^e$
At P_3 : $C^a \;>\; L^b \;>\; D^b \;>\; ß^b \;>\; E^b$

Specific concentration/pressure effects

P_1C_1 : $C^a \;>\; L^b \;>\; D^b \;>\; ß^b \;>\; E^b$
P_2C_1 : $L^a \;>\; C^a \;>\; ß^b \;>\; D^b \;>\; E^c$
P_3C_1 : $C^a \;>\; L^a \;>\; ß^{ab} \;>\; D^b \;>\; E^b$
P_1C_2 : $C^a \;>\; D^a \;>\; ß^b \;>\; L^b \;>\; E^c$
P_2C_2 : $C^a \;>\; L^b \;>\; D^{bc} \;>\; E^{cd} \;>\; ß^d$
P_3C_2 : $C^a \;>\; L^a \;>\; D^a \;>\; E^a \;>\; ß^b$

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[1] C=control, ß=ß-carotene, L=lycopene, E=lycopene-5,6-epoxide, D=lycopene-5,6-diol.
P_1 = 2% oxygen, P_2 = 10% oxygen, P_3 = 20% oxygen.
C_1 = 10 μg carotenoid/g squalene, C_2 = 100 μg carotenoid/g squalene.
Highest weight-gain on the left, decreasing to the right.

[abcd] Treatments in a row having the same letter are not statistically different at $\alpha=0.05$.

Table A12.　Significant trends in Peroxide Value experiments[1]

Compounds averaged over pressure and concentration

$$ß^a > C^b > D^c > E^d > L^d$$
$$P_1^a > P_3^b > P_2^b$$
$$C_1^a > C_2^b$$

Compounds assessed at C_1 and C_2 averaged over pressure

At C_1 : $\quad ß^a > C^b > D^c > E^c > L^c$
At C_2 : $\quad C^a > ß^a > D^b > E^c > L^c$

Compounds assessed at P_1, P_2, P_3 averaged over concentration

At P_1 : $\quad ß^a > D^b > C^b > L^c > E^d$
At P_2 : $\quad ß^a > C^a > D^b > E^b > L^b$
At P_3 : $\quad C^a > ß^a > D^b > E^b > L^c$

Specific concentration/pressure effects

P_1C_1 : $\quad ß^a > D^b > C^c > L^d > E^e$
P_2C_1 : $\quad ß^a > C^b > L^c > E^d > D^e$
P_3C_1 : $\quad ß^a > C^b > D^c > E^d > L^e$
P_1C_2 : $\quad ß^a > D^b > C^c > L^d > E^e$
P_2C_2 : $\quad C^a > ß^b > D^c > E^d > L^e$
P_3C_2 : $\quad C^a > ß^b > D^c > E^d > L^e$

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[1]　C=control, ß=ß-carotene, L=lycopene, E=lycopene-5,6-
epoxide, D=lycopene-5,6-diol.
P_1 = 2% oxygen, P_2 = 10% oxygen, P_3 = 20% oxygen.
C_1 = 10 μg carotenoid/g squalene, C_2 = 100 μg
carotenoid/g squalene.
Highest weight-gain on the left, decreasing to the
right.

[abcd]　Treatments in a row having the same letter are not
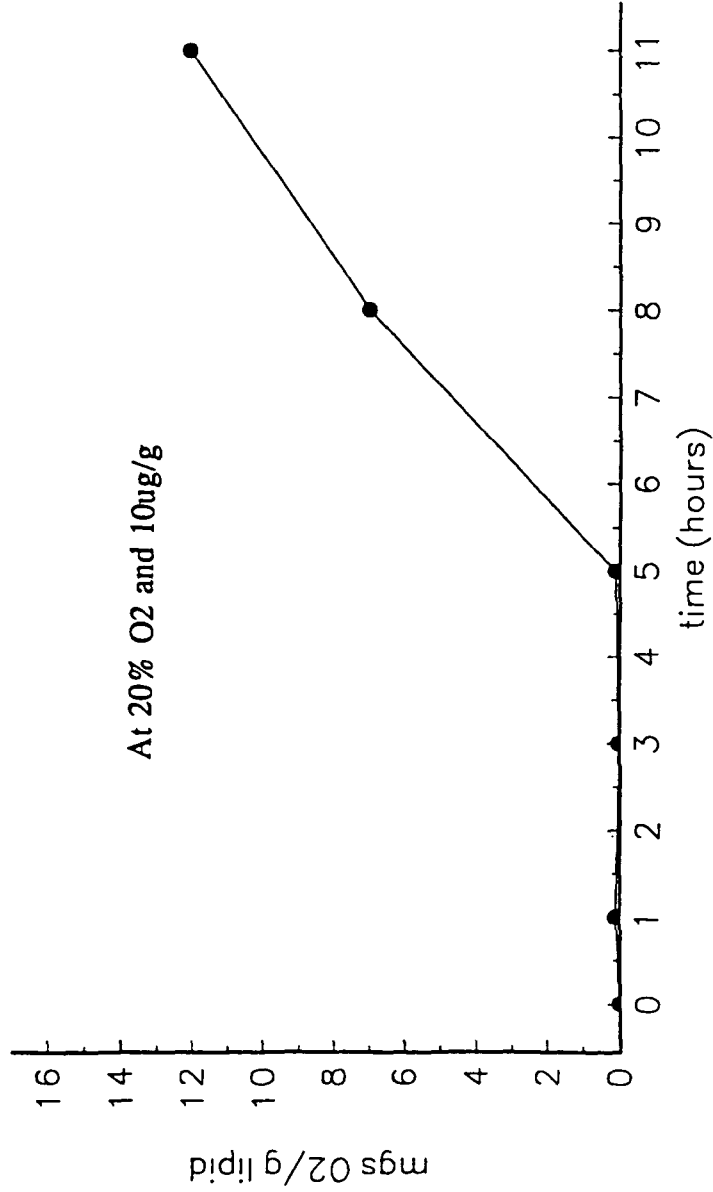statistically different at $\alpha=0.05$.

Figure A1. Oxygen weight-gain of squalene as influenced by lycopene-5,6-diol from time 0 to 12 hours