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**Role and control
of antioxidants
in the tomato
processing industry**

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**The White Book on antioxidants in tomatoes
and tomato products and their health benefits**

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Introduction

The European Commission Concerted Action FAIR CT97-3233 was set up in 1997 following the publication of several scientific papers showing a possible role of lycopene in the prevention of diseases such as cancer and cardiovascular diseases. Epidemiological studies showed that these risks increased with low tomato consumption, the main source of lycopene.

A consortium of 15 tomato research institutes and tomato processors from all around Europe was formed, with funding from the European Commission within the framework of the FAIR RTD programme, to review the scientific literature available in order to produce a "white book" on the antioxidants in tomatoes and tomato products. This data would help the industry improve the nutritional quality of the tomato-based products and the scientists to understand better the relationship between nutrition and health.

The researchers were organised into 4 working group to study different aspects:

- **Working Group 1 : Composition of tomatoes and tomato products in antioxidants** (methods of analysis; breeding and biotechnology to increase the antioxidant content; evolution of the composition during ripening and storage), chaired by Dr. Pascal Grolier (INRA, Clermont-Ferrand, France)
- **Working Group 2 : Processing and bioavailability** (effects of mechanical and thermal treatments and storage conditions on the antioxidants content and their bioavailability), chaired by Dr. Carlo Leoni (SSICA, Italy)
- **Working Group 3 : Epidemiological observational studies** (protection offered by antioxidants on cardiovascular diseases, cancer and ageing), chaired by Dr. Mariette Gerber (INSERM-CRLC, Montpellier, France)
- **Working Group 4 : Mechanisms and biomarkers** (mechanisms involved in the protection and biomarkers), chaired by Prof. Rod Bilton (Liverpool John Moores University, UK).

Project management and co-ordination were provided by Prof. Gérard Bartholin (CTCPA, Avignon, France), Mr. Bernard Bièche & Mrs Sophie Colvine (AMITOM, Avignon, France).

Composition of tomatoes and tomato products in antioxidants

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1. Introduction

Several epidemiological studies suggest that the consumption of fruit and vegetables would be beneficial to human health by reducing the risk of developing cancer and cardiovascular diseases (Koo 1997, Mayne 1996). However, this protective aspect is more difficult to estimate when one particular group of vegetables or one particular family of nutrients or micro-nutrients is considered (WCRF/AICR 1997). Fruit and vegetables are rich in constituents likely to contribute to the prevention mechanisms and the use of only one group of the constituents cannot totally demonstrate the potential interaction amongst them.

Recently a great deal of attention has been focused on the tomato and its products. Studies from Giovannucci *et al.*(1995) and Franceschi *et al.*(1994) reported that the consumption of tomatoes, tomato sauce and pizza was associated with a reduced risk of developing digestive tract and prostate cancers. Tomatoes are also one of the main part of the Mediterranean diet which has been associated with a low mortality from cardio-vascular troubles. Because tomatoes constitute the almost exclusive source of lycopene, this pigment could be one of the active agents of this protection.

Experimental studies reported that lycopene exhibited antioxidant activities (Di Mascio *et al.*, 1989), suppressed cell proliferation (Levy *et al.*, 1995) and interfered with the growth cancer cells (Clinton 1998). However, tomatoes are also rich sources of the essential nutrients vitamin C, potassium and folic acid, as well as beta-carotene, gamma-carotene, phytoene, selenium, flavonoids and phenolic acids which may exhibit antioxidant, immunostimulant, photoprotector or even chemopreventive activities on *in vitro* and animal models. In consequence, several of these constituents would contribute to the disease-preventive properties.

To date, limited information is available about specific composition of fruits and vegetables, including tomatoes. Mangels *et al.*(1993) published a food composition database for the 5 major dietary carotenoids, but no adequate table exists covering the composition in other antioxidant compounds.

The quality of a product can also be related to its composition in antioxidants. Thus, with tomatoes, the stability of the processed products, the nutritional value, the colour retention, the organoleptic qualities could be changed as a function of β -carotene, lycopene, flavonoids and vitamin C contents. Further insight into the antioxidant composition of tomatoes and the factors likely to modify this composition should help to better define the quality of tomatoes, to evaluate the intake of each antioxidant of humans and to assess the potential interactions among each of them during nutrition and public health studies. Such investigation would also contribute to improve information available to breeding and biotechnology companies, processing industries, health institutes and consumers.

2. Objectives

Our working group focused on the data collection of antioxidant composition in tomato and tomato products. A first database contains average values of all antioxidants we have found in literature. The antioxidant levels were reported for fresh tomatoes, but only few papers have reported values for specific varieties. So, information has been collected about the tomato varieties mainly used for the processing industry, and the concentrations of carotenoids and vitamin C have been collected in a second database.

Beside fruit ripening, there are a number of factors that influence taste, colour and antioxidant levels in tomato products. The effects related to tomato processing are reported by the working group two.

Different strategies can be applied in order to improve or to modulate the tomato quality and especially the carotenoid and ascorbic acid levels. Biosynthesis pathways of these compounds are described with a specific attention on the genes and enzymes involved in the metabolic steps. Breeding selection and biotechnology approach are presented.

The fruit colour and the antioxidant levels can progress during ripening. Cultural practices can also modulate both parameters. Thus, influences of cultural practices and ripening are described in this report.

Through epidemiological studies, associations between diseases and antioxidant consumption or blood antioxidant levels have been examined by the working group three. Thus, discussion about epidemiological data largely depends on the measured antioxidant values and on the method used. The different current methods are listed and critically described. Physical and chemical properties of lycopene, beta-carotene and vitamin C are described.

3. Composition of tomatoes in antioxidants

In the United States, about 29% of daily lycopene intakes (0.5-5 mg/day) come from tomato sauces, 12% from ketchup, 8% from juice, 8% from pizzas (total from processing tomato products: 57%) and only 12% from fresh tomatoes (Chug-Ahuja *et al.* 1993). Thus, it appears necessary to collect composition data not only for the market tomatoes but also for processing tomatoes.

Composition tables show that the tomato (*Lycopersicon esculentum*) contains 93-95% water and low levels of solid matter. Tomatoes are relatively rich in antioxidants: vitamin C (160-240 mg/kg), provitaminic A carotenes (6-9 mg/kg) (Table 1) and phenolic compounds (Table 2): flavonoids (5-50 mg/kg) and phenolic acids (10-50 mg/kg). Also present in small quantities are vitamin E (5-20 mg/kg), flavonoids (5-50 mg/kg) and trace elements such as copper (0.1-0.9 mg/kg), manganese (1-1.5 mg/kg) and zinc (1-2.4 mg/kg) which are present in several antioxidant enzymes. Most often the tomato variety is not indicated and the reported values are a mean concentration of the constituents in tomatoes found in local markets. In addition, tables contain values for the whole fruit but heterogeneous distribution of the compounds may occur in the fruit. Thus, antioxidant intakes may be different if skin or seeds are discarded. Hence, the “Campbell 146” variety was shown to contain 4 and 59 mg/kg whole fruit of β -carotene and lycopene respectively. But the concentration of β -carotene was 4-fold higher in the locular cavity than in the pericarp whereas the concentration of lycopene was 2-fold higher in the pericarp than in the locular cavity (Davies and Hobson 1981). Flavonoids and phenolic acids also seem more concentrated in the skin than in the flesh (Table 2) and vitamin E appears specifically located in seeds (Table 1).

Table 1. Basic analytical data for ripe tomato fruit

Composition	Database			
	1	2	3	4
(/kg fresh matter)				
<u>in g</u>				
Water	931	942	933	ni
Protein	7.0	9.5	9.0	9.6
Fat	3.0	2.1	2.0	ni
Carbohydrate	31.0	34.5	32.0	ni
<u>in mg</u>				
Fe	5.0	5.0	4.0	6.0
Cu	0.1	0.9	0.6	0.9
Zn	1.0	2.4	1.4	1.5
Mn	1.0	1.4	1.1	0.9
Vitamin C	170	242	180	160
Vitamin E	12.2	8.0	10.0	ni
Carotene	6.4	8.2	6.0*	7.6
Folates	0.17	0.39	0.23	ni
<u>in µg</u>				
Se	tr	6.0	tr	ni

ni : not indicated; * : beta-carotene Equivalent

- 1 B. Holland, A.A. Welch, I.D. Unwin, D.H. Buss, A.A. Paul and D.A.T. Southgate. The composition of foods. Fifth revised and extended edition. McCance and Widdowson's, UK, 1992 (flesh, skin and seeds)
- 2 H. Scherz and F. Senser. Food composition and nutritional tables 1989/90.5th revised and completed edition. Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1989.
- 3 M. Feinberg, J.C. Favier and J. Ireland-Ripert. Répertoire général des aliments. Table de composition REGAL. Technique et Documentation, Lavoisier, 1991.
- 4 H.C. Price *et al.* University of California. Veg. Crops. Series N°178, 171, 1976.

Table 2. Phenolic compounds in fresh red tomato fruit

	Concentration (mg/kg)	Part of fruit
<u>FLAVONOIDS</u>		
Quercetin glycosides ^a	3-7	whole
Kaempferol glycosides ^a	0.2-0.8	whole
Naringenin	8-42 0.8 64	whole flesh skin
Rutin	10-15	whole
<u>PHENOLIC ACIDS</u>		
Chlorogenic acid	13-38	whole
Caffeic acid ^a	29 56	flesh skin
<i>p</i> -Coumaric acid ^a	16	whole
Ferulic acid ^a	7	whole
Sinapic acid ^a	2	whole
Vanillic acid ^a	1	whole
Salicylic acid ^a	1	whole

a : Calculated as the aglycone

After the review made by J. N. Davis and G. E. Hobson, Critical Reviews in Food Science and Nutrition, 15, 205-280, 1981.

Recent chromatographic methods have led to identify in tomatoes at least 17 different carotenoids of which the colouring and antioxidant properties may vary. Khachick *et al.*(1992) reported that tomatoes contain mainly lycopene and also β -carotene, ζ -carotene, phytofluene and phytoene and traces of lutein, α -carotene etc. The composition of carotenoids greatly depends on the variety. Such a variation is reported in Table 3 for fresh market tomato. Some red varieties, like Flavourtop and Beefsteak, contain up to 50 mg/kg of lycopene, of which 87% in the former and 56% in the latter of trans-isomers and very little β -carotene (Hart and Scott 1995). By contrast, Tigerella contains similar quantities of the 3 pigments. In the yellow varieties β -carotene is the major pigment. Finally, it should be noted that the wild varieties can contain up to twice the lycopene and vitamin C quantity of the cultivated varieties (Stevens and Rick 1986)

The composition of processing tomato vairetiee will be described in paragraph 6.1.4.1.

Table 3. The content of lutein, lycopene and β -carotene in some tomato varieties
(mg/kg)

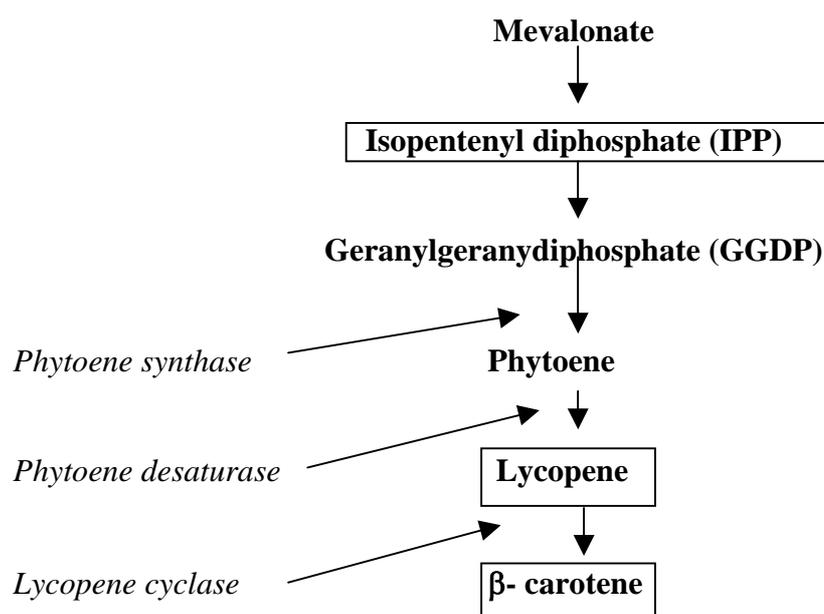
	Lutein	<i>trans</i> -Lycopene	Total Lycopene	<i>trans</i> - β -carotene
<i>Red varieties</i>				
Cherry	1.0	26.9	37.8	4.7
Flavourtop	0.5	49.6	56.5	4.3
Tigerella	1.9	12.2	15.8	17.0
Beefsteak	0.9	27.3	48.3	8.8
Craig	1.5	29.5	39.1	10.9
<i>Yellow varieties</i>				
Sungold	2.0	3.9	5.3	22.3
Gold sunrise	1.1	0.2	0.2	0.9

Adapted from Hart and Scott, Food Chem. 54: 101-111, 1995.

4. Breeding and biotechnology methods for high lycopene content

The biochemical pathway involved in lycopene production has been largely elucidated. Until recently, the mevalonate (MVA) (Fig 1) pathway was believed to be the only route for the provision of the key isoprenoid intermediate, IPP (Britton 1990).

Figure 1. Main stages of carotenoid synthesis in plants



Recently a non-MVA pathway has been identified (Schwender *et al.*1996). This is localised in the plastid in contrast to the MVA pathway which is localised in the cytoplasm of the cell. In this pathway, glyceraldehyde 3-phosphate (GAP) and pyruvate are the first precursors. Pyruvate is first decarboxylated and then condensed on the carbonyl of GAP yielding 1-deoxy-d-xylulose 5-phosphate and finally IPP. This novel mevalonate-independent biosynthesis pathway was first demonstrated in bacteria and then was shown to be involved in the carotenoid synthesis in higher plants.

Then condensation of IPP molecules results in Geranylgeranyl diphosphate (GGDP) formation. The first C₄₀ carotenoid phytoene is formed from two molecules of GGDP. The non-coloured phytoene undergoes a series of sequential desaturation reactions to produce phytofluene, ζ-carotene, neurosporene and finally the coloured lycopene. It was suggested that during these reactions *cis-trans* double bond isomerization occurred. Finally, cyclization

of the two end-groups results in β -carotene formation. A collection of genes for the early isoprenoid/carotenoid specific pathway have been isolated from bacterial, yeast, and plant sources (Bird *et al.* 1981, Sandman G 1994). Up to date details of this are being prepared as part of the “Neodiet” Concerted Action.

Table 4. Genes that influence the biosynthesis of carotenoids in tomato fruits

Genes	Abbreviation	Gene action
t, t-v	tangerine	Structure (<i>cis</i> , <i>trans</i>) of the polyene chain of carotenoid molecules
B	Beta-carotene	Cyclization of lycopene in β -carotene
Del	Delta-carotene	Cyclization of lycopene in δ -carotene
gh, gh-2	ghost	Block in the dehydrogenation of phytoene
vo	virescent orange	Block in dehydrogenation of ζ -carotene
hp, (hp1, hp2, bs, dr)	high pigment	high total carotene content
Ip	Intense pigment	Idem
og (Cm, Cr, crn-2, cr2)	old gold	Idem
gf	green flesh	Decrease in chlorophyll degradation with slightly increase in β -carotene
r (r:3, r-2, r2)	yellow flesh	Low total carotene content
at	apricot	Idem
nor	non-ripening	Low total carotene content and fruit ripening greatly retarded
rin	ripening inhibitor	Idem
Nr, Nr-2	Never ripe	Idem
alc	alcobaca	Idem
Gr	Green ripe	Idem
u, u2	uniform ripening	Uniform fruit colour

The availability of the genes (table 4) now opens the way to enhance levels of lycopene in tomato fruit using the following general strategies:

4.1 Breeding

In a breeding programme it is possible to accumulate the genes that increase the lycopene content and to eliminate those genes that decrease the lycopene content. There are several ways to accomplish this:

First of all, it is very important to have a good genetic base to start with. All the desired agronomic traits need to be available in the breeding nursery. Incorporation of the genes that increase lycopene content and elimination of genes that decrease the lycopene content can be done by pedigree selection and backcross programme. That this approach can be successful has been proven by the Israeli company LycoRed. Together with the Volcani Institute they developed hybrids with 3 or 4 folds content of lycopene in tomato fruits.

During natural or breeding selections, certain mutations can be produced on genes which regulate the synthesis of enzymes in the metabolism of carotenoids. For example, the mutants *t* accumulate the metabolic precursors of lycopene : phytoene, and ζ -carotene but contain no lycopene (Table 5) (Tomes 1963).

Table 5- The carotenoid composition of tomato mutants (g/kg dry matter)

Mutant	Phytoene	Lycopene	β -carotene	ζ -carotene	δ -carotene
normal	2.9	4.4	0.5	-	-
<i>r</i>	1.0	-	trace	-	-
<i>t</i>	5.1	-	0.1	4.5	
<i>del</i>	1.3	1.7	0.6	0.1	3.3
<i>hp</i>	1.5	5.6	0.7	-	-
<i>og</i>	2.5	5.0	0.3	-	-

After Tomes 1963.

In this case, the phytoene desaturase is apparently absent from the fruit. The mutant *Del* accumulates mainly the δ -carotene which is an intermediary in the synthesis of carotene. Certain types tested, for example the mutant *hp*, accumulate lycopene. This can either be the consequence of over-expression of phytoene desaturase (lycopene synthesis) or of repression of lycopene cyclase (β -carotene synthesis).

The variations reported for the composition of tomatoes might hinder interpretations of epidemiological studies in nutrition. At the opposite, such variations can be a welcome tool for breeding and biotechnology selections. Breeders can, by pedigree selection and backcross programmes, propose new tomato varieties. Marker assisted breeding (MAB) could be a welcome tool to fasten the breeding programme. Tomato is very well developed in this regard. There is a gene map available. Genes are well described and markers are available (Iso-enzymes, RAPD's, RFLP's, AFLP's and micro-satellites).

4.2 Genetic modification approach

Another strategy to increase carotenoid levels may be to introduce into a plant one or several genes coding for carotenoid biosynthesis using bacteria vectors such as *Agrobacterium tumefaciens*. Such strategy produces new plants, genetically modified, enriched or depressed in lycopene content (Bird *et al.* 1991, Fray and Grierson 1993) with:

1. overexpression of IPP generating enzymes
2. over-expression of late general isoprenoid enzymes
3. over-expression of carotenoid biosynthesis specific enzymes
4. inhibition of lycopene cyclase.

The preferred strategy uses the following principles:

1. Expression of new genes should be fruit/ripening specific
2. Overexpression of rate-limiting genes is desirable.
3. The use of enzymes which are not subject to metabolic control are desirable.
4. Combination of genes likely to yield highest levels of lycopene deposition.

Therefore, the use of DX Synthase, IPP Isomerase GGPP Synthase, Phytoene Synthase and the inhibition of lycopene cyclase offer the most promising strategy.

However, there are also other considerations:

1. Tomato mutants exist which deliver high lycopene. One route chosen is to increase plastid number. Therefore an alternative approach could be the increase of chromoplast number within the tomato.
2. Another approach could be to store lycopene in the vacuole.
3. A further approach is to use carotenoid associated proteins to increase deposition of lycopene.

These approaches are more technical challenging as the basic enabling research has not yet been carried out.

5. Influence of maturity stages and storage on the antioxidant composition of tomatoes

The colour and the composition of the fruit greatly vary during ripening. In the unripe fruits, the green colour is associated with chlorophylls and a low quantity of carotenoids. It should be noted that in these fruits the biosynthesis of carotenoids is active but the carotenoids are immediately broken down due to photoprotective and antioxidant properties (Giulano *et al.* 1993, Fraser *et al.* 1994). During the ripening period, the chlorophylls gradually disappear and become undetectable 7 days after the breaker stage. Simultaneously, even though the enzymatic activities of phytoene synthetase, phytoene desaturase and lycopene cyclase are reduced, both the expression of genes coding for phytoene synthetase and phytoene desaturase and the content in lycopene and β -carotene increase. At the turning stage, lycopene content considerably increases and can reach 80-100 mg/kg fresh matter at red stage (Giulano *et al.* 1993, Fraser *et al.* 1994).

The content in chlorogenic acid is the highest in earlier stages of fruit development and rapidly decreases during fruit ripening reaching less than 20 mg/kg fresh matter at red stage (cv Ailsa Craig and Pik-Red) (Aziz *et al.* 1973). This evolution is similar for numerous fruits (Buta and Spaulding 1997). While quinic conjugates of phenolics, like chlorogenic acid, are declining, glucoside conjugates (p-coumaric-glucose) become high in mature stages, especially in pulp (around 60 mg/kg fresh matter at red stage for the two cultivars previously cited). Glucose derivatives were suggested as biomarkers for maturation by Fleuriet and Macheix (1985). Rutin (or quercetin-3-rutinoside), specially located in tomato pericarp and

well known for its antioxidant properties, also decreases during ripening, especially between the immature green and mature green stages.

The maximum concentration of ascorbic acid is estimated in tomato fruits at yellow turning stage (around 250 mg/kg fresh matter for numerous cultivars). This content decreases during ripening. This decrease is certainly due to its antioxidant function when the ripening cells absorb high amount of oxygen; this is the result of an increase in respiration, characteristic of climacteric fruits (with an autocatalytic synthesis of ethylene) (Macheix *et al.*1990).

Changes in tocopherols are different among α , β and γ forms. There is an increase in α and β -tocopherol during ripening reaching 3 mg/kg fresh matter for α and 0.5 mg/kg fresh matter for β isomer at the red stage; γ -tocopherol reaches a maximum at colour break stage, between 1 and 1.5 mg/kg fresh matter. Its level declines in a similar manner than ascorbic acid (Abushita *et al.*1997).

6. Influence of cultural practices and agronomic aspects on antioxidants content of tomato fruit

6.1. Lycopene, β -carotene and total carotene

6.1.1. Introduction

The colour of red tomatoes is determined primarily by their lycopene content (Stevens and Rick, 1986). Johjima and Matsuzoe (1995) have shown that the fruit colour values (a/b) were highly correlated with lycopene content, including *cis* and *trans* forms. But according to Koskitalo and Ormrod (1972) surface colour could not be used to specify accurately the internal colour of fully ripened tomatoes. At lycopene concentrations between 32 and 43 mg/kg fresh matter, fruit colour turns from orange to red and satisfactory red coloration required a total carotene concentration in excess of 55 mg/kg fresh matter of which lycopene accounted for 90% of the carotenes. Giovanelli *et al.*(1999) measured the colour changes at seven stages during vine and post-harvest ripening on two genotypes (Normal Red and

Crimson) of the tomato cv. Moneymaker grown in a greenhouse. Instrumental determination of red colour and evaluation of a^*/b^* index (Hunter's ratio of red-to-green component of colour measured with a tristimulus Minolta Chromameter mod CR-210) made on the whole fruit skin were the most sensitive and significant indicators for fruit maturity, but they did not show a direct, unequivocal correlation with the lycopene content, since the same a^*/b^* value could correspond to lycopene contents differing by 100%.

From various sources of data in the 70's, tomato lycopene content may range from 43 to 181 mg/kg fresh matter, with most frequent values between 55 and 80 (Davies and Hobson, 1981). Sharma and Le Maguer (1996b) established that skins contained about five times more lycopene (540 mg/kg fresh matter; wet basis) than the whole tomato pulp (110 mg/kg). Most of the lycopene is probably attached to the insoluble and fibre portion of the tomatoes. The lycopene content in tomato pulp ranged from 64.6 to 107 mg/kg fresh matter (according to cultivars) whereas it ranged from 354 to 536 mg/kg in the wet insoluble fraction and from 0.074 to 0.34 mg/kg in the soluble fraction. It would be expected that tomatoes with high insoluble solids would contain more lycopene. In addition, Moretti *et al.* (1998) have shown that fruit bruising at the breaker stage could significantly decrease (-37%) the total carotenoids content in the locular tissue of tomato fruit at the ripe stage.

Many factors may influence the concentration of lycopene in tomato fruit: environmental factors (light, temperature) and cultural practices (variety, mineral nutrition, water alimentation, harvest date through ripening stage...).

6.1.2. Influence of temperature

McCullum (1954) had observed that tomato fruits exposed to direct sunlight in the field often developed poor colour due primarily to a low content of lycopene, because of the high temperatures of the exposed fruits. Lycopene synthesis in excised fruits from cultivars with various strains of pigments was drastically inhibited at 32°C in every strain which produced this pigment (Tomes, 1963).

From the breaker point, Koskitalo and Ormrod (1972) distributed greenhouse-grown tomato plants (c.v. Early Red Chief) in controlled environment chambers at 4 night/day

temperature regimes respectively. Twenty-one days later, the fruit carotenoids contents were decreased for lower regimes, except for β -carotene (Table 6).

Table 6. Effect of night/day temperature on carotenoid pattern in tomato (mg/kg fresh matter) (after Koskitalo and Ormrod, 1972)

Temperature (°C)	Phytoene	Phytofluene	β -Carotene	Lycopene
17.8/25.6	19.2	8.2	2.2	64.8
7.2/18.3	19.1	7.2	3.4	51.5
4.4/15.6	16.0	6.0	3.6	31.9
2.8/13.9	13.2	4.6	3.7	24.2

Baqar and Lee (1978) showed that 30°C temperature drastically reduced carotene synthesis in cultivar Rouge de Marmande, except for β -carotene (Table 7) and Grierson and Kader (1986) confirmed that the synthesis of lycopene, but not β -carotene, was inhibited by temperatures within the range 30-35°C.

Table 7. Temperature effect on carotene synthesis (after Baqar and Lee, 1978)

Carotene	Carotene content (mg/kg fresh matter)	
	20°C	30°C
Phytoene	18.4	5.6
Phytofluene	10.5	0.5
β-Carotene	7.5	6.6
ξ-Carotene	1.0	0.1
γ-Carotene	0.5	0.1
Neurosporene	0.4	0.1
Lycopene	40.5	1.1
Total	78.9	14.1

In fruit pericarp sections stored at various temperatures, Hamauzu *et al.*(1998) have observed that, at 20°C, the biosynthesis and accumulation of phytoene and lycopene were fast, and those of β -carotene was slow; by contrast, at 30°C, the biosynthesis and accumulation of lycopene and β -carotene were fast and phytoene accumulation was slow; at 35°C the biosynthesis and accumulation of β -carotene was fast but slower than at 30°C and accumulation levels of phytoene and lycopene were very slow. Hamauzu *et al.*(1998) have postulated that high temperature (35°C) inhibits the accumulation of lycopene in tomato fruit because of the stimulation of the conversion of lycopene to β -carotene.

In conclusion, the formation of lycopene depends on temperature range and seems to occur between 12°C and 32°C (Leoni, 1992). It is optimum in cell suspension culture of VFNT tomato for temperature between 18 and 26°C (Robertson *et al*, 1995). It is also optimum in the temperature range 16-26°C for tomato fruits (cv. Sunny) harvested at the pink-ripe stage and stored for ripening (Türk *et al.*1994) as presented in Table 8. The production of lycopene is hindered by excessive sunlight and the best conditions are temperature high enough together with dense foliage to protect fruit from direct exposure to the sun (Leoni, 1992). However in processing tomatoes, lycopene accumulation was enhanced by 5% at incubation temperatures of 30 and 34°C and by 33% at 37°C while lycopene accumulation declined in salad tomatoes above 30°C (Boothman *et al*, 1996). Indeed, processing tomatoes may often be exposed to temperatures of 35-40°C during the storage period prior to processing and over-ripening may occur accompanied with high lycopene content.

Table 8. Total lycopene formation (mg/kg fresh matter) in tomato fruits (cv. Sunny) harvested at the pink-ripe stage and stored under the conditions of 0 and 18°C and 90% Relative Humidity (after Türk *et al*, 1994)

	Storage temperature (°C)	Storage duration (weeks)					
		1	2	3	4	5	6
Total lycopene	0	3.58	3.50	4.87	5.63	15.37	15.66
	18	3.58	4.79	7.75	37.83	-	-

6.1.3. Influence of light

At favourable temperatures (22-25°C) the rate of development of lycopene is increased by illumination. Light is effective until chlorophyll disappears. Carotene is increased by illumination of tomato fruits during ripening. If exposed to direct sunlight during development, the fruits will be higher in carotene when ripe than shaded fruits (McCollum, 1954).

Lipton (1970) found that the incidence of defective coloration of the shoulders or sides of fruits was higher in fruit exposed to the sun than in fruit shaded by foliage and seemed to be influenced by infrared and short-wave radiation. Thomas and Jen (1975) established that red light and the intensity of red light had a positive effect on carotenoid synthesis of detached mature-green tomato fruit and this effect was not a temperature effect. Below saturation but above a critical minimum, the phytochrome-mediated response to red light depended linearly on the log of the incident energy. The involvement of phytochrome in carotenoid biosynthesis was suggested. The far-red light suppressed carotenoid production, as compared to dark control.

Cabibel and Ferry (1980) compared the content in carotenoids of tomato fruits produced under glass, plastic tunnel or in open field. β -Carotene content was equal or lower under glass or plastic tunnel than in open field. The lycopene content increased from glass to plastic tunnel to field. Carotenoid's contents were higher in open field than under glass or plastic tunnel. The level of intercepted light may influence carotenoid content. But interactions with high temperatures in protected cultivation may also have occurred.

Adegoroye and Jolliffe (1987) observed that in tissues of fruit directly exposed to radiation (650 W m^{-2} from incandescent lamps inducing visible injury), lycopene accumulation was blocked. Lycopene is the carotenoid mainly affected by exposure to intense solar radiation. It has been suggested that radiation injury in tomato fruit might be due to generalised effects of overheating in irradiated tissues.

6.1.4. Influence of cultural techniques

6.1.4.1. Variety

The main tomato varieties cultivated for processing in the E.C. are : Perfect Peel (PS 1296), Hypeel 244, Titano M, Rio Grande, Heinz 7151, Heinz 3044, Isola, Guadalete (PS 121), Snob (EXH 98063), Soprano, Rio Fuego, Avalon, Cannery Row.

Abushita *et al.*(1997) analysed tomato fruit from various poorly known cultivars cultivated in Hungary and found variations on more than 1 to 3-fold: β -carotene varied from 1.15 to 3.7 mg/kg fresh matter and total carotenoids from 18.5 to 60.7 mg/kg fresh matter.

Data from P. Spigno (Cirio Ricerche, Acerra, Naples; personal communication of unpublished data 1998, 1999) give fruit lycopene content for 24 varieties analysed from not replicated samples in 1998 and for 29 varieties (not all the same as the previous year) cultivated in the same conditions in 1999. The mean value was 86.0 mg/kg fresh matter in 1998 and 87.0 mg/kg fresh matter in 1999. The maximum range of values was 34 to 150 mg/kg fresh matter in 1998 and 45 to 163 mg/kg fresh matter in 1999, i.e. about 1- to 4-fold.

Table 9 gives data extracted from the Degree Thesis 'Carotenoid content in tomato and its processing products' (Zanetti, 1996/97).

Table 9. Carotenoid content in fresh tomatoes (mg/kg fresh matter) (after Zanetti, 1997)

Cultivar	Lycopene	Neolycopene(*)	β-Carotene	Phytofluene(*)
Isola	93.8	17.3	2.4	4.5
EXH 98063	87.9	17.6	2.7	4.3
PS 1296	94.9	18.1	2.5	4.1
PS 121	90.9	17.9	2.6	4.7
Calroma	89.1	17.4	2.4	4.2

(*) as lycopene equivalent

Siviero *et al.*(2000) have measured fruit lycopene content in hybrids “HP” (high pigment) cultivated in open field in comparison with Perfectpeel a hybrid not “HP” as control (Table 10). They found variations ranging almost from 1- to 2-fold.

Table 10. Lycopene content in tomato hybrids “HP” compared with Perfectpeel not “HP” (after Siviero *et al.*, 2000)

Hybrid	Gardner colour a/b	Lycopene (mg/kg fresh matter)	Lycopene (mg/kg dry matter)
H-01	2.39	118	2510
H-04	2.35	111	2620
DRD 8133	2.51	170	3750
DR 10747	2.60	188	3460
DR 10750	2.40	125	2570
ES 85/95	2.25	113	2520
Nun 1365	2.53	137	3020
Nun 4994	2.40	129	2930
PLX 1129	2.36	101	2440
PS 1617	2.49	175	4210
Forum	2.57	146	3540
Perfectpeel (control)	2.34	116	2710

6.1.4.2. Water

In a study of the effect of 4 irrigation regimes (40, 50, 60 and 70% depletion of available soil moisture) on tomato cultivars Pusa Ruby, Pusa Early Dwarf and Sioux, the lycopene content in tomato fruit was reduced by moisture stress (Naphade, 1993). If this were verified, there could be an antagonism between improving some basic technological characteristics of the tomato fruit juice (dry matter content, °Brix, acidity) and improving lycopene content by means of water management at the field level.

On the contrary, in red and pink cherry tomato cultivars, Matsuzoe *et al.*(1998) found that total carotene of fully ripe fruits and the amount of lycopene were increased by soil water deficit. In red and pink large-fruited tomatoes, soil water deficit also tended to increase the amount of lycopene per fresh matter in the outer pericarp region but it had no effect on the amount and distribution of β -carotene and xanthophylls (Zushi and Matsuzoe, 1998).

6.1.4.3. Mineral nutrition

Effect of Nitrogen

Aziz (1968) has cultivated tomato cv. Moneymaker in nutrient solution at three levels of nitrate nitrogen (1.0, 12.9 -considered as normal level- and 15.8 milli-equivalents). Fruit under the low N level were earlier and higher in gaining both total carotene and lycopene than those under the normal or the high N levels (1.0, 12.9 and 15.8 m.e. yielded 68, 44 and 38 mg/kg fresh matter fruit of lycopene content respectively). On the contrary yield increased with N level. It was concluded that for good colour in tomato fruit, N supply should be as low as possible without reducing fruit yield.

Montagu *et al.*(1990) cultivated tomatoes in pots filled with peaty loam soil. Various forms (nitrate, ammonium, compost, blood and bone) of nitrogen were applied at four rates (0, 150, 300, 600 kg N/ha). Fruit lycopene content tended to increase in average of 30% when N applied increased, except with compost.

More generally, nitrogen fertilizers have been presented as able to increase the concentrations of carotenes in plants (Mozafar, 1993), but few specific information seems to be available.

Effect of Phosphorus

Saito and Kano (1970) found that increased P supply (from 0 to 100 mg/l of nutrient solution) in hydroponic culture greatly improved the fruit colour and lycopene content. Few specific information seems to be available.

Effect of Potassium

For tomato plants (var. Fireball) grown in sand culture in a growth chamber (16-h light period at 24°C and 8-h dark period at 18°C, 65% relative humidity) and at any particular stage

of maturity, the fruit carotenoid contents were increased by increasing levels of K (0 to 10 meq per litre, applied when the first fruit were 1.0-1.5 cm diameter) in the nutrient solution, except β -carotene content (Trudel and Ozbun, 1970, 1971, Table 11). Lycopene content sharply increased when the K level in the nutrient solution was increased with a maximum at 8 meq. Chlorophyll (a and b) content of tomato fruit regularly decreased during ripening, from 8 mg/kg fresh matter to 1.5 mg/kg fresh matter in average. The decline of the green pigment was more pronounced in high K fruit, and disrupted the grana to a greater extent than in K-deficient fruit where carotenoid synthesis was lower.

Table 11. Tomato fruit carotenoid content (mg/kg fresh matter) in response to levels of K in the nutrient solution (after Trudel and Ozbun, 1970, 1971)

K level (meq/l)	Total carotenoids	Phytoene	Phytofluene	β-carotene	Lycopene
0	72	11.8	4.1	3.5	36.8
1	75	12.7	4.1	3.6	41.9
2	91	16.2	5.4	3.1	53.6
4	92	15.2	4.9	2.8	52.7
6	110	14.7	5.0	2.8	59.3
8	111	15.1	4.8	2.6	61.5
10	104	16.3	5.3	2.4	52.4

Winsor (1979) showed that increasing rates of potassium applied resulted in a striking decrease in the incidence of ripening disorders (mainly colour uniformity) as well as in increasing levels of titratable and total acidities (Table 12).

Table 12. Effect of potassium addition on ripening disorders (after Winsor, 1979)

	Cultivar	Potassium applied (kg/ha)		
		404	807	1614
% unevenly ripened (all forms)	Moneymaker	38.9	14.9	2.1
	J 168	26.1	8.6	1.4
% unevenly ripened (severe forms only)	Moneymaker	29.5	9.2	1.9
	J 168	18.0	5.0	1.7

It clearly appears that K deficiency may lower the rate of carotenoid synthesis, particularly lycopene. However, the rates of K supply applied in the study presented above and which led to responses in ripening uniformity are very high. They should be considered as non adapted to cultural practices, particularly in modern agriculture which is supposed to reduce inputs, spare resources and respect environment.

Effect of Calcium

In a soil-pot culture experiment, Subbiah and Perumal (1990) tested the influence of sources of calcium (calcium oxide, calcium chloride, calcium sulphate), concentrations (0.0%, 0.1%, 0.2% and 0.3% Ca), stages (S1 = 30th day after transplanting, S2 = 45th day after transplanting, S3 = 60th day after transplanting), and number of sprays (S1, S2, S3, S1+S2, S1+S3, S1+S2+S3, S2+S3) on physico-chemical properties of tomato fruits, cv. Co.3. Moreover, all the pots received also 120 kg N/ha, 100 kg P₂O₅/ha and 100 kg K₂O/ha. They could significantly increase the lycopene content (from 85 to 263, 300 and 340 mg/kg fresh matter) of the tomato fruit by calcium sprays at 0.0, 0.1, 0.2 and 0.3% Ca concentration respectively, whatever the source of calcium. It would be interesting and necessary to verify these results.

On the contrary, Paiva *et al.* (1998) cultivated tomato plants cv. Jumbo hydroponically in a greenhouse and imposed different calcium doses in the nutrient solutions. Total carotene and lycopene levels (expressed in fresh matter) in the tomato fruit decreased with increasing

Ca concentration, possibly due to the simultaneous reduction in potassium absorption because of competitive effect between the two cations.

Effect of combined Nitrogen-Phosphorus-Potassium and others

Some studies indicate an influence of combined fertilizers on fruit lycopene content. Bagal *et al.*(1989) obtained higher lycopene content (cultivar Pusa Ruby) with combined nitrogen, phosphorus and particularly potassium application (Table 8). Lycopene content increased even when expressed in relation to soluble solids. Increasing supply of phosphorus together with nitrogen might result in a decrease in lycopene content. But in this experiment the cultivar was ancient, it is surprising that °Brix largely varied while fruit moisture remained constant and, moreover, the values of lycopene content were so low that there might be a unit error or the data are not reliable. Lacatus *et al.*(1994) also found that increasing nitrogen supply (100, 200, 300 kg/ha) combined to various rates of phosphorus (75 and 150 kg/ha) or of potassium (75 and 150 kg/ha) or of half-fermented organic manure (20, 40, 60 t/ha) influenced positively the fruit lycopene content in average : from 61.8 mg/kg fresh matter for 100N and 200N to 66 mg/kg fresh matter for 300N. But the cultivar used is not well known.

Table 13. Effect of combined nitrogen, phosphorus and potassium on tomato lycopene levels (after Bagal *et al.*, 1989)

Fertilizer treatments (kg/ha)			Fruit moisture (%)	Soluble Solids SS (°Brix)	Lycopene (mg/kg fresh matter)	Lycopene (mg/kg SS) ⁽¹⁾
N	P ₂ O ₅	K ₂ O	(%)	SS (°Brix)	(mg/kg fresh matter)	(mg/kg SS) ⁽¹⁾
0	0	0	94.8	4.2	2.0	47.6
100	50	0	94.9	5.4	2.6	48.1
200	100	0	94.8	5.3	2.5	47.2
400	200	0	94.6	4.8	2.1	43.7
100	0	50	94.4	5.6	3.0	53.6
200	0	100	94.1	5.9	3.3	55.9
400	0	200	94.8	6.1	3.4	55.7
100	50	50	94.6	5.6	3.6	64.3
200	100	50	94.7	6.0	3.9	65.0
400	200	200	94.7	5.7	3.7	64.9

(1) added calculation

In conclusion, the available results are either rare (nitrogen, phosphorus) or not always reliable (calcium, combined fertilizers) or usable (cultivar) or, when they are well established, they hardly correspond to current cultural practices.

6.1.5. Ripening stage

Laval-Martin *et al.*(1972) have reported that during cherry tomato growth and maturation, the metabolism of carotenoids and of chlorophylls is first preferentially situated in the center of the fruit and then in the external areas. The increase in foliar-type pigments is followed by a stagnation or even a reduction (chlorophylls, neoxanthin) while lycophyll and lycoxanthin continue to increase and new pigments appear which are characteristics of the tomato fruit (phytofluene and mainly lycopene). Several studies present pigment changes in tomato fruit during ripening.

Fruits of green Homestead variety were harvested and ripened at 22°C and carotenoids were analysed at 6 stages of maturity (Meredith and Purcell, 1966). Lycopene content increased from breaker stage to red stage (Table 14).

Table 14. Lycopene concentrations in tomato during ripening

(after Meredith and Purcell, 1966)

Stage of maturity	Lycopene content (mg/kg fresh matter)
Mature green	-
Breakers	0.41
Turning	0.44
Pink	9.01
Light red	22.3
Red	82.8

Trudel and Ozburn (1970) grew tomato plants (var. Fireball) in sand culture in a growth chamber (16-h light period at 24°C and 8-h dark period at 18°C, 65% relative humidity), and observed that fruit total carotenoids increased constantly during ripening from 0.1 to 70 mg/kg fresh matter.

Tomato plants (c.v. Early Red Chief) grown in the greenhouse in soil containers were placed in a controlled environment at 17.8/25.6°C night/day temperature from the breaker point and fruit were harvested at 7, 14 and 21 days (Koskitalo and Ormrod, 1972); the concentrations of various carotenes were measured and are reported in Table 15.

Table 15. Time course of carotene levels (mg/kg fresh matter) in tomatoes at various harvest dates (after Koskitalo and Ormrod, 1972)

Harvest time	Phytoene	Phytofluene	β-Carotene	Lycopene
7 days	18.1	5.9	3.0	43.5
14 days	23.0	7.9	2.2	57.8
21 days	19.2	8.2	2.2	64.8

(1) f. m. : fresh matter

In tomato fruit cv. Moneymaker cultivated in greenhouse, Rabinowitch *et al.*(1975) observed a rapid fall in chlorophyll content with the onset of the mature-green stage and chlorophyll disappeared completely at the beginning of the pink stage. During the same period of time, the tissue content of β -carotene was doubled. Lycopene and its colourless precursors phytoene and phytofluene began to accumulate following the breaker stage. At the ripe-red stage, lycopene constituted 95% of the coloured carotenoids, or 73% of the total carotenoids including phytoene and phytofluene.

Cabibel and Ferry (1980) considered 5 stages : 1 (mature green), 2 (white), 3 (yellow), 4 (pink), 5 (red). The β -carotene content increased regularly in open field while lycopene increased sharply between the stage pink and the stage red. Lycopene content could be a good index of maturation degree (Table 16).

Table 16. Carotenoids content of tomato fruit in open field

(after Cabibel and Ferry, 1980)

	Stage of ripeness	Dry matter content (%)	β -carotene (mg/kg f. m.)	β -carotene (mg/kg d. m. ⁽¹⁾) ⁽²⁾	Lycopene (mg/kg f. m.)	Lycopene (mg/kg d. m.) ⁽²⁾
First truss	1	6.79	6.75	99.41	-	
	2	6.93	7.09	102.31	-	
	3	7.01	10.99	156.78	-	
	4	6.72	26.06	387.80	47.13	701.34
	5	6.86	30.12	439.07	106.55	1553.2
Sixth truss	1	8.34	9.69	116.19	-	
	2	7.75	10.42	134.46	-	
	3	8.08	15.07	186.51	-	
	4	8.10	23.91	295.18	28.46	351.36
	5	8.33	28.48	341.90	129.15	1550.42

(1) d. m. : dry matter

(2) added calculation

López-Andréu *et al.* (1986) considered 7 stages of ripeness : I (mature green), II (green yellow), III (yellow-orange), IV (orange-yellow), V (orange-red), VI (red), VII (deep red). Lycopene increased from 0.14 mg/kg fresh matter, slightly until stage IV then by a sharp rise up to 80.77mg/kg at VII. β -carotene increased from 1.05mg/kg (I) to 4.55mg/kg (III) then decreased to 0.42mg/kg (VII). The changes in lycopene percentage are presented in Table 17.

Table 17. Relative content of lycopene during ripening (after López-Andréu *et al.*, 1986)

Stage	I	II	III	IV	V	VI	VII
Lycopene % total carotenoids	12	55	72	82	90	95	99.5

Giovanelli *et al.*(1999) evaluated the variation in the antioxidant content at seven stages during vine and post-harvest ripening on two genotypes (Normal Red and Crimson) of the tomato cv. Moneymaker grown in a greenhouse. In vine-ripened tomatoes lycopene and β -carotene concentrations progressively increased linearly during the ripening process. Conversely, in post-harvest-ripened fruit lycopene and β -carotene accumulation followed an exponential trend. At the end of the experiments, the lycopene and β -carotene concentration (roughly 125-130 and 12 mg/kg fresh matter respectively) in post-harvest-ripened tomatoes was almost twice the value reached in vine-ripened tomatoes (roughly 75-80 and 5-7 mg/kg fresh matter respectively) having the same colour (a^*/b^*) index. As a consequence, adequate post-harvest storage can result in increased fruit lycopene content. β -carotene synthesis was observed on both genotypes under both ripening conditions for a longer time than that usually considered in tomato ripening studies, particularly from Koskitalo and Ormrod (1972) who observed that β -carotene synthesis stopped after tomato colour had changed from orange into red.

All the studies indicate the same trend of lycopene content to sharply increase during the last period of ripening (from pink stage). However it remains difficult to compare the results of the previous studies because the cultivars used were different and the stages considered were not the same and described with subjectivity. The data available in the papers do not generally allow to calculate temperature sums which could allow comparisons or modeling.

6.1.6. Growth and development regulators

The following growth and development regulators have been experimented on tomato plants in various conditions :

DCPTA (2-(3,4-dichlorophenoxy)triethylamine) is a bioregulator of lipid and protein synthesis which has been shown to increase the harvestable yield of numerous crop plants by maintaining a balanced partitioning of photosynthetate between plant growth and crop yield.

CPTA (2-(4-Chlorophenylthio)triethylamine hydrochlorid) is a bioregulator considered as an enhancer of protein synthesis and a carotenoid inducer, stimulator of the disappearance of chloroplasts and the accumulation of chromoplasts (Robertson *et al*, 1995).

Ethephon ((2-chloroethyl)phosphonic acid) is a plant growth and development regulator (flower induction, dwarfing effect) with systemic properties, progressively decomposed to ethylene in plant tissues. It is also known to enhance the ripening of green tomatoes by accelerating colour development.

Gibberellins are plant growth promoters through stimulation of stem elongation, promotion of seed germination, inducement of flowering and fruit formation, changes of plant metabolism.

CCC or Cycocel (2-chloroethyl trimethylammonium 3-chloride), Phosphon (2,4-dichlorobenzyl tributyl phosphonium chloride) and Alar (succinic acid 2,2-dimethyl hydrazide) are growth retardants generally inducing dwarfing effects.

Duraset (N-metatolyl phtalamic acid) and the cytokinin Adenin (6-aminopurine) are growth substances exhibiting auxin.

Rabinowich and Rudish (1972) dipped full sized green tomato fruits cv. Moneymaker during one minute into CPTA and/or ethephon solutions at various concentrations (0 to 4800 mg/l) and observed colour development at room temperature or at 32°C. At room temperature, ethephon treatments enhanced fruit ripening and combining ethephon with CPTA resulted in faster and higher lycopene accumulation. At 32°C, only both CPTA and the combined ethephon and CPTA treatment resulted in an accumulation of red colour. CPTA appeared to prevent from the inhibition of lycopene synthesis by high temperature. The hypothesis of a pathway of lycopene synthesis different from the natural pathway in these conditions was proposed.

Detached fruit at the breaker stage of normal red and high-beta tomato genotypes were dipped by Chang *et al.*(1977) for 1 min in an aqueous solution containing CPTA (4300 µg/mL) and 1% Tween 80 as a surfactant, then were allowed to ripen at 21 or 32°C for 6 or 12 days. Treatment of normal red fruit generally resulted in an increased synthesis of phytoene, phytofluene, ξ-carotene, lycopene (up to 550-580 mg/kg dry matter) and of γ-carotene with a concomitant decrease of the synthesis of β-carotene when ripened for 6 and 12 days at 21°C. At 32°C, lycopene synthesis was much lower (up to 100 and 230 mg/kg dry matter for 6 and 12 days respectively) and not stimulated by CPTA treatment. The high-beta fruit produced relatively large amounts of β-carotene (about 1700 and 700 mg/kg dry matter at 21 and 32°C respectively) and small amounts of lycopene (1 and 120 mg/kg dry matter at 21°C/6 and 12 days respectively). CPTA reduced high-beta fruit β-carotene content of 31 to

50% at 21°C and of 11 to 22% at 32°C and increased high-beta fruit lycopene content up to 300 and 500 mg/kg dry matter at 21°C/6 and 12 days respectively.

Baqar and Lee (1978) immersed fruit of the cultivar Rouge de Marmande at the mature green stage in 5000 mg/l CPTA solution. It increased carotene synthesis at 20°C except for β -carotene (Table 18). At 30°C CPTA appeared to be a direct antagonist of the high temperature effect of lowering the total carotene content of the fruit, except for β -carotene. It could partially remove the block imposed by a temperature of 30°C on lycopene synthesis in red tomato fruit.

Table 18. Effect of CPTA exposure on carotene contents in tomato

(after Baqar and Lee, 1978)

Carotene	Carotene content (mg/kg fresh matter)			
	Control		CPTA	
	20°C	30°C	20°C	30°C
Phytoene	18.4	5.6	35.8	8.6
Phytofluene	10.5	0.5	18.6	1.0
β-Carotene	7.5	6.6	4.3	4.1
ξ-Carotene	1.0	0.1	2.1	0.2
γ-Carotene	0.5	0.1	0.7	0.1
Neurosporene	0.4	0.1	0.8	0.1
Lycopene	40.5	1.1	88.2	11.5
Total	78.9	14.1	150.5	25.7

CPTA was tested by Fosket and Radin (1983) for its ability to bring about carotenogenesis in suspension cultures of tomato cells (*Lycopersicon esculentum* cv. EP-7). Untreated dark-grown cultured tomato cells (from callus tissues) contain low levels of carotenoids. The addition of CPTA to the culture medium brought about a 60-fold increase in their total carotenoids (from 0.26 to 16.8 mg/kg fresh matter) during a 14-day culture period (lycopene content increased from 0.21 to 15.8 mg/kg fresh matter).

Keithly *et al.*(1990) applied DCPTA to tomato (cv. Pixie) as a pre-germination seed treatment, at 5 rates ranging 0-3-15-30-150 μ M (0-1-5-10-50 mg/kg) for 6h at 24°C with Tween 80 (0.1%, w/v). The seedling were then cultivated in soil pots in greenhouse at 24/18°C day/night temperature. Ripe fruit weight per plant was doubled from 0 to 10 mg/kg DCPTA, while the total soluble solids increased from 3.75 to 4.68 % fresh matter. Lycopene and β -carotene contents increased like 58.5-81-98-112-118 mg/kg and 2.2-3.2-4.3-5.2-5.7 mg/kg fresh matter respectively when DCPTA increased from 0 to 50 mg/kg. So 10 mg/kg was most efficient.

Hsu and Yokohama (1991) cultivated tomatoes (cv. UCD-82) in soil pots under greenhouse. Plants were treated either by seed imbibition or by foliar application at the three-leaf stage with 0, 5, 10, 20 and 50 mg/kg of DCPTA solutions plus 0.01% Tween 80 only once. DCPTA at 10 mg/kg almost doubled fruit yield and gave a 28% increase in lycopene (from 1454 to 1857 mg/kg dry matter).

CPTA induced an increase in lycopene synthesis of in vitro cultured VFNT Cherry tomato fruit which already naturally contained about 10-fold more lycopene than field-grown tomatoes (Ishida *et al.*, 1998, Table 19).

Table 19. Effect of CPTA on lycopene levels in Cherry tomatoes (after Ishida *et al.*, 1998)

	Fruit at 26°C		greenhouse	
	-CPTA	+CPTA	green fruit	red fruit
Lycopene				
(mg/kg f. m.)	340	640	1.1	200

In field grown tomatoes cultivated by Graham and Balletero (1980), Gibberellic acid and above all Cycocel increased fruit β -carotene content.

Field tomato plants were sprayed twice (25 and 40 days after transplanting) with chlormequat (CCC) or alar separately at 250, 500 or 1000 mg/kg by Gabr *et al.*(1985). Chlorophylls a and b and carotenoids content (dry matter basis) of tomato plants were

increased (the highest i.e. +10% after 15 days, +9% after 30 days and +14% after 45 days in carotenoids for 500 mg/kg) by the application of CCC or alar. Yields were increased by 250 mg/kg CCC (+7%) and by all doses of alar (+28% for 250 mg/kg, then with decreasing influence). Mean fruit weight was reduced by 5% by CCC and increased by 9% by alar. Most levels of CCC and alar seemed to increase total soluble solids in fruits, mainly alar (+10% at 135 days and +5% at 150 days after transplanting). No data about fruit carotenoids content were provided.

In conclusion, the results about DCPTA, CPTA or CCC influence on carotenoid concentration in tomato fruit seem to be interesting. They probably could hardly be directly applied to field processing tomatoes. Studies of feasibility in open field and of the various consequences of applications on the crop response should be undertaken.

6.2. Vitamin C

6.2.1. Introduction

Vitamin C concentration in tomato fruit may be influenced by many factors. Various studies performed by Hamner *et al.*(1945) have demonstrated that very large variations in ascorbic acid content of tomatoes may be associated with growing conditions, more than with varietal differences, and have indicated that a factor of primary importance in determining the ascorbic acid level may be the light intensity a few days previous to harvest.

From various sources of data collected in the 70's, Davies and Hobson (1981) mentioned a main influence of season, nutrition and environment on ascorbic acid content of tomatoes and they indicated that current mean values ranged from 150 to 230 mg/kg raw edible part of tomato fruit with a normal range from 84 to 590 mg/kg. Shi *et al.*(1999) demonstrated that for plum tomatoes (var. Heinz 9478) the ascorbic acid content in the juice in the locule section was 10% and 33% higher than in the mesocarp and pole sections respectively at full maturity. According to Stevens and Rick (1986), there is large variations in vitamin C levels among tomato cultivars and species (from 80 mg/kg in cultivated varieties to 1190 mg/kg in the wild species *L. peruvianum*). In spite of considerable effort to develop cultivars with high vitamin C levels, little success has been achieved. There appears to be a relationship between high vitamin C levels and relatively poor yields. Indeed Murneek *et al.*(1954) found that in the cultivar Marglobe and Stokesdale the larger the fruit the lower the

vitamin C content (20 mg for mean fruit weight above 85g and 27 mg for mean fruit weight 25-85 g). Thus, cultural practices may have a great impact on tomato fruit vitamin C content. Moreover, Grantz *et al.*(1995) demonstrated that total ascorbate contents were greatest in leaves, with decreasing amounts in stems and roots and relatively constant levels in all stages of fruit. Ascorbic acid content in developing fruit peaked at the light-red stage before full colour was reached. Ascorbate Free Radical (AFR) reductase activity was inversely correlated with enzyme activity in tissues examined. AFR reductase mRNA abundance increased dramatically in response to wounding. AFR reductase may contribute to maintaining levels of ascorbic acid for protection against wound-induced free radical-mediated damage. Indeed, Moretti *et al.*(1998) have demonstrated that vitamin C content of fruit bruised at the breaker stage was decreased of at least 15% in locular tissue and in pericarp tissue at the ripe stage in comparison to unbruised fruit. Citing the U.S. Department of Agriculture (1997), Beecher (1998) reported a Vitamin C content of cooked tomatoes of 230 mg/kg.

An investigation of literature knowledge of the influence of the main environment factors and of the main cultural factors is presented below.

6.2.2. Influence of temperature

Little information was found about the influence of temperature on vitamin C content. However, from various studies performed in control chambers by Hamner *et al.*(1945) ascorbic acid content was only slightly, if at all, influenced by storage of the fruit for 10 to 14 days at temperatures from 19.5 to 32°C, at various relative air humidity. Relatively high temperature of maturing or ripening fruit, whether on or off the plant, coupled with low light intensity, may probably lead to reduction in ascorbic acid due to oxidation (Murneek *et al.*1954). However, in greenhouse, Liptay *et al.*(1986) observed seasonal variations of cv. Jumbo fruit vitamin C content from 70 to 230 mg/kg fresh matter at the mature-green stage, in direct relation with temperature variations.

6.2.3. Influence of light

In experiences conducted by Hamner *et al.*(1945) on tomato cv. Bonny Best, a great influence on fruit ascorbic acid content was produced by variations in light intensity previous to harvest. Increases in the ripe fruit of 66% in ascorbic acid (from about 160-170 to 240-270

mg/kg fresh matter) resulted when plants were transferred from shade to sunshine at the time the fruit was mature green.

For tomatoes grown in sand culture (cv. Bonny Best) Somers *et al.*(1951) found that the ascorbic acid content of the fruits was associated with the degree to which they were shaded inside the plants: vitamin C content increased significantly from 298 mg/kg fresh matter in “full shade” fruit to 344 mg/kg fresh matter in “fully exposed” fruit. This was confirmed by Murneek *et al.*(1954): greenhouse-grown tomatoes (cvs. Marglobe and Stokesdale) were usually lower in vitamin C than those grown outdoors, due chiefly to lower light intensity and shorter days during late fall, winter and early spring. There seemed to be a seasonal increase in concentration in vitamin C in field-grown fruit from early summer to late summer (250 to 350 mg/kg fresh fruit). There was a strong positive correlation between vitamin C concentration and light intensity. In shade situation (by foliage) might reduce it by 15-20% compared to in light situation. The side of a tomato that was directly exposed to light was invariably higher in vitamin C than the shaded side. Brown (1954) also reported that fruit receiving direct sunlight were higher in ascorbic acid than fruits shaded by leaves or artificial cover, showing that, like many previous studies, light is the predominant factor in ascorbic acid production and accumulation in plant material. Venter (1977) demonstrated also that vitamin C content of tomato fruit (cv. Sieger) increased (from 250 to 400 mg/kg fresh matter) with the length of the radiation period, with differences between shaded or unshaded fruits on the same plant or between shaded or unshaded fruit sections. López-Andréu *et al.*(1986) also found lower fruit vitamin C values in greenhouse with less direct sunlight than for field cultivated fruit. Another field study on the effects of shading with netting (0, 35, 51 or 63% shade) conducted in Egypt on two tomato cultivars showed that ascorbic acid content decreased with increasing shading while the best yield components were obtained from plants grown under 35% shading (El-Gizawy *et al*, 1993). Adegoroye and Jolliffe (1987) found that in tissues of fruit directly exposed to radiation (650 W m⁻² from incandescent lamps inducing visible injury), ascorbic acid content was decreased, although treated fruit exhibited some capacity for ascorbic accumulation during subsequent storage. It has been suggested that radiation injury in tomato fruit might be due to generalised effects of overheating in irradiated tissues.

Light exposure seems to be favourable to vitamin C accumulation in the tomato fruit, somewhat like for carotene synthesis in fruit. Thus fruit vitamin C and β -carotene contents might be affected positively by not too close plant spacing to provide radiation and the use of

cultivars naturally presenting a somewhat poor foliage as well as the use of moderate nitrogen rates to avoid excess of vegetative growth.

6.2.4. Influence of cultural techniques

6.2.4.1. Variety

Hamner *et al.*(1945) estimated that variations of tomato fruit vitamin C content due to variety were small in comparison with variations caused by growing conditions.

Abushita *et al.*(1997) analysed tomato fruit from various poorly known cultivars cultivated in Hungary and found variations on about 1 to 2-fold : from 250 to 480 mg/kg fresh matter.

No data seem presently available for varieties currently cultivated in the European Community. Genetic improvement might be possible as the wild species *Lycopersicon peruvianum* reaches a fruit vitamin C content of 1190 mg/kg fresh matter.

6.2.4.2. Water

In tomatoes (var. Pusa Ruby) obtained from field experiments in winter and summer on a sandy loam medium fertility soil, Dastane *et al.*(1963) observed that fruit vitamin C content was increased by soil moisture depletion (40% and 50%) from 21 to 25.5 mg/100ml of juice mainly in winter. Rudich *et al.*(1977) confirmed that low water tensions maintained in soil by daily drip irrigation during the period of fruit development decreased fruit vitamin C concentration together with soluble solids content while yield increased. Sanchez Conde (1984) grew two cultivars of tomato (Marglobe and Moneymaker) in nutrient solution at three levels of osmotic pressure (0.65 -normal-, 3.5 and 5.0 atmospheres) obtained by increasing NaCl concentration. Fruit pulp vitamin C content decreased (231, 225 and 195 mg/kg fresh matter for Marglobe and 196, 181 and 154 mg/kg fresh matter for Moneymaker respectively) when osmotic pressure increased. But the total solids contents increased simultaneously (5.5, 9.5 and 9.6% for Marglobe; 5.4, 6.5 and 7.8% for Moneymaker respectively). An opposite trend was reported by Albu-Yaron *et al.*(1993): in tomatoes (cv. VF M82-1-8) grown in an aeroponic system in a greenhouse, the vitamin C levels were slightly positively affected (variations between 8.5 and 12 mg/100ml juice) by the increase of the osmotic potential

(between 0.06 and 0.45 Mpa) of the nutrient solutions depending on either Cl^- or/and NO_3^- . In a study of the effect of 4 irrigation regimes (40, 50, 60 and 70% depletion of available soil moisture) on tomato cultivars Pusa Ruby, Pusa Early Dwarf and Sioux, Naphade (1993) observed that ascorbic acid content in tomato fruit increased as moisture stress increased, together with total soluble solids, acidity, sugars, sugar:acid ratio, and percentage of cracked fruits. The 60% depletion regime produced also fruits of good size and the highest water use efficiency. However Zushi et Matsuzoe (1998) reported that the effects of soil water deficit on vitamin C content (fresh matter basis) varied, depending on the cultivar; in some cultivars vitamin C content increased, whereas in others it was unaffected. In tomato plants of two cultivars (Matador and Elin) grown in rockwool slabs under greenhouse, Petersen *et al.* (1998) showed that increased salinity (3 to 9 mS/cm) in the root zone increased the concentration of vitamin C in the tomato fruit.

Even if the varieties used in the previous studies are poorly known, it appears that water shortage tends to increase fruit vitamin C content, as well as dry matter and soluble solids content ($^{\circ}\text{Brix}$).

6.2.4.3. Mineral nutrition

Effect of Nitrogen

A number of studies over more than the last 50 years are consistent to demonstrate that increasing rates of nitrogen fertilizer tend to decrease the fruit vitamin C content.

According to Hamner *et al.* (1945) the influence of mineral nutrition on ascorbic acid content has been found to be of relatively minor importance for different varieties, although heavy application of nitrogen might cause some decrease; this decrease might be an indirect effect since it might be associated with the influence of the nitrogen application in producing greater foliage and resultant shading of the fruit on not pruned plants grown in the field in rows 3 feet apart; in previous studies, no influence of nitrogen supply was found in plants cultivated outside in sand pots 4 feet apart, with vines supported on poles, axillary growth removed and thus evenly illuminated by direct sunshine.

For tomatoes grown in sand culture (cv. Bonny Best), nitrate supply for the month prior the onset of ripening influenced fruit production and the ascorbic acid content of the

fruits (-10% out of 300 mg/kg fresh matter); high nitrate supply was associated with high fruit production, but with low ascorbic acid content; on the contrary, the nitrate supply after the onset of ripening influenced neither fruit production nor acid ascorbic content (Somers *et al.*, 1951). Working on cvs. Marglobe and Stokesdale, Murneek *et al.* (1954) observed that with increasing nitrogen supply there was a decrease in vitamin C concentration in the fruit and that differences due to nitrogen supply may be slight when the tomato crop is grown under a prevailing low light intensity but considerable in the presence of bright sunlight (250 to 320 mg/kg in ripe fruit). In field and pot trials established by Neubert (1959), high N-applications (160 or 320 kg N/ha) were followed by a decrease of vitamin C content in ripe fruits (up to -25 or -30% according to the seasons), bound to the existence of monosaccharides which were also decreased, due to a concurrency coming from the increased synthesis of N-compounds and organic acids, in spite of an enlarged surface of assimilation. Dastane *et al.* (1963) obtained tomatoes (var. Pusa Ruby) from field experiments in winter and summer on a sandy loam medium fertility soil; fruit vitamin C content was not influenced by increasing nitrogen supply (45 to 135 kg N/ha) with a tendency to decreasing in winter (21.5 mg/100ml juice against 24 in summer). Kaniszewski *et al.* (1987) demonstrated on cv. New Yorker cultivated in field that increasing nitrogen fertilization (preplant and/or top dressing at the rates 37.5, 75, 75 + 75, 150, 150 + 75, 225 and 150 + 150 kg N ha⁻¹) reduced fruit vitamin C concentration (3136, 2723, 2494, 2218, 2169, 2068, 1786 mg/kg dry matter respectively) while yield increased. According to Müller and Hippe (1987), in tomato fruits the vitamin C content (variations from 300 to 450mg/kg fresh matter) was inversely correlated with the N supply (pot culture in a mixture of sand and soil) and there was only very small amounts of dehydroascorbic acid present. Montagu *et al.* (1990) cultivated tomatoes in pots filled with peaty loam soil. Various forms (nitrate, ammonium, compost, blood and bone) of nitrogen were applied at four rates (0, 150, 300, 600 kg N/ha). Fruit vitamin C content decreased almost linearly from about 320 mg/kg fresh matter to about 230-250 mg/kg fresh matter when N applied increased from 0 to 600 kg/ha. A general review from Mozafar (1993) reports that increased application of nitrogen fertilizers has been shown to decrease the content of ascorbic acid in many plants; there are also reports indicating that nitrogen fertilizer may have no effect or may even increase the content of vitamin C; there are both types for tomato but nitrogen fertilizers, especially at high rates, often seem to decrease the concentration of vitamin C in tomato fruit. However, urea sprays (6 applications on foliage at fortnight intervals) on field grown tomato "Marglobe" increased vitamin C content from 200 to 240 mg/kg (Varma, 1970) and in sandy soils, fresh fruit vitamin C content was increased with the

higher level of N fertilization, like yield and yield components, but in the same time the percentage of soluble solids decreased (Merghany, 1997).

In conclusion it generally seems that nitrogen supply, especially at heavy rate, tends to decrease the vitamin C content of tomato fruit and that it could be an indirect effect of increased shading caused by a greater development of plant foliage favoured by high nitrogen availability. The present tendency to reduce N supply in agriculture as much as possible for objectives of food and environment quality and cost lowering would thus be favourable to keeping high vitamin C content in tomato fruit.

Effect of Phosphorus

Increased P supply in hydroponics did not influence the vitamin C content of fruit which yielded variations from 150 to 220 mg/kg (Saito and Kano, 1970). The effect of phosphorus cannot be discussed because of the lack of studies. Further research would be necessary.

Effect of Potassium

Very little was found about the specific effect of potassium on the tomato fruit vitamin C content. Further research would be necessary.

Effect of Calcium

In a soil-pot culture experiment, Subbiah and Perumal (1990) tested the influence of sources of calcium (calcium oxide, calcium chloride, calcium sulphate), concentrations (0.0%, 0.1%, 0.2% and 0.3% Ca), stages (S1 = 30th day after transplanting, S2 = 45th day after transplanting, S3 = 60th day after transplanting), and number of sprays S1, S2, S3, S1+S2, S1+S3, S1+S2+S3, S2+S3) on physico-chemical properties of tomato fruits, cv. Co.3. All the pots received also 120 kg N/ha, 100 kg P₂O₅/ha and 100 kg K₂O/ha. They could significantly increase the ascorbic acid content (from 180 to 258, 268 and 265 mg/kg fresh matter) of the tomato fruit by calcium sprays at 0.0, 0.1, 0.2 and 0.3% Ca concentration respectively. This is in agreement with Bangerth (1976) and with a suggested hypothesis of a positive correlation of Ca and vitamin C levels in tomato fruit presented in Table 20 (Premuzic *et al*, 1998).

Table 20. Vitamin C concentration (mg/kg) and Calcium concentration (mg/kg) in tomato fruit obtained in two different growing environments (rough values estimated from a graph) (after Premuzic *et al*, 1998)

Growing environment	Vitamin C concentration	Calcium concentration
Hydroponic	60	100
Organic	150	500

Effect of combined Nitrogen-Phosphorus-Potassium...

In several papers it has been reported that fruit vitamin C content could increase with the supply of combined fertilizers. Sharma and Mann (1971) obtained about 10% significant increase in ascorbic acid content of tomato juice, from about 20 to 22 or from about 21 to 23 mg/100 ml juice according to the season (not significant in tomato fruit) for N fertilization increase (50 to 150 kg/ha) combined with phosphorus or P₂O₅ fertilization increase (30 to 90 kg/ha) combined with nitrogen respectively in open field (Table 21). However, when the ascorbic acid content was expressed in relation to total soluble solids, P effect seemed to result in an increase but N effect did not.

Table 21. Main effect of increasing rate of phosphate fertilizer combined with nitrogen and of increasing rate of nitrogen fertilizer combined with phosphorus on ascorbic acid concentration in tomato fruit juice (after Sharma and Mann, 1971)

Fertilizer treatment	Total soluble solids (%)			Ascorbic acid (mg/100 ml juice)			Ascorbic acid (mg/kg total soluble solids) ⁽¹⁾		
	Winter		Summer	Winter		Summer	Winter		Summer
	1963-64	1964	1965	1963-64	1964	1965	1963-64	1964	1965
30 P₂O₅	5.86	6.17	5.93	20.15	21.06	21.20	3438	3413	3575
60 P₂O₅	5.91	6.09	5.81	21.78	22.14	21.99	3685	3635	3784
90 P₂O₅	5.99	6.02	6.02	22.19	22.76	22.42	3704	3780	3724
P effect	NS	NS	NS	**	**	**			
50 N	5.41	5.83	5.72	20.21	21.14	20.83	3735	3626	3641
100 N	6.14	6.13	5.94	21.60	21.67	21.86	3517	3535	3680
150 N	6.20	6.31	6.10	22.31	23.15	22.92	3598	3668	3757
N effect	**	**	**	**	**	**			

(1) added calculation

NS : non significant

** : significant at p = 0.01%

In tomato cultivated in sand pots, vitamin C content was reduced to one third in the less sulphur (65 mg/kg) and nitrogen (70 mg/kg), and to half in -Mg (110 mg/kg), -K (114 mg/kg), -P (135 mg/kg) and -Ca (140 mg/kg) compared to control (210 mg/kg) (Dube and Misra, 1975). In field experiments tomato fruit vitamin C content was significantly increased by increasing levels of both nitrogen (up to 150 kg/ha) and phosphorus (up to 140 kg/ha), the variation range being from 24 to 29mg/100ml juice (Patil and Bojappa, 1984).

Audisio *et al.*(1993) have cultivated tomato cv. Arletta in non heated greenhouse on sandy-loam soil poor in organic matter. Organic fertilizing yielded a low production of fruit rich in vitamin C (414 mg/kg fresh matter) while fertilizing with mineral or mineral + organic fertilizer yielded a high production of fruit poorer in vitamin C (280 mg/kg fresh matter). In glasshouse, Premuzic *et al.*(1998) have grown tomatoes cv. Platense on two substrates (pure sand and peat-perlite) hydroponically irrigated with a complete nutrient solution and on two organic substrates (100% vermicompost and 50% vermicompost:50% soil) irrigated with

water. Vitamin C concentration was higher in fruit grown on vermicompost (about 150 mg/kg) than in fruit grown on the hydroponic substrates (about 60 mg/kg). This could be related to a shading effect, as high rates of fertilizers on the hydroponic substrates promoted foliage growth and shading of the developing fruit.

6.2.4.4. Ripening stage

In cv. Marglobe and Stokesdale, Murneek *et al.*(1954) showed that vitamin C content of greenhouse tomatoes picked “green” and allowed to ripen at room temperature (22-24.5°C.) increased from mature-green stage (110 mg/kg) to ripe (red) stage (200 to 260 mg/kg according to fruit size). López-Andréu *et al.*(1986) graded the stages of ripeness of the cultivar Vemone as follows : I (mature green), II (green yellow), III (yellow-orange), IV (orange-yellow), V (orange-red), VI (red), VII (deep red). From I to VII, vitamin C content increased from 84 to 180 mg/kg of fresh matter. The values were lower than those given in the literature because in greenhouse with less direct sunlight the fruit contains less vitamin C than field cultivated fruit. Normally the rise is sudden from the stage II. With plum tomatoes (var. Heinz 9478) Shi *et al.*(1999) found that ascorbic acid content of whole fresh fruit increased from the stage mature-light pink (175 mg/kg) to the stage mature-pink (209 mg/kg) and to the stage mature-red stage (256 mg/kg). However, Abushita *et al.*(1997) found that for Floriset cultivar, vitamin C content increased from green stage (130 mg/kg) to yellow stage (240 mg/kg) then decreased until red stage (120mg/kg), most likely due to its antioxidant function when the ripening cells absorb high amounts of oxygen as a result of increasing rate of cell respiration (characteristic physiological change in climacteric fruits).

Giovanelli *et al.*(1999) evaluated the variation in the vitamin C content at seven stages during vine and post-harvest ripening on the tomato cv. Moneymaker grown in a greenhouse. Ascorbic acid content presented different patterns of variation with the ripening conditions : in post-harvest-ripened fruit it decreased from about 200 mg/kg fresh matter to 150 then increased to about 200 mg/kg fresh matter. In vine ripened fruit it first increased from about 200 mg/kg to 250 then decreased to roughly 150-200 mg/kg fresh matter.

6.2.4.5. Growth and development regulators

DCPTA (2-(3,4-dichlorophenoxy)triethylamine) is a bioregulator of lipid and protein synthesis which has been shown to increase the harvestable yield of numerous crop plants by maintaining a balanced partitioning of photosynthetate between plant growth and crop yield.

Gibberellins are plant growth promoters through stimulation of stem elongation, promotion of seed germination, inducement of flowering and fruit formation, changes of plant metabolism.

CCC or Cycocel (2-chloroethyltrimethylammonium chloride), Phosphon (2,4-dichlorobenzyl tributyl phosphonium chloride) and Alar (succinic acid 2,2-dimethyl hydrazide) are growth retardants generally inducing dwarfing effects.

Duraset (N-metatolyl phtalamic acid) and the cytokinin Adenin (6-aminopurine) are growth substances exhibiting auxin.

Sprays of plant growth-regulating substances (naphtalene acetamide, naphtoxyacetic acid, indolbutyric acid, P-Chlorophenoxyacetic acid) used to improve fruit set and fruit size under subnormal conditions of light and temperature, did not seem to have an appreciable direct influence on fruit vitamin C concentration which varied between 190 to 250 mg/kg, according to the season for the cultivars Marglobe and Stokesdale (Murneek *et al*, 1954).

Irulappan and Muthukrishnan (1974) increased fruit ascorbic acid content of 25% together with total soluble solids content of field grown tomatoes by Alar treatment at 1500 and 3000 mg/kg. In field grown tomatoes cultivated by Graham and Ballesterro (1980), gibberellic acid, cycocel and phosphon increased fruit ascorbic acid content.

Gabr *et al.*(1985) sprayed field tomato plants twice (25 and 40 days after transplanting) with chlormequat (CCC) or alar separately at 250, 500 or 1000 mg/kg. Yields were increased by 250 mg/kg CCC and by all doses of alar, with decreasing influence. Most levels of CCC and alar seemed to increase slightly fruit vitamin C content (+5%; values ranging from 200 to 230 mg/kg according to successive harvests).

Majeed *et al.*(1991) found that the stage and number of sprayings of DCPTA did not influence fruit yield and vitamin C content. Increased concentration of DCPTA from 0 to 80 mg/kg doubled the fruit yield per plant and increased fruit vitamin C content from 386 to 549 mg/kg fresh matter and total soluble solids content from 4.53 to 5.48 %.

6.3. Vitamin E

6.3.1. Introduction

Booth and Bradford (1963) reported that, in not peeled tomato fruit, the mean vitamin E content was 60 mg/kg dry matter or 3 mg/kg fresh matter, with 35-100 mg/kg (dry matter basis) as 95% confidence limits. From various sources of data in the 70's Davies and Hobson (1981) indicated the following values of vitamin E concentration : 0.3, 2.7, 3.0, 12 mg/kg raw edible part of tomato fruit. They mentioned the influence of season, nutrition and environment. The tocopherol content of tomato fruit was found by Piironen *et al.*(1986) to be moderately high 6.6 mg/kg fresh matter at 95.4 % moisture. Citing the U.S. Department of Agriculture (1997), Beecher (1998) reported a vitamin E content of tomatoes of 3.2 mg/kg fresh matter.

But little has been found in the literature about the influence of temperature, light and some aspects of cultural techniques (water, mineral nutrition, growth regulators and other molecules).

6.3.2. Influence of cultural techniques

6.3.2.1. Variety

Abushita *et al.*(1997) analysed tomato fruit from various poorly known cultivars cultivated in Hungary and found variations of vitamin E content on about 1 to 3-fold : from 0.96 to 3.15 mg/kg fresh matter for α -tocopherol and from 1.2 to 4.0 mg/kg fresh matter for total tocopherol.

6.3.2.2. Ripening stage

Abushita *et al.*(1997) analysed tomato fruit from various poorly known cultivars cultivated in Hungary and found an influence of the ripening stage on the concentration of various forms of tocopherol : for Floriset cultivar, α -tocopherol increased from 1.5 (green stage) to 3.2 mg/kg fresh matter (red stage); β -tocopherol increased from nearly 0 (green stage) to 0.3 mg/kg fresh matter (red stage); γ -tocopherol increased from 0.7 (green stage) to

1.4 mg/kg fresh matter (yellow stage) then decreased to 1 mg/kg fresh matter (red stage) due to a high antioxidative activity in the fruit and thus for food protection, but it is biologically inactive as vitamin E in the human body.

6.4. Phenolic compounds

6.4.1. Introduction

Citing the U.S. Department of Agriculture (1997), Beecher (1998) gave estimates of tomato content in quercetin (8 mg/kg fresh matter), one of the flavonoids that has very high antioxidant activity relative to alpha-tocopherol.

Senter *et al.* (1988) have shown that in tomato fruit the quantities of total phenols in the epidermal tissue, the placental tissue, the radial and inner wall of the pericarp and the outer wall of the pericarp were $30.2 \cdot 10^3$, $25.2 \cdot 10^3$, $20.8 \cdot 10^3$ and $19.4 \cdot 10^3$ mg/kg dry tissue respectively. Since the greater quantities of these compounds are concentrated predominantly in the epidermal tissue of the tomato, they might function as natural barriers to microbial invasion.

6.4.2. Influence of light

In a review, Herrmann (1976) reported that nearly the total flavonol content was in the outer parts of the tissues, which were 4-5% of the fruit. Since the formation of flavonol (e.g. kaempferol and quercetin) glycosides depends on light, it is not surprising that they are found mainly in the skins of fruit. For the tomato cv. "Ronald V" cultivated in open air and in glasshouse, values of kaempferol and quercetin glycoside (estimated and calculated as mg aglycon per kg of fresh matter) fruit contents are given respectively : 0.2 and 0.2 mg/kg kaempferol, 7 and 2.5 mg/kg quercetin; leaf contents are respectively : 20 and 4 mg/kg kaempferol, 420 and 155 mg/kg quercetin.

Hunt and Baker (1980) have identified chalconaringenin, naringenin, naringenin-7-glucoside, and *m*- and *p*-coumaric acids as phenolic constituents of tomato fruit cuticles of three cultivars (Aisa Craig, Alicante and Grower's Pride). The composition of the flavonoid fraction was controlled by the spectral quality of incident radiation, red light favouring the formation of chalconaringenin.

Wilkens *et al.*(1996) investigated the influence of light resources on the accumulation of the soluble phenolics rutin and chlorogenic acid by Cherry tomato plants in greenhouse, together with the influence on growth. Plants grown under high light had approximately a two-fold higher concentration of soluble phenolics than low-light plants.

6.4.3. Influence of cultural techniques

6.4.3.1. Variety

Senter *et al.*(1988) have shown that in tomato fruit the quantities of total phenols in the epidermal tissue, the placental tissue, the radial and inner wall of the pericarp and the outer wall of the pericarp (presented in § 6.4.1.) did not vary significantly among three tested cultivars (Patriot, Floridade, Walter).

6.4.3.2. Mineral nutrition

Effect of Nitrogen

The pigment absorption spectrum of leaf ethanol extracts was analysed on tomato seedlings (cv. UC82) which were cultivated until fruit-setting by Dumas *et al.*(1993) in sand pots in glasshouse to study the influence of N availability (0, 2, 6 and 18 meq N L⁻¹ nutrient solution) in the root environment on growth and development. Leaf polyphenol content giving a yellowish colour (absorbency measured between 300 and 350 nm) was considerably increased by low N availability (2 meq compared to 6 and 18), which also resulted in a slower rate of appearance of new trusses.

Wilkens *et al.*(1996) investigated the influence of nitrogen resources on the accumulation of the soluble phenolics rutin and chlorogenic acid by Cherry tomato plants in greenhouse, together with the influence on growth. Plants grown with low N resources showed low levels of soluble phenolics and low plant mass. Plants grown with intermediate levels of N resources showed high phenolic concentrations but inhibited growth. Plants grown with high N resources had high growth but no increases in phenolic concentrations. The phenolic concentrations were higher in younger leaves. The differences in phenolic

concentrations were large enough to have potential consequences for insect herbivores feeding on tomato plants.

Nitrogen availability was also found by Stout *et al.*(1998) to have a strong effect on constitutive levels of phenolics in tomato seedlings grown in sand in a greenhouse. Leaflets from a given position on 3- to 5-leaf stage plants in the low-N treatment ($[N] \leq 2$ mM) had levels of phenolics more than twice those found in corresponding leaflets from high-N plants ($[N] \leq 8$ mM), with plants in the intermediate-N treatment ($[N] \leq 4$ mM) having intermediate levels.

Effect of Phosphorus

In tomato plants, cv. Marglobe, cultivated by Peñalosa *et al.*(1989) in sand pots in glasshouse, deficient P levels (0.2 and 0.5 mmol P/l) in the nutrient solution resulted in a flavonol glycosides decrease in leaves in comparison with adequate P supply (2.0 mmol P/l). High P level (4.0 mmol P/l) in nutrient solution also led to a decrease in flavonol glycosides content of leaves at flowering.

6.4.3.3. Fruit development and ripening stage

Hunt and Baker (1980) have identified chalconaringenin, naringenin, naringenin-7-glucoside, and *m*- and *p*-coumaric acids as phenolic constituents of tomato fruit cuticles of three cultivars (Aisa Craig, Alicante and Grower's Pride). The phenolic content of the cuticles increased substantially during fruit development, those from immature green and mature ripe fruit of cv. Ailsa Craig yielding respectively 2.8 and 61 $\mu\text{g}/\text{cm}^2$ (1.4 and 6% of the total membrane wt). Phenolics were minor constituents (<5%) of the surface extracts from green fruit. In contrast, the surface extracts obtained from ripe fruit of the three cultivars contained much higher proportions of phenolics ranging from 7-14% at the onset of the climacteric to 21-26% at the post-climacteric stage and chalconaringenin was mainly present (6.1-7.1 $\mu\text{g}/\text{cm}^2$). The amount of cuticular phenolics, composed entirely of flavonoids, increased steadily (0.9-5.7 $\mu\text{g}/\text{cm}^2$) during fruit development whereas the proportion of these constituents in the extracts remained low (0.7-3.5%) due to the steady production of sugars and fatty acids. Naringenin was dominant and increased steadily throughout the climacteric

(3.7-5.7 $\mu\text{g}/\text{cm}^2$). Coumaric acids, present only in the 'cutin-bound' phenolics, increased from 2 to 24 $\mu\text{g}/\text{cm}^2$ during fruit development.

Senter *et al.*(1988) have shown that for the whole tomato fruit the quantities of total phenols tended to increase from green stage to midripe stage, then to decrease to the green stage level at the ripe stage.

Buta and Spaulding (1997) have related changes in phenolics in the pulp and pericarp of tomato fruit cv. Ailsa Craig and Pik-Red according to the stage of development. The highest quantities of chlorogenic acid were found in the pulp and pericarp at the earliest stage of fruit development, and they declined during fruit ripening. It was the same for rutin found only in the pericarp. The *p*-coumaric acid was always low. High levels of *p*-coumaric acid glucoside were detected in the pulp continuously during fruit maturation and ripening.

Giovanelli *et al.*(1999) evaluated the variation in the total phenolics content at seven stages during vine and post-harvest ripening on two genotypes (Normal Red and Crimson) of the tomato cv. Moneymaker grown in a greenhouse. Total phenolic content was higher in post-harvest-ripened samples (about 100 to 200 mg/kg fresh matter, with a mean moisture content of 92.5% in all conditions) than in vine-ripened fruit (about 70 to 110 mg/kg fresh matter). There were no significant differences in phenolics in the two genotypes.

6.4.3.4. Influence of the season

In fresh market tomatoes, Hertog *et al.*(1992) found only quercetin in noticeable quantity. Fruit quercetin content was 4.6, 11.0, 8.2 and 4.9 mg/kg of fresh edible part of fruit in April 1991, August 1991, December 1991 and April 1992 respectively. Kaempferol content was always <2 mg/kg of fresh edible part of fruit.

6.4.4. Conclusion

For phenolic compounds and from the agronomic point of view, information found in literature revealed many gaps. Studies about phenol secondary metabolism have often been performed on young tomato seedlings and on vegetative organs, but few studies give information on phenolic changes in tomato fruit according to the main agronomic factors like

temperature, light, water availability, fertilizer resources (except nitrogen) and variety. Another question is what could also be the influence of plant growth and development regulators on phenolic concentration since there could exist important interactions : some phenolics (chlorogenic acid and rutin) have been suggested as regulants of auxin (like indole-3-acetic acid) metabolism (Buta and Spaulding, 1997).

6.5. Conclusions on the influence of cultural practices and agronomic aspects

Many studies and results are old and can hardly apply to the current cultivars used now in processing tomato cropping. Recent studies apply to cultivars poorly known and not utilized in the European area. Moreover, in many cases the experiment conditions (e.g. soil characteristics) were not precisely detailed and it is difficult to consider the results as general enough.

The contents in antioxidants were generally calculated relative to the fresh matter and the papers rarely give indications about the fruit dry matter content or the juice solids content. However it should be more accurate and more reliable to express the antioxidant concentrations relative to the dry matter in order to really understand the influence of the main involved factors because these factors often modify also the dry matter content of tomato fruit.

Some factors seem antagonist with regard to the development of different antioxidants. For example, water shortage during cropping could be favourable to tomato fruit vitamin C content and possibly unfavourable to tomato fruit lycopene content. It seems that direct sunlight is favourable to vitamin C and phenols accumulation in fruit while lycopene would develop better in fruit protected by crop foliage. Few data have been found about the influence of some cultural factors, for example nitrogen and phosphorus/lycopene, water/lycopene, temperature, phosphorus and potassium/vitamin C, temperature, water and P-K nutrition/phenols, temperature, light, water and mineral nutrition/vitamin E.

It is important to get a good understanding of the influence of water availability on antioxidant accumulation in tomato fruit because water is a major agronomic factor of great influence on the quantitative and qualitative characteristics of processing tomato production. Maybe fertilization could not be the most efficient way to influence the level of antioxidants. It is surely more difficult to explore the influence of fertilizers. Knowledge of soil nutrient availability by soil analysis after chemical extractions remains insufficient because it gives an

idea of the nutrient stock, total or "available" but not of the soil feeding capacity (Dumas, 1990). Indeed for nitrate there is a great variability within time and space according to environmental conditions (mineralisation, reorganisation, leaching). For P and K it has been shown that tomato roots could have a very high extraction capacity : 80% of P or K absorbed by plants can come from the soil P or K part which was not given as "available" by chemical analysis (Maertens and Bosc, 1988). Interactions exist between ions (antagonism between potassium and calcium-magnesium, or synergism nitrogen-phosphorus). Buffering properties of soils often moderate nutrient relations. Fertilization must be related to the whole crop management . It is an evidence that fertilization practice and efficiency are most dependent on irrigation mode for instance. But it also depends on soil structure and rooting conditions. Indeed P uptake has been found to be proportional to root surface area and K is above all absorbed by young parts of growing roots. Fertilization has to be reasoned according to the situations.

Various growth and development regulators seem to positively influence the tomato fruit content in carotenes and vitamin C. But before planning to apply such substances to processing tomato fields, studies should be undertaken about the feasibility of such interventions in open field, about possible consequences of applications on the crop response and about possible modifications of fruit quality (other properties, residues?).

As a result of the present literature review it appears very difficult and hazardous to define optimal growing conditions which would enable farmers to maximise the biosynthesis and the storage of lycopene, vitamin C, vitamin E and phenolic compounds in the tomato fruit during ripening. These issues have not been much addressed. It might be of great interest to understand better the effects of light and temperature -influenced themselves by water relations, mineral nutrition and cultural techniques like the date of crop installation and plant spatial distribution, which alter the crop canopy structure- on the concentrations of these compounds in different cultivars or genotypes during the fruiting period.

7. Physico-chemical characteristics and properties

7.1. Lycopene

7.1.1 Chemical structure

Lycopene is the common name of ψ - ψ -carotene. It is a carotenoid which belongs to the subgroup of carotenes consisting only of hydrogen and carbon atoms, its molecular formula is $C_{40}H_{56}$ (MW 536.85 g/mol).

Lycopene is characterized by a symmetrical and acyclic structure containing 11 linearly arranged all-*trans* form conjugated double bonds in its central part.

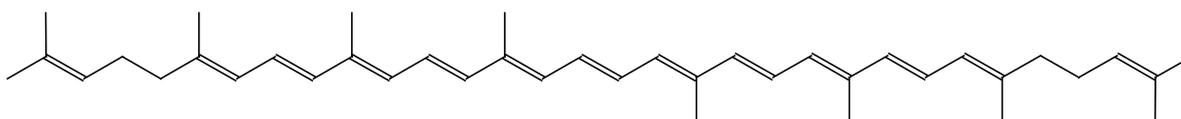


Figure 2 : Structural formula of lycopene

The structure of a carotenoid is a key determinant of the physical properties, chemical reactivity and biological functions or actions observed.

7.1.2 Physico-chemical characteristics and properties

7.1.2.1. Melting point

Lycopene in pure form is a red crystal with a melting point of 172-173 °C.

7.1.2.2. UV-vis spectrophotometrie

The system of 11 conjugated double bonds of lycopene constitutes a chromophore. It is responsible for bright red colour and determines the visible absorbing parameters. The UV-visible spectra of lycopene is characterized by a vibrational fine structure with 3 maximums of absorption in the visible region (see figure 3). Table 22 gives the values of these 3 peaks in different solvents. The molar extinction coefficient ϵ can be determined for each wavelength; the most intense is usually used (see values in Table 22).

The fine structure is characterized by a comparison between the size of the 2 bigger peaks of the absorption spectra (figure 4). The numerical notation is %III/II. In this notation, the baseline or zero value is taken as the minimum between the 2 peaks. The peak height of the longest-wavelength absorption band is designated as III, that of the middle absorption band as II. Spectra fine structure is then expressed as the ratio of the peak heights III/II as a percentage. The table 22 gives the value of % III/II for lycopene in ethanol and light petroleum.

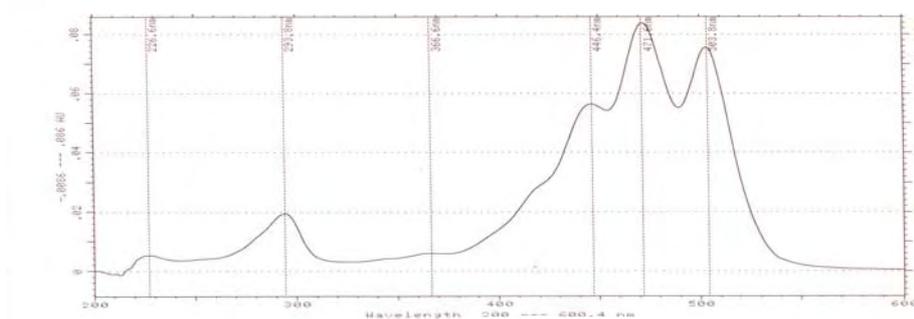


Figure 3: UV-visible absorption spectra of lycopene

λ max (nm)	% III/II	ϵ_{mol}	solvent
448 474 505			acetone
455 487 522		180600 (at 487 nm)	benzene
458 484 518			chloroform
446 472 503	65		ethanol
444 470 502	65	184900 (at 470 nm)	light petroleum

Table 22 : UV/vis spectroscopic data of lycopene.

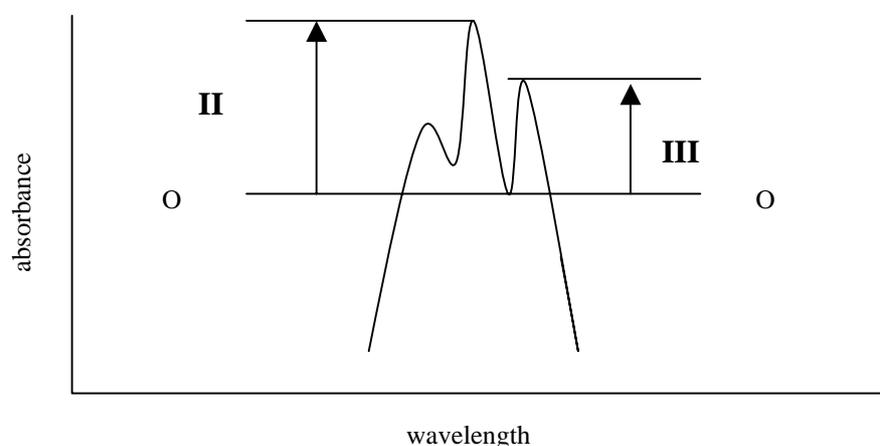


Figure 4 : spectral fine structure. Calculation of %III/II for a carotenoid

7.1.2.3 IR Spectroscopy

The polyene structure of lycopene gives rise to 2 weak bands at 1629 and 1559 cm^{-1} , (C=C stretching), and a quite strong band at 960 cm^{-1} (CH out-of-plane deformation).

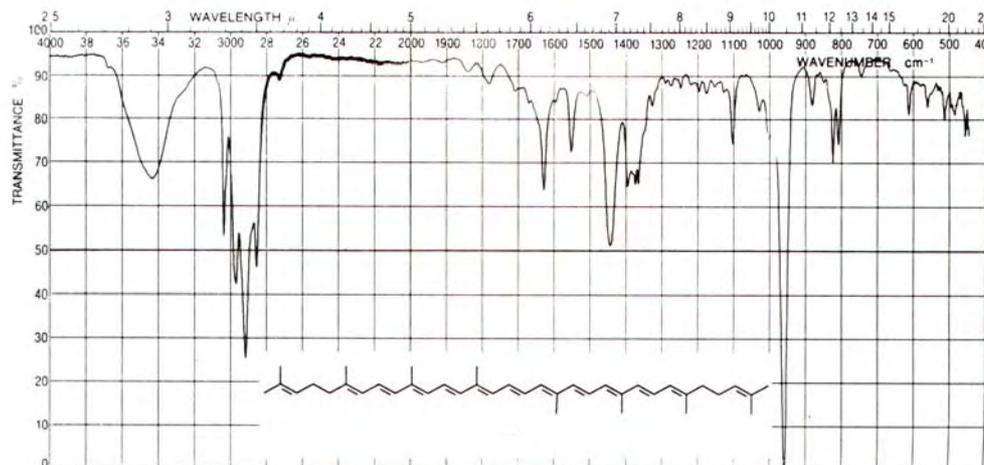


Figure 5 : IR spectra of lycopene

7.1.2.4 Solubility

Due to its hydrocarbon structure, lycopene is very lipophilic. It is then soluble in apolar solvents : hexane, light petroleum, benzene, dichloromethane, chloroform and THF. It is slightly soluble in acetone, ethanol. It is completely insoluble in water.

Its partition coefficient (log P in octanol/water) has been calculated (Cooper et al. 1997): the value is 17.64. Among the carotenoids, it is one of the most apolar compounds, the xanthophylls have a calculated log P between 5.82 and 16.

7.1.2.5 Sensitivity

As a highly conjugated polyene, lycopene is particularly susceptible to oxidative degradation. Physico-chemical factors known to degrade carotenoids in general including lycopene are :

- exposure to oxygen
- elevated temperature
- exposure to light
- extreme pH

As an example of sensitivity to oxygen, Black (1998) has measured the decrease in absorbance of a solution of carotenoid in hexane when bubbled with oxygen. In these experimental conditions, lycopene is shown to be more sensitive to degradation than β -carotene (figure 6).

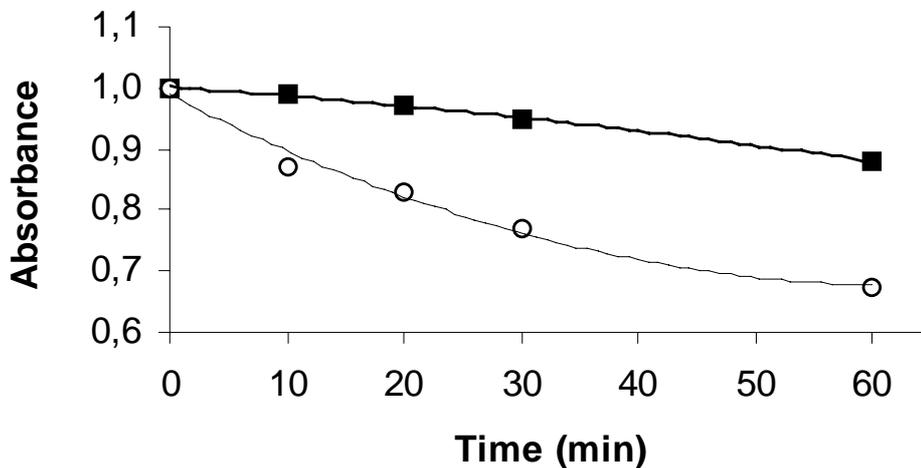


Figure 6 : Decrease in absorbance of a solution of carotenoid in hexane when bubbled with oxygen with ■ : β -carotene ; ○ : lycopene

Sharma and Le Maguer (1996a) have followed the time-dependent loss of lycopene in tomato pulp when heated at 100°C (figure 7)

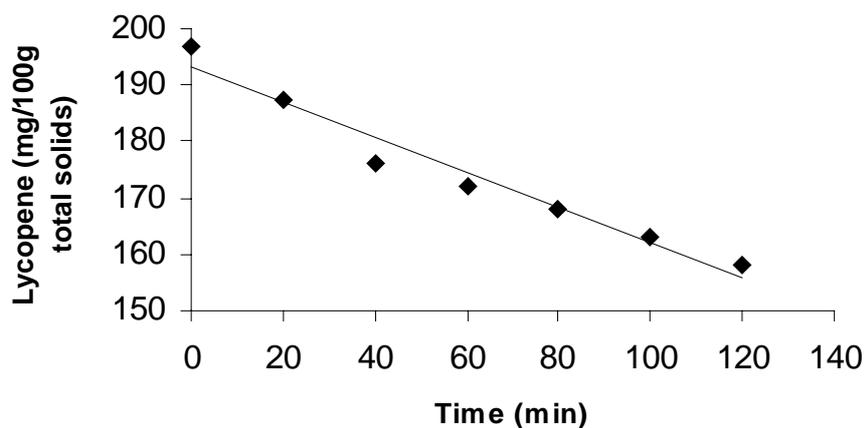


Figure 7 : Kinetics of lycopene degradation in tomato pulp heated at 100°C

7.1.2.6 Influence of the environment on physico-chemical properties.

The chemical and physical properties of lycopene and carotenoids in general are strongly influenced by other molecules within the microenvironment, particularly associated proteins and membrane lipids.

These interactions usually improve stability of lycopene, which is then less susceptible to degradative oxidations. Indeed lycopene appears to be relatively stable during food processing and cooking (Tonucci et al. 1995); the food matrix might protect lycopene. Also lycopene appears to be relatively stable in blood samples collected using standard clinical techniques and stored at -70°C over several years (Comstock et al. 1995).

7.1.3 Lycopene *cis* isomers.

7.1.3.1 Structures

Lycopene contains 13 carbon-carbon double bonds, among which 11 are conjugated. The most thermodynamically stable form of lycopene is the all-*trans* (or all-E) form where all the double bonds are in E configuration. With very few exceptions, lycopene from natural plant sources exist predominantly in the all-*trans* configuration.

Theoretically, each one of the 11 carbon-carbon conjugated double bonds can undergo isomerization to produce an array of mono- or poly-*cis* isomers of lycopene, all together $2^{11}=2048$ isomers are theoretically possible. Practically, because of steric hindrance, only certain ethylenic groups can participate in *cis-trans* isomerization. In fact, about 72 lycopene *cis* isomers are structurally favourable and only some have been observed (figure 8).

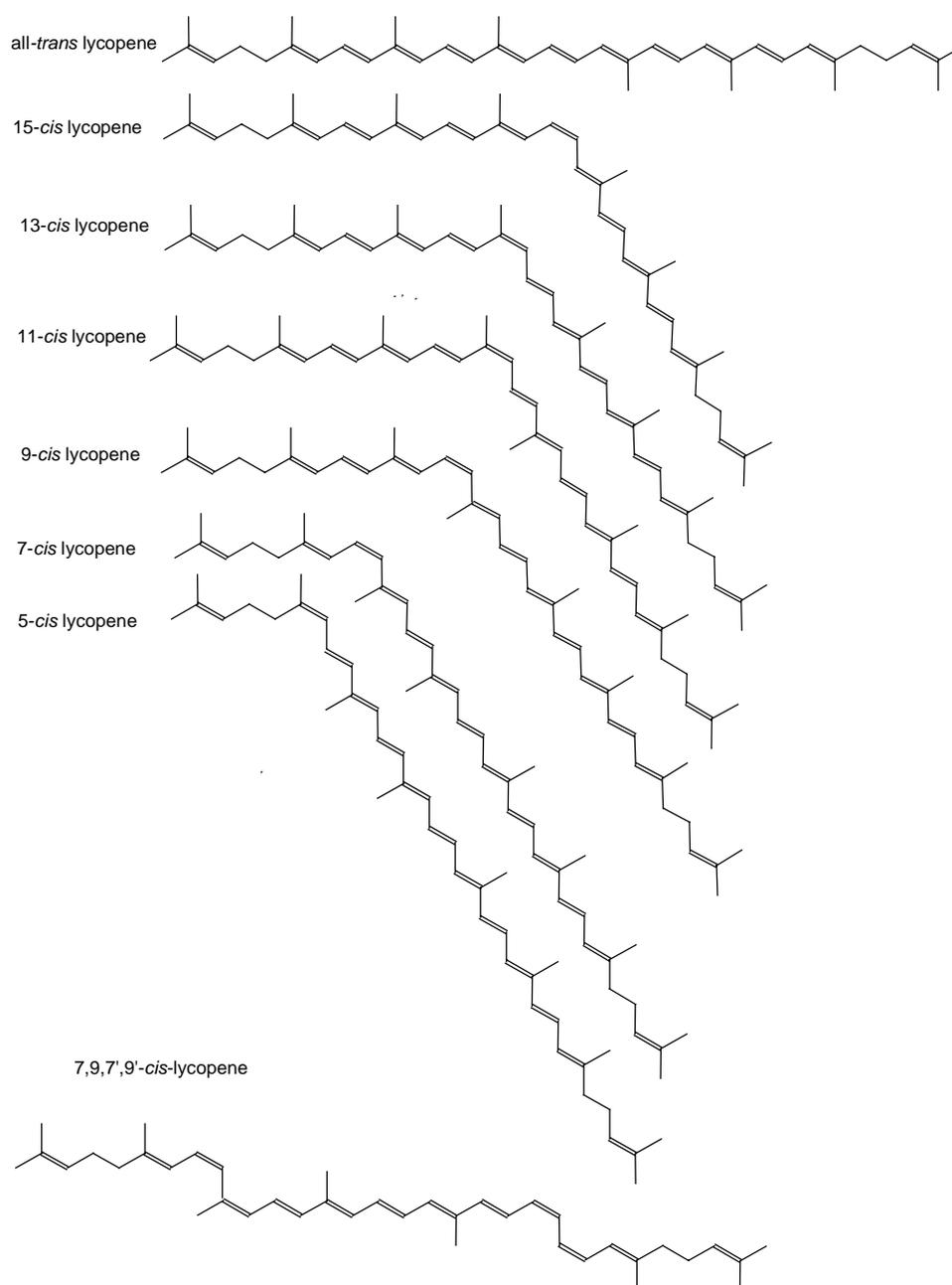


Figure 8 : Natural lycopene geometrical isomers.

7.1.3.2 Occurrence

All-*trans* lycopene is the predominant isomer in tomatoes and tomato products (~95%) (Clinton et al. 1996), 15-*cis*, 13-*cis*, 9-*cis* and 5-*cis*-lycopene have been found in human serum, making up approximately half the total lycopene content, sometimes even more. The predominant *cis* isomer is the 5-*cis*-lycopene. An even greater proportion, approximately

80% of total lycopene is found as *cis* isomers in prostate tissue. 7,9,7',9'-*cis*-lycopene is the naturally occurring form of lycopene in fresh tangerine-type tomatoes.

The *trans-cis* interconversion is thought to occur with the absorption of light, exposure to heat, or by participation in specific chemical reactions, for instance during metabolism *in vivo*. Very little is known about formation and specific roles of *cis* isomers in the vertebrate biology.

7.1.3.3 Physico-chemical properties

Cis isomers of lycopene have distinct physical characteristics and chemical behaviours from their all-*trans* counterpart. Some differences observed as a result of a *trans* to *cis* isomerization reaction include :

- lower melting points
- decreased color intensity
- lower extinction coefficient (hypochromic effect)
- a displacement of λ_{\max} to shorter wavelength (hypsochromic effect)
- a reduction in vibrational fine structure
- the appearance of a new absorption band in the ultraviolet region so-called the « *cis*-peak ». It is at a characteristic position about 142 nm below the longest-wavelength absorption maximum (III) in the spectrum of the all-*trans* compound, when measured in hexane.

For di-*cis* and poly-*cis* carotenoids, a large hypsochromic shift in the main absorption band may be seen. Prolycopene (7,9,7',9'-*cis*-lycopene, see fig 8), for example, has λ_{\max} at 414, 436, 463 nm, in contrast to 444, 470, 502 nm for all-*trans*-lycopene, and the spectral fine structure is also greatly reduced.

Among the observed *cis*-isomers, some have been characterized spectrophotometrically (Table 23).

Carotenoid	λ_{\max} (nm)				E 1%
5- <i>cis</i> -lycopene	362	443	<u>470</u>	502	3466
7- <i>cis</i> -lycopene	362	443	<u>470</u>	502	2901
15- <i>cis</i> -lycopene	362	443	<u>470</u>	502	2072
13- <i>cis</i> lycopene	360	437	<u>463</u>	494	-
9- <i>cis</i> -lycopene	360	438	<u>464</u>	495	-
7,9,7',9'- <i>cis</i> -lycopene	-	414	<u>436</u>	463	

Table 23 Spectrophotometric parameters of *cis* isomers of lycopene

(in hexane containing 2% (v/v) dichloromethane or toluene), main maxima are underlined

From Hengartner et al. 1992; Aebischer et al. 1999; Giger 1994; Schierle et al. 1997

Cis isomers tend to crystallize or aggregate much less easily than all-*trans* isomers, this improves their solubility in lipophilic organic solvents.

7.1.4 Antioxidant properties

7.1.4.1 Quenching of singlet oxygen

Because of its highly unsaturated structure, lycopene has the capacity of accepting energy from various electronically excited species in particular singlet oxygen : $^1\text{O}_2$, thus acting as an antioxidant molecule.

The mechanism of quenching of singlet oxygen by carotenoids (figure 9) is mainly a physical process involving the transfer of excitation energy from singlet oxygen to the carotenoid, resulting in ground state oxygen and excited carotenoid. In turn, the energy is dissipated through physical interaction (rotation and vibration) between the excited carotenoid and its surroundings, yielding ground state carotenoid and heat.

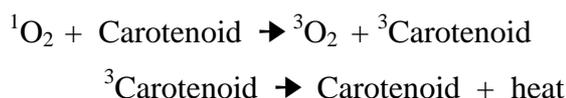


Figure 9 : Physical quenching of singlet oxygen by a carotenoid

Among a series of carotenoids tested, lycopene was shown to be the most efficient quencher of singlet oxygen (Table 24). The explanation for the somewhat enhanced reactivity of lycopene towards singlet oxygen is not established.

Carotenoid	K_q ($\times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)	Source
Lycopene	17	Tomato, watermelon
All- <i>trans</i> β -carotene	13	Carrots
Astaxanthin	14	Salmon
Zeaxanthin	12	Maize
Lutein	2.9	Green vegetable
Violaxanthin	1	Green vegetable
Bixin	9.2	Annatto seeds

Table 24 : Singlet oxygen quenching constants, from Conn et al. (1991)

7.1.4.2 Quenching of free radicals

Radicals are present *in vivo* in various forms, they are activated species of oxygen like hydroxyl radical (HO^\bullet), superoxide ($\text{O}_2^{\bullet-}$) or peroxy radical (ROO^\bullet). The unpaired electron, characterizing the radical, gives them their instability. As molecules rich in electrons, carotenoids can react with a radical in order to fill its gap of electron, thus acting as a radical-quencher.

Two mechanisms of radical quenching can occur. It is either a transfer of electron from the carotenoid to the radical (1), or an addition reaction (2). In both cases, the radical formed is delocalised along the polyunsaturated chain of the carotenoid, making it more stable and then less reactive. It should be noted that upon physical quenching of $^1\text{O}_2$ the carotenoid molecule stays intact. It is destroyed by chemical quenching of $^1\text{O}_2$ and by radical interactions (figure 10).



Figure 10 : mechanisms of radical quenching by a carotenoid

In an *in vitro* experiment the relative antioxidant activities of a range of carotenoids were assessed by the extent of their ability to scavenge the ABTS radical cation (Miller et al. 1996). The results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values. In the ability to scavenge the radical cation under the conditions of this experiment, lycopene was the most effective (Table 25).

Carotenoid	TEAC (mM)
lycopene	2.9
β -carotene	1.9
Lutein	1.5
Canthaxanthin	0.02

Table 25 : TEAC values of some carotenoids (from Miller et al. 1995)

Mortensen and Skibsted (1997) have shown that lycopene is among the most efficient carotenoid free radical scavengers, because the lycopene radical cation is more stable than many other carotenoid radical cations.

Conclusion for lycopene.

In summary, lycopene is the main carotenoid in tomato, it is the characteristic pigment of this fruit conferring its red colour. It is a hydrocarbon molecule containing 11 conjugated double bonds, this particular chemical structure is responsible for the physico-chemical properties of the molecule. It is a lipophilic molecule, completely insoluble in water. When isolated, lycopene is sensitive to oxygen and light and can isomerize easily. Like other carotenoids, it presents antioxidant properties demonstrated *in vitro*: inhibition of singlet oxygen, quenching of free radicals.

7.2 Vitamin C

Vitamin C is used as the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid (AA). Therefore, this term refers to either or both of the common biologically active forms: ascorbic acid (AA) and dehydroascorbic acid (DHAA) (figure 11).

7.2.1 Chemical structure

Ascorbic acid (AA) is the enolic form of an α -ketolactone. The molecular structure contains 2 ionizable enolic hydrogen atoms that give the compound its acidic character (pKa1 at carbon 3 = 4.17 ; pKa2 at carbon 2 = 11.57). The asymmetric carbon atom 5 allows two enantiomeric forms, of which the L form is naturally occurring.

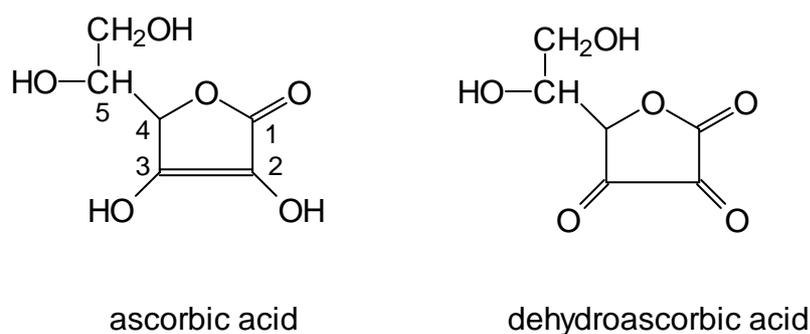
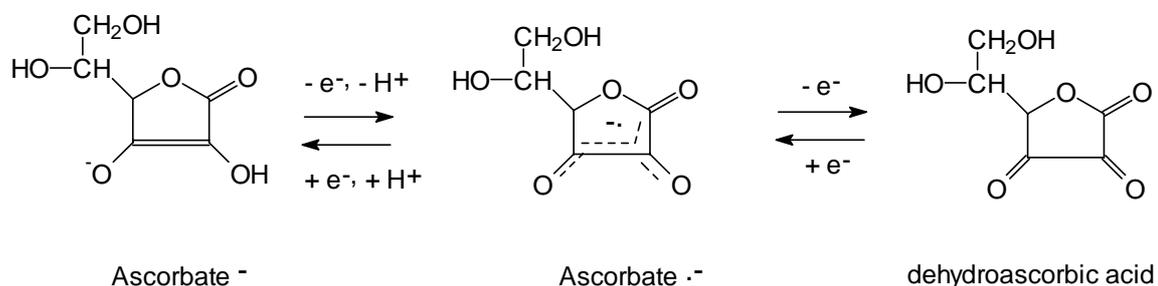


Figure 11 : Chemical structure of ascorbic acid and dehydroascorbic acid

7.2.2 Physico-chemical characteristics

- Molecular formula C₆H₈O₆, Molecular weight 176.12 g/mol
- White crystals (usually plates, sometimes needles), mp 190-192°C, d 1.65
- λ_{\max} 245 nm (acid solution), 265 nm (neutral solution)
- Solubility : soluble in water (1g in 3 ml), absolute ethanol (1g in 50 ml). Insoluble in diethyl ether, chloroform, benzene, petroleum ether, oils, apolar solvents.

- E^0 (pH7) = 280 mV for $\text{ascorbate}^{\cdot-} + \text{H}^+ + \text{e}^- \rightarrow \text{ascorbate}^-$
- E^0 (pH7) = -174 mV for $\text{dehydroascorbic acid} + \text{e}^- \rightarrow \text{ascorbate}^{\cdot-}$



- Stability :

- Stable to air when dry
- It is extremely unstable in aqueous solution. Indeed it is easily oxidized by air to dehydroascorbic acid (DHAA), and further compounds. The oxidation of AA to DHAA is reversible, but oxidation beyond DHAA is irreversible and is enhanced by alkaline pH and metals, especially copper and iron. The degradation processes of ascorbic acid are very complex and contain a number of oxidation/reduction and intermolecular rearrangement reactions (Deutsch 1998; Yuan and Chen 1998). Hence, procedures for stabilizing the vitamin in biologic samples involve acidification with the addition of a reducing agent and a metal chelator.

7.2.3 Antioxidant properties

Ascorbic acid is a well known reducing agent both *in vivo* and *in vitro*, thus being an antioxidant compound. In contrast to this role, AA also appears to act as a prooxidant under certain conditions *in vitro*, in particular in presence of ferric iron (Fe^{3+}). Indeed ascorbate can reduce ferric iron to ferrous iron (Fe^{2+}) which, in turn, can enter the Fenton reaction (figure 12) to produce the very toxic hydroxyl radical.



Figure 12 : role of ascorbic acid in the production of hydroxyl radical

The pro-oxidant activity of vitamin C *in vivo* is still controversial (Halliwell 1996; Podmore et al. 1998).

Conclusion for vitamin C

In summary, ascorbic acid is a water soluble vitamin, but unstable in aqueous solutions. It is a reducing agent, property which confers the molecule its antioxidant character. However, the pro-oxidant property of the molecule has also to be considered under certain conditions (presence of iron salt...).

7.3 Vitamin E

Vitamin E is a generic name for 8 naturally occurring compounds with a characteristic biological activity. Four vitamers are members of the tocopherol family and four are tocotrienols. Moreover, the stereochemistry of commercially synthesized tocopherols further complicates structural considerations because there are three asymmetric carbon atoms and therefore numerous stereoisomers.

The most abundant and active isomer is RRR- α -tocopherol (figure 13). Natural α -tocopherol as found in foods is RRR- α -tocopherol, whereas chemical synthesis produces a mixture of eight epimers.

In addition to the α -vitamer, three other tocopherols with biological activity are present in foods: β -tocopherol, γ -tocopherol, and δ -tocopherol. They differ from α -tocopherol only in regard to methyl substitutions on the benzene ring. The tocotrienols consist of four compounds similar to the corresponding tocopherols but with unsaturated side chains.

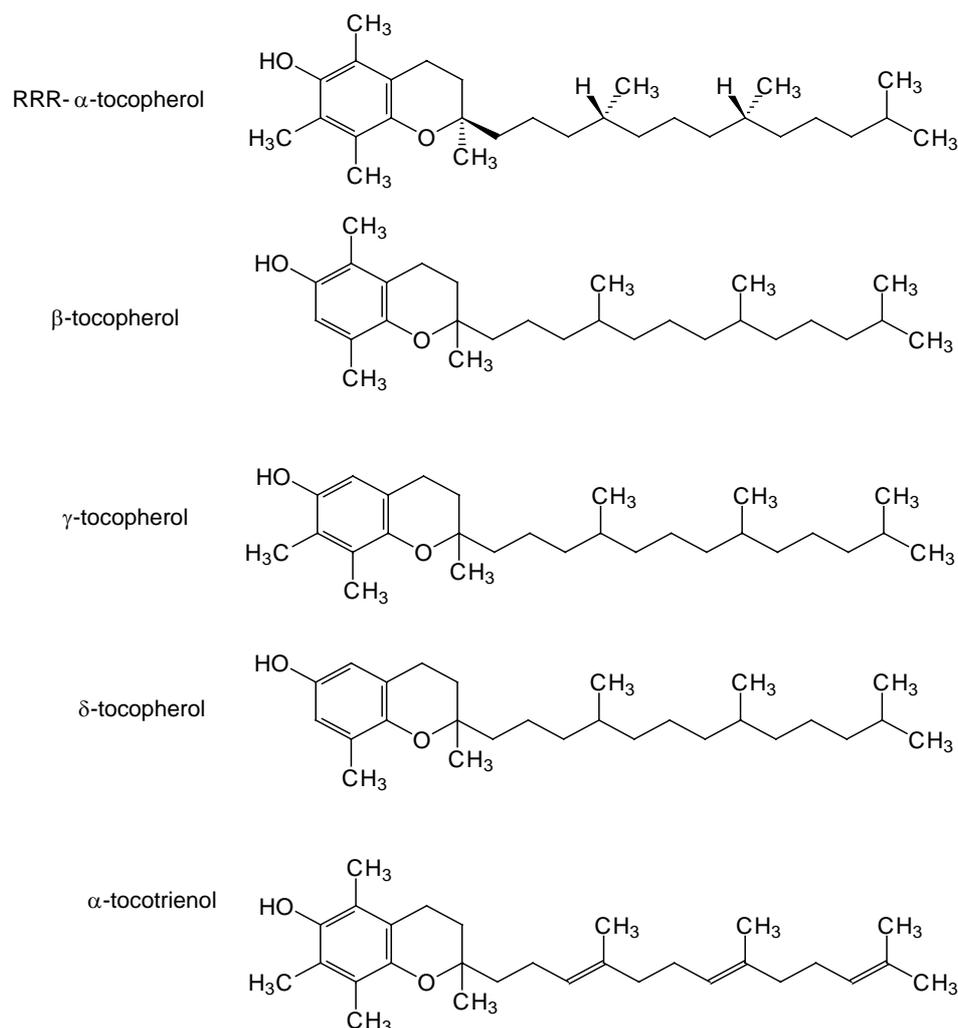
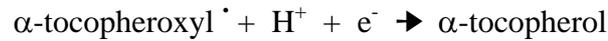


Figure 13 : Main naturally occurring vitamin E compounds

7.3.1 Physico-chemical characteristics

- Molecular formula $C_{29}H_{50}O_2$, Molecular weight 430.9 g/mol
- Slightly viscous, pale yellow oil.
- Natural α -tocopherol has been crystallized, its physical characteristics are :
mp 2.5-3.5°C, d_4^{25} 0.950 ; $bp_{0.1}$ 200-220°C ; n_d^{25} 1.5045
- λ max = 294 nm ($E_{1\%}^{1\text{cm}} = 71$)
- Solubility : practically insoluble in water. Highly soluble in oils, fats, acetone, alcohol, chloroform, ether, other lipophilic solvents.
- E^0 (pH7) = 500 mV



- Stability :
 - stable to heat and alkalies in the absence of oxygen
 - not affected by acids up to 100°C
 - slowly oxidized by atmospheric oxygen, rapidly by ferric and silver salts
 - gradually darkens on exposure to light
- The International Unit (IU) of vitamin E is equal to one mg of standard dl- α -tocopheryl acetate

7.3.2 Occurrence

Found largely in plant materials, present in highest concentrations (0.1-0.3 %) in wheat germ, corn, sunflower seed, rapeseed, soybean oils, alfalfa and lettuce. Natural α -tocopherol is usually found together with β -tocopherol and γ -tocopherol.

7.3.3 Antioxidant properties

Vitamin E is the major chain-breaking antioxidant in body tissues and is considered the first line of defense against lipid peroxidation, protecting cell membranes and lipoproteins at an early stage of free radical attack through its free radical-quenching activity (Ingold et al. 1987). Upon the chemical repair of, for example, a chain carrying lipid peroxy radical, α -tocopherol undergoes a one-electron oxidation to form the α -tocopheroxyl radical (Burton and Ingold 1986). Although the α -tocopheroxyl radical is generally considered to be poorly reactive and to serve as a radical sink, under certain *in vitro* conditions the radical can slowly abstract a hydrogen atom from the bisallylic methylene groups of polyunsaturated fatty acids (Ingold et al. 1993). Consequently, as well as being an antioxidant, α -tocopherol can also be considered to possess pro-oxidant properties, which has been confirmed experimentally (Yamashita et al. 1998).

Conclusion for vitamin E

In conclusion, vitamin E is a rather stable lipophilic molecule. It is the main chain-breaking antioxidant in the process of lipid peroxidation.

8 Antioxidant analysis in foods and biological tissues

Numerous analysis parameters have been shown to influence the extraction yield and the quantification of tocopherol and carotenoid levels from tomato products and human biological samples. Such variations could lead to an imprecise estimate of tomato quality or estimate of association between tomato product consumption and biological responses. So it is important to choose the most reliable method and this method will depend on the research objectives.

8.1 Samples

It has been reported that when parallel serum and plasma samples of blood were collected from one subject, the plasma extract showed 70% and 50% lower retinol and carotenoid peak heights respectively, as compared with the serum extract (Nierenberg 1984; Stacewicz-Sapuntzakis et al.1987), suggesting problems with plasma extraction. Because similar effect was observed for the internal standards, the corrected concentration of these micronutrients in plasma and serum were in the same range. Additionally, Aebischer *et al.* (1999) in a complete quality assurance study have reported that although no differences could be observed statistically for vitamins and carotenoids, the plasma levels for all analytes were slightly, but systematically, 2% lower than serum levels.

No degradation of tocopherol or carotenoids was observed during plasma processing (Gross et al.1995) confirming that plasma samples collected by “typical” clinical blood collection and processing procedures can be used for carotenoid and tocopherol analysis. Moreover, the concentrations of carotenoids and tocopherol were similar in samples of plasma frozen immediately and those that were maintained at room temperature for up to 24 h collected, then frozen (Craft et al.1988).

When tissues are analyzed, depending on the lipid and the collagen levels, digestive enzymes such as lipase and collagenase must be added to improve further homogenisation and extraction (Nierenberg and Nann 1992).

In tomato, the antioxidants are not distributed homogeneously. Seeds contained most of the vitamin E in the fruit and skin and pericarp contained about five times more lycopene than the whole tomato pulp (Le Maguer et al.1998) indicating that probably most of the lycopene is bound to the insoluble fraction of tomatoes. Thus it seems necessary to homogenize all the fruit and to use accurate seed and skin treatments to extract efficiently both vitamin E and lycopene.

8.2 Storage of the samples

Stability studies showed that at -20°C plasma β -carotene was only stable for 6 months whereas tocopherol and vitamin A levels remained unchanged for 2 years, (Fig 14) (Aebischer et al. 1999). However all carotenoids and vitamin E were shown to be stable in plasma stored at -70°C for 28 months (Craft et al. 1988). At -80°C plasma β -carotene and α -tocopherol levels were not affected up to 5 years (Aebischer et al. 1999). When plasma were protected from light during processing and stored under nitrogen, vitamins A and E, and carotenoids did not undergo any significant degradation after repeated freezing and thawing cycles (Hsing et al. 1989, Zaman et al. 1993).

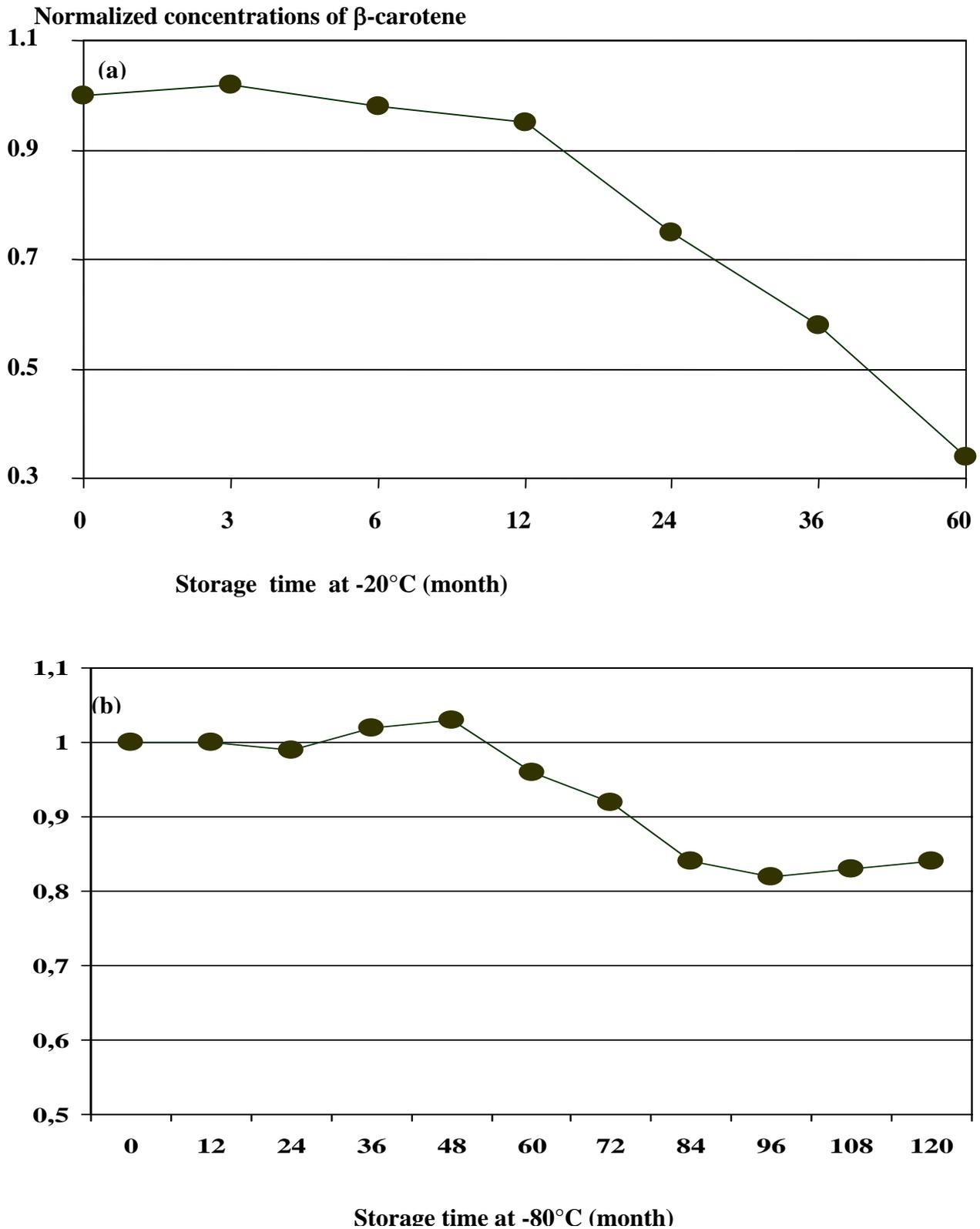


Fig. 14 Stability of retinol, α -tocopherol and β -carotene at -20°C (a) and -80°C (b) in human plasma. (Adapted from Aebisher et al.1999.)

8.3 Standards

When High Performance Liquid Chromatography (HPLC) is used, identification, then quantification of the different analytes needs external and internal standards. These standards must be as pure as possible and must remain stable during standard solution preparation and storage, during extraction and analysis. Impurities can affect the standard calibration and the final quantification of the analytes becomes imprecise. There are actually no 100% pure standards that are commercially available. In consequence when purity is less than 97%, it is suggested to purify the compounds on open columns or specific cartridges just before their use. Osberg et al. (1999) have suggested that lycopene from Sigma Chemical was not always of sufficient quality and that lycopene from Indofine appeared to be better even if more expensive. It is generally accepted that the purity of standards would be calculated through HPLC from the specific area of the standard/sum of the areas before preparing calibration curves.

Unlike tocopherol, carotenoids were shown to be weakly soluble in alcohols and acetonitrile at room temperature. At -20°C , α -carotene, β -carotene and lycopene in ethanol precipitated out at concentrations above $1\ \mu\text{g/ml}$ and did not redissolve when returned to room temperature (Zaman et al. 1993). The more diluted standards were shown to be very stable, except for lycopene, which had to be diluted to about $0.2\ \mu\text{g/ml}$. More apolar solvents such as methylene chloride, hexane, toluene, benzene, or tetrahydrofuran (THF) were thus required to prepare stock solutions. However, chlorinated solvents were shown to degrade *trans*-lycopene and to form several *cis*-isomers. Hence, Scott (1992) observed that over a 20 day period and at -20°C , the concentration of lycopene in chloroform declined by 46-48% (Fig. 15-16).

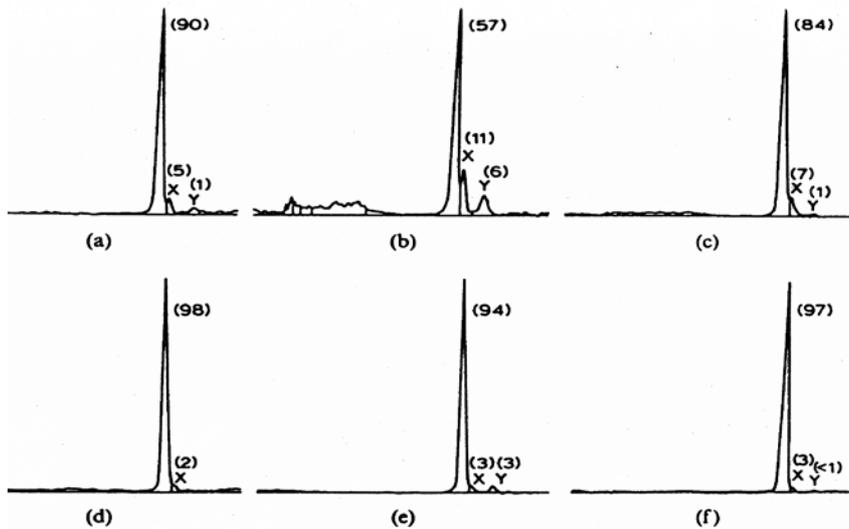


Fig. 15 HPLC profile of lycopene after storage of stock solution in chloroform with and without Butyl hydroxy Toluene (BHT) at -20°C :

a-d, Chloroform solution with (d) and without (a) 0.1% BHT, day 0

b-e, Chloroform solution with (e) and without (b) 0.1% BHT, day 20

c-f, Evaporated chloroform solution which was (f) or not (c) containing 0.1% BHT, day 20

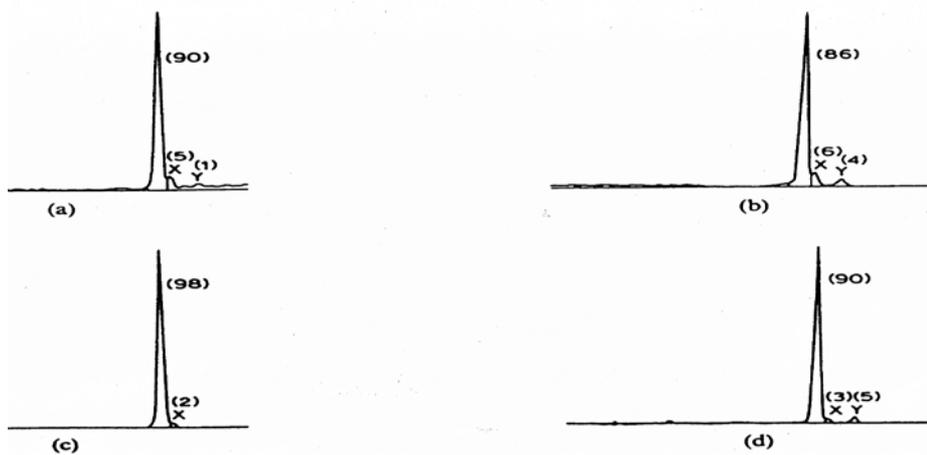


Fig 16. HPLC profile of lycopene after storage in mobile phase.

a-b, working solution at day 0 (a) and 20 (b)

c-d, working solution with BHT at day 0 (c) and 20 (d).

In parentheses, the areas of peaks as % of total area.

(From Scott, 1992)

When 0.1% of the antioxidant BHT was added in the stock solution, the % of lycopene degradation was limited to 7-11%. When diluted in the mobile phase (working solution), lycopene concentration remained unchanged over 20 days. At 27°C, lycopene isomerized readily in organic solvent and the content of *cis*-lycopene reached 50% after 3h of incubation. Lowering the temperature to 4°C or addition of BHT limited the isomerization rate but did not modify the final equilibrium distribution of lycopene isomers (Nguyen and Schwartz 1998). The BHT effect suggested that free radicals might be involved in the isomerization process of lycopene (Gao et al. 1996). Because of the lycopene instability, it has been proposed to evaporate lycopene solution under nitrogen before storing at -20°C, to prepare daily working solution and to inject it separately on the chromatographic system (Hart and Scott 1995, Porrini et al. 1998).

8.4 Extraction

In a comparative study of extraction procedure on 2 g tomato juice, Taungbodhitham et al. (1998) reported that the methods validated by the Association of Official Analytical Chemists (AOAC) were 2 fold more efficient for lycopene than the other methods based on chlorinated solvents, which were usually applied to extract total lipids (Table 26, Fig 17).

Table 26. Extraction methods for comparative study using canned tomato juice.

Methods		
1	Association of Official Analytical Chemists, 1984, Section 43.015	2-5 g extracted 5 min with 100 ml acetone:hexane (4:6)
2	Folch et al., 1957	2 g sample extracted with 34ml chloroform:methanol (2:1)
3	Hara and Radin, 1978	2 g sample extracted with 36 ml hexane:isopropanol (3:2)
4	Hsieh and Karel, 1983	2-5 g sample extracted with acetone:petroleum ether (50:50)

(Solvent ratios in the parentheses are by volume ratio)

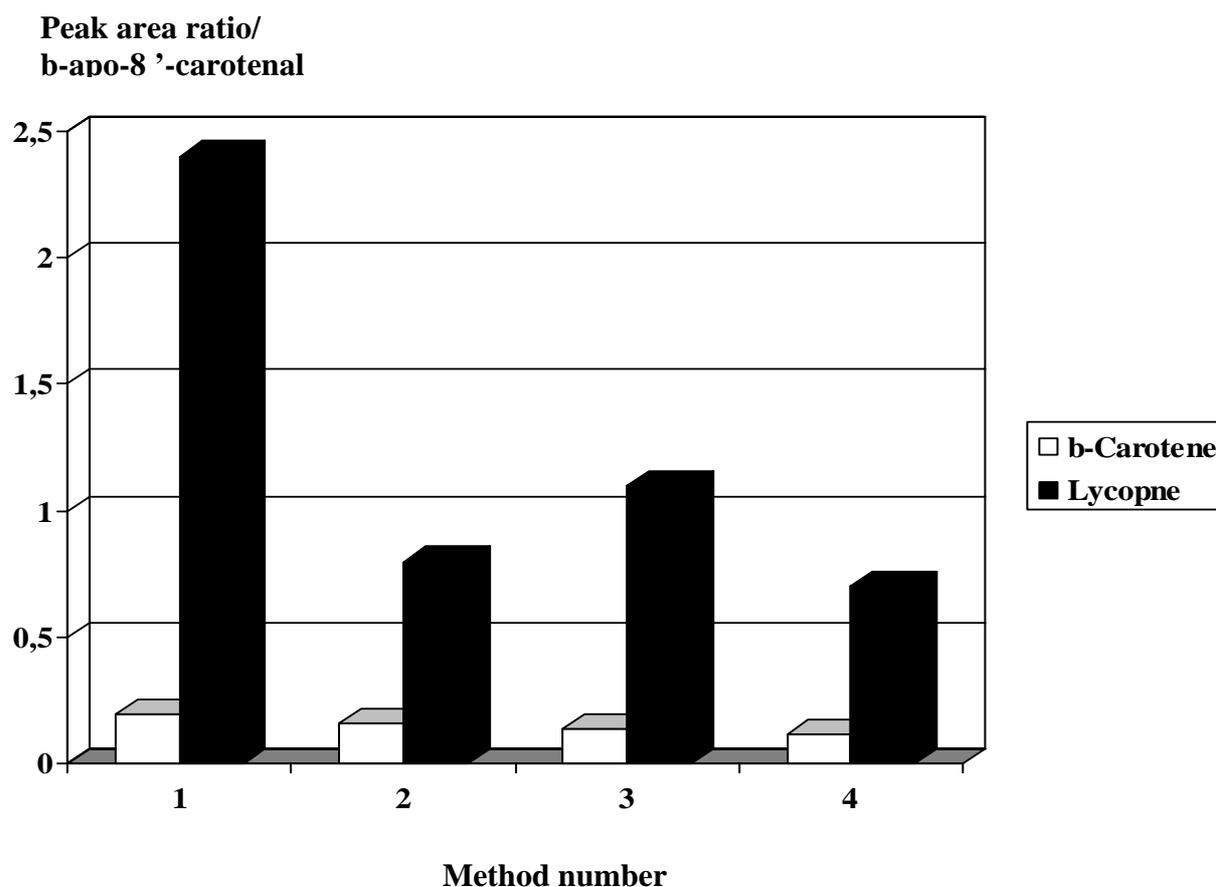


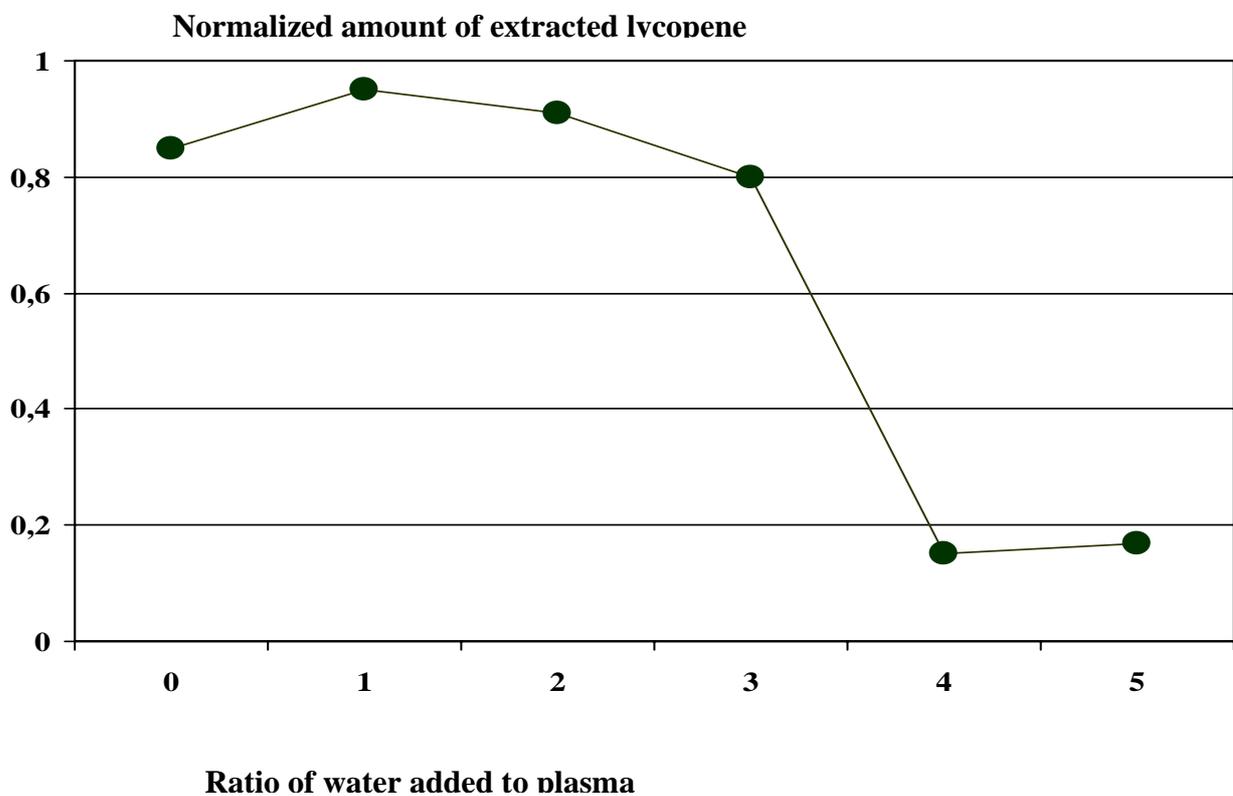
Fig 17. Recovery of tomato carotenoids using different extraction phases.

(method numbers in the figure correspond to numbers in table 26)

However β -carotene was extracted with the same yield whatever methods was used. The effectiveness of different solvents in the extraction of carotenoids was tested on tomato soup (Scott 1992). With the dried material, alcohols were more effective than THF, acetone and hexane. When the tomato soup was dispersed in water, the combination of THF and methanol was found to result in efficient extraction. This mixture was proposed as a first step of extraction of a reference material (vegetable powder) during a certification study financed by the European community (MAT-CT 93-0022) undertaken by J.Scott, R.Seale and P.M.Finglas. The extraction was achieved by addition of petroleum ether and methylene chloride.

For plasma and serum, lipid soluble vitamins and carotenoids were usually extracted after protein precipitation by ethanol, by an organic solvent such as hexane. The extraction was improved if the plasma had been previously diluted by a same volume of water or physiologic buffer (Fig. 18) (Aebisher et al.1999).

Fig 18. Effect of water addition in plasma on retinol, α -tocopherol and carotenoid extraction efficiency.



(From Aebisher et al; 1999)

In several studies, magnesium carbonate was initially added combined with the biological material (Scott 1992, Taungbodhitham et al. 1998, Tonucci et al. 1995). The best extraction yields were obtained when carbonate/solvent ratios were between 1/1 and 1/2

Higher proportions of solvents resulted in a 19% reduction of lycopene extraction (Taungbodhitham et al. 1998).

Extractions of vitamin E and carotenoids in plasma were shown to be efficient (93-100%) after only one extraction (Table 27) (Aebisher et al. 1999). A second extraction leads to a total extraction of all tested compounds. Similar results have been reported for carotenoid extraction from tomato juice, carrots, and spinach (Taungbodhitham et al. 1998). In contrast, Zaman et al. (1993) found that in contrast to vitamins A and E, β -carotene and lycopene from plasma were only extracted around 80% after a single extraction and they extracted a second time to achieve a yield of 100%. Moreover, Aebischer et al. (1999) reported that mixing the plasma/water/ethanol/n-hexane emulsion on a vortex was not sufficient to extract all the vitamin A and E and carotenoids quantitatively and they suggested to horizontally mix 4 ml tubes in order to have at least a void volume of 2 ml.

Table 27. Effect of the number of extraction procedure on the vitamin A and E, and carotenoid recovery.

	Extraction 1	Extraction 2	Extraction 3
Lycopene	97.2	2.8	0
all-trans β-Carotene	97.2	2.8	0
cis-β-Carotene	100	0	0
α-Tocopherol	95.7	4.3	0

To assess the extraction efficiency of the procedure the same plasma has been extracted three times with n-hexane.

(Adapted from Aebisher et al. 1999)

When chlorophylls or high lipid levels were present in the biological material it would be necessary to use alkaline treatment (saponification). The objective of this procedure is to remove chlorophylls and to hydrolyze triglycerides in water-soluble fatty acid salts. However incubation with 30% methanolic KOH for 3 h at room temperature under nitrogen resulted in

significant losses of xanthophylls (37% for lutein) but not carotenes (Khachick et al. 1986, Scott 1992). Granado et al. (1992) reported that these losses could be partially corrected if the sample was quantified on the basis of a calibration curve, which was also subjected to a process of saponification. Similarly, tocopherol from biological tissues and tocol (internal standard) were shown to be largely degraded during saponification (Sommerburg et al. 1997). However, when mild conditions of saponification were used, no marked losses of lutein levels from broccoli were observed (Heinonen et al. 1989). Recently, an enzymatic digestion alternative was proposed (Lietz and Henry 1997, Sommerburg et al. 1997). Hence, plasma samples were saponified by ethanolic KOH (30%) at 60°C for 30 min or were incubated with lipase and cholesterol esterase at room temperature in the dark for 1h. Without saponification or lipid digestion, lutein and α -tocopherol were well extracted whereas lycopene, β -carotene and γ -tocopherol were only moderately extracted by hexane. The enzymatic digestion of lipids resulted in higher recoveries for all analytes than the KOH saponification (Table 28). Because tocol and BHT were degraded during the chemical hydrolysis, the enzyme digestion was preferred.

Table 28. Effect of saponification and lipolysis enzymes on the recovery of plasma vitamin E and carotenoids.

	Direct n=5	KOH n=6	Enzymes n=6
Lycopene	80.7\pm 0.3	91.7\pm 10.8	96.7\pm 4.2
β- Carotene	66.1\pm 1.8	92.7\pm 3.9	99.1\pm 4.5
α- Tocopherol	99.9\pm 0.9	96.4\pm 7.1	104.7\pm 4.2

(Adapted from Sommerburg et al. 1997)

8.5 Analysis

Total carotenoid levels can be estimated through the red color determination. By using the L*a*b colorimetric system, the red (a) and yellow (b) colors are measured and the lycopene level is evaluated.

Total carotenoid levels could be measured by spectrophotometric assessment of a hexane extract at 450 nm. However such simple methods cannot provide information on the carotenoid profile since most of these compounds absorb between 440 and 505 nm. Thus other methods must be used for analysis of plasma and fruits which contains a mixture of carotenoids. However in red tomatoes in which lycopene is the predominant carotenoid and β -carotene and xanthophyll levels are very low, this method could represent a rapid, cheap and simple alternative to high performance liquid chromatography (HPLC) methods to determine the pigment concentration. Moreover the maximum absorption of lycopene occurs around 472 nm and that of β -carotene occurs around 450 nm. Thus shifting the absorbance measurement to the red radiations would result in sufficient response of lycopene and a very low if not undetectable response of β -carotene. Hence lycopene from tomato puree was extracted with hexane containing 0.08% BHT. Optical density of the hexane extract was measured on spectrophotometer at 502 nm against a hexane blank. For validation purposes, hexane extract of lycopene was analyzed by HPLC. HPLC data confirmed that quantitative determination of lycopene by direct absorbance measurement could be an accurate method (Fraile et al. 1998). However this method should not be recommended for tomatoes which contained both β -carotene and lycopene.

When a mixture of tocopherol isomers and carotenoids are present in the sample, chromatographic analyses allowed for progress. The oldest methods described separation of these analytes on paper, thin-layer and open column (AOAC method) chromatographies. They were generally time consuming and resulted in low sensitivity. There are numerous HPLC methods published in literature using normal (Columns with polar solid phase) (Khachick et al. 1992, Schmitz et al. 1994), reverse (columns with non-polar solid phase) (Olmedilla et al. 1990, Schierle et al. 1997), and supercritical conditions (Lesellier et al. 1993). Similarly isocratic mobile phase as well as gradient of mobile phase and flow rate were used. Some of these methods used single UV/Visible, diode array, fluorescence and electrochemical detectors. It should be noted that the detection limits for β -carotene by

electrochemical detection were measured as low as 10 fmol representing a 100- to 1000-fold increase over conventional UV-Visible detections (Ferruzzi et al. 1998). However because electrochemical response was shown to be dependent and sensitive to operational conditions, proper manipulation of the different variables is required. Different carotenoids including *cis* and *trans* isomers can be detected by this method.

We will not here describe specifically all these methods but rather we will try to present several of the main factors that could influence the separation and the quantification of the analytes.

Reverse phase analyses with isocratic conditions lead to fast separation of main plasma carotenoids but they did not allow efficient separation of lutein and zeaxanthin and *trans* and *cis* isomers of lycopene (Aebischer et al. 1999). When normal phases were used, 12 *cis* isomers of lycopene were resolved from the same plasma sample and they consisted around 60% of the total lycopene area. These isomers exhibit different coefficient of absorption (E1%/cm) (Table 29). Thus in plasma, quantification as a single peak (reverse phase) of lycopene would result in an overestimation of its concentration in the sample as compared with the quantification of both *trans*-lycopene and *cis*-lycopene (normal phase) using their respective E1%/cm. However, very few *cis*-lycopene was detected in tomato and tomato products suggesting that quantification of one peak of lycopene would be an adequate measurement.

Table 29. Absorption coefficients of lycopene isomers

Analyte	λ max (nm)	E(1%/cm)	Solvent
all- <i>trans</i> -Lycopene	472	3450	n-Hexane
5- <i>cis</i> -Lycopene	470	3466	n-Hexane
7- <i>cis</i> -Lycopene	470	2901	n-Hexane
15- <i>cis</i> -Lycopene	470	2072	n-Hexane

(Adapted from Aebischer et al.1999)

Some specific columns were shown to separate efficiently and in a short time isomers of lycopene and β -carotene in reverse HPLC conditions. Five *cis*-lycopene peaks were detected in human serum using a 5- μ m Suplex pKb 100 (4.6x250 mm) column (Fig 19) (Stahl and Sies 1992). When monomeric were changed for polymeric C18 columns (Vydac 201TP54) lutein was separated from zeaxanthin and some *cis*-isomers of β -carotene could be detected (Fig 20) (Scott 1992).

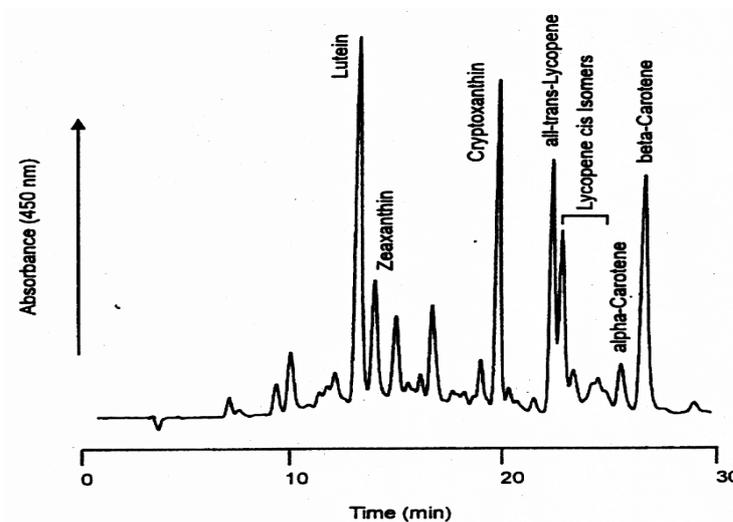
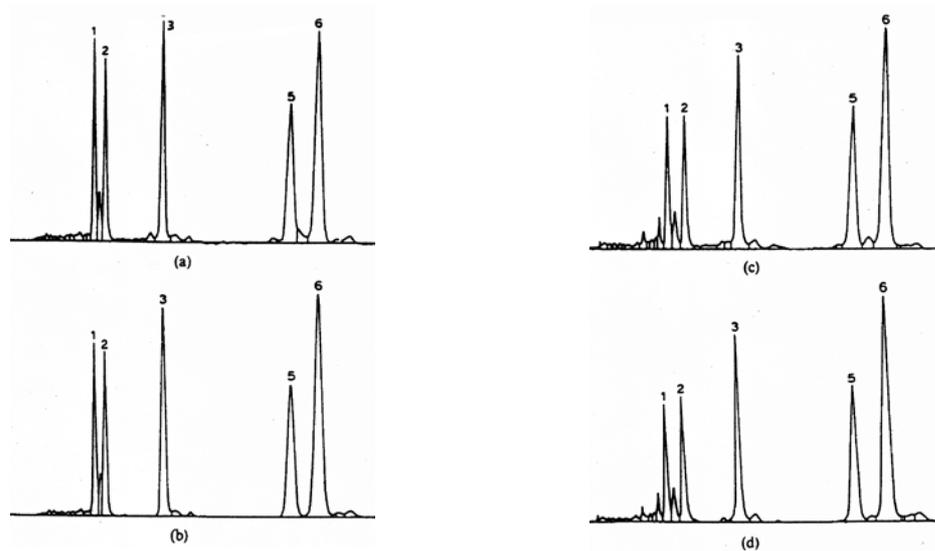


Fig 19. Plasma carotenoids eluted on a Suplex pKb100 column

(From Stahl and Sies 1992)

Fig 20. plasma carotenoids eluted on a Vydac TP 54 column.

(From Scott 1992)



a: column 2, metal frits

b: column 2, metal free frits

c: column 3, metal frits

d:column 3, metal free frits

When a new C30 polymeric column was used, a large number of carotenoids including *cis* isomers were detected in human plasma and vegetable samples (Emenhiser et al. 1996). However when a complex mixture of carotenoids was present in the samples, it was necessary to previously elute different fractions on Sep-Pak cartridges. It must be noted that the recovery of α - and β -carotene from the column were less than 65% probably due to 1) the higher interaction of these pigments with the C30 stationary phase as compared with C18 polymeric and 2) the lack of encapping of the C30 column that gives unreacted silanol groups which are thought to degrade partially carotenoids on the column. Addition of triethylamine (TEA), increased the carotenoid recovery by 21-55 % The mechanism is not known but several hypotheses, including buffering of acid groups of silanols by the basic properties of TEA, were proposed. The same positive effect on the carotenoid recovery was reported for ammonium acetate and a mixture of TEA/ammonium acetate (Scott 1992, Emenhiser et al.

1996). However TEA concentration must not exceed 0.1 % in the mobile phase because tailing process occurred on peaks leading to misquantification.

Of course, the mobile phase was shown to influence the separation efficiency of the different peaks but it can also modify the carotenoid recovery. Hence methanol or buffered acetonitrile mobile phase produced the higher recoveries (Epler et al. 1993). Switching from an unbuffered acetonitrile-based mobile phase to a methanol-based phase gave the most striking increase in recovery (Fig 21).

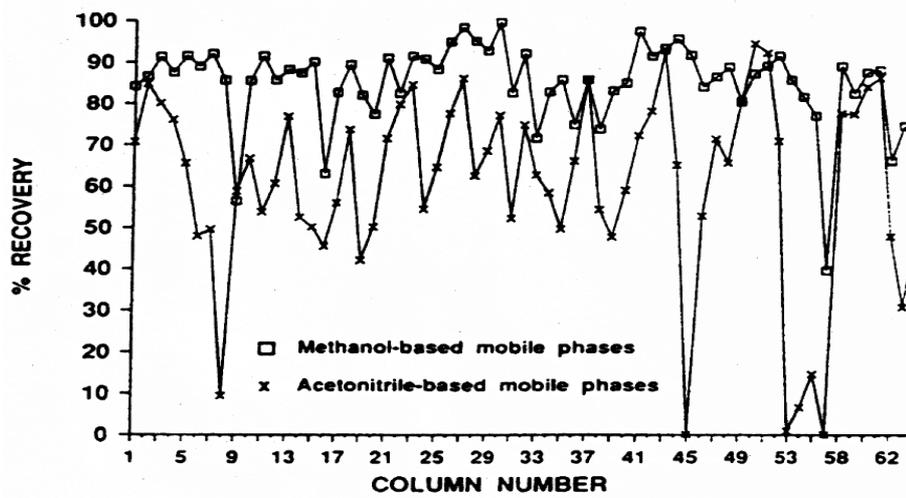


Fig 21. Total recovery of a mixture of plasma carotenoids from 64 commercially available reverse phase columns using methanol and acetonitrile based mobile phases.

(From Epler et al; 1993)

The metal frits of HPLC columns were thought to increase recrystallization or to increase oxydative degradation of carotenoids during analysis (Scott 1992). Replacing metal frits by metal free biocompatible (teflon or ceramic) frits resulted in a reduction of minor contaminant peaks and an increase of the peak response (Fig 20).

Finally, Scott (1992) reported that interactions between carotenoids, injection solvents and mobile phase can result in peak distortion and the production of artefacts which can lead to misinterpretation of the chromatogram. Depending on the nature of the injection solvent, single or multiple peaks can be produced from a single compounds (Khachick et al. 1988). Hence we observed that THF solubilized very well the dry residue of the extraction organic

phase but if the injection volume exceeds 10-15 μ l, then the individual peaks tended to double. The best solution is probably to dissolve the dry residue with a mixture of methylene chloride or THF with ethanol or methanol, and add mobile phase.

9. Description of specific methods for tomato antioxidant analysis

9.1 Lycopene analysis on tomato matrix

9.1.1. Fresh tomatoes

Ripe fruits (Quantity 3.5-5 Kg) are well washed and dried, crushed and cold homogenized in an industrial cutter with variable speed.

All the system is maintained under vacuum; this is important to avoid dangerous air soaks with subsequent oxidation.

The following phase is the analysis of the sample obtained right after homogenization with pigment extraction. If it is impossible to analyze immediately, it is necessary to stock the homogenized sample in under vacuum plastic bags hermetically sealed and placed in a light air safe freezer as soon as possible.

9.1.2 Tomato Puree (light) (Passata di pomodoro) and tomato paste

Industrial tomato puree and tomato pastes have usually a high degree of homogeneity and for this reason the product is analyzed without procedure.

9.1.3 Peeled tomatoes and crushed tomatoes

Before the extraction of pigments, canned peeled tomatoes and crushed tomatoes (whole and pulp) are finely crushed with a hand crusher (homogenizer) and immediately analyzed.

9.1.4 Peels from peeled tomatoes

Peels discarded during processing are taken directly from skin eliminator .

Peels are appropriately diluted with distilled water and treated in a Waring Blender homogenizer maintained under vacuum to obtain very small pieces. Right after, peels are broken using colloid grinding mill.

Peels are suddenly disaerated and analyzed or stocked under proper conditions.

The amounts of fresh tomatoes or preserved tomatoes to weight for the extraction are considered on the basis of the theoretical concentrations of pigments. In the previous examples we have :

2 – 5 g for tomato peels; 5 g for tomato puree; 10 g for crushed tomatoes and for peeled tomatoes; in the case of tomato paste we must dilute the sample to reach the same soluble solids concentration of tomato puree (8°Bx), and to weight the same amount (5g).

9.1.5 Extractions

The extraction method considered is similar to the D.J.Hart and K.J. Scott's method (1995) with some modifications devised to obtain better separations of pigments in tomato matrix and is well described in the work published in *Industria Conserve*, 74, 1999, (341-357) by R. Tamburini, L. Sandei et al.

9.1.6 Recommendations for working with lycopene

- a) Lycopene and its derivatives are sensitive to sunlight, artificial light and oxidation. Therefore, it is necessary to avoid exposure to light and oxygen as much as possible while preparing sample for testing;
- b) it is recommendable to work with amber glassware;
- c) during all stages of determination in the laboratory, all the solutions will contain the antioxidant BHT in a similar concentration to that of lycopene;

- d) the solutions diluted are not stable overnight even in the refrigerator (should be kept in the freezer).

9.1.7 Differences Between our Analytical Method and One of Hart & Scott.

- Quantity of tomato derivatives to weigh . Particular extraction for peels.
- Use of THF instead of CH_2Cl_2 to redilute samples after evaporation with rotavapor: THF seems to be the best solvent to extract lycopene in tomato matrix and secondary CH_2Cl_2 is responsible of decreasing in lycopene concentration after storage (even just 1 day).
- Temperature of columns: not thermostated at 22.5 (from 20 to 24°C air conditioned ambient temperature).

9.2 Vitamin C in tomatoes and in tomato products.

Vitamin C, the antiscorbutic vitamin necessary for normal body functions, is correctly associated by consumers with tomato juice and other tomato products. Whole red-ripe tomatoes contain nearly all the vitamin C activity in the reduced ascorbic acid form. Dehydroascorbic acid has been reported to be from 1 to 5% of the total ascorbic acid in tomatoes.

The ascorbic acid concentration in fresh ripe tomatoes is about 25 mg per 100g.

9.2.1 Processing effects on Vitamin C.

The maintenance of high levels of ascorbic acid products during processing has received considerable emphasis by food technologists. In the manufacture of tomato juice, ascorbic acid is destroyed, mainly by oxidation. Ascorbic acid is oxidized to dehydroascorbic acid, which is further oxidized to degradation products with no vitamin C activity.

The oxidation may be enzymatic or non enzymatic, and is catalized by copper ions. The rate of oxidation is dependent on the dissolved oxygen, enzyme content, dissolved

copper, and temperature of the juice. The longer the tomato juice is held at optimum conditions for oxidation the lower will be the retention of ascorbic acid after processing.

The temperature to which tomato products such as tomato juice are heated in the presence of air is the most important factor in the rate of ascorbic acid destruction; it has been found that the rate of ascorbic acid destruction increases with increased temperature in the presence of air. It is therefore important that juice be brought to the desiderated temperature as quickly as possible and held for only a short period at high temperature. Concentrated products present a further problem in retaining vitamin C.

Tomato paste has been reported to have ascorbic acid content of 49 mg per 100 g on a solid percentage equal to that of tomato juice; however, concentrated products usually contain less ascorbic acid than do whole tomatoes or tomato juice (Lamb et al. 1951; Hummel and Okey 1950).

Bolcato (1936) reported less loss of ascorbic acid in the preparation of tomato concentrates by heating at 60°C to 70 °C than at temperatures either higher or lower.

It is evident from literature on canned tomato products that a good deal of ascorbic acid may be lost during the processing unless care is taken, but that most of the vitamins potency may be preserved if suitable precautions are taken . Tomatoes lend themselves to the production of juice high in ascorbic acid more readily than many other fruits, but the importance of proper processing methods is obvious. It is also clear that an important factor in improving the vitamin C content of commercial products is the utilization of fruits in the canning process, which are initially high in ascorbic acid.

9.2.2 Determination of ascorbic acid in tomato products

9.2.2.1 Volumetric titration

Reagents: 2,6 dichlorphenolindophenol 0.05%
 Ascorbic acid (pure)
 Solution 3% of metaphosphoric acid in acetic acid at 8%

Apparatus: Flasks 200ml
Pipettes 2 ml
Burette 50 ml
Burette 25ml precision 1/20

Preparation of titrated solution of 2,6 dichlorphenolindophenol :

0.1 g of 2,6 dichlorphenolindophenol are diluted in distilled water, then filtered and diluted to 200 ml. 0.1 g of pure L-ascorbic acid weighed precisely and dissolved with 100 ml of solution made of metaphosphoric acid 3% and acetic acid 8% (already prepared).

2 ml of standard solution are transferred in a 50 ml flask and suddenly titrated with the 2,6 dichlorphenolindophenol solution until a mild pink color remain for 5 seconds.

From the media (A) of 3 of these titrations it is possible to calculate the concentration of 2,6 dichlorphenolindophenol solution :

$$x = \frac{2}{A}; \quad x = \text{Amount of ascorbic acid titrated by 1 ml of 2,6 dichlorphenolindophenol}$$

Extraction of tomato samples and determination:

50 ml of fresh tomatoes are diluted to 100ml with solution made of 3% of metaphosphoric acid in acetic acid at 8%. Mix and filter, then 10 ml of filtered solution are titrated with the 2,6 dichlorphenolindophenol solution until a mild pink color remain for 5 seconds.

Ascorbic acid concentration is expressed in mg/L

9.2.2.2 Determination of ascorbic acid by HPLC

Samples are analyzed with HPLC after dilution with metaphosphoric acid to have the complete solubilization of ascorbic acid present .

Reagents: Solution of metaphosphoric acid (33.5-36.5) 6%.L+Ascorbic acid

Apparatus HPLC with spectrophotometric detector UV-Vis wavelength: 254nm
HPLC Column Merck Lichrosorb RP18 (10 μ m) 250x4 mm
Paper filter and 0,45 μ m Millex (Millipore)

Standard preparation:

0,1 g of L+ascorbic acid are weighed and directly diluted with 100 ml of redistilled water (sol.1000 ppm). Then prepare 2 solutions with note concentrations 20 and 50 ppm for the calibration curve.

Sample (tomato derivatives) preparation

Fresh tomatoes are diluted with metaphosphoric acid (6%) to obtain the final concentration of ascorbic acid not over the standard max concentration. Filter with paper filter and with Millipore 0.45 μ m.

Dilutions:	Tomato juice :	x5
	Tomato puree :	x10
	Tomato paste :	x20

HPLC Conditions :

Detector	UV 254 nm
Column	Merck Lichrosorb RP18 10 μ m 250x4 mm
Temperature	Ambient
Flow	1 ml/min
Injection volume	20 μ l
Retention time	10 min
Mobile phase	metaphosphoric acid 0,3% (Isocratic)

The concentration of the ascorbic acid is calculated with external standard and peak area related.

9.3 polyphenols

All information about polyphenol analysis was taken from the very good review of K. Robards and M. Antolovich, *Analyst*, vol 122, 11-34, 1997.

Many procedures have been developed for flavonoid compounds. The design of the analytical procedure will depend very much on the analysis.

9.3.1 Colorimetric method

Total phenolics were most conveniently assessed by spectrophotometric measurement on a simple extract of plant. Many difficulties are associated with this system. First exhaustive extraction with alcoholic and aqueous alcoholic solvents is likely to leave behind much tannin and other bound at the cell wall. Second, the diversity of phenolics means that the selection of a reagent and absorbing wavelength will be a compromise. Colorimetric methods rely on the reaction of the flavonoid with one of a number of reagent of varying selectivity. Folin-Ciocalteu and vanillin are the classic reagents however Folin reagent react with compounds other than the target phenols and interfering reductants must be removed prior the assay.

9.3.2 HPLC Determination

Sample preparation

Flavonoids are generally stable compounds and may be extracted from the dried, ground plant material with cold or hot solvents. Suitable solvents for this purpose are aqueous mixtures with ethanol, methanol, acetone and dimethylformamide. This procedure is unsuitable for anthocyanins and the less polar aglycones such as flavanones, isoflavones and flavonols. The latter are more soluble in chloroform, ethoxyethane and ethyl-acetate-methanol.

The need of a clean-up step depends on the sample type and method of extraction. Carotenoid and chlorophyll pigments can be removed by liquid-liquid extraction with hexane

of the aqueous extract after removal of the organic solvents. Fractionation of phenolics can also be applied on silica and polyamide minicolumns.

Hydrolysis of glycosides may be applied prior the analysis. Three types of hydrolytic treatments are used for this purpose, acidic, enzymatic and alkaline. The rate of acid-base hydrolysis of glycosides depends on acid-base strength, the nature of the sugar and the position of attachment to the flavonoid nucleus.

HPLC analysis

HPLC combines the advantages of simultaneous separation and quantification without the need for preliminary derivatization in most cases. Normal-phase chromatography has been used for the quantification of flavonoids in skin of ripe tomatoes. Non polar components were removed from the plant material by extraction, following which the aqueous phase was suitable to clean-up on a polyamide column. Flavonoids were eluted with methanol prior to acetylation. The recovered acetates were separated isocratically on Lichrosorb Si60 using benzene-acetonitrile, benzene-ethanol or octane-ethanol-acetonitrile solvent systems and detection either 312 or 270nm. As an alternative to HPLC bare adsorbent, supercritical fluid chromatography allows excellent separation of polymethoxylated flavones. Carbon dioxide modified with methanol gave rapid elution of these compounds as sharp, well resolved peaks. For these normal-phase systems, there is the concern of highly polar materials that may be retained irreversibly on column, with the result that the separation characteristics may be gradually altered. Thus reversed-phase chromatography has invariably been the method of choice.

The separation of flavonoids usually eluted on C8 or C18 columns with aqueous mobile phase with methanol, acetonitrile or tetrahydrofuran as organic modifiers. Under the usual reversed-phase conditions, diglycosides precede monoglycosides which precede aglycones. The elution pattern for flavonoids containing equivalent substitution patterns is flavanone followed by flavonol and flavone. Hence, Hertog et al. (1993) published a method to quantify flavonols (quercetin, kaempferol, myricetin) and flavones (apigenin, luteolin) on tomato juice. Glycosides were hydrolyzed under acidic conditions and aglycones were extracted. This products were isocratically eluted on a Nova-Pak C18 with acetonitrile-phosphate buffer as mobile phase and detected at 370 nm.

Phenolics can be detected under UV wavelengths, 280 nm for flavanone glycosides and 313 nm for the polymethoxylated flavones. Fluorescence detection is an obvious mean of improving sensitivity and selectivity in flavonoid analysis. Electrochemical detection of phenolics has been described. Eluted species were characterized by retention data and voltammetric responses.

10. Conclusions about analysis data

Many methods were described to measure lycopene, polyphenols and vitamin C in tomatoes. For lycopene, the measurement of red colour by a chromatometer is quite related to the lycopene content, however this method is not sensible enough and cannot determine all carotenoids.

Determination of lycopene at 502 nm using a spectrophotometer is more specific than the first one but less than high performance liquid chromatography (HPLC). Numerous HPLC analysis were described including normal, reverse and supercritical phase. Reverse phase methods appear more reproducible and more adequate for routine analysis.

Total polyphenols can be determined by the classical method of Folin but HPLC methods are again more specific.

Total vitamin C can be measured by a colorimetric method but both ascorbic and dehydroascorbic acids can be measured by HPLC.

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Effects of mechanical and thermal treatments and storage conditions on antioxidants content and their bioavailability in processed tomatoes

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Introduction

The lycopene in tomato products has always been considered very important, although until the '80s the interest in it was confined to its characteristic as a red pigment and thus responsible for the red colour of tomato products. The colour has been recognised by regulatory authorities in establishing standards of quality. A bright red colour, typical of the mature raw tomato, is taken as evidence of quality by processor and consumer alike.

Millardet in 1875 obtained a crude mixture containing a red pigment, referring to it as *solanorubin*. The name was given by Schunck in 1903 after he had shown that the pigment from tomatoes (*Solanum Lycopersicum* L.) had a different absorption spectrum than carotenes from carrots (Nguyen and Schwartz, 1999).

A considerable body of information is available regarding pigment types and levels in a wide range of tomato genotypes (Zscheile *et al.*, 1996; Davis, 1948; Goodwin, 1952; Liu and Luh, 1977; Yamaguchi *et al.*, 1960).

During ripening, tomatoes change in colour from green, typical of chlorophylls, through pink-orange to bright red, due to the development of carotenoids. These are polyenes, in particular tetraterpenes, which originate from a head-to-tail condensation (with 1,4-bonds) of several isoprenic units; they are divided into xanthophylls, which are oxygen-containing carotenoids, and carotenes, consisting solely of hydrogen and carbon atoms. They present a long chain of double bonds, most of which are conjugated. This chain is responsible for their typical absorption of light in the visible region.

Of the carotenoids occurring in ripe tomatoes, lycopene, red in colour, is the last to form and its formation increases especially after the breaker stage (colour change from green to pink) of the berry. Earlier literature reported that lycopene was found only in the red coloured strains (Zscheile, 1947). So far, little is known as to the effect of agricultural practices and soil/climate factors on the oxidant content of tomatoes. It is plain, however, that factors such as water, fertilisation, temperature and light have a bearing on carotenoid level in tomatoes, as have variety, degree of maturity, harvest date, fruit growth and post-harvest storage (Sies *et al.*, 1998b).

Lycopene formation, for example, is inhibited at temperatures above 30°-32°C, whereas it is favoured at temperatures from 16° to 21°C. The other carotenes occur in concentrations lower than that of lycopene which, in ripe tomatoes, accounts for 85% of total carotenoids (Leoni). Within the berry, lycopene level is higher in the outermost part of the mesocarp's

cells; here it builds up in vesicles (aged chloroplasts) which originate from chloroplast transformation-degeneration and which form, with the carotenoid molecules, the so-called LHC (light-heaping complexes). These complexes consist of sequences of hydrophobic membrane-linked proteins containing several pigment molecules coagulated in the form of elongated needle-shaped crystals (Voet *et al.*, 1991). Laval-Martin (1974) categorized tomato chromoplasts into two types: globulous chromoplasts, containing mainly β -carotene found in the jelly part of the pericarp and other chromoplasts found in the outer part of the pericarp containing voluminous sheets of lycopene. The development and ultrastructure of these sheets of lycopene were studied by Ben-Shaul *et al.*, and named crystalloids (or *coagula*, or clots).

In the last decade, the red pigment has also been seen as a possible natural antioxidant compound, with promising implications for human nutrition and health.

Vegetable products, including tomatoes, contain many substances which may have beneficial effects on the health, providing protection from certain pathologies correlated to oxidative processes. These substances have differing functions, such as free radical scavengers, singlet oxygen quenchers, metal chelants and inhibitors of enzymes involved in the formation of the active species of oxygen (Di Mascio *et al.*, 1989). Several antioxidants are present in tomatoes (carotenoids, vitamin C, polyphenols, vitamin E), although, thanks to its specificity, lycopene has been the main centre of attention. Tomato is the main dietary source of lycopene, the typically red-coloured carotenoid. Other carotenoids, such as β -, γ - and ζ -carotene, lutein, phytoene and phytofluene, are present, though in much lower concentrations. Tomato is an important source of ascorbic acid, which exerts a well-known antioxidant and nutritional effect. Like other vegetables, the tomato contains a number of polyphenolic compounds, which can exert antioxidant activity. Finally, tocopherols are also found in tomatoes, though in low concentrations. The antioxidant composition of the tomato is complex and rich, and optimisation criteria of processing and storage technologies should take into account the preservation of the whole antioxidant pool and of its functional properties.

In most Western diets the majority of carotenoids are derived from green vegetables, carrots, tomatoes and tomato products (Foreman *et al.*, 1993, Bolton-Smith *et al.*, 1991), although in some parts of the world other food items may be a major source, e.g. sweet potatoes. Lycopene concentration in plasma normally ranges from around 50-800 ng/ml (0.1-1.5 μ mol/l) depending upon intake. This lycopene is almost exclusively derived from

tomatoes and tomato products. Large acute doses of lycopene from tomato juice (12 and 80 mg lycopene) failed to cause any measurable change in plasma lycopene concentration (Stahl and Sies, 1992b), while acute doses from tomato puree (16.5 or 7 mg lycopene) resulted in significant increase in plasma (Porrini *et al.*, 1998, Riso *et al.*, 1999) and in chylomicrons (Gärtner *et al.*, 1997); chronic dosing always elevates plasma (Sakamoto *et al.*, 1994, Porrini *et al.*, 1998, Riso *et al.*, 1999) as well as lymphocyte (Porrini and Riso, 2000) concentrations.

However, it is frequently seen that large acute doses of lycopene fail to cause any measurable change in plasma lycopene concentration, whereas chronic dosing does elevate plasma levels (Sies and Stahl, 1998). It is estimated that the intake of lycopene from fresh tomatoes is around 0.92 mg/day in older UK women (Scott *et al.*, 1996) and that in the United States adult population is 3.7 mg/d (Foreman *et al.*, 1993).

Epidemiological studies have demonstrated that tomato consumption provides a protective effect against some types of cancers and ischaemic heart disease; this protective effect has mainly been ascribed to the antioxidant activity of some tomato components. These findings, which are extensively reported in other sections of this document, introduce novel optimisation criteria and goals for processed tomato products. If it is clear that the starting point for the optimisation of tomato nutritional properties is raw material, great attention must also be paid to avoid or minimise the detrimental effects induced by technological processing and by the storage of processed products.

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Sub Group 1 : Processing

Effects of mechanical and thermal treatments and storage conditions on the lycopene content

C. Leoni, G. Bartholin, G. Giovanelli and T. Van Boekel

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Lycopene from processed tomato products

Of the more than 50 dietary carotenoids, lycopene is the most prevalent in the Western diet and the tomato is the primary, and usually the only, source of dietary lycopene.

It is, however, important to note that a large proportion of tomatoes are eaten in the form of industrially-processed products. Yearly, between 25 and 30 million tonnes of tomatoes are processed, more than a third of the 70 millions produced. The average per capita consumption is nearly 3.5 kg (on a fresh tomato basis), with variations from zero for some populations, to 14-15 kg in the EU, and to more than 30 kg in Italy and the USA.

The tomato is now the most important vegetable product used in the making of industrial preserves. The "traditional" tomato-growing nations are the USA, Italy, Greece, Spain, Portugal and France, to which were gradually added Turkey, the countries of North-Africa, Israel, Canada, Mexico, Chile and Brazil and, more recently, China, the Southern republics of the former USSR, Australia, Thailand, India and South-Africa. Table 1 shows the production data for the most recent production campaigns, from which it can be seen that, although this is an industry which produces products of relatively low added value, the USA and the EU alone process 70% of the world's entire tomato production..

Table 1 - Quantity of processed tomatoes in the more important countries (000 tons) in the last 25 years ()*

Country	1975	1980	1985	1990	1995	1999
USA	7715	5646	6525	9307	10235	11724
Italy	1575	3083	3785	3850	3535	4900
Turkey	520	600	1100	1500	1920	1800
Spain	827	499	819	1134	916	1480
Greece	979	1500	1180	1150	1178	1200
Brazil	*	*	*	600	930	1100
Portugal	800	454	716	760	831	996
China	*	*	*	420	550	900
Chile	*	275	*	609	822	900
Tunisia	*	*	-	100	435	720
Canada	350	379	476	580	524	480
France	280	416	392	340	281	370
Argentina	*	*	-	267	190	330
Mexico	210	220	230	365	275	310
Israel	163	166	257	300	315	287
<i>total</i>	*	*	*	21507	22937	27497
World	*	*	*	22821	24959	29592

(*) some data have been excluded because of uncertainty

Tomatoes can be processed into a number of different products, such as:

- canned tomato preserves (such as whole peeled tomatoes, tomato juice, tomato pulp, tomato sauce, tomato paste). Whatever the technological flow-sheet, these products are finally canned and stabilised by heat treatment. Only salt, sugar and citric acid are usually added to standardise the final products;
- tomato-based foods (such as tomato soup, tomato ketchup, etc). In this case many other ingredients can be added to make up the final product, which is canned and stabilised by heat treatment;
- dried tomatoes (tomato powder, tomato flakes, whole, halved and sliced dried tomatoes). These products are dehydrated by different techniques, and low moisture content represents the stabilising factor;
- tomato-containing foods. Tomato is a major ingredient of many typical foods (such as pizza, bolognese sauce and chili, to name but a few). The variety of tomato-containing foods makes it impossible to identify a general processing flow-sheet. Products can be heat-treated, refrigerated, frozen or dehydrated, and can be stored in different conditions, depending on their stability.

In addition, many of the above-mentioned products require further home processing before consumption, such as cooking, baking or rehydration.

Finally, some new processing and preservation technologies have been experimented on in recent years in order to minimise the technological damage and improve the sensory properties of final products. These aspects are treated elsewhere in this document.

It is thus important to evaluate clearly the behaviour of lycopene during industrial processing and the other stages prior to consumption (storage, cooking processes and preparations). In particular, consideration must be made of those processes which may accelerate the degradation process (rises in temperature, contact with oxidatives) and of those which destroy the tissue structure, thus rendering lycopene less protected and therefore more open to attack, but at the same time probably more readily assimilated by man.

The latter factor is of vital importance in the evaluation of whether the potential antioxidant property of the lycopene contained in the tomato and its industrial derivatives can actually be assimilated by man and to what extent it can thus effectively exert its protective role against the considerable number of serious degenerative diseases correlated to the presence of free radicals.

The activity of the WG 2 - Subgroup Processing

Analysis of scientific literature on the matter

The aim of Working Group 2 of the Concerted Action was to examine the scientific literature regarding the lycopene content of tomato products, lycopene degradation during processing and lycopene bioavailability for humans; a critical examination of what is reported in the scientific literature on the matter has been carried out.

We thought to use a critical analysis of the literature collected, similar to the one used in HACCP. In other words, to analyse those factors which may give rise to lycopene modification during the stages from harvesting to consumption, in terms both of degradation and/or loss, and of other modifications (in particular isomerisations) which may influence its reactivity and bio-availability.

In this way, the various factors analysed are viewed as possible causes of degradation, loss or isomerisation; an evaluation is obtained of the potential effect of the preventive measures already in use to optimise the technological processes, and of further corrective measures to adopt for the assignation to tomato products of a new role, that of a "nutraceutical food".

The HACCP Plan can be summarized as follows:

1. Develop and describe the whole flow sheet of the process
2. Describe the product
3. Prepare a list of steps where significant hazards occur
4. Describe the preventive measures
5. Identify the CCPs
6. Establish critical limits for preventive measures with each identified CCP
7. Establish CCP monitoring requirements
8. Establish procedures for using the results of monitoring to adjust the process
9. Establish corrective actions to be taken
10. Establish procedures for verification that the system is working correctly.

Obviously, since we did not analyse a process and did not have the aims of the HACCP (which are fundamentally those of hygiene and health), in our case the system was considerably simplified:

1. Develop Flow Diagram

Describe the whole chain from field to the consumer table

2. Hazard Analysis

Prepare a list of steps where significant lycopene modification hazards occur

Describe the preventive measures normally used, when they may have an effect on lycopene content

Identification of the Relevant Control Points for lycopene content reduction.

Instead of HACCP, we introduced the *HA-RCPL Hazard Analysis - Relevant Control Point for Lycopene content reduction*.

A *Relevant Control Point for Lycopene content reduction (RCPL)* can be defined as a point, processing step or technological operation at which control can be allied and a reduction, degradation or loss of lycopene hazard can be prevented, eliminated or reduced to acceptable levels. The information developed during the hazard analysis should enable us to identify which steps in the process are RCPLs.

1. Develop Flow Diagram - Describe the whole chain from field to the consumer table. Summarising, we can consider these points in the whole chain from field to the consumer table

A. Agricultural production (see WG1)

- Climate, soil and geography
- Varieties
- Cultural practices
- Fertilising
- Harvesting

B. First processing

B.1 Operations common to all types of products

- Handling and transport
- Raw material storage
- Thermal stabilisation treatments
- Packaging
- Storage of finished products

B.2 Operations specific for each type of product

- Crushing and breaking of tomatoes (juice, puree, paste)
- Refining (juice, puree, paste)
- Homogenisation (juice, puree)
- Evaporation (puree, paste)
- Peeling (whole and non-whole peeled tomatoes - non-whole includes chopped, diced, crushed, strained, etc.)
- Drying (powder , flakes)

C. Second processing

- Ingredients (lipophilic or hydrophilic)
- Cooking (industrial and domestic)
- Freezing
- Packaging
- Storage of finished products

D. New processes

- High pressure
- Microwave heating
- Ohmic heating
- Pulsed electric fields

2. Hazard Analysis

2.1 - Prepare a list of steps where significant hazards occur and describe the preventive measures

The Hazards for Lycopene are identified as **L, D, O, I**

- **L** means Quantitative **L**oss
- **D** means Thermal **D**egradation
- **O** means Chemical **O**xidation
- **I** means **I**somerisation

L occurs when a considerable quantity of tomatoes or portion of fruit rich in lycopene is lost.

D occurs when the thermal treatment causes a lycopene degradation

O occurs when lycopene reacts with oxygen. Oxidation is an irreversible step, since volatile hydroxylic and ketonic groups are formed.

I occurs when all-*trans*-lycopene isomerises to *cis*-lycopene. Lycopene may alter in one of two ways: the first involves the transformation (reversible) from the all-*trans* to the *cis* form, which is less coloured and more readily oxidisable; whereas the second is practically the oxidation (irreversible) of both all-*trans* and *cis*-lycopene to a series of volatile compounds with a consequent discolouring of the product. Both mechanisms are described in the section relating to the activity of WG1.

Referring to Section WG1 for the analysis of the themes sub A, for the Technological aspects (Themes B and C), we have identified the hazards for lycopene maintenance in processed tomatoes; these are reported in Table 2 below.

In the same Table 2 we have also identified the **preventive measures** normally used to reduce damage of quality characteristics. Generally, the same preventive measures allow for a reduction in lycopene content degradation.

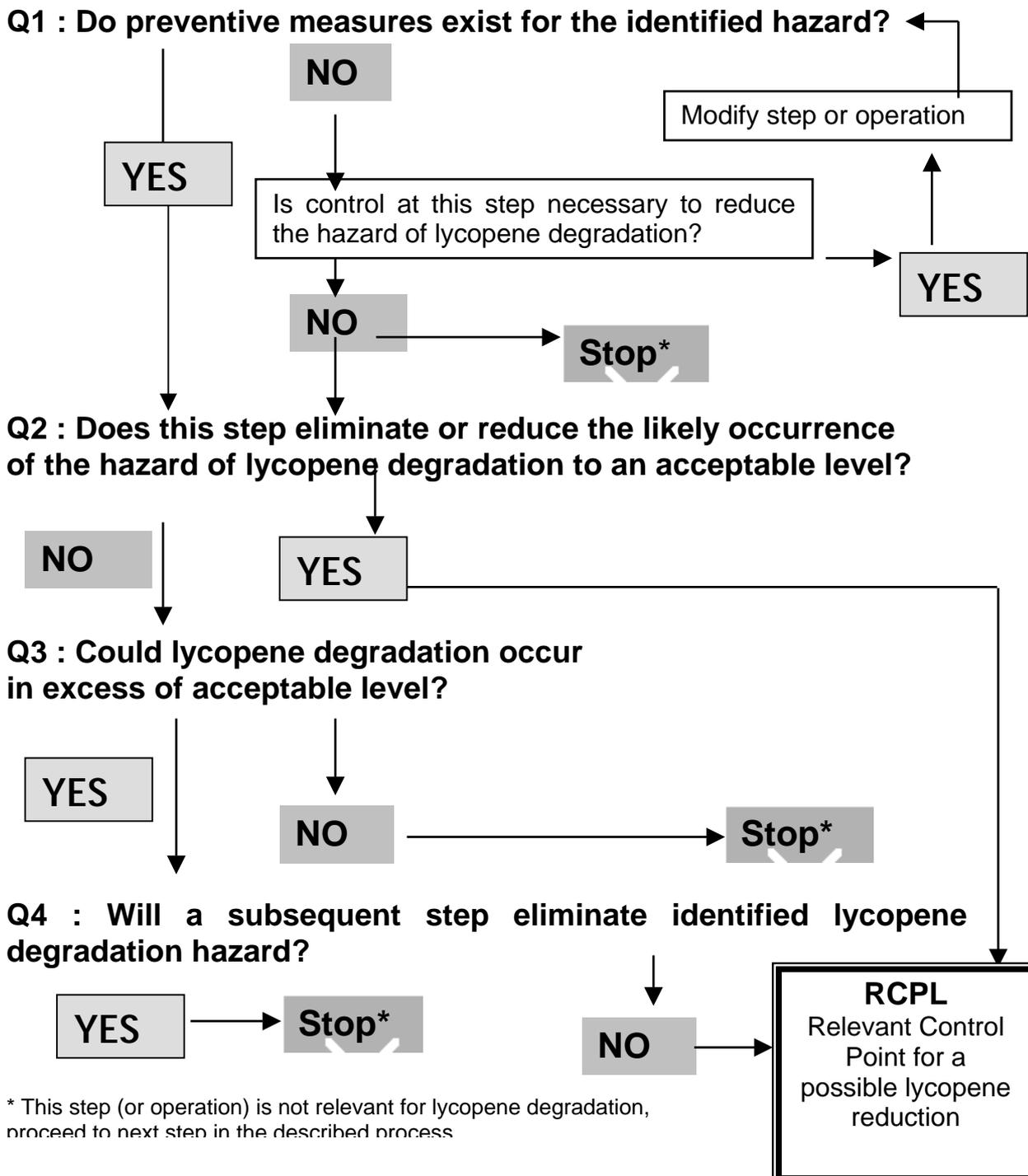
		<i>Step</i>	<i>Hazard</i>	<i>Preventive measures</i>
B1	1	Handling and transport	L	Harvesting in good conditions - Avoid tomato height in truck higher than 120 cm.
	2	Raw material storage	L	Avoid storage in sunny hours and areas
	3	Thermal stabilisation treatments	D I	Avoid fouling in heat exchangers Reduce holding time if not necessary Use temperature as low as possible
	4	Packaging	O	Supply with oxygen barrier material
	5	storage of finished products	O I	O Use barrier packaging I use storage temperature as low as possible
B2	6	Crushing and Breaking of tomatoes (Juice, puree, paste)	D	Avoid breaking at high temperatures if not strictly necessary for consistency characteristics
	4	Refining (juice, puree, paste)	L D O	L Avoid too high refining pressures D Avoid storage at high temperatures if not strictly necessary O Reduce the holding time before the evaporation (air presence in juice)
	5	Other mechanical actions (homogenisation, pumping, etc.) for juice, puree, paste)	O	The operation must be carried out under vacuum
	6	Evaporation (puree, paste)	D	Avoid fouling
	7	Peeling (whole and unwhole peeled tomatoes)	L D	L Reduce the loss of under-peel pulp D Use steam temperatures as low as possible
	8	Drying (powder , flakes)	D O	D Use air temperatures as low as possible O Reduce the time of contact with warm air
C	9	Addition of ingredients (lipophilic or hydrophilic)	D O	D, O Avoid the solubilisation caused by lipid because in soluble forms the reactions are easier
	10	Cooking (industrial and domestic)	D O I	D, O, I Avoid too long cooking time, especially in presence of lipids.
	11	Freezing	O	Protect products from oxygen exposures
D	14	High pressures	unknown	
	15	Microwave heating	D	Use temperatures as low as possible
	16	Electric heating	D	Use temperatures as low as possible

2.2 - Identification of the Relevant Control Points for Lycopene.

The identification of each RCPL can be facilitated by the use of an RCPL decision tree.

Lycopene degradation Tree

Apply at each step of processing to determine if a particular step is important in order to prevent lycopene degradation (RCPL): answer in the indicated order.



* This step (or operation) is not relevant for lycopene degradation, proceed to next step in the described process

Literature review and critical analysis

The aim of this Concerted Action is to examine the scientific literature on antioxidant content and their degradation. Several antioxidants are present in tomatoes (carotenoids, vitamin C, polyphenols, vitamin E), although, thanks to its specificity, lycopene has been the main centre of attention. The HA-RCPL will be carried out with a critical examination of what is reported in the scientific literature on the matter.

B. First processing

B.1 Operations common to all types of products

1	Handling and transport	L	Harvesting in good conditions - Avoid tomato height in truck higher than 120 cm
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Survey of references:

Any

Comments and Topics

It can be assumed that there is no degradation or oxidation of lycopene if normal conditions of handling and transport are used. In the case of fruit crushing, there is obviously a loss of tomatoes and a quantitative loss of lycopene. All other causes of lycopene reduction are precluded if the preventive measures indicated are respected.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	Result
Yes	No	No		No

2	Raw material storage	L	Avoid storage in sunny hours and areas In pools, storage longer than 6-8 hours must be avoided
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Survey of references:

Any.

Comments and topics

Also in this case only a loss of raw material can be presumed, and consequently a quantitative loss of lycopene. All other causes of lycopene reduction are precluded if the preventive measures indicated are respected.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	No	No		No

3	thermal stabilisation treatments	D I	Avoid fouling in heath exchangers Reduce holding time if not necessary Use temperature as low as possible
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Survey of references:

The frequently cited studies by Cole and Kapur do not refer to industrially processed products, since they study degradation with the lycopene dissolved in an organic solution. Results show the importance of temperature in the breakdown of lycopene by oxygen, but in experimental conditions, different from those used in industrial processing. Important losses were observed, but with lycopene in organic solutions. In their pioneer study on the stability of lycopene, Cole and Kapur (1957a, 1957b) investigated the degradation of lycopene extracted from tomatoes by heating hexane and light petroleum ether lycopene solutions at 65° and 100°C under a slow current of oxygen. The effects of the presence of copper (copper stearate 1 mg/L) were also investigated. Lycopene was determined by spectrophotometric analysis, and oxidation products were separated by paper chromatography.

In pure solution, apparent lycopene losses of 26% and 35% were recorded after 3 h at 65 °C and 100 °C respectively. Lycopene degradation was at an almost constant rate at 100 °C, while an induction period was observed at 65 °C. The presence of copper increased lycopene loss in the above-mentioned conditions to 54% and 88.5% respectively. In the same study, the authors studied lycopene degradation due to heating of serum-free tomato pulp. Products were heated at 100 °C, under oxygen or CO₂ flux for up to 3 h, both in the dark and in the light. Lycopene apparent loss was slightly higher in the daylight, and equal to 11.35 and 33.1%, under CO₂ and oxygen respectively. The effects of increasing temperature and increasing light intensity were also investigated. Both light intensity and temperature influenced the rate of lycopene loss, and the effect of increasing temperature was particularly marked. Lycopene loss after 3 hours' heating under oxygen was 18.9%, 34.9% and 53.9% at 60°, 100 and 110 °C (all trials with 100 ft. candle light intensity). Some lycopene degradation products were detected, such as acetone, methyl-heptenone, laevulinic aldehyde and a α -carbonyl, probably glyoxal. From these first studies, it was concluded that lycopene is quite sensitive to heat treatments in the presence of oxygen. It also appeared that in these conditions (soluble form) the food matrix did not provide a protective effect against lycopene oxidative degradation.

During the heating of tomato pulp at 100°C for 120 min at atmospheric pressure, Sharma and Le Maguer (1996a) found that the lycopene content in pulp decreased from 185.5 to 141.4 mg/100 g of d.w. (equation of pseudo first order, with an apparent reaction rate constant K of 0.0023 min⁻¹). The experimental values suitably followed the fitted data (Figure 1) with residual mean sum of squares (RMSS) less than 0.1%

The experimental conditions were very severe and far from the ones used in a usual industrial process.

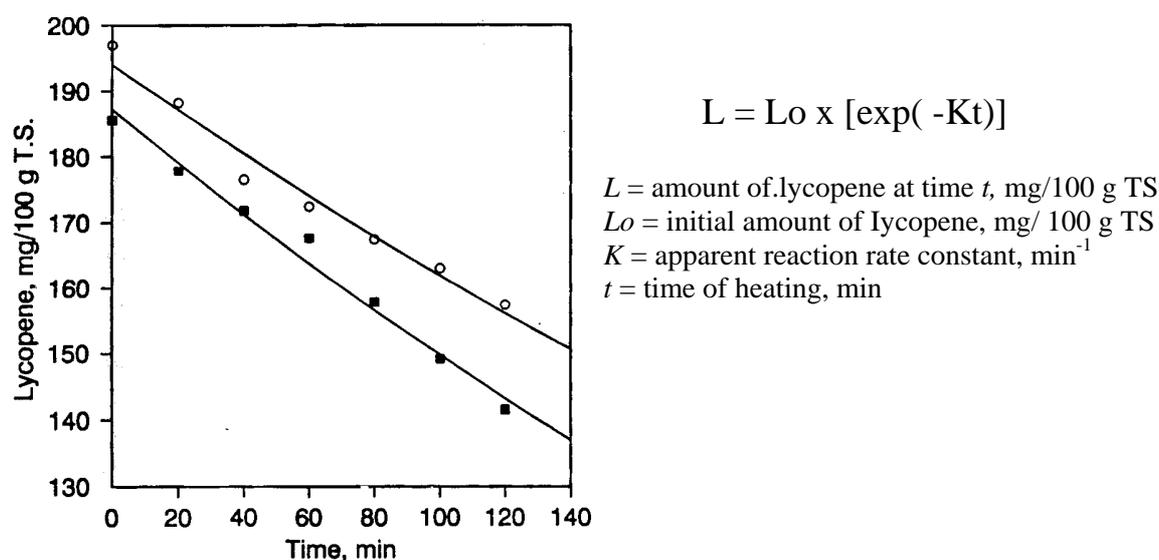


Fig. 1 - Decrease in lycopene content in pulp during heating.

Nicoli *et al.* (1997) studied the effect of heat treatment on the complex of global antioxidant properties of a tomato juice. The information is aggregate and non-specific for lycopene. After 50 hours at 95°C, the antioxidant property of the juice was practically unaltered, and the authors hypothesize the formation of a compound with novel antioxidant properties (MRP). The lack of data relating to carotenoid concentrations renders the study invalid for the evaluation of the effect of heat treatment on the degradation of lycopene

Similar results were obtained regarding the Lerici *et al.* (1997) study

Zanetti studied the global effect of the industrial process. For the cold break production of concentrate manufactured under aseptic conditions and thus using medium-intensity heat treatments in the initial stages (extraction of the juice and concentration), becoming more intensive in the final stage, that of stabilisation, a 28% reduction of lycopene was obtained (with reference to the t.s. content.) as against a 38% β -carotene loss and a 49% phytoene loss.

In the case of diced tomato, no decrease was observed in the case of a filling under aseptic conditions (heating to a high temperature, but for relatively short times and with immediate cooling), or in a hot filling into 500 g cans (bulk heating and quite rapid cooling), whereas there was a decrease of around 10% in the case of a filling into 5 kg cans (slow and prolonged heating of the already canned product and slower cooling).

Comments and topics

The kinetics of lycopene degradation in an aqueous matrix is extremely slow and thus the rise in temperature which occurs during pasteurisation treatments does not cause significant reductions in lycopene content. The decline in quality (colour) is greater than the lycopene decrease, and this is probably due to the fact that the part which degrades is the outer surface of the coagula, i.e. that which is most exposed to the action of the oxidative agents, but also that which is responsible for the "visible" effect of pigmentation.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	Yes			Yes

4	Packaging	O	Supply with oxygen barrier material
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Survey of references:

A thesis by Zanotti showed that in the case of a tomato puree, the best packaging material would seem to be unlacquered tinfoil; in any case, the tests carried out reveal the practical suitability of all the packaging materials utilised (glass, lacquered tinfoil, polypropylene with Al foil), and the high chemical inertia of lycopene in this type of product, substantially devoid of lipidic components, allowing for good maintenance in time also under improper storage conditions.

Comments and topics

Also on account of the substantial chemical inertia of lycopene in an aqueous matrix, oxidation (easier with the use of packaging materials which are partially permeable to oxygen) is rather slow, and of scant significance in quantitative terms. The reductions in colour are more evident. It would be interesting to observe what happens in the presence of oils or fats, which solubilise lycopene and make it more reactive and thus more susceptible to attack

Is this step an RCPL ?

Q1	Q2	Q3	Q4	Result
Yes	Yes			Yes

5	Storage of finished products	O D	O use of barrier packaging D avoid high storage temperature
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Survey of references:

Some research works deal with the effects of storage on the stability of tomato antioxidant components: most of these studies concern dried tomatoes. This is principally due to the fact that dried tomato products are very sensitive to colour fading, and this effect is mostly due to lycopene isomerisation and oxidation and to non-enzymatic browning.

The tests carried out by Zanetti (1997) on industrially produced diced tomato lead to conclusions which are not always easy to explain: They yield a sizeable lycopene reduction only in the product packaged in raw tinplate cans (- 27% after 400 days), and a less noticeable reduction in the product packaged in glass bottles (- 13%)

In their study on foam-mat-dried tomato powder, Lovric *et al.* (1970) made some fundamental observations on the mechanism of lycopene degradation during the storage of dehydrated products. The percent retention of lycopene at various temperatures at high temperatures and in the presence of oxygen and/or nitrogen are shown in Table 3. The main conclusions of this study were the following. With regard to colour stability, a much faster colour loss occurred in the presence of air. The predominating mechanism of colour fading is autoxidation of lycopene. The extreme desiccation achieved with in-package desiccant (<1% moisture) had a pronounced negative effect on colour retention. At 20°C an increase in soluble colour was observed, and this corresponded to a re-isomerisation of *cis*-lycopene into *trans*-lycopene, which is a more coloured form. On the whole, total soluble colour and total lycopene were retained better at 20° than at 2°C. With regard to individual pigments, the detrimental effects of air and desiccation were again observed. After about 150-200 days of storage, *cis* isomers were no longer present, in any sample. The *cis* isomers were lost partly by oxidation and partly by re-isomerisation. Re-isomerisation of lycopene was favoured at 20°C and was hindered by very low moisture content. With regard to lycopene degradation, the total lycopene retention was about 53% and 66% in nitrogen-stored samples and 8% and 22% in air-stored samples at +2° and +20°C respectively. After prolonged periods of storage, samples kept at -10° and +2°C were bleached and emitted hay- or grass-like odours due to oxidation products. In samples stored at +37°C, non-enzymatic browning occurred with subsequent colour browning. The effect of light was also briefly examined. In the presence of air the effects of daylight exposure were negligible, whereas small differences were observed

in nitrogen-packed samples. The authors concluded that while lycopene reversion and consequent colour deepening were favoured by increased storage temperatures (up to 20°C), oxidation was accelerated by higher temperatures. Carotenoid stability in dehydrated tomatoes thus depended upon a number of favourable and unfavourable factors, which had to be taken into account when selecting storage conditions.

A study on vacuum-dried tomato powder showed that in-package desiccation and packaging in an inert atmosphere (e.g. nitrogen) favoured colour retention, while the presence of air caused a loss of lycopene and colour fading by oxidation (Kaufman *et al.*, 1957). Analyses of the storage-study samples for lycopene (Wong and Bohart, 1957) showed that air-packed samples retained the lowest lycopene levels, and all air-packed samples showed a progressive loss of lycopene throughout the storage period. The most important factor contributing to degradation is availability of oxygen during storage. With careful selection of storage conditions to protect the products from such factors as air by storing in an inert atmosphere or under vacuum, it is possible to retain initial color levels during storage.

Table 3 - Total lycopene retention in tomato powder stored in different atmospheres and temperatures for different time lengths.

<i>Storage period (days)</i>	<i>Storage conditions</i>	<i>Total lycopene retention. (%)</i>
Fresh tomato powder		100
30	N ₂ , 2°C	85.5
	N ₂ , 20°C	90.0
	air, 2°C	37.0
	air, 20°C	46.3
80	N ₂ , 2°C	66.3
	N ₂ , 20°C	78.5
	air, 2°C	11.3
	air, 20°C	28.7
160	N ₂ , 2°C	54.2
	N ₂ , 20°C	76.5
	air, 2°C	9.35
	air, 20°C	25.5
210	N ₂ , 2°C	53.3
	N ₂ , 20°C	69.8
	air, 2°C	8.55
	air, 20°C	23.0
385	N ₂ , 2°C	53.0
	N ₂ , 20°C	65.8
	air, 2°C	8.2
	air, 20°C	21.8

from Lovric *et al.* (1970)

Also for Zanoni *et al.* (1998) lycopene appeared to be stable during drying, but not during the storage of the dried product, and thus storage conditions also require optimisation for product moisture, time and temp. of storage, and exposure of product to air and light. The authors observed a marked total lycopene loss (>50% and >70% after 90 days) during storage of powdered air-dried tomatoes ($\cong 10\%$ moisture) at 37°C in the dark in the presence of air.

In a recent work, Anguelova and Whartesen (2000) affirmed that in tomato powders lycopene was degraded 30% and 60%, during 6 weeks of storage at 6°C 45°C respectively (Fig.2).

Reviewing available data on the fate of lycopene during the production and storage of tomato powders, Boskovic (1979) proposed a pathway for all-*trans* lycopene degradation. This pathway involves an initial reversible stage, when all-*trans* lycopene isomerises into mono-*cis* and/or poly-*cis* lycopene. These isomers are energy-richer and relatively more reactive than the all-*trans* form, which is a fully stretched, planar molecule. The reversibility of the isomerisation reaction is explained by the return from an unstable, energy-rich state to the more favoured and stable ground state. Both *cis* isomers and all-*trans* lycopene can proceed to the reaction with an irreversible autoxidation, which is favoured by low a_w and low temperature. Since *cis* isomers are more readily oxidised than the all-*trans* forms; the overall extent of oxidative degradation is related to the isomerisation rate. Even if this pathway was proposed for dehydrated tomato products, it could be assumed that the same reactions could occur during the processing and storage of other tomato products, favoured by oxygen availability and by temperature.

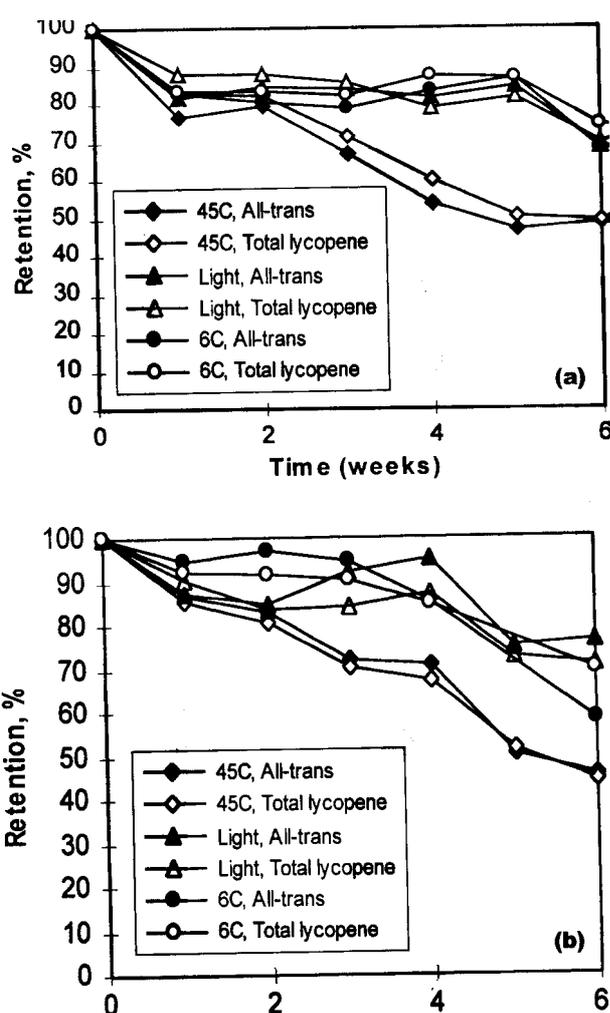


Fig.2 - (a) All-*trans* lycopene and total lycopene Degradation during storage of two tomato powders. Anguelova and Whartesen (2000)

According to Rao and Agarwal ((1999), lycopene contents in canned tomato juice stored at room temperature for 2 months did not change significantly. At 3 different storage temperatures (4, 25 and 37 °C), lycopene levels did not change after 1 month of storage

Sharma and Le Maguer (1996a) studied the kinetics of lycopene degradation during the storage of tomato pulp solids. Samples of fibre-rich tomato pulp, obtained by decanter centrifugation, were stored under different conditions (vacuum and dark, dark and air, and air and light) at -20° , $+5^{\circ}$ and $+25^{\circ}\text{C}$ for 60 days. Lycopene loss was maximum (77.6%) at 25°C in the presence of air and light. Lycopene degradation followed a pseudo-first-order kinetics. The apparent reaction rate constant increased with the increase in storage temperature from 20° to 25°C under different storage conditions, and were the lowest under vacuum and dark, confirming the potential detrimental effect of oxygen and light on lycopene loss. The authors calculated the activation energy (E_a) values for lycopene degradation under the three different storage conditions, finding non-statistically different E_a values ranging from 20 to 28 kJ/mol. Freeze-drying and oven drying (25, 50 and 75°C) of fibre-rich tomato pulp did not cause any loss in lycopene content. However, after storage of dried samples at room temperature in the dark, lycopene loss reached 97% in freeze-dried samples and 73-79% in oven-dried ones. Although the moisture content of the differently dehydrated samples was not reported, it can be assumed that freeze-dried samples were more readily oxidised because of the higher surface exposure to oxygen and the lower moisture content.

Tamburini *et al.* (1998) found that the hot-break juice extraction technique lowers, but less than 2%, the initial lycopene content because of the severe heat effect, although allowing the tomato cell structure to remain almost unchanged preserves lycopene more efficiently over time against the reactions that cause its destruction; in contrast, with other juice extraction techniques (cold-break); owing to the lack of this protective effect on account of the triggering of pectolytic reactions, an 8-15% decrease in lycopene content already occurs after 4-6 months of storage.

A thesis study by Zanotti (1999) reveals that although lycopene remains substantially unaltered in time, even under rather drastic storage conditions, a marked degradation of colour occurs (see Fig. 3)

Time (days)	Lycopene \pm std dev.. (ppm at 9°Bx)	Lycopene \pm .std dev. (mg/Kg of d.w.)
0	187.4 ^{ab} \pm 9.3	2082 ^{ab} \pm 103
30	183.8 ^b \pm 10.4	2042 ^b \pm 115
90	178.5 ^c \pm 15.3	1983 ^c \pm 170
165	180.5 ^c \pm 9.9	2006 ^c \pm 110

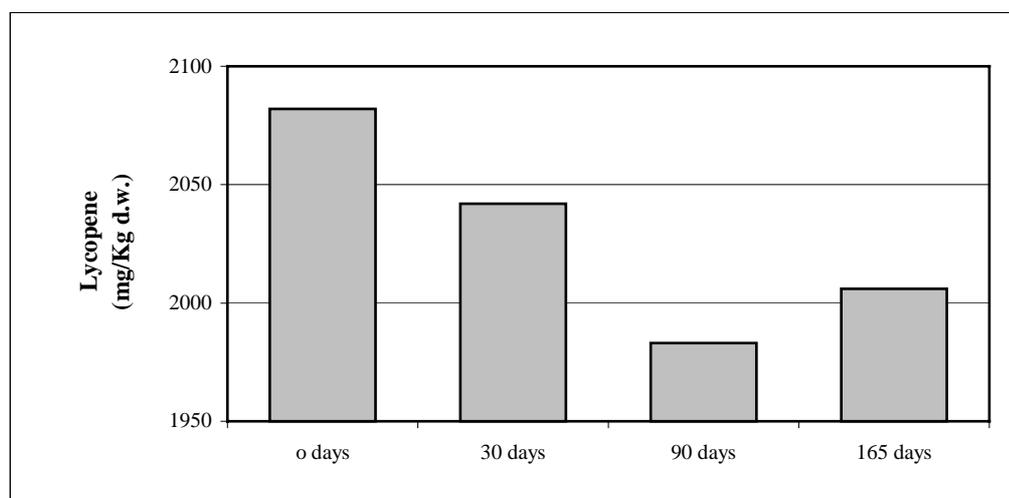


Figure 3: Variation in the a/b ratio with time

Comments and topics -

As long as lycopene remains within the original hydrophilic matrix and, most of all, within a whole cell, it is considerably stable. The hot break juice extraction technique does not lessen the initial lycopene, but allows the lycopene concentration to remain almost unchanged during the storage of finished products, probably because of the more intact tomato cell structure.

Very few studies concern the fate of tomato antioxidant constituents other than carotenoids during the storage of tomato products.

Lee *et al.* (1977) studied ascorbic acid stability in tomato as a function of temperature (from 10° to 38°C), pH (from 3.53 to 4.36) and copper concentration under anaerobic conditions. The degradation reaction followed a pseudo-first-order kinetics, with activation energy of 13.8 kJ/mol at pH 4.06. The rate of ascorbic acid destruction was significantly influenced by pH, reaching a maximum near the pK_a of ascorbic acid ($pK_a = 4.08$). The presence of copper also increased the rate of ascorbic acid degradation, with a linear correlation between copper concentration (from 2 to 10 mg/L) and the rate constant.

Mesic *et al.* (1993) studied the variation in redox potential, ascorbic acid and HMF concentrations during storage of tomato paste in aluminium tubes. Ascorbic acid degradation was followed in sample stored in various materials at 4°, 20° and 40°C. In all cases, residual ascorbic acid after 10 months of storage was approximately 20%-35% of the initial content,

with the lowest values in samples stored at 40°C, and with higher retention in lacquered tubes than in unlacquered ones.

Giovanelli *et al.* (2000) studied the variations in heat damage indexes (HMF and colour) and oxidative damage indexes (lycopene, β -carotene, ascorbic acid, total phenolics, antioxidant activity of the hydrophilic and lipophilic fractions) during 3 months' storage of commercial tomato pulp, tomato puree and tomato paste. Variations in heat damage indexes were significantly higher in tomato paste than in puree and pulp. Colour changes, measured as ΔE values, followed a zero-order kinetics, although darkening was only visually perceptible in tomato paste. Ascorbic acid decreased following pseudo-first-order kinetics, with higher rate constants in tomato paste and puree than in tomato pulp. Ascorbic acid total loss was 40% in tomato pulp (which had the highest initial concentration), 55% in tomato puree, and 60% in tomato paste (which had the lowest initial concentration). Total phenolics content increased slightly in tomato pulp and puree and was stable in tomato paste, which showed a higher initial concentration (about 6000 mg/kg d.w.). Lycopene was almost stable in all products, whereas β -carotene decreased to a final 30-40% reduction. The antioxidant activity, measured as the inhibition of oxidative reactions in model systems, had a similar decreasing trend in all tomato products: the final reduction was about 35%-40% for the hydrophilic fractions and 30% for the lipophilic fractions.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	Yes			Yes

B.2 Operations specific for each type of product

6	Crushing and breaking of tomatoes (juice, puree, paste)	D	Avoid high temperatures if not strictly necessary (HB)
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Survey of references:

Rao and Agarwal (1999) detected a 15.8% lycopene depletion on a production line of tomato juice, although they expressed doubts as to the effective representativity of the sample. They found no further lycopene loss in any subsequent processing operations.

Reference has already been made to the study by Tamburini *et al.* (1999), according to which the hot-break juice extraction technique lessens the initial lycopene content because of the severe heat effect, although allowing the tomato cell structure to remain almost unchanged preserves lycopene more efficiently over time.

Comments and topics

The operation would not appear to give rise to lycopene reductions; in the case of hot-break products, the high heating temperature may cause lycopene reductions, but this is offset by the greater integrity of the cells and thus by the better resistance of the lycopene granules to oxidation.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	No	No		No

7	Refining (Juice, puree, paste)	L O	L Use high refining pressures O Reduce the holding time before the evaporation (air presence in juice)
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Survey of references:

For Tamburini *et al.* (1999) lycopene concentration in puree obtained with a pulper having 1.3 mm diameter holes was higher than in puree obtained with one having 0.8 mm diameter holes; however, the difference, albeit statistically significant, was of negligible practical interest (1%).

Shi and Le Maguer (2000) hypothesise a possible lycopene reduction related to mechanical cell disruption.

Comments and topics-

In the field of refinement currently in use (0.6 - 2 mm), the operation would not appear to be capable of causing lycopene reductions.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	No	No		No

8	Other mechanical actions (homogenisation, pumping, etc.) for juice, puree, paste)	O	Operate under vacuum, if possible
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Survey of references:

The composition and structure of food may affect the release of lycopene from the tomato tissue matrix, with an impact on the bioavailability (absorption) of lycopene. Fine grinding of foods could increase the bioavailability of lycopene by disrupting or softening plant cell walls and disrupting lycopene-protein complexes. Giovannucci *et al.* (1995) compared the differences in lycopene bioavailability from fresh tomatoes and processed tomato products, and found that the lycopene serum concentration was greater with the consumption of heat-processed tomato-based foods than with that of unprocessed tomatoes.

Comments and topics

These operations are very delicate, since many factors suspected of reducing the lycopene content (cell fragmentation, air, temperature) are involved. It would be preferable to avoid homogenisation (otherwise favourable to prevent syneresis) if the product is intended for the manufacture of sauces with oil/lipid as ingredients.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	No	No		No

9	Evaporation (puree, paste)	D	Avoid fouling
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Survey of references

In general, data are reported which reveal the values of lycopene in fresh tomato and in concentrates. Unfortunately, the products are hardly ever comparable, either because the fresh tomato is not the same as that utilised for the concentrate or because the concentration ratios are not fixed, or else because measurements are taken of all the processes and not only at the concentration stage. It must, however, be borne in mind that the concentration by evaporation occurs under vacuum and at temperatures lower than those utilised at the breaking and stabilisation stages, albeit for greater times.

Abushita *et al.* (2000) evaluated the changes in carotenoids and antioxidant vitamins (ascorbic acid and tocopherols) in tomatoes during industrial tomato paste production. Products were sampled and analysed (solvent extraction followed by HPLC determination) at 3 processing stages: raw tomato, crushed-sieved puree, and pasteurised paste (28°Bx). The authors observed an increase in the *all-trans* lycopene and total carotenoid content (on a dry weight basis) during processing, which was ascribed to the removal of seeds and other by-products. *All-trans* β -carotene concentration decreased from 37.2 in raw tomatoes to 26.3 mg/kg d.w. in tomato paste, while *cis* β -carotene increased (from <1 to 9.7 mg/kg d.w.). In contrast, no lycopene isomerisation was observed (*cis* lycopene accounted for 1.7% and 1.5% total lycopene in raw tomatoes and tomato paste respectively). With regard to other vitamins, tomatoes lost about 38% of their original ascorbic acid content during hot-break extraction (90°C for 5-10 min), and a further 16% loss was caused by concentration (60-70°C for 4 h). Tocopherols were not affected by hot-break extraction, but α -tocopherol decreased by 20%, α -tocopherol quinone by 46% and γ -tocopherol by 33% during the thermal processing of tomato paste. The authors concluded that lycopene was stable during tomato paste processing, whereas β -carotene and other antioxidant vitamins were lost to a considerable extent.

Liu and Luh (1977) studied the total effect of paste processing (pilot plant), starting from tomatoes at different stages of ripeness. Unfortunately, they did not report the carotenoid contents of raw material and limited the analyses to the pastes obtained.

The data reported by Tavares and Rodriguez-Amaya (1994) are not utilisable either, since they compare the carotenoid content in fresh tomatoes and in industrially produced derivatives taken from outlet shelves; besides, they do not specify the dry weight values at all. The same consideration must be made for the study by Rao *et al.* (1998) and for that of Tonucci *et al.*

(1995) who reported the aggregate result of processing. Again, it was observed that fresh and differently processed tomatoes contained the same carotenoid compounds with the exception of lutein, which was not detected in ketchup and tomato sauce. Since the data were expressed on a fresh weight basis and solid content was not given, it is only possible to compare quantitative ratios of the individual components in the various products. These ratios did not vary significantly for processed products and whole fresh tomatoes (with the exception of lutein, as previously mentioned), indicating that processing did not cause important degradation of carotenoids. Isomerisation of carotenoids was not investigated in this work. The lycopene content ranges from 93 ppm in fresh tomatoes to 167 in puree and 555 in paste, although the d.w. values are not reported. Normally, the paste/fresh concentration ratio is 6 (the same as that between the content of paste and fresh tomatoes).

The thesis by Zanetti (1997) yields a datum that can be utilised in part. In the production process of HB double concentrate, he observed a progressive lycopene reduction (with reference to 1 kg of dry weight) from 3.7 g (juice at 7 °Brix prior to concentration) to 2.75 (concentrate 24 °Brix), which he attributes to the concentration phase by evaporation, but which is also influenced by the heat stabilisation stage (aseptic packaging)

Comments and topics

Evaporation would not appear capable of causing reductions in the lycopene content higher than those due to heat treatments in general. The prevention of fouling allows for quicker operating times and thus reduces the possibility of heat damage to the lycopene

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
No	No	No		No

10	Peeling (whole and non-whole peeled tomatoes)	L D	L Reduce the loss of under-peel pulp D Use steam temperatures as low as possible
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Survey of references:

Sharma and Le Maguer (1996b) observed that in the fresh tomato lycopene is located above all in the insoluble fraction and that the concentration in the outermost layer (pulp thick fraction, rather vaguely defined) is 5 times greater than in the pulp, although the ratio goes down to 1:2 if referring to dry weight.

Nguyen and Schwartz (1998) reported that the lye peeling of tomatoes (treatment in 18% NaOH at 82 °C for 15 sec) did not cause significant lycopene loss or isomerisation (results were expressed on a d.w. basis). The lycopene content and isomeric distribution found in tomato products produced at the University centre showed that lye peeling of tomato, maceration prior to juicing, pasteurisation of the juice (82°C for 15 sec), canning and heating at 104°C for 50 min of whole peeled tomatoes did not significantly affect either the formation of lycopene isomers or their relative abundance.

Comments and topics.

It is rather difficult to isolate the various tissues (esocarp and mesocarp) completely, hence the analytical data remain uncertain. The peeling technique now almost universally adopted (only in the USA are there a few NaOH peeling lines still in operation) involves scalding the tomatoes with high pressure steam (2-3 bar) for a few tens of seconds to heat the water content of the cells under the peel, followed by a sudden reduction in pressure, which causes the heated water to evaporate and the cells to break, so that the peel comes away from the pulp. The pressure can be lowered by the addition of cold water, or else by connection to a vacuum system; in the first case, the surface of the tomato is washed away, with a consequent loss of substance and thus also of lycopene.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
Yes	Yes			Yes

11	Drying (powder , flakes, halves)	D O	D Use air temperatures as low as possible O Reduce the time of contact with warm air
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Survey of references

A few studies deal with the effects of drying technologies on the antioxidant components of tomatoes. Tomatoes are usually dried by drum- or spray-drying for the production of tomato powder, and by sun- or air-drying for the production of tomato pieces (tomato halves, quarters and slices). Drying is a critical technology in terms of oxidative damage because the product is in most cases exposed to high temperatures and high oxygen levels for long periods. In addition, solid concentration increases during drying, thus increasing the rate of many oxidative and degradation reactions.

Lovric *et al.* (1970) produced foam-mat tomato powder (starting from tomato paste) to study the *cis-trans* isomerisation of lycopene and colour stability during storage. Although drying conditions were not specified, the authors observed a retention of soluble colour in freshly dehydrated powder from nitrogen- and air-foamed puree of 94% and 82% respectively. Data on carotenoid composition (determined by Al₂O₃ column separation followed by spectrophotometric evaluation) of the fresh foam-mat-dried powder showed that nearly 17% lycopene was represented by *cis* isomers.

More recently, Zanoni *et al.* (1999) evaluated oxidative and heat damage induced by air-drying of tomato halves, which were dried using air at 80°C and 110 °C to approximately 10% final moisture content. Lycopene, ascorbic acid, 5-hydroxymethyl-2-furfural (HMF) contents and colour were measured during drying. A marked loss of ascorbic acid occurred during drying: 40% and 80% ascorbic acid losses were already observed at 80%, residual moisture at 80°C and 110 °C respectively. No ascorbic acid remained in 50% moisture samples at 110 °C, whereas a 10% residual ascorbic acid content was detected in final products dried at 80 °C. In contrast, total lycopene had a high stability under the drying conditions applied: no significant lycopene loss occurred in tomato dried at 80°C, and a maximum 12% loss was observed in samples dried at 110 °C. Colour changes became evident when the HMF content had reached 20 mg/kg d.w., that is, when the temperature of tomato halves had reached 80°C.

Shi *et al.* (1999) compared the effects of different dehydration techniques on lycopene degradation and isomerisation. In this study, whole mature tomatoes were perforated with a set of fine needles to create pier holes on the surface (20 holes/cm²); the fruits were then

dehydrated by: a) conventional air-drying at 95 °C for 6-10 h; b) vacuum-drying at 55 °C for 4-8 h; dehydration by an osmotic treatment at 25°C in a 65 °Bx sucrose solution for 4 h followed by vacuum-drying at 55 °C for 4-8 h. HPLC analysis of lycopene isomers on the dehydrated samples showed that no *cis* isomers were detected in raw tomatoes, while 6.5% *cis* isomers were found in osmo-vacuum-dried samples, 10.1% in vacuum-dried samples and 16.6% in air-dried samples. Maximum (3.9%) and minimum (2.4%) lycopene losses were detected in the air-dried and osmo-vacuum-dried samples respectively. The authors concluded that the osmotic treatment could reduce lycopene losses in comparison with other dehydration methods: the protective effect was ascribed to the fact that sugar permeation into the tomato tissues strengthened the bonding force of lycopene in the tomato matrix.

Data from other studies indicated that lycopene *cis* isomers accounted for 5.23% and 6.25% in commercial spray-dried and drum-dried tomato powders respectively (Nguyen and Schwartz, 1998); in the above-mentioned study by Lovric *et al.* (1970), *cis* lycopene represented 17% of total lycopene in foam-mat-dried tomato powder.

Comments and topics

The data confirm the considerable stability of lycopene during industrial transformation, even in the case of extreme conditions (prolonged contact with hot air). Besides, the drying process did not significantly affect the carotenoid content.

Is this step an RCPL ?

Q1	Q2	Q3	Q4	result
No	Yes			No

C. Second transformation

12	Addition of ingredients (lipophilic or hydrophilic)	D O	D The lipid ingredients solubilize the lycopene O In soluble forms the reactions are easier
13	Cooking (industrial and domestic)	D O	D The lipid ingredients solubilize the lycopene O In soluble forms the reactions are easier and the high temperatures activate the cinetics

The two points are treated together, because the domestic or industrial preparations of sauces forecast the cooking with fatty ingredients and the references do not distinguish the two possibilities (with or without).

Survey of references

Most of the scientific literature examined shows that industrially processed products, above all those which are formulated (i.e. those in which the tomato is not the only ingredient) contain more lycopene, and in a form more readily assimilated by man.

In particular, the studies by Rao *et al.* (1998), Gärtner *et al.* (1997), and Stahl and Sies (1992a) underline the greater assimilation properties of lycopene as a result of a diet based on concentrate rather than on an equivalent quantity of fresh tomatoes.

The work of Böhm (1999) is more complex, and is linked to a complete diet, whereas that of Lerici *et al.* (1997), refers in general to the antioxidant property. The study by Lindley (1998) is also of a general nature.

Schierle *et al.* (1997) evaluated the amount of lycopene isomers (all-*trans* lycopene, 5-*cis* lycopene, 9-*cis* lycopene, 13 and 15-*cis* lycopene) in various tomato-based commercial products. Samples were solvent extracted, the extracts were purified by aluminium oxide column chromatography and then injected in the HPLC system, which allowed for the separation and quantification of the lycopene and β -carotene isomers. The effects of the analytical procedure on lycopene isomerisation were also evaluated, showing that the percentage of all-*trans* lycopene increased or decreased by approximately 2-7%, depending on the initial concentration. Food analysis showed that the proportion of all-*trans* lycopene varied from 96% of total lycopene in preserved tomato paste down to 77% in tomato ketchup, 67% in bolognaise sauce and 35% in a long-term cooked spaghetti sauce prepared from canned tomatoes. In order to study the effects of the presence of oil on lycopene

isomerisation, tomato paste dispersed both in water and olive oil was heated for more than 3 h at about 75°C. Heating increased the percentage of *cis* isomers from 7.4% to 16.6% in the aqueous dispersion and from 12.6 to 23.3% in the oily dispersion. The higher initial *cis* isomer proportion in the oily sample (12.6%) was ascribed to a greater isomerisation occurring during sample extraction in the presence of oil. It was concluded from these data that lycopene isomerisation can occur to a significant extent during tomato-based food preparation.

It was found that 20% to 30% of total lycopene consisted of *cis*- isomers when tomatoes were heated at 100 °C for 1 h (Stahl and Sies, 1992a). Görtner *et al.* (1997) found that lycopene bioavailability from paste and processed tomato juice was significantly higher than from unprocessed fresh tomatoes.

Khachik *et al.* (1992a) examined the effect of various methods of cooking (microwaving, boiling, steaming, stewing) on the carotenoid content of green vegetables, but only of stewing in ripe tomatoes. Major carotenoids isolated from raw and processed tomatoes were lycopene, ζ - and β -carotene, lycopene 5,6-epoxide, phytoene, phytofluene, neurosporene and lutein. Most of these components were present both in the all-*trans* and *cis* forms. The authors' main conclusions were that tomato processing (8 minutes' stewing and industrial production of tomato paste) did not modify the carotenoid composition of the products to a great extent, since the same compounds were detected in the three samples. However, analysis of individual data shows that percentages of minor carotenoids (such as β - and ζ -carotene, lutein, phytoene and phytofluene) with respect to lycopene were much lower in tomato paste than in raw and stewed tomatoes. This could be due both to the different carotenoid composition of raw materials and to the effects of processing.

Labrador *et al.* (1998) found that tomato sauces prepared in an open stirred kettle (15 min at 95 °C, in the open air) had significantly better colour attributes and lycopene content than that obtained in a tubular pasteuriser (105 °C for a non-specified time sufficient to reach an F_0 value ensuring product stability), but showed a faster change in lycopene content during storage, indicating lower stability. Also in this case, cooking in an open stirred kettle may have given rise to greater cell destruction, and consequently to a more intense colour, greater aeration of the mass, and a better possibility of extraction (and thus the highest value of lycopene, not otherwise accountable for), although loss of stability during the subsequent storage phase would also occur .

In a recent study, Nguyen and Schwartz (1998) investigated the effects of various processing technologies on the isomerisation rate of tomato carotenoids. The analytical

method (HPLC analysis using a specially developed C30 column) allowed for the separation and identification of 8 different lycopene isomers. In this research study, several commercial products were analysed for their carotenoid profile. In addition, a number of tomato products were produced at the University production centre to further investigate the formation of *cis* isomers as a result of thermal processing. Data collected both on commercial products and on home-cooked tomato products showed that *cis* lycopene levels were not significantly different, despite the different technological processes applied. For commercial tomato products, such as tomato juice, tomato sauce, tomato soup, tomato paste, pizza sauce, spray-dried and drum-dried tomato powder, and sun-dried tomatoes in oil, the total *cis* lycopene percentage varied between 2.5% and 10.1%, with no relationship with the intensity of heat treatment applied to the products (Table 4).

Table 4 - Relative abundance of lycopene isomers in various thermally-processed tomato products prepared at the Food Industry Centre

	lycopene, mg /100 g d.w.	lycopene cis-isomer, %	β -carotene cis-isomer, %
Tomato (fresh)	152.98	4.16	21.77
Peeled tomato	148.89	5.37	23.83
Tomato juice (hot-break)	161.23	5.98	57.55
Tomato juice (retorted)	161.23	3.56	78.28
Tomato (whole, retorted)	183.49	3.67	62.03
Tomato paste (concentrated)	174.79	5.07	57.82
Tomato paste (retorted)	189.26	4.07	85.85
Tomato soup (retorted)	136.76	4.34	55.57
Tomato sauce (retorted)	73.33	5.13	56.14

From Nguyen and Schwartz, 1998.

Even the tomato soup and sauce, which were made from tomato paste and processed at 104°C for 50 min, and the presence of olive oil at 2% and 15% in the tomato soup and sauce, did not affect the isomer content. Although lycopene did not isomerise under any of the processing conditions, significant levels of β -carotene *cis* isomers were formed. The authors found that the formation of *cis* lycopene was induced by severe heat treatments, such as heating a thin film of tomato puree at 200°C for a few seconds to remove water (*cis* lycopene level increased from 4.2 to 19.1%), as well as incubation in organic solvents. For example, 3 hours' incubation of pure lycopene in 1:1 methanol and methyl-t-butyl ether in the dark at 27°C resulted in a *cis* isomer content of 50% compared to 4.5% in the initial solution.

Comments and topics

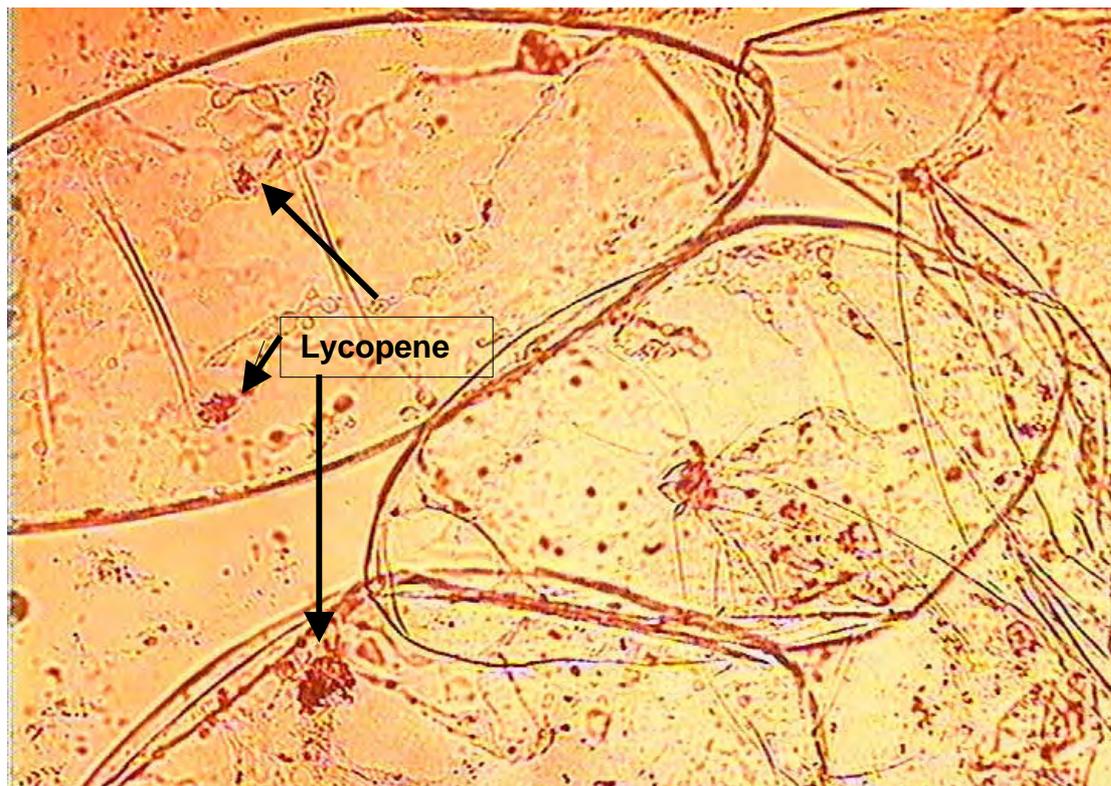
It is quite clear that any industrial transformation will exert a demolitive action on the cells of the fruit; since lycopene is in the state of lipophilic coagulation, it is more readily solubilised by the lipids taken up in a varied diet and thus is in a condition to be more easily assimilated. Lycopene availability from tomato-based food is significantly higher than from fresh tomatoes when co-ingested with oil. Ingestion of tomato juice cooked in an oil medium resulted in a two- to threefold increase in lycopene serum concentrations 1 day after ingestion, but an equivalent consumption of unprocessed tomato juice caused no rise in plasma concentration (Stahl and Sies, 1992b). This indicated that heat treatment and an oil medium are required to extract lycopene into the lipophilic phase. It was assumed that heating tomato juice in the presence of corn oil for 1 h converts lycopene from the *trans* to the *cis* form, thereby increasing its absorption by the body (Stahl and Sies, 1992a).

The food matrix (i.e., the lipid and other constituents of chromoplasts as well as the fibre contained within the tomato fruits) may contribute greatly to the stability of the all-*trans* form of lycopene in the fruits. This is supported by the observation that when whole tomatoes are heat processed, no isomerization is noted. For example, tomato sauce and tomato paste contain about 90% *trans*-isomers (Nguyen and Schwartz, 1998). The food matrix that surrounds lycopene when it is present within the tomato seems to prevent this isomeric equilibrium from occurring.

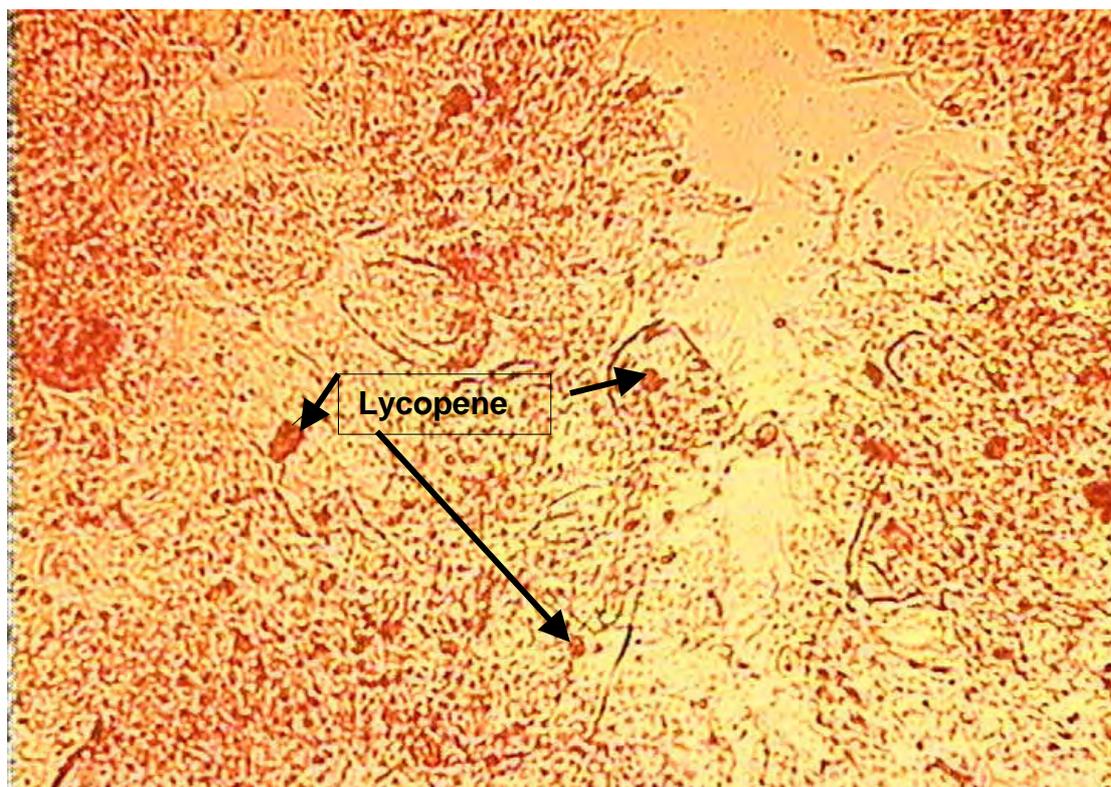
Lycopene is more easily extracted from cooked meals than raw material, where it is bound to the matrix. Thermal processing such as cooking and mechanical cell disruption such as chopping are convenient ways to enhance availability by breaking down sturdy cell wall structures, disrupting chromoplast membranes, and reducing cellular integrity, thus making lycopene more accessible (Fig. 4).

Is this step an RCPL ?

This paragraph includes aspects which vary widely and which cannot be grouped together, hence a hazard analysis does not appear to be applicable.



(a)



(b)

Fig. 4 - Microscope-photographs of a fresh tomato sample (a) and tomato juice sample (b) -
Courtesy Friedrich-Schiller University Jena

14	Freezing	O	
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Survey of references

Urbany and Horty (1989) evaluated colour and carotenoid changes during frozen storage of quick-frozen tomato cubes. Total carotenoids (determined by solvent extraction, column chromatography on magnesium oxide and spectrophotometric evaluation) decreased by approximately 40% during 6 months' storage at -20°C. Lycopene degradation was almost linear in time. Residual lycopene concentrations in 3 tomato varieties ranged from 75% to 12% after 1 year's storage. Colour intensity was linearly correlated to residual carotenoid concentration in the products.

Labrador *et al.* (1998) studied the effect of processing technique on the colour and lycopene content of tomato sauces for pizza during frozen storage. As stated above, sauces prepared in an open stirred kettle (o.s.k.) (15 min at 95 °C, in the open air) had significantly better colour attributes and lycopene content than those obtained in a tubular pasteuriser (105 °C for an unspecified time adequate to reach an F_0 value ensuring product stability) but showed a faster change in lycopene content during storage; this indicates lower stability (in the product obtained in o.s.k., the lycopene content diminishes from 110 to 16 ppm in 6 months of storage at -10 °C, while for sauces obtained with the pasteuriser, the lycopene reduction is from 95 to 30 mg/kg).

Comments and topics

The scant bibliography on the subject makes it impossible to make any affirmations or to carry out an analysis of the hazards.

Is this step an RCPL ?

This aspect includes extremely varying aspects which cannot be grouped together, hence a hazard analysis would not appear to be applicable.

D. New processes

15	High pressures	None	
16	Microwave heating	D	Use temperatures as low as possible
17	Electric heating	D	Use temperatures as low as possible

References:

No data are reported on the effect of non-traditional heating processes such as microwave and ohmic treatments and non-thermal pasteurisation processes by high-pressure technology on the lycopene and more in general on the antioxidant content.

The analysis of microwave processed juice made by Charanjit-Kaur *et al.* (1999) revealed that the juice obtained had low electrolytes, high viscosity and high retention of ascorbic acid, total carotenoid and lycopene content compared to conventionally processed juice.

Comments and topics

It is important to distinguish between microwave heating at the production stage, for enzyme inactivation in the tomato prior to chopping, and that used in home cooking. In the first case, maintaining optimal cell integrity, optimal quantitative and qualitative lycopene conservation is achieved but the lycopene is less readily available (see above comments regarding hot-break).

In the case of microwave heating of industrial products in the home, the shorter cooking time compared to traditional heating methods (cooking or stewing) should reduce the possibility of isomerisation and oxidation and thus allow for greater lycopene retention with the same bioavailability found in the industrially processed product.

Is this step an RCPL ?

This paragraph includes extremely varying aspects which cannot be grouped together, and thus a hazard analysis would appear to be inapplicable.

We can now summarise the result of the RCPL analysis (Table 5).

Table 5. - Hazard Analysis for RCPL (Relevant Control Point for Lycopene content reduction)

	<i>Step</i>	<i>Hazard</i>	<i>RCPL</i>
Operations common to all types of products	Handling and transport	L	No
	Raw material storage	L	No
	Heat stabilisation treatments	D	Yes
		I	
	Packaging	O	Yes
	Storage of finished products	O	Yes
I			
Operations specific for each type of product	Crushing and Breaking of tomatoes (juice, puree, paste)	D	No
	Refining (juice, puree, paste)	L	No
		D O	
	Other mechanical actions (homogenisation, pumping, etc.) for juice, puree, paste)	O	No
	Evaporation (puree, paste)	D	No
	Peeling (whole and non-whole peeled tomatoes)	L	Yes
		D	
drying (powder, flakes)	D O	No	
Selected Processes	Ingredients (lipophilic or hydrophilic)	D	n.d.
		O	
		I	
	Cooking (industrial and domestic)	D	n.d.
O			
I			
Freezing	O	n.d.	
New processes	High pressures	?	n.d.
	Microwave heating	D	n.d.
	Electric heating	D	n.d.

Other aspects

At this point, in order to provide a more detailed view various topics must be addressed which are of importance for quality (ratio between lycopene content and colour of tomato conserves) and for the tomato/health aspect, which is the specific aim of this CA.

1. Lycopene and colour

Various studies have been carried out on the relationship between chemical-physical and sensory parameters of processed tomato, and several methods have been set up to evaluate the quality of products. Despite this, data on the relationship between lycopene content and sensory attributes, in particular colour, are poor, although this relationship may be useful to select products and to set up rapid methods of measuring the lycopene content in raw and processed tomatoes.

Since lycopene is responsible for the red colour of tomatoes (95% of the carotenoids in the ripe fruit), it might seem natural, at first sight, to presume that a correlation exists between lycopene content and the colour of the tomato and its products; colour is normally measured by means of reflectance with *tristimulus* colorimeters (Gardner, Hunterlab, Minolta) which provide measurements with three parameters: L (visual lightness), a (red colour) and b (yellow colour) and combinations of these (a/b , $L*b/a$, $(\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ with respect to a reference value, etc). In effect, this does not occur (Koskitalo, Zanotti), the attempts made yielding correlation coefficients lower than 70%. This is probably because the comparison made is between an objective measurement (absolute lycopene content) and an "artificial" instrumental one. Furthermore, an analytical measurement of lycopene yields the value of all the lycopene present, and not that which is responsible for the colour of the tomato or tomato product, on account of its physical state (solid coagula, of varying dimensions depending on the degree of crushing, in a liquid matrix). Stated in simple terms, the same red colour may be produced by a cumulus of small red granules, either empty or full of pigment.

The same problem occurs with tomato powders, in which the same powder reduced to a different physical state (granulometry) yields, even to the naked eye, completely discordant results, despite starting from the same product and hence the same lycopene content.

2. Heat damage and antioxidant power

When evaluating the effects of heat treatments on the antioxidant activity of tomato products, the formation of Maillard reaction products must also be considered. HMF and furosine (ϵ -N-(2-furoyl-methyl-L-lysine) formation in heat-treated tomato products has been studied by Hydalgo *et al.* (1999). In this study, HMF and furosine reaction kinetics were examined in four tomato products with differing dry matter content (from 10.2 to 34.5%) over a temperature range of 80-120°C. The reactions followed a pseudo-zero order kinetics; E_a values were 139.9 kJ/mol for HMF and 93.9 kJ/mol for furosine formation. Furosine concentration was also determined in many commercial products (detecting values from 43 to 140 for tomato pulp, from 93 to 132 for tomato sauce, and from 220 to 468mg/100 g protein for tomato paste) and during the industrial production of tomato pulp and paste (Hydalgo *et al.*, 1998). This study led to the conclusion that furosine can represent a sensitive heat damage index in tomato products. The interest in Maillard reaction products is due to their activity as pro-oxidant and antioxidant compounds.

Anese *et al.* (1999) studied the antioxidant properties of tomato juice as affected by heating (70° or 95°C for up to 50 h). The antioxidant activity was measured as peroxy radical quenching and oxygen scavenging activity. A decrease in the antioxidant potential was found for short heat treatments; this decrease was ascribed to both degradation of natural antioxidant components (such as ascorbic acid) and the formation of early Maillard reaction products with pro-oxidant properties. However, prolonged heating caused a recovery of the initial antioxidant activity and then an increase in the overall antioxidant activity. This was ascribed to the formation of melanoidins, which exert antioxidant activity during the advanced steps of the Maillard reaction.

Conclusions

An important aspect of this Concerted Action was to verify the contribution of the processed tomato to the human diet; the tomato being the primary, and usually the only, source of dietary lycopene. An attempt has been made to establish accurately the content, type and bioavailability of lycopene intake through industrial derivatives, also taking into account the events occurring during the transformation processes and/or resulting from subsequent storage, distribution and final handling on the part of the consumer.

An initial observation is that very few data are available on the antioxidant components of tomato other than lycopene and carotenoids. The interest in lycopene is justified by its high concentration in tomatoes, which represent its main dietary source, and by its demonstrated antioxidant activity both *in vitro* and *in vivo*. Nevertheless, it is well known that the antioxidant activity of food is usually due to the combined action of many food constituents, which often act with synergistic mechanisms. A more profound knowledge of the other antioxidant components of the tomato and of their fate during processing and storage is needed to understand and optimise the nutritional properties of tomato products.

Data and information supplied by scientific literature on lycopene degradation during common tomato processing such as heat sterilisation, concentration by evaporation and dehydration and also for storage of processed tomato products, though sometimes inconsistent or not completely clear, allow for some general conclusions and comments. Since the operating conditions applied to the tests are either not well defined or do not correspond to those used for industrial treatments, the results should be considered as being often unreliable. Also, the above mentioned studies are incomplete because they only aimed to measure the lycopene content, not its bioavailability, which is most important for the nutritional quality of the product.

The data seem to suggest that lycopene is stable to heat treatments for tomato concentration and cooking but less stable during processed tomato storage even at low temperatures and in the absence of oxygen, particularly for powders and for products submitted to treatments which have damaged the cell walls and which have consequently reduced the protective effect concerning lycopene coagula. Exposure to oxygen, high temperature and low water activity may cause lycopene degradation. With regard to lycopene, researchers substantially agree in considering this compound stable to commercial production processes, in terms of both degradation and isomerisation rate. Even air-drying, which is a

really severe treatment in terms of oxidative stress, does not cause serious lycopene losses. Some of the studies reviewed witnessed a relatively high lycopene loss and isomerisation in heat-treated tomato products; a possible reason for these results, which are in contrast with other data, could be the differing analytical methods and procedures were applied. Various authors have demonstrated that lycopene is much less stable towards isomerisation and oxidation when it is solubilised in organic solvent, and that degradation is promoted by various factors, such as oxygen and light exposure, high temperature, or acidic pH. In order to compare data and results from different studies, standardised analytical methods should be defined and applied, and the use of antioxidant preservative agents (for example BHT) should be recommended.

Other carotenoids, particularly β -carotene, are much more sensitive to oxidative and heat damage than lycopene and are partly isomerised and lost during tomato processing. This is probably due to the fact that different carotenoids have different bond energies and kinetics for isomerisation and oxidation reactions. With regard to other tomato antioxidants, research studies have shown that ascorbic acid is lost to a great extent during tomato processing, and the degradation rate is correlated to the intensity of heat treatments. Changes in polyphenolic compounds and tocopherols have not been sufficiently investigated; consequently, no indications can be given about these components.

Storage of tomato products can represent a critical condition for antioxidant stability. This is particularly true in the case of dehydrated products. Many studies have demonstrated that lycopene is substantially stable to dehydration technologies, while other carotenoids and ascorbic acid are degraded to a considerable extent. However, serious lycopene losses have been observed during the storage of dried tomato products in differing conditions. Exposure to oxygen or light, increasing temperature and very low moisture content increase the rate of lycopene degradation. High total carotenoid and lycopene losses have also been observed during frozen storage of quick-frozen tomato cubes; these products can be considered as low- a_w samples. The effects of oxygen, light and temperature can be easily explained considering the mechanism of lycopene autoxidation; the effect of low moisture has not been clearly explained. It is known that small amounts of water exhibit an important protective action and prevent lipid oxidation in general.

However, many studies led to contradictory conclusions, relating to a presumed marked degradatory effect of storage, probably because measurements were taken on second-transformation industrially processed products with high percentages of oil and/or fats

(sauces) involving a partial solubilisation of the lycopene and consequently greater reactivity and degradation.

With regard to other tomato antioxidants, it has been shown that ascorbic acid concentration decreases during storage in various conditions.

As a final consideration, it seems that more research work is needed to understand the behaviour of the whole antioxidant pool in processed tomato products. Relationships between individual components should be studied, in order to reveal mutual protective or pro-oxidative effects. Researchers usually evaluate the antioxidant power of products in terms of concentration of the various antioxidant constituents: this kind of determination is useful but not exhaustive in explaining the antioxidant effectiveness of food. The antioxidant activity of tomato products should be measured through “functional methods”, that is, methods allowing for the evaluation of the protective effects against oxidative reactions. These methods should use model oxidative reactions similar to those occurring *in vivo* and in food products.

The most stimulating objective of the Concerted Action was to evaluate the presence and the actual bioavailability of lycopene and other antioxidants (in the forms in which they are present in processed tomato products) for humans.

Although there are a number of comparative studies on the bioavailability of lycopene from tomato products, there are no validated methods for the quantitative assessment of bioavailability of the carotenoids; not even for β -carotene, which is the most well studied. There are only few studies on the bioavailability of lycopene in man, one indicating that absorption of lycopene is greater from heat treated tomato juice than from untreated juice and the other indicating that absorption from tomato paste is greater than from fresh tomatoes.

What is known, however is that the physical state and processing history of food has a very marked effect on the availability of these compounds for absorption, hence, disruption of the food matrix and thermal history via processing technique could be the most important factor determining bioavailability. It is also known that bioavailability of carotenoids is markedly affected by the fat content of the diet, since the presence of lipid is essential for the extraction of the carotenoid from the aqueous bulk of the food and the formation of mixed micelles via which the carotenoids are absorbed by enterocytes and transfer to the tissues (via plasma lipoproteins). Carotenoids are passively absorbed lipophilic compounds and their bioavailability is therefore affected by those factors that influence the mass transfer of the carotenoids from the food into the mixed micelles, which can be absorbed by the gut. Interestingly, absorption can be improved by cooking and homogenising the food to break down the cell structure, and by cooking in the presence of oil or fat.

The level of absorption determined in these studies is hugely different, and so we have little reliable information at present to pass on to the industry and to the consumer. However, the involvement of scientists undertaking E.U. funded research in this area will facilitate access to new results which can be disseminated to the relevant industries via the activities of this Concerted Action.

The currently available experimental data are not sufficient to recommend either the form of the food, processing strategy or dietary composition which will result in defined bioavailability.

The results of the review prompt us to state that, so long as lycopene remains within the original hydrophilic matrix and, most of all, within a whole cell, it is considerably stable but that, precisely because of the subsequent scarce reactivity, it is probably less bioavailable and therefore it may be practically ineffective in exerting its potential antioxidant activity. Conversely, when lycopene solubilizes in a lipophilic matrix, it is considerably reactive and more available; therefore, it could efficiently perform its antioxidant activity. However, it is obvious that this higher reactivity renders it much more unprotected against the degradation activity of environmental conditions (air, biological matrix components, temperature).

In conclusion, we can affirm tomato products represent an important, indeed the only, source of lycopene consumption both quantitatively (in amounts higher than those of fresh fruits which, in addition, are often eaten at an incomplete stage of ripeness, when the lycopene is present at lower levels) and qualitatively (i.e., in a form easier to attack and therefore more available). The preparation of sauces in the presence of oil or fat allows lycopene to be solubilized (particularly if the clot has no physical suitable defences, i.e. whole cell walls) and to be more available for humans.

But it is also important to remember that, while lycopene in an aqueous matrix is very stable and therefore able to keep its potential for a long time, in a lipid matrix it is much more reactive and thus available, but also much more rapidly degradable.

Sub-Group 2 : Bioavailability***The bioavailability of carotenoids, with special reference to lycopene*****R.Faulks, S.Southon, V. Böhm and M. Porrini****Contents:**

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Introduction

Most dietary carotenoids are derived from food plants where they serve as photoprotectants (quenchers of singlet oxygen), accessory light harvesting pigments and colourants. In most cases, the carotenoid (normally present as the all trans form) is associated intimately with the light harvesting complex in the thylakoid membranes of the chloroplast where it is found as an ordered structure in association with binding protein (Cogdell, 1988). In the case of the carrot root and tomato fruit the β -carotene and lycopene, respectively, occur as membrane bounded semi-crystalline structures derived from chromoplast, or chloroplast structures. It might be expected that in plants the lipophilic carotenoids would be found in association with sub-cellular lipid structures but it is also known that there are associated binding proteins (Cogdell, 1988). Such a complex environment has implications for their extraction, analysis and behaviour during digestion.

The hypothesis, based on epidemiological evidence, that health benefits arising from the consumption of fruits and vegetables are at least in part due to their carotenoid content, has led to considerable interest in these compounds. However, at present, there are no quantifiable biochemical or physiological markers of carotenoid 'status' (other than in relation to the vitamin A activity). Carotenoid 'deficiency' is not recognised (Mathews-Roth, 1988) but, increasingly, plasma carotenoid concentration is used as an indicator of those 'at risk' of chronic disease based on the direct association between the intake of carotenoid containing vegetables and fruits, plasma and tissue concentrations of carotenoids (Foreman *et al.*, 1993, Bolton-Smith *et al.*, 1991) and the development of chronic disease states, particularly cardiovascular disease (Kohlmeier *et al.*, 1997) and cancer of various sites (Block *et al.*, 1992).

Most recently, a prospective cohort study (Giovannucci *et al.*, 1995) indicated a strong negative correlation between the intake of tomato and tomato products, but not tomato juice, and the incidence of prostate cancer. A similar association was found with plasma lycopene concentration. Thus, it is concluded that lycopene is the effective agent. But why should different tomato products exhibit different strengths of association between intake and efficacy? A currently favoured hypothesis is that the bioavailability of lycopene varies between products, such that lycopene is better absorbed from tomato sauce than from tomato juice. [Bioavailability is defined as the proportion of a nutrient (or other food component) which is absorbed into the body and used in normal metabolism].

Carotenoids are passively absorbed along with lipids. The efficiency of absorption of the carotenoids is, therefore, dependent on releasing the lipophilic molecules and crystals of carotenoids from the food microstructure and dissolution into dietary lipids during processing or domestic preparation, and during the digestive processes. It is now recognised that release and dissolution are the most important factors governing carotenoid bioavailability; so it is not surprising to find greater lycopene bioavailability from heat-treated tomato products that have been comminuted or co-processed with oils (Gärtner *et al.*, 1997, Porrini *et al.*, 1998, Stahl and Sies, 1992a).

Why is the study of carotenoid bioavailability so important ?

- Knowledge of the total amount of a carotenoid in a food does not predict the amount of carotenoid available to the body; this will depend intimately on food microstructure and diet composition.
- Epidemiologic associations between dietary carotenoids and disease incidence cannot be seen as causal; demonstration that the carotenoid is absorbed in sufficient and dose responsive amounts is required.

Processing and storage

Lycopene like the other carotenoids is normally present in fruits and vegetables as the all-trans (E) form. During processing there are a number of physical and chemical changes that need to be considered for their possible impact on bioavailability.

Giovanelli *et al.* (2000a) compared various tomato products (tomato pulp, tomato puree, tomato paste and air-dried tomato halves) in terms of their antioxidant content (lycopene, β -carotene, ascorbic acid and total phenolics) and antioxidant activity of the hydrophilic and lipophilic fractions. Significant differences were observed in ascorbic acid content, according to the severity of the technological process applied (ascorbic acid concentration was 2000, 621 and 400 mg/kg d.w. in tomato pulp, tomato paste and dried tomatoes respectively). Conversely, total phenolics showed an opposite trend, with higher concentrations in tomato paste (6720 mg/kg d.w.) and dried tomatoes (5600 mg/kg d.w.) than in tomato pulp and puree (\cong 4000 mg/kg d.w.). Total lycopene and β -carotene concentrations were similar in tomato pulp, puree and paste, indicating that the different processing technologies did not affect these components.

The main conclusions of Nguyen and Schwartz work are that heat and shear stress during typical industrial food processing operations did not produce lycopene isomerisation or loss, whereas β -carotene readily isomerised during the processing of tomato products. This conclusion agrees with a previous study carried out using the same analytical methods by Lessin *et al.* (1995), who observed an increase in β -carotene *cis* isomers from 15% (raw tomatoes) to 45% (canned tomatoes) and 48% (tomato juice).

Thermal processing is normally undertaken to render the product edible, to eliminate any spoilage/pathogenic organisms and to inactivate enzymes. Cooking therefore softens the cell walls so that they are easily separated or broken mechanically, all cellular membranes are destroyed and proteins denatured. The carotenoid, stable within the original structure (Schierle *et al.*, 1997, Nguyen and Schwartz, 1998), is then exposed to the external environment where it may be subject to light, atmospheric oxygen and reactive products of other components. Lycopene appears to be quite stable in the fresh tomato and during processing, however, once extracted it is readily oxidised. It has been shown that excessive thermal processing may also create *cis* (Z) isomers, particularly 5(Z)-lycopene although isomerisation can also occur at positions 9, 13 and 15 (Schierle *et al.*, 1997, Nguyen and Schwartz, 1998). *Cis* isomers with their kinked structure tend to be more soluble in organic

solvents and this change in physical properties may have an influence on the ease with which they are absorbed by the gut and on their partitioning between the various lipoprotein carriers.

Any losses of lycopene that occur after thermal processing may be a result of light exposure, or oxidation by compounds formed enzymatically or thermally during processing. Processing however, increases the availability of lycopene for absorption, particularly if processed in the presence of lipid. (G•rtner *et al.*, 1997, Porrini *et al.*, 1998, Stahl and Sies, 1992b). It is also recognised that a small amount of dietary fat (10 g) itself improves carotenoid bioavailability (Jayarajan *et al.*, 1980, Dimitrov *et al.*, 1988, Prince and Frisoli, 1993, Reddy *et al.*, 1995).

Intake

In most Western diets the majority of carotenoids are derived from green vegetables, carrots, tomato and tomato products (Foreman *et al.*, 1993, Bolton-Smith *et al.*, 1991), although in some parts of the world other food items may be a major source e.g. sweet potatoes. Lycopene concentration in plasma normally ranges from around 50-800 ng/ml (0.1-1.5 $\mu\text{mol/l}$) depending upon intake. This lycopene is almost exclusively derived from tomatoes and tomato products. Large acute doses of lycopene from tomato juice (12 and 80 mg lycopene) failed to cause any measurable change in plasma lycopene concentration (Stahl and Sies 1992b), while acute doses from tomato puree (16.5 or 7 mg lycopene) determined a significant increase in plasma (Porrini *et al.*, 1998, Riso *et al.*, 1999) and in chylomicrons (Gärtner *et al.*, 1997); chronic dosing always elevates plasma (Sakamoto *et al.*, 1994, Porrini *et al.*, 1998, Riso *et al.*, 1999) as well as lymphocyte (Porrini and Riso, 2000) concentrations. Additionally, apricots, guavas, watermelons, papayas, and pink grapefruits contain lycopene. Table 6 shows the lycopene contents of some foodstuffs rich in lycopene.

Table 6. Contents of lycopene in selected foodstuffs (modified from Nguyen *et al.*, 1999)

Food	Type	Amount (mg/100 g)
Apricots	fresh	0.005
Apricots	dried	0.86
Chili	processed	1.08 - 2.62
Grapefruit	pink, fresh	3.36
Guava	pink, fresh	5.40
Guava juice	pink, processed	3.34
Papaya	red, fresh	2.00 - 5.30
Pizza sauce	canned	12.71
Pizza sauce	from pizza	32.89
Spaghetti sauce	processed	17.50
Tomato juice	processed	7.83
Tomato paste	canned	30.07
Tomato soup	canned	3.99
Tomatoes	red, fresh	3.10 - 7.74
Tomatoes	whole, peeled, processed	11.21
Vegetable juice	processed	7.29
Watermelon	red, fresh	4.10

Recently, some capsulated products became commercially available, containing tomato extract or lycopene as single compound as well as mixed with other antioxidants.

However, it is frequently seen that large acute doses of lycopene fail to cause any measurable change in plasma lycopene concentration, whereas chronic dosing does elevate plasma levels (Sies and Stahl 1998). It is estimated that the intake of lycopene from fresh tomatoes is around 0.92 mg/day in older UK women (Scott *et al.*, 1996) and that in the United States adult population is 3.7 mg/d (Foreman *et al.*, 1993).

Digestion

The carotenoids are insoluble within the aqueous environment of the GI tract and therefore to be absorbed by the body they must be released from the food matrix and solubilised within a lipophilic pool. Within the gastric environment the principal pool is the lipid ingested within the meal. The lipid forms droplets (an emulsion) within the chyme and thus provides a large surface area of dissolution of the carotenoids. The droplets are emptied into the duodenum where the pancreatic enzymes, principally lipase, hydrolyse the lipid into free fatty acids and monoglycerides. These lipolysis products form a complex phase at the droplet surface that dissociates to form a micellar (molecular aggregate) phase with bile constituents such as the phospholipids and bile salts. It is the micellar phase that is the diffusion vehicle of the carotenoids to the point of absorption at the mucosal surface.

The carotenoids released from the food matrix can be solubilised within the emulsion droplets in the stomach or directly into the micellar phase within the duodenum. The ease of incorporation of lycopene within the micellar phase is determined not only by its ease of release from the food matrix itself but also by the ease of inclusion into the micellar phase either directly or via the emulsion droplets. Release from the food matrix is enhanced by efficient particle breakup of the food material (Rich *et al.*, 1998) either by separation of the plant cells or by their complete disruption. Heating of foods prior to ingestion can also improve carotenoid availability as a result of the dissociation of the protein-carotenoid complexes or dispersion of the crystalline aggregates. (Grtner *et al.*, 1997, Porrini *et al.*, 1998).

Similarly, if the solubilisation of the carotenoid within a lipid phase is allowed to occur during processing, for example by heating tomato juice with supplemental lipid then the measured absorption of lycopene is enhanced (Stahl *et al.*, 1992b). It should be noted that the solubility of the carotenoids is still very limited within bulk lipid (0.112-0.141% for hydrocarbon carotenoids and 0.022-0.088% for hydroxyl carotenoids). Transfer of the carotenoid from the emulsion to the micellar phase may also be influenced by the hydrophobicity of the carotenoid. The hydroxy-carotenoids (eg. lutein) are located preferentially at the surface of the lipid droplets hence exchange with the micellar phase will occur. For the hydrocarbon carotenoids, (eg. lycopene) which are located within the random core of the lipid droplets, exchange with the micellar phase does not occur and lipolysis is

necessary for transfer to the micellar phase (Borel *et al.*, 1996). Little information is currently available as to the solubilisation capacity of mixed micelles for lycopene. The structures of the micellar phases are generally closed packed and hence inclusion of a substrate is geometrically constrained. Thus the solubility of hydroxy carotenoids will differ to those of the hydrocarbon carotenoids. Initial results from in vitro digestive systems (Garrett *et al.*, 1999) suggest the ease of solubilisation of selected carotenoids can be ranked in the following manner: lutein greater than β -carotene greater than lycopene. From this short discussion it can be recognised that the digestion and absorption of the carotenoids is a multistage and multiphase process. The relative ease of dissolution in each phase will be determined by the structure and physicochemical properties of the carotenoid in question, the food matrix it is incorporated within and the other components of the meal, principally the composition and level of lipid. These differences in physical properties and their preferred lipid domains will also control the possible transfer of carotenoids between lipid structures both in the gut lumen and post absorption.

Carotenoids are passively absorbed from the micellar phase (Parker, 1997). However, it is not known if all the carotenoids present in a mixed micelle is absorbed, or whether some is left behind in association with unabsorbed bile salts and cholesterol, perhaps to be absorbed more distally or lost to the large intestine. Factors that increase the thickness of the unstirred layer on the surface of the gut, for example soluble dietary fibre, act as a barrier to the absorption of dietary fats and may, therefore, also inhibit the absorption of carotenoids (Gee *et al.*, 1983, Rock and Swendseid, 1992). Disease states which impair lipid absorption, for example cystic fibrosis and coeliac disease, also lead to low plasma carotenoid levels, although in some cases persistent inflammation may be a significant factor in reducing plasma levels of carotenoids (Homnick *et al.*, 1993). The mass transfer of the carotenoids from digesta to absorbable species is clearly a limiting step in the bioavailability of carotenoids on the basis that free carotenoids given orally either as supplements, or as oil solution, or suspension are much better absorbed (Faulks *et al.*, 1997) than those from foods and the evidence that homogenisation of the food and heat treatment enhance absorption.

Various types of dietary fibre were found to reduce the bioavailability of carotenoids in foods (Erdman *et al.*, 1986). Matrix effects were proposed as an explanation for the lack of improvement in vitamin A status in Indonesian women fed green leaf vegetables compared with a manufactured wafer containing a similar amount of carotene in oil solution (De Pee *et al.*, 1995). Rock and Swendseid (1992) tested the inhibitory effect of pectin, a typical dietary fibre, and their results showed that this type of dietary fibre affected the absorption of dietary

carotenoids in humans. High-methoxyl pectin is especially associated with the hypocholesterolemic effect of dietary fibres and the low absorption of lycopene because of promoting high-viscosity conditions

Absorption: methods of measurement

Whilst the emphasis in the concerted action 'Role and control of anti-oxidants in the tomato processing industry, FAIR CT 97-3233' is on the 'tomato' carotenoid lycopene, data on the bioavailability of this carotenoid are very limited. This chapter is therefore dependant on examples related to beta-carotene, the most widely studied carotenoid. The points raised with regard to the importance of understanding factors affecting bioavailability, and methods for estimating carotenoid absorption are, however, applicable to the study of lycopene.

Various approaches have been used to assess carotenoid absorption and in each case assumptions are made in order to estimate values, either absolute or comparative. The sections below describe the most widely used approaches for assessing carotenoid absorption. It should be remembered, however, that whilst absorption is a major element of carotenoid bioavailability, it is only part of the story; it take no account of their metabolic fate.

Metabolic balance techniques

Metabolic balance studies are commonly used to measure the absorption of minerals because they are still measurable as minerals even if they do undergo changes in oxidation state, or are associated with other molecular species during processing or passage through the GI tract. Such studies are normally carried out over a period (5-8 days) known as the balance period.

Balance studies measure the difference between intake of a compound and its excretion. This may involve several meals given over several days (chronic dosing) or a single meal given after an overnight fast (acute dose).

The 'chronic' method is dependent on establishing an equilibrium between intake and excretion and requires faecal markers at the beginning and end of the balance period to ensure that all relevant faecal samples are collected. Because absorption is the difference between intake and excretion, both values need to be determined with great accuracy. The use of food tables is insufficient and foods and or supplements need to be assayed accurately.

If the method is applied to measuring absorption of a carotenoid from a single acute dose, the diet needs to be carotenoid free for 5 days before and during the test period. Faecal collections need to be continued until no further carotenoid from the test dose is lost in the faeces. The collection period is usually 3-5 days giving a total study period of 6-10 days

during which there will almost certainly be changes in the 'normal' plasma concentration of carotenoids due to the modification of the diet. As with the 'chronic' approach, intake and excretion values need to be assayed accurately.

If these methods (acute or chronic) are used for the measurement of absorption of carotenoids, then it must be assumed that: faeces are the only significant excretory mechanism of unabsorbed carotenoids; there is no enterohepatic recycling; the carotenoids recovered from the faeces are of dietary origin; and none of the unabsorbed carotenoid has undergone biotransformation, or otherwise been lost, due to the activity of the colonic microflora.

The latter assumption gives cause for concern. The carotenoids are likely to be susceptible both to microbial degradation in the large bowel and to oxidative degradation. Thus, it is likely that unabsorbed carotenoids are not quantitatively recovered from the faeces and that this approach does not give meaningful values for amounts absorbed.

Unfortunately, much of the carotenoid absorption data from foods and isolates are based on either acute or chronic faecal mass balance methods (Klauri and Bauernfeind, 1981) and show great variability.

Ileostomy mass balance

In individuals who have undergone ileostomy, the colon has been surgically removed and the terminal ileum brought to a stoma on the abdominal wall. Ingested food passes through the stomach and ileum in around 6 h as it would in the intact individual. The digesta (ileal effluent) can be recovered at regular intervals (2 h) and all the residue from a test breakfast can be recovered in 12 h if the volunteers are given carotenoid free midday and evening meals. Test meals of either an isolated carotenoid or food can, therefore, be given to an overnight fasted volunteer at breakfast (without dietary modification) and the unabsorbed carotenoid recovered from the ileal effluent in real time without the delay of the colon and rectum, or the confounding influence of the colonic microflora. The model has the added advantage that an excretion profile can be obtained, the timing of which gives a time span for the absorption, which can in turn be compared to changes in plasma concentration over the 12 h test period. This approach is described in Faulks *et al.* (1997).

Gastrointestinal Lavage Technique(TGWM)

In this technique the entire gastrointestinal track is washed out by consuming a large volume (1 gallon/4.5 litres) of 'Colyte' containing polyethylene glycol (PEG) and electrolyte salts. Washout is complete with the production of clear rectal effluent (2.5-3.5 h) and the

volunteers then consume the test meal and are permitted only water or 'diet' soft drinks (non-caloric) for the next 24 h. All the effluent is collected and pooled with the effluent collected on the following day when another dose of 'Colyte' is given to wash out the remainder of the test meal. The carotenoid recovered in the stool is subtracted from that fed to obtain an absorption figure. Absorption values for isolated β -carotene of 17 % were obtained in the absence of a meal and 52-29 % with a meal (Shiau *et al.*, 1994). Difficulties associated with the method is that it is relatively time consuming, can only be applied to healthy individuals, and may give an underestimation of absorption if absorption is compromised or normal transit time is reduced due to the use of Colyte. In addition, as with the faecal mass balance, the method depends upon there being no degradation or loss of unabsorbed carotenoids. On the other hand, it has the advantage of standardising the residence time of carotenoids in the GI tract.

Plasma and plasma fraction concentration methods

Measurements of absorption are usually carried out by the administration of an acute or chronic oral dose of isolated carotenoid, or carotenoid containing food, and following the changes in plasma concentration of the carotenoid of interest. Changes in plasma concentration are then interpreted as measures of absorption. In assessing absolute absorption from plasma responses, however, it is essential to understand that the plasma response curve is composed of two, possibly three, elements. These are (i) newly absorbed carotenoids appearing in the blood (absorption); (ii) disappearance of carotenoids from the blood to tissues (clearance); and (iii) carotenoids appearing in the blood from the tissues (re-exportation).

Acute doses

Such tests are usually carried out in fasted individuals who have restricted their dietary intake of the carotenoid of interest (and other carotenoids) for several days before the test day and for a period of days following. Blood samples are drawn at various time intervals after the test meal and the plasma/serum analysed for the carotenoid(s) of interest. Plasma concentration is then plotted as a function of time to produce a response curve. The Area Under the Curve (AUC) is calculated as concentration x time (C.t)

This method cannot determine absolute absorption but it is possible to compare different doses and foods and derive some information as to the relative absorption by comparison to a

standard dose, normally the isolated carotenoid. Such studies cannot normally be carried out 'blind' because of the problem of disguising the treatment. A crossover design, with an adequate period of washout between treatments, is the most suitable approach so that each individual can act as their own control (Brown *et al.*, 1989) and data can be compared using a paired t-test. Each volunteer acting as their own control is essential, since the AUC for the same dose in different individuals will be very variable. Such variability does not only depend upon the amount absorbed but on the absorption and clearance kinetics which may vary widely between individuals.

The measurement of absolute absorption of a carotenoid, calculated from the changes in plasma concentration following a single acute dose, is difficult and frequently misunderstood. The first point to deal with is the form and duration of the plasma response curve. Peak plasma concentration occurs at between 6 h and 48 h, depending upon the dose and the frequency of making the measurements. Since it is evident that the dose passes through the ileum in about 6 h, the advent of plasma peaks found beyond this time can only result from delayed passage of carotenoid into the blood, or rapid absorption of carotenoid into the body, followed by rapid sequestration from the circulation, and then re-exportation to the plasma.

Evidence cited for the first case is a frequently found second plasma peak occurring following a meal. However, this is countered by lack of evidence for temporary storage in the enterocyte. There is no known storage mechanism, no 'tailing' of ileal loss in ileostomy patients (Faulks *et al.*, 1997) and radio labelled β -carotene absorption appears complete in less than 12 h (Goodman *et al.*, 1966; Blomstrand and Werner, 1967). The second peak could simply result from an increase in the plasma lipids following a fat-containing meal. This would provide the lipoprotein and triglyceride needed to transport the carotenoid in the plasma. Alternatively, and most probably, the first peak in plasma concentration is due to the newly absorbed carotenoid present in chylomicrons and the second peak, or prolonged duration of the first peak, results from carotenoids exported from the liver in lipoproteins (very low density lipoprotein, VLDL and low density lipoprotein, LDL).

The transfer of carotenoids from the short lived chylomicrons to the longer lived LDL and high density lipoprotein (HDL), which carry most of the carotenoids in the blood of fasting individuals, would explain why the plasma concentration remains elevated for up to and beyond 10 days post dose (Dimitrov *et al.*, 1986; Brown *et al.*, 1989).

Under such circumstances, the plasma AUC approach is not appropriate for the calculation of absolute absorption because changes in the carotenoid concentration in the different plasma fractions (the chylomicrons, VLDL, LDL, HDL) are different and cannot be interpreted from

a ‘gross’ plasma response. Even comparative studies of two sources of the same carotenoid within a single individual may not be valid. This is because the AUC after a carotenoid dose may not be linear (i.e. a dose twice the size of another dose may not produce an AUC twice as large). The equation of the dose response curve needs to be obtained. In addition, it is not known if attenuated delivery or absorption caused by different physical characteristics of the meal can effect the AUC.

Table 7 summarises recent studies on the intestinal absorption of lycopene using a single dose.

Table 7. Human studies on the intestinal absorption of lycopene using a single dose

Dosage	Matrix	Result	Reference
12 mg, single dose	· Tomato juice (180 ml)	No effect	Brown <i>et al.</i> , 1989
16.5 mg, single dose	· Tomato purée (60 g/d) · Tomatoes (300 g/d)	Lycopene better available from purée	Porrini <i>et al.</i> , 1998
23 mg, single dose	· Tomato paste (40 g) · Tomatoes (400 g)	Lycopene in chylomicrons after tomato paste ↑	Gärtner <i>et al.</i> , 1997

Chronic dosing

Chronic dosing with supplements or foods (Micozzi *et al.*, 1992) needs to be carried out until the plasma concentration reaches a plateau. This normally takes a period of weeks when supplementing with dietary achievable amounts (15 mg/d) and may increase the plasma concentration of β -carotene up to 10-fold, with other common carotenoids, particularly lycopene, showing smaller increases. Again, absolute absorption cannot be measured but the data may allow comparisons between isolated compounds and foods, and between different foods. Dose response should also be considered (see acute dose AUC). Decay curves of falling plasma concentration of carotenoids, when supplementation is discontinued, may also provide some data on the half life of the body carotenoid pool (Dimitrov *et al.*, 1988, Micozzi *et al.*, 1992).

Table 8 summarises recent studies on the intestinal absorption of lycopene using multiple doses.

Table 8. Human studies on the intestinal absorption of lycopene using multiple doses

Dosage	Matrix	Result	Reference
5 mg/d, 6 w	<ul style="list-style-type: none"> · Tomatoes (80 - 230 g/d) · Tomato juice (59 g/d) · Capsules 	Lycopene better available from tomato juice and capsules	Böhm <i>et al.</i> , 1999
12 mg/d, 6 w	<ul style="list-style-type: none"> · Tomato juice (180 ml/d) 	No effect	Micozzi <i>et al.</i> , 1992
16,5 mg/d, 7 d	<ul style="list-style-type: none"> · Tomato purée (60 g/d) · Tomatoes (300 g/d) 	Lycopene better available from purée	Porrini <i>et al.</i> , 1998
39 - 75 mg/d, 1 w	<ul style="list-style-type: none"> · Spaghetti sauce (126 g/d) · Tomato juice (540 ml/d) · Lycopene capsules 	Lycopene in serum ↑	Agarwal <i>et al.</i> , 1998
40 mg/d, 2 w	<ul style="list-style-type: none"> · Tomato juice (330 ml/d) 	Lycopene in plasma ↑	Müller <i>et al.</i> , 1999
70 - 75 mg/d, 4 w	<ul style="list-style-type: none"> · Tomato juice (476 g/d) · Capsules 	Lycopene in plasma ↑	Paetau <i>et al.</i> , 1998

Figure 5 shows the plasma response after multiple doses of 5 mg/d lycopene comprised in capsules, tomatoes or tomato juice. Lycopene is significantly better absorbed from tomato juice and oleoresin capsules compared to raw tomatoes.

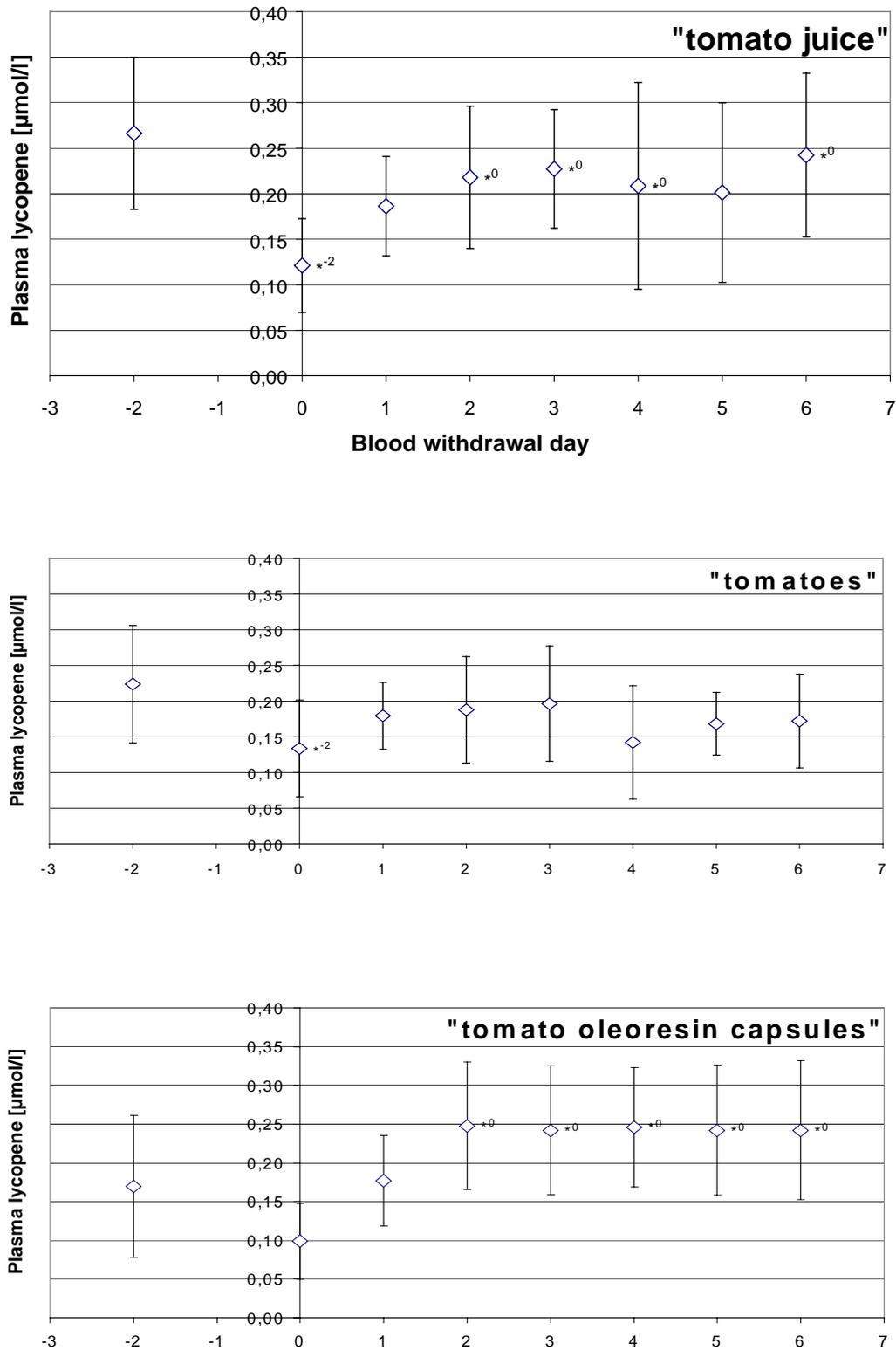


Figure 5. Plasma lycopene contents (mean \pm s) of all groups, *-2 significantly ($p < 0.05$) lower than the basal value (T-2), *0 significantly higher than the value before supplementation (TO)

Plasma Triglyceride Rich Lipoprotein (TRL) Fraction

Newly absorbed carotenoids are initially present in plasma chylomicrons before they are sequestered by body tissues and then re-exported in, or transferred to, other lipoprotein fractions. Thus, measurement of carotenoids in this fraction, and a knowledge of the rate of clearance of the chylomicrons, should permit the calculation of rates of absorption, disposal and overall absorption based on AUC measurement.

This method has the advantage that chylomicrons present in fasting plasma are few and they are almost devoid of carotenoids. The disadvantage is that the plasma has to be ultracentrifuged to separate the lipoprotein classes. Ultracentrifugation, however, does not normally permit the separation of the chylomicron fraction free of other low density lipoproteins, particularly the VLDL, which may be the primary vehicle for the hepatic re-export of absorbed carotenoids. Selective or sequential centrifugation may be used to obtain 'cleaner' fractions (Dubois *et al.*, 1998) but this may also lead to incomplete collections.

In addition, absorption data based on oral AUC in TRL, and the theoretical AUC that would be obtained if the dose had been administered intravenously (using plasma volume and chylomicron clearance half life), give results that differ. For β -carotene, van Vliet *et al.* (1995) calculated an absorption figure of 11 % (central cleavage) or 17 % (eccentric cleavage), whereas O'Neill and Thurnham (1998) calculated 3.9 % and 2.5 % absorption in males and females, respectively, assuming only central cleavage. Both authors assume a cited chylomicron remnant half life of 11.5 min., however, a true clearance rate of carotenoid in the TRL fraction can also be obtained from the graph of TRL carotenoid concentration vs time and this could also be used to provide a true carotenoid half life term which would be independent of assumptions based on lipid kinetics.

It is also worth noting that much shorter half lives (2.5-7.9 min) have been reported for the clearance of chylomicron triglyceride (Grundy and Mok, 1976) and use of these values (Faulks *et al.*, 1997), rather than those of chylomicron remnant clearance (Cortner *et al.*, 1987), would have the effect of proportionally increasing the apparent absorption (% absorption doubles every time the half life is halved). The plasma chylomicron concentration will depend both on the lipid load and the ability of the individual to clear the chylomicrons from the plasma and unless this is known it will introduce errors by the use of inappropriate half life values.

The calculation of absorption, using a theoretical plasma concentration excursion based on plasma pool size and a theoretical intravenous dose (van Vliet *et al.*, 1995, O'Neill and

Thurnham, 1998), must therefore be treated with caution unless exact clearance kinetics of the carotenoids are known.

Some difficulties in explaining carotenoid kinetics may arise from: the observations that the triglyceride in the TRL peaks at 2 h whereas the β -carotene peaks at 5-6 h (van Vliet *et al.*, 1995); individuals are highly variable in their plasma and TRL response to oral β -carotene (Dimitrov *et al.*, 1986; Johnson and Russell, 1992; Borel *et al.*, 1998a); and individuals with a high concentration of plasma β -carotene appear to be those that show the greatest increment in plasma carotenoid concentration on supplementation (Faulks *et al.*, 1998).

Isotope Methods

The use of radioactive tracers in human volunteers, to determine the bioavailability of the carotenoids, is not now possible because of ethical constraints. There are, however, 2 studies (Goodman *et al.*, 1966, Blomstrand and Werner, 1967) in males using ^{14}C and ^3H . These studies provide useful information on the duration and extent of absorption of β -carotene and the degree of conversion to retinol. Absorption of radio-labelled β -carotene was found to be in the range 8.7-16.8 % but most of this was recovered as the retinyl esters. This indicated that β -carotene absorbed by this route was largely converted to retinol. Peak absorption was found to be at 3-4 h and 6-7 h for each of 2 volunteers, respectively, and that this time coincided with maximum lactescence in the lymph as assessed visually. In both cases, despite the relatively low absorption, no further radio-label was found in the lymph after 12 h. Transitory storage in the enterocytes, prior to transfer to the serosal side, would probably have been detected as a tailing of the absorption curve and the high level of conversion may explain why elevation of plasma β -carotene is not always seen in volunteers given small acute doses.

The use of stable isotopes is more ethically acceptable. Highly labelled β -[^{13}C] carotene has been used to study the metabolism of β -carotene in man (Parker *et al.*, 1993; Parker 1997). The single acute oral dose used in these studies was 1-2 mg of purified labelled (>95% ^{13}C), dissolved in tricaprylin or safflower oil and given with a standard meal. Blood samples were drawn at intervals and the β -carotene, retinol and retinyl esters separated, quantified and purified by HPLC. The β -carotene (converted to the perhydro derivative by hydrogenation over platinum oxide), and the retinol and retinol derived from retinol esters, were subjected to gas chromatography-combustion-isotope ratio mass spectrometry (GCC-IRMS). The method was sufficiently sensitive to track the ^{13}C in retinol esters up to 2 days and β -carotene and retinol up to 25 days.

Potentially, the use of [^{13}C] carotenoids, either as an isolate or within a food, should permit the measurement of absolute absorption and the kinetics of disposal and conversion to other metabolites.

An alternative to the use of β -[^{13}C] carotene is octadeuterated β -carotene (β -carotene- d_8), an isotopomer that can be separated from natural abundance β -carotene by HPLC, thus, avoiding the use of mass spectrometry (Dueker *et al.*, 1994). The retinol- d_4 derived from β -carotene- d_8 has to be separated from the plasma using a solid phase (Dueker *et al.*, 1993) and derivatised to the tert-butyldimethylsilyl ether (Handleman *et al.*, 1993) before measurement by gas-chromatography-mass spectrometry. The method has been applied successfully to the tracking of both β -carotene- d_8 and retinol- d_4 in human volunteers for up to 24 days after an oral dose of 73 μM (40 mg) (Dueker *et al.*, 1994). Application of a compartmental model (Novotny *et al.*, 1995) indicated that 22 % of the carotenoid dose was absorbed; 17.8 % as carotenoid and 4.2% as retinoid. This result is close to the 11 % absorption of β -carotene found by van Vliet (1995) but indicates much lower percentage conversion to retinol than that found using very small oral doses of b-[^{13}C] carotene (Parker, 1997).

Interactions

Single acute oral doses of lutein and β -carotene have shown that the two carotenoids interact to reduce the apparent absorption of lutein as measured by the plasma area under the curve (AUC), and in some instances lutein has been shown to reduce the AUC for β -carotene (Kostic *et al.*, 1995). A combined dose of β -carotene and lycopene does not appear to affect the absorption of β -carotene but enhances the absorption of lycopene (Johnson *et al.*, 1997). Short term supplementation of volunteers with β -carotene, either as a pure compound or as the major constituent of a natural β -carotene source, has also been found to reduce the plasma concentration of lutein (Micozzi *et al.*, 1992; Hughes *et al.*, 1997; Faulks *et al.*, 1998). However, in a long term study (4 years) of β -carotene supplementation, although there was a trend, the reduction of plasma lutein was not significant (Nierenberg *et al.*, 1997). The other carotenoids do not appear to affect the apparent absorption of lycopene, possibly because only β -carotene and lycopene are the two main hydrocarbon carotenoids. Although they use the same carriers, both in the gut lumen and *in vivo*, carrier capacity (LDL) is probably not limiting (Romanchik, 1997).

With respect to other carotenoids and vitamin E, β -carotene supplementation of volunteers with colorectal adenomas for 2 y resulted in highly significant increases in plasma lycopene and α -carotene in both men and women (Wahlqvist *et al.*, 1994). However, short-term supplementation (15 mg/d β -carotene for 35d) of apparently healthy volunteers had no effect on the plasma concentration of lycopene, or on plasma Vitamin E (Faulks *et al.*, 1998).

Isomerisation and cis-isomers

Human blood plasma contains mainly the all trans forms of the common dietary carotenoids but 5(Z)-lycopene (up to 50% of the total plasma lycopene) and 9(Z) and 9'(Z) lutein and 9(Z)- β -carotene (Khachik *et al.*, 1992b) are also commonly found in human blood plasma. In some cases 5(Z)-lycopene appears in plasma in a much greater proportion than in the food (Schierle *et al.*, 1997). This could suggest that the 5(Z)-lycopene is preferentially absorbed, or less rapidly cleared from the plasma, or that all trans lycopene undergoes isomerisation as a result of some biochemical interaction since simulated digestion *in vitro* does not cause significant acid catalysed isomerisation.

It has been reported that 9(Z)- β -carotene preferentially accumulates in the lipoprotein carriers (Stahl *et al.*, 1995) but Gaziano *et al.* (1995) found that there was a marked preferential absorption of the all trans- β -carotene in man. Supplementation of female volunteers with 15 mg/d of palm oil carotenoids (a mixture of trans and cis β -carotenes) elicited a plasma response where the ratio of cis:trans forms was much lower than in the supplement (Faulks *et al.*, 1998). This indicates that the trans form is better absorbed than the cis form, or that the cis form is cleared from the plasma more rapidly. However, in ileostomists given an acute oral dose of all-trans- β -carotene and 9(Z)- β -carotene, both isomers appeared to be equally well absorbed from the gut and cis-trans isomerisation did not occur during passage of the β -carotene through the GI tract (Faulks *et al.*, 1997). Currently it is unclear at what stage of absorption (mass transfer from food, dissolution in the luminal lipid structures, absorption from the micelle, transport within the enterocyte and incorporation into chylomicrons) these effects occur.

In humans, concurrent feeding of single acute doses of β -carotene and canthaxanthin was found to inhibit the plasma appearance of canthaxanthin but there was no converse interaction (White *et al.*, 1994).

The observation that the carotenoid profile in the triglyceride rich (chylomicron) fraction is not the same as in a supplement (Gärtner *et al.*, 1996) clearly indicates that in order to assess bioavailability of any one carotenoid the carotenoid profile of the supplement or food needs to be defined, as does the amount and type of fat in the test meal (Borel *et al.*, 1998b). The use of TRL is, currently, a useful option for the calculation of β -carotene bioavailability, but its use for the other carotenoids has yet to be tested.

These findings suggests that β -carotene has a sparing effect on lycopene and that if this is the case other 'interactions' *in vivo* may also occur which will need to be considered when looking at plasma carotenoid profiles and dietary habits.

A general problem investigating the isomer pattern of carotenoids is the lack of reference substances. Besides the (E)-isomers of the main carotenoids, which are commercially available, there is no (Z)-isomer available. To study the isomerisation in food and plasma, a source of highly purified carotenoid isomers is needed.

Transport

As has already been stated, the carotenoids are passively absorbed from mixed micelles at the brush border along with dietary lipids, lipid hydrolysis products, sterols and bile salts. The absorbed carotenoids are transported through the enterocyte from the luminal side to the serosal side, where they are re-excreted in chylomicrons into the thoracic duct and hence find their way into the circulating blood. The cleavage of some of the retinol precursor carotenoids by 15, 15' dioxygenase occurs in the enterocyte. The resulting retinal is reduced to retinol and subsequently esterified to the retinyl ester (mainly palmitate) which also enters the mesenteric lymph with the chylomicrons (Krinsky *et al.*, 1993). It is probable that some of the retinol produced in the enterocyte is excreted into the portal blood in association with retinol binding protein. It is not known if any of the non-provitamin A carotenoids are competitive inhibitors of 15,15' dioxygenase or if the other carotenoids can give rise to bioactive retinol analogues.

The chylomicrons have a biologically controlled composition with regard to protein (2%, consisting of apoproteins A-1, A-2, A-4 and B-48) and lipid (dietary derived), and appear to carry the carotenoids (and other lipid soluble components) passively in the mesenteric lymph. The chylomicrons are acted upon by endothelial lipoprotein lipase in the extrahepatic capillary bed. Some of the lipid is absorbed as free fatty acids and the glycerol is metabolised. It is not known if any of the carotenoid is also absorbed at this point, although clearance kinetics (Faulks *et al.*, 1997) would imply that some does get absorbed, perhaps by adipocytes. The chylomicron remnants, including residual carotenoids, are then cleared from the circulation by passage through the liver. However, lycopene in the rat and monkey model does appear to concentrate in the liver and gut (Mathews-Roth *et al.*, 1990) and in the human to a range of tissues (Schmitz *et al.*, 1991, Kaplan *et al.*, 1990, Stahl *et al.*, 1992b) indicating that it disperses to tissues differently to β -carotene and this may help to explain why it is so difficult to increase plasma concentration of lycopene with chronic supplementation of 15mg/d (Hughes *et al.*, 1997)

The liver re-exports lipids in the form of very low density lipoproteins (VLDL) and these contain carotenoids dissolved in the lipid portion. No carrier proteins have been identified. The VLDL is acted upon by endothelial lipoprotein lipases in the extrahepatic capillary bed and this removes lipid and glycerol to produce intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). The IDL is converted to LDL by receptor mediated

endocytosis in the liver and the LDL is cleared from the plasma by receptor mediated endocytosis in both liver and other tissues. The hydrocarbon carotenoids appear to remain associated with these lipoproteins, such that around 80 % of β -carotene and lycopene found in fasting plasma are carried by LDL (Krinsky *et al.*, 1958; Vogel *et al.*, 1996; Lepage *et al.*, 1994). How much of the carotenoid (and how quickly it) is distributed to the tissues by these processes is unknown but endocytosis of LDL particles by adipose tissues is likely to be a major carotenoid 'sink'.

The hydroxy carotenoids (lutein, zeaxanthin) are found almost equally distributed between the LDL and the high density lipoproteins (HDL) in fasting subjects (Vogel *et al.*, 1996). HDL particles are produced in the liver and intestine and are secreted into the blood and mesenteric lymph, respectively. Other HDL particles may arise from chylomicrons as the triacylglycerol is removed by lipoprotein lipase. The carotenoids associated with HDL may, therefore, be derived from the liver, or directly from the gut, or by exchange between lipoproteins. Unlike LDL, where the bulk of the particle is lipid, the bulk of HDL consists of cholesterol esters. However, both particles have a hydrophobic core in which the hydrocarbon carotenoids may be carried. It is unclear, therefore, why the polar hydroxy carotenoids partition almost equally between LDL and HDL, unless both particles have similar interfacial characteristics.

If the currently accepted model of carotenoid absorption and transport is correct, then dietary carotenoids should first appear in the chylomicrons and then VLDL, LDL and HDL, depending upon the amounts of each lipoprotein and their residence time (half life) in the plasma. It would be expected that the chylomicrons would show a peak at around 4-6 h post ingestion, as the meal passes through the ileum, and would be clear at about 12 h. Studies of β -carotene absorption, using thoracic duct cannulation, found this to be the case (Goodman *et al.*, 1966; Blomstrand and Werner, 1967). Studies of the absorption of α -carotene, lycopene and lutein in the triglyceride rich lipoprotein (TRL) fraction of plasma also show a peak plasma concentration around 4-6 h which returns to baseline by 12 h post dose (van den Berg and van Vliet, 1998). This would be followed by peaks in VLDL then LDL as the hepatically absorbed carotenoid is re-excreted into the circulation.

State-of-the-art: Summary

- For **mass balance measurements**, the ileostomy model is preferred. It offers measurement of the absorption of any of the carotenoids (by difference between intake and ileal loss), not confounded by microbial degradation of carotenoids which may occur in the lower intestine. If performed with parallel blood sampling, ileal loss can be related directly to the appearance of carotenoids in blood fractions. This provides data related to both carotenoid absorption and metabolism. Such studies have been performed for β -carotene and are in progress for lycopene (from food sources).

- **Plasma Area Under the Curve** may not be an appropriate method of measuring carotenoid bioavailability, because of the probable re-export of carotenoid from the liver into the plasma while absorption from the gut is still active.

For the plasma response to hydrocarbon carotenoids like β -carotene and lycopene, the use of the tricycleride-rich lipoprotein (chylomicron) fraction offers the best prospect of quantifying absorption, especially if the chylomicron fraction can be isolated from contaminating VLDL. For the more polar carotenoids (xanthophylls) the chylomicron fraction may not be appropriate, especially if they are not exclusively carried by the chylomicrons when freshly absorbed.

Application of metabolic modelling techniques to plasma, and plasma fraction, response curves provides a powerful tool for quantifying the kinetics of carotenoid absorption and clearance to other tissue pools.

- Newer methods using stable isotopes indicate that they have the potential to permit measurements of absolute absorption and subsequent metabolism in human subjects in the presence of both dietary and endogenous carotenoids. Ideally, the use of stable isotope labelled food materials coupled with the measurement of the isotopomers of the native carotenoid and retinol in plasma fractions should provide the best possible measure of bioavailability.

- Although this review draws heavily on work with β -carotene, this is because as yet there is little work on lycopene. However, the methods are applicable to the carotenoids in general and are currently being used to measure lycopene bioavailability from raw and processed tomatoes (Modem, FAIR CT97-3100)

- Future studies will have to look at the isomerisation and metabolism of carotenoids to get further insight in possible protection mechanisms. As recently observed with regard to the antioxidant activity, there are differences between the isomers.

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The relationship between tomatoes and their constituents, and diseases

Working Group 3: Observational epidemiological surveys

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Introduction

Although not originally found in the Mediterranean area, tomato is one of the most used vegetables of the Mediterranean diet. This diet is known to be beneficial for health especially with regard to the development of chronic degenerative diseases (Corpet and Gerber, 1997. Gerber and Corbet, 1997). Therefore, tomatoes appear especially important in terms of public health since they are consumed in large quantities and rich in several compounds believed to protect or reduce the risk for chronic degenerative diseases. Carotenoids are among the first compounds to have attracted the attention of scientists on the effect of fruit and vegetables, and tomato is especially rich in one of them: lycopene. β -carotene is also present together with vitamin C, and vitamin E in the seeds. Moreover, there is a growing interest for other compounds present in tomatoes like folates and phenolics though not enough human studies are available to really estimate the effect of phenolics particularly on human health. Finally, tomatoes can be easily processed in several products which are consumed in large amounts, and during this processing, the main components of tomatoes (carotenoids) are preserved and even concentrated. Because of this widespread and large consumption, tomatoes appear as one of the most interesting food in terms of health.

Objectives

The objective of Working Group 3 was to summarise the state of the art with regards to the potential protective (or risk-reducing) effect of tomatoes and their constituents with regard to chronic degenerative disease, especially cancers, cardio-vascular disease, lung function, lens opacities and ageing.

It was decided:

- 1) to focus on studies reporting on tomatoes (fresh, cooked, processed whenever specified) intake
- 2) to summarise all studies related to fruit and vegetables because most studies do not distinguish between the various vegetables
- 3) to cover the studies related to the specific nutriment and microcompounds found in tomatoes
- 4) to give the most important place to the antioxidants carotenoids, vitamins C and E because, so far most of the previous studies report on these compounds, but also to give the main results -yet limited- on folates and phenolics which are certainly very important with regard to

prevention of disease. Beside, a study on the contribution of tomatoes in the microcompounds of interest to the intake of a Mediterranean population will be presented.

Methodology

A large body of data concerning cancers and cardio-vascular disease in particular has been reviewed and summarised. For cancers, we decided to use four books as a basis (CNERNA, 1996; World Cancer Research Fund-WCRF-, 1997; COMA Food and Nutrition Policy, 1998; Carotenoids, IARC, 1998) which were published in three different countries. For cardio-vascular disease Ness and Powles review (1997) up to 1996. We updated these reference books and reviews with studies published since 1996. When no background reviews existed for the remaining diseases and for the microcompounds we had to cover, all the reported studies were analysed as long as they satisfied methodological criteria. We did not make any attempt to obtain unpublished data.

The criteria of selection of analysis were as follows :

- Design of the study: case-control and prospective studies were reviewed.
- Assessment of the exposure: dietary intake, plasma or tissue nutrient concentration. Quality of nutritional data was estimated and based on number of items of the questionnaire, type of quantitative assessment, and validation of the questionnaire. Quality of biological measurements was estimated by conditions of storage (-70°C) and of the method used (results based on total anti-oxidant capacity of the plasma were excluded).
- Size of the sample: At least 100 cases for prospective studies and 400 cases in case-control studies for high incidence cancers (lung, breast, colorectal cancers) were analysed; 50 and 100 cases respectively for less frequent cancers. The studies based on plasma or tissue nutrient concentration were limited to 50 cases.
- Statistical analysis had to include potential dietary and non-dietary confounders, and energy had to be included in the logistic regression model.
- Significance of the results (indicated with 95% confidence interval) and presence of a dose-response effect.

Rejection of paper has been justified.

All the analysis of data must be based on the actual published report which is available for the reader.

Evaluation of epidemiological data will be based on the causal inference criteria as described first by Hill (1965) as follows:

- Consistency of the results
- Strength of the association
- Dose- response
- Biological plausibility
- Analogy

We did not use either experimental data, because animal models are not necessarily relevant to the study of nutritional risk factors in humans, or temporality relationship because of the difficulty in assessing retrospective nutritional intake. However, we included large intervention trials, in which epidemiological observations could be made, and they represent some types of experimental data.

Vocabulary

To characterise the relationship between cancer and food or nutriment we used the vocabulary of the three books mentioned above:

- *no data reported*: the authors ignored any data related to this relationship
- *no relationship*: no effect of the food or nutriment was considered on cancer
- *insufficient*: not enough data to draw a conclusion (less than three studies)
- *consistent*: good agreement of data
- *inconsistent*: heterogeneity of data, no major trends in the results.
- *convincing*: the evidence of a causal relationship is conclusive and sufficient for being the basis for dietary recommendations
- *probable*: the causal relationship is likely and sufficient for being the basis for dietary recommendations
- *possible*: a causal relationship may exist, but the evidence is not strong enough to be a basis for recommendations. This qualification covers inconsistent results, but with a tendency of more results showing an effect.

Part 1: Tomatoes and tomato products, lycopene, other tomato microcompounds and cancers

This part consisted of:

1. Summary of the data provided by the four reference books (CNERNA, 1996; World Cancer Res Fund, 1997; COMA Food and Nutrition Policy, 1998; Carotenoids, IARC, 1998) accompanied with comments and comparison and conclusion.
2. Up-dating of these data with studies reported since 1996.
3. Major intervention trials
4. Synthetic conclusion.

1-1 SUMMARY OF THE DATA PROVIDED BY THE THREE REFERENCE BOOKS

1-1-1 Tomatoes and tomato products (Table 1)

Comments on table 1

The specific effect of tomatoes was never considered in the COMA and was once considered in the CNERNA (a multicentric study conducted in Latin America showed a protective effect of tomatoes and other fruit-vegetables). Tomatoes were specifically quoted for 11 cancer sites in the WCRF book. However, there were less than three studies for most cancer sites and the conclusion was *insufficient*. For mouth and pharynx, there were three studies mentioning tomatoes; of these, two showed an inverse relationship, and one study showed a positive relationship. Altogether, the three studies for oesophagus and the four studies for lung and respiratory tract showed a reduced risk associated with a high tomato intake. Among the 11 studies considering the relationship between a high tomato intake and stomach cancer, 9 studies demonstrated a reduced risk, one study showed no relationship, and one study showed a positive association. Thus for these three localisations, results were said to be *convincing*. Only one of these studies (Giovanucci *et al.*, 1995) stated whether the tomatoes were raw, cooked or processed.

Table 1: Level of evidence of protection provided by studies on the relationship between tomatoes, tomato products and cancers

cancer sites	CNERNA (France, 1996)	World Cancer Res Fund (USA, 1997)
mouth and pharynx	NM	possible
larynx	insufficient	insufficient
lung and resp. tract	NM	convincing

oesophagus	NM	convincing
stomach	NM	convincing
rectum	NM	inconsistent
pancreas	NM	insufficient
breast	NM	insufficient
endometrium	NM	insufficient
cervix	NM	insufficient
prostate	insufficient	insufficient

NM: not mentioned

1-1-2 Other fruit and vegetables (Table 2)

We report below the summary of the three reference books with regard to « fruit and vegetables » because tomatoes are most often included in this foods group.

Comments on table 2

The level of agreement among the reference books was generally good:

perfect (3/3 or 2/2) : • For **oesophagus** and **pancreas** cancers for which a high intake of fruit and vegetables is recommended. For **cervix** and **bladder** cancers, results are also consistent but the data are limited.

moderate (2/3): • For the tobacco-related cancers, **mouth** and **pharynx, larynx, lung** and **respiratory tract**, the disagreement comes from COMA. The members of this group noted that the alteration of the diet might be tobacco-induced, and hence, that tobacco could be the only cause for these cancers.

• For **stomach** cancers the disagreement comes from COMA, because only half of the cohort showed a decreased risk which was due to a potential confounding factor: *helicobacter pylori*.

Table 2: Level of evidence of protection provided by studies on fruit and vegetables and cancers

cancer sites	CNERNA (France, 1996)	World Cancer Res Fund (USA, 1997)	COMA Food and Nutrition Policy (UK, 1998)
mouth and pharynx	consistent	convincing	fruit: weakly consistent; vegetables: inconsistent
larynx	consistent	probable	moderately consistent
oesophagus	consistent	convincing	strongly consistent
lung and respiratory tract	consistent	convincing	fruit: moderately consistent; vegetables: weakly consistent
stomach	consistent	convincing	moderately consistent
colon-rectum	vegetables: moderately consistent	vegetables: convincing	vegetables: moderately to weakly consistent
pancreas	consistent	probable	consistent but limited
liver	ND	vegetables only: possible	ND
breast	inconsistent	green vegetables: probable	green/yellow vegetables: moderately consistent
ovary	inconsistent	possible	inconsistent
endometrium	inconsistent	insufficient	inconsistent
cervix	ND	possible	consistent but limited
prostate	inconsistent	vegetables: possible	vegetables: moderately consistent but limited
kidney	ND	vegetables only: possible	ND
bladder	ND	probable	moderately consistent but limited
thyroid	ND	possible	ND

ND: Not Done

- For **colon** and **rectum** cancers, the three sources agreed that fruit had no relationship with these cancers. However, there was a disagreement on the level of risk reduction provided by vegetables. The reading of the original articles showed that WCRF proposed convincing effect for vegetables although half of the cohort studies did not show reduction of risk with a high intake of vegetables.

- For all of the hormone-dependent cancers, **breast**, **ovary**, **endometrium** and **prostate**, CNERNA found *no relationship* with intake of vegetables. This opinion was shared by COMA for ovary and endometrium cancers. The relationship is judged *possible* by WCRF for ovary cancer although they quoted that not all studies were significant. For endometrium cancer, results are more *inconsistent* than insufficient. For breast cancer, there was a total agreement on the absence of effect from fruit. For vegetables, the results of a few cohort studies overwhelmed those of case-control studies in WCRF and COMA. However, certain studies indicated that compounds other than carotenoids are the likely agents of reduction of risk. For prostate cancer, the findings mainly differed by type of vegetables, therefore they

may appear *inconsistent* or *weakly consistent* if all types of vegetables are considered. It has to be noted that several studies attributed the risk reduction effect of vegetables specifically to vegetable fibre.

1-1-3 Lycopene (Table 3)

1-1-3-1 studies based on dietary intake

The specific effect of lycopene was never considered in the COMA. There are very few data on dietary lycopene and cancers in the two other reference books.

Table3: Level of evidence of protection provided by studies on dietary intake of lycopene and cancers*

cancer sites	CNERNA (France, 1996)	WCRF (USA, 1997)	Carotenoids (I A R C, 1998)
lung and respiratory tract	NM	NM	inconsistent 1:-; 2: (-); 1: 0
pancreas	insufficient 1: -	NM	NM
breast	NM	NM	insufficient 1: 0
prostate	insufficient** 1: (+); 1-	insufficient 1:(+)1:-	inconsistent 1:-; 3: 0.

*the figures indicate the number of studies, -: a significant risk reduction; (-): a non significant risk reduction; 0: no relationship; + an increased risk.

**the study showing an increased risk of prostate cancer with high intake of lycopene, was significant only when papaya was the source of carotenoids.

NM: Not Mentioned

The data were scarce and inconclusive. However, studies based on lycopene intake are hampered by lack of availability of complete and reliable food composition tables, which might explain the unexpected inconsistency of the relationship between lycopene intake and lung and respiratory tract cancers. Alternatively, it might be that compounds other than lycopene are responsible for a protective effect of tomatoes.

1-1-3-2 Studies based on plasma levels (Table 4)

Neither CNERNA nor COMA reported on plasma levels of lycopene and cancers. They were summarized in the IARC book and were shown to be too few to draw any conclusions except for breast (inconsistent) and skin (no relationship) cancers (Table 4). However, it is known that lycopene is characterised by instability and fast turnover in plasma (see WG2), and this might explain these inconclusive results.

Table 4: Level of evidence of protection provided by studies on plasma lycopene and cancers*

cancer sites	World Cancer Res Fund (USA, 1997)	Carotenoids (I A R C, 1998)
mouth and pharynx	NM	insufficient 1: - ; 1: 0
larynx	NM	insufficient 1: -
oesophagus	NM	insufficient 1: (-)
lung	NM	insufficient 1: 0
colon-rectum	NM	insufficient 1: -
pancreas	NM	insufficient 1: -
breast	NM	inconsistent 2: (-); 1: 0
ovary	NM	insufficient 1: (+)
cervix	NM	inconsistent 1: (-); 1: 0
prostate	insufficient 1:+	insufficient 1:(-)
bladder	NM	insufficient 1:+
skin	NM	no relationship 4: 0

*the figures indicate the number of studies, -: a significant risk reduction; (-): a non significant risk reduction; 0: no relationship; +: a significant increased risk; (+): a non significant increased risk.
NM: Not Mentioned

Comparison between tables 1, 3 and 4

The evidence for a protective effect of tomato intake was stronger than for lycopene, especially for oesophagus, stomach and lung cancers, where this effect is judged « *convincing* ». Studies with lycopene have been less frequent, and the comment was mostly « *insufficient* ». But, when there were three studies (breast cancer) or more (skin cancer), the results were inconsistent. Therefore it is possible that other compounds present in tomatoes also play a protective role. These other compounds are essentially β -carotene, vitamins C and E, folates and phenolics. We will describe the relationship of cancers with β -carotene, vitamins C and E as reported in epidemiological studies.

1-1-4 β -Carotene (Table 5)

Only the IARC book systematically differentiated between studies on dietary intake and plasma levels, therefore both methods are presented in table 5.

Table 5: Level of evidence of protection provided by studies on dietary intake and plasma levels of β -carotene and cancers

cancer sites	CNERNA (France, 1996)	World Cancer Res Fund (USA, 1997)	COMA Food and Nutrition Policy (UK, 1998)	Carotenoids (I A R C, 1998)*
mouth and pharynx	insufficient	insufficient	insufficient	1-inconsistent 2-consistent but limited
larynx	consistent but limited	insufficient	moderately consistent but limited	1- consistent but limited 2-insufficient
oesophagus	moderately consistent	possible	consistent	1- consistent 2-insufficient
lung and respiratory tract	strongly consistent	probable	1-consistent 2-strongly consistent	1-strongly consistent 2-strongly consistent
stomach	consistent	possible	1-moderately consistent 2-strongly consistent	1-consistent 2-consistent but limited
colon-rectum	inconsistent	possible	inconsistent	1-inconsistent 2-inconsistent
pancreas	insufficient	inconsistent	insufficient	1-insufficient 2-insufficient
liver	ND	inconsistent	ND	1-insufficient 2-insufficient
breast	inconsistent	possible	weakly consistent	1- inconsistent 2-inconsistent
ovary	insufficient	insufficient	insufficient	1-inconsistent 2-insufficient
endometrium	insufficient	insufficient	insufficient	1-insufficient 2-insufficient
cervix	ND	possible	weakly consistent	1-inconsistent 2-consistent but limited
prostate	inconsistent	inconsistent for all and each carotenoids .	inconsistent	1-inconsistent 2-inconsistent
kidney	ND	insufficient	ND	1-inconsistent 2-insufficient
bladder	ND	insufficient	inconsistent	1-inconsistent 2- inconsistent
thyroid	ND	no data	ND	ND
skin	ND	ND	inconsistent	1-inconsistent 2-inconsistent

*1: conclusion from food intake; 2: conclusion from plasma level

ND: Not Done

Comments on table 5

The level of agreement was generally good. All of the four sources agreed on the relationship between carotenoids and **lung** cancer. The WCRF said « *probable* » and not « *convincing* »

because of the results of intervention trials and the possibility that it is another fruit and vegetable compound which is responsible for the risk reduction.

For **stomach** cancer, CNERNA, COMA and IARC agreed on the reduction of risk by carotenoids. WCRF only said « possible » because there was no certitude that it is not another fruit and vegetable compound which is responsible for the risk reduction. For **larynx** and **oesophagus** cancers, results consistently showed a reduction of risk but there are few studies which explain the « possible » of WCRF instead of the expected « probable ». For **cervix** cancer, there are few studies, but they tend to agree with a reduction of risk. For **colon** and **rectum** cancers, CNERNA, COMA and IARC agreed on the inconsistency of the results. The WCRF sources indeed are very heterogeneous, in spite of the statement « possible ». CNERNA and IARC found results on **pancreas** cancer more insufficient than inconsistent, which was chosen by WCRF and COMA. CNERNA and IARC agreed on the inconsistency of findings related to **breast** cancer. The « weakly consistent » of COMA was due to the including carotenoids with vitamin A activity. This point demonstrates the difficulty of assessing carotenoids in nutritional data base, since many of them evaluate carotenoids in vitamin A equivalent. WCRF noted that one cannot ascertain that carotenoids are the marker of other bioactive compounds. All of the four sources agreed to say that studies on **prostate** cancer are too heterogeneous to bring any type of conclusion and that studies are insufficient on **ovary** and **endometrium** cancers. Data are insufficient and/or inconsistent for all of the other cancer sites.

Comparison between tables 2 and 5

The evidence that β -carotene is inversely associated to the risk of larynx, oesophagus, lung and respiratory tract and stomach cancers was consistent. Thus it is possible that the reduction of risk which is attributed to fruit and vegetables is mediated through carotenoids for these cancers or alternatively that carotenoids are the markers of fruit and vegetables intake. For bladder and pancreas cancers, data were insufficient. Colon, rectum, breast, ovary and endometrium cancers appeared not related to carotenoids. Thus, the possible effect of vegetables on the risk of these cancers might be not related to carotenoids.

1-1-5 Vitamin C (Table 6)

The conclusions of the three reference books, which are summarised in this part, do not differentiate between dietary intake and plasma levels, and are presented as such in Table 6.

Table 6: Level of evidence of protection provided by studies on vitamin C and cancers

cancer sites	CNERNA (France, 1996)	World Cancer Res Fund (USA, 1997)	COMA Food and Nutrition Policy (UK, 1998)
mouth and pharynx	consistent	possible	no data reported
larynx	consistent but limited	insufficient	no data reported
oesophagus	consistent	possible	moderately consistent
lung and respiratory tract	moderately consistent	possible	inconsistent
stomach	consistent	probable	strongly consistent
colon-rectum	inconsistent	insufficient	inconsistent
pancreas	consistent	possible	moderately consistent but limited
liver	ND	no data reported	ND
breast	inconsistent	insufficient	insufficient
ovary	inconsistent	insufficient	no data reported
endometrium	no data reported	insufficient	no data reported
cervix	moderately consistent	possible	moderately consistent
prostate	no data reported	no relationship	inconsistent
kidney	ND	insufficient	ND
bladder	ND	insufficient	insufficient
thyroid	ND	no data reported	ND

ND: Not Done

Comments on table 6

There was a high level of agreement either to show *consistent* reduction of risk for **stomach** cancer, and, *moderately consistent*, for **oesophagus, mouth, pharynx, larynx, pancreas**, and **cervix** cancers, or to acknowledge the *inconsistency* or *insufficiency* of findings on **colon, rectum** and **hormone-dependent cancers**.

One disagreement was found for **lung** cancer, COMA denied the possible risk reduction because of the few studies showing an increase in risk. However, the OR are all non significant.

Comparison between tables 2 and 6

It appears that vitamin C can play a role in the reduction of risk of larynx, oesophagus, lung and respiratory tract, stomach, pancreas and cervix cancers; or alternatively that vitamin C is a marker of fruit and vegetables intake.

1-1-6 Vitamin E (Table 7)

The conclusions of the three reference books, which are summarised in this part, did not differentiate between dietary intake and plasma levels, and are presented as such in Table 7.

Table: 7 Level of evidence of protection provided by studies on vitamin E and cancers

cancer sites	CNERNA (France, 1996)	World Cancer Res Fund (USA, 1997)	COMA Food and Nutrition Policy (UK, 1998)
mouth and pharynx	insufficient	insufficient	no data reported
larynx	no data reported	no data reported	no data reported
oesophagus	insufficient	insufficient	moderately consistent
lung and respiratory tract	moderately consistent	possible	insufficient
stomach	insufficient	no relationship	inconsistent
colon-rectum	insufficient	insufficient	inconsistent
pancreas	insufficient	insufficient	insufficient
liver	no data reported	no data reported	ND
breast	inconsistent	no relationship	insufficient
ovary	no data reported	no data reported	no data reported
endometrium	no data reported	no data reported	no data reported
cervix	insufficient	possible	moderately consistent but limited
prostate	no data reported	insufficient	inconsistent
kidney	ND	no data reported	ND
bladder	ND	insufficient	insufficient
thyroid	ND	no data reported	ND

ND: Not Done

Comments on table 7

None of the reference books had data on **larynx**, **liver**, **ovary**, **endometrium**, **kidney** and **thyroid** cancers. They agreed that data are insufficient for **mouth** and **pharynx**, **oesophagus**, **colon**, **rectum**, **pancreas** and **prostate** cancers. For **stomach** and **breast** cancers, the prevalent opinion is that there is no relationship. The only possible reduction of risk provided by vitamin E could be for **lung** and **respiratory tract** cancers (COMA said it was insufficient because they took into account intervention trials), and for **cervix** cancers, although data were limited.

Comparison between tables 2 and 7

There was little overlapping between Table 2 and Table 7, the only exception being the possible effect of vitamin E on lung and respiratory tract cancers. This discrepancy is largely explained by the food source of vitamin E which was mainly seed oils and not fruit and vegetables.

1.1.7 Concluding Statements

- Tomatoes:

⇒ *convincing* for oesophagus, stomach and lung cancers; *possible* for mouth and pharynx cancers. For most of the other cancers, the results were

insufficient because the studies did not differentiate among the various vegetables and fruit. To be noted, the beneficial effect of processed tomato in Giovanucci *et al.* study on prostate cancer.

- Fruit and vegetables:
 - ⇒ *convincing* evidence of a reduction of risk for all cancers of the upper aerodigestive tract including lung and stomach cancers, as well as pancreas, cervix and bladder cancers;
 - ⇒ *possible* reduction of risk by vegetables only for hormone-dependent cancers, colon, rectum liver, kidney and thyroid cancers.
- Lycopene (dietary and plasma levels):
 - ⇒ one might wonder whether it is possible to bring a conclusion on lycopene at this point of research because of the paucity of studies and the difficulties in assessing lycopene levels by whatever method. Limited results on pancreatic and prostatic cancers.
- β -carotene (dietary and plasma levels):
 - ⇒ *convincing* evidence of a reduction of risk for oesophagus, lung and stomach cancers; data are *consistent but limited* for larynx and cervix cancers; *insufficient* for pancreas, mouth and pharynx cancers;
 - ⇒ data were inconsistent or insufficient for all of the other cancers.
- Vitamin C
 - ⇒ *convincing* for stomach cancer, *probable* for oesophagus, mouth, pharynx, larynx, lung and respiratory tract, pancreas, and cervix cancers;
 - ⇒ data were inconsistent or insufficient for all of the other cancers.
- Vitamin E
 - ⇒ *possible* reduction was found for lung and respiratory tract and cervix cancers;
 - ⇒ *no relationship* demonstrated, *inconsistent or insufficient* data for all other cancers.

1-2- RECENT STUDIES

About 57 studies have been reported on the relationship between fruit and vegetables or carotenoids, vitamin C and E since the reference books were published: 40 case-control

studies and 17 prospective studies, most of them based on dietary intake, one study was based on blood concentration and three studies were based on tissue concentrations. We excluded the study by Harrisson *et al.*, (1997) a case-control study on gastric adenocarcinomas with only 91 cases subdivided in intestinal (60) and diffuse type (31). This study concluded to the protective effect of fruit, and, only vitamin C for the diffuse type after energy adjustment. We also excluded the study by Huang *et al.* (2000) because the dietary assessment was scanty (frequent/less frequent). The results showed a significant but moderate risk reduction of gastric cancer risk by fruit and fresh vegetables. The study by Vlajinac *et al.* (1997) with 101 cases with prostate cancer was excluded because the adjustment for potential confounders appeared unclear and insufficient. This study concluded on the protective effect of vitamin E. One study (Baldwin *et al.*, 1997) quoted in the review by Giovanucci (1999) was not used because it was referenced as an abstract and was not yet published. Two case-control studies reported on cervical dysplasia. One reported in 1996, (Palan *et al.*, 1996) was based on plasma concentration of lycopene. This study was not considered in the reference books because the samples were small and there is no estimation of the relative risk but the calculation of an inverse trend between the plasma lycopene levels of normal subjects (82), subjects with increasing severity of cervical intraepithelial neoplasia (52 grade 1; 37 grade 2 and 25 grade 3) and subjects with actual cervical cancer (14). The trend was significant ($P=0.0023$) from $530 \pm 24.5 \mu\text{g/liter}$ to $33.7 \pm 12.0 \mu\text{g/liter}$. However the limitations of this study precluded any conclusion. Kanetsky *et al.* (1998) reported also on lycopene blood levels and intake in relation to cervical dysplasia. It was excluded because of the small number of cases (32) and the non-significance of the results after adjustment. We excluded the study by Farrow *et al.* (1998) on naso-pharynx carcinoma because we were more interested in epithelial cancers, and the OR were only borderline significant for β -carotene. We made exception for the case-control studies by Negri *et al.*, (1996-b) and d'Avanzo *et al.* (1997) in which the dietary questionnaire was limited to 29 food groups, because they report on less frequent cancers, i.e. endometrium and thyroid cancers; and for the study by Zhang *et al.* (1997) which reports on adipous breast tissue from 46 cases only because of the well known quality of the authors. Also, the study by Galanis *et al.* (1998) on gastric cancer using a short food frequency questionnaire of 13 food groups was retained because it is a prospective study. We present tables as summaries of these recent results on fruit and vegetables, carotenoids, vitamin C and vitamin E.

1- 2-1 Tomatoes (Table 8)

Comments on table 8:

The recent studies:

- **confirm the beneficial effect of tomato intake against larynx, lung and stomach cancers** and indicate an absence of relationship between raw tomatoes and colo-rectal and breast cancers,
- indicate a possible beneficial effect of cooked tomatoes against prostate cancer,
- do not bring any new data on mouth and pharynx, oesophagus, endometrium and cervix cancers.

The data reported by Giovanucci in his recent review (1999) were in agreement with these observations, although his conclusion (anticancer properties of tomatoes) tends to generalize data which, by now, appear to be restricted to some cancers.

1-2-2 Fruit and vegetables (Tables 9 and 10)

Comments on tables 9 and 10

The recent case-control and prospective studies totally agreed with the previous results on the risk reduction of stomach and lung cancers conferred by fruit and vegetables intake with the exception of the Netherlands cohort study (Botterweck *et al.*, 1998). However, in this study, adjustment on previous stomach disorders might represent an overadjustment and decrease the estimation of the effect. With regards to cancers for which the effect of fruit and vegetables appeared less pronounced (colon, rectum and hormone-related cancers) the recent case control studies tend to reinforce the inverse association with vegetables especially carotenoids-(but not lycopene) rich vegetables. It has to be noted that the studies showing this trend were based on a wide range of intake with high upper quantile (about two servings per day versus less than one) and that for these cancers, the prospective studies are only borderline significant and only for categories of plant food (e.g. only fruit, green leafy vegetables) or of patients (premenopausal breast cancers found in two studies). For prostate, cruciferous vegetables appear to reduce the risk in one study (Cohen *et al.*, 2000) but not in another one (Norrish *et al.*, 2000).

Table 8: Characteristics and results of recent case-control studies on tomatoes and cancers

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Larynx	De Stefani <i>et al.</i> , 2000	Uruguay cases : 148 controls :444	continuous OR for increment of 17.3g/day: 0.32 (0.17-0.58)	-	
Lung	Agudo <i>et al.</i> ,1997	Spain cases:103 controls: 206	H vs L (not defined) 0,45 (0,22-0,91)	0.026	women
	Nyberg <i>et al.</i> , 1998	Sweden cases: 124 controls: 235	H(daily) vs L(2-4 /week) 0,79 (0,43-1,46)	0.04	consumption expressed in frequency.
	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls :540	H(>2/week)vs L<1/week) 0.76 (0.55-1.07)	0.09	
	Brennan <i>et al.</i> 2000	multicentric European cases :256 controls : 599	H (daily) vs L(≤1/week) 0.5 (0.4-0.6) adenocarcinoma	<0.05	in non smokers, OR for squamous cell and small cell carcinomas NS
Stomach	Ekström <i>et al.</i> , 1998	Sweden cases: 567 controls: 1165	H(≥ 4/week) vs L(≤3/month) cardia :0.6 (0.3-1.1) non cardia 0.8 (0.6-1.2)	0.06 0.09	
Colon	Franceschi <i>et al.</i> , 1998	Italy cases: 1225 controls: 5155	raw: H (80th percentile) vs L (20th) 0.9 (0.8-1.0)		no data on cooked tomatoes
Rectum	Franceschi <i>et al.</i> , 1998	Italy cases: 728 controls: 5155	raw: H (80th percentile) vs L (20th percentile) 0.9 (0.8-1.1)		no data on cooked tomatoes
Breast	Franceschi <i>et al.</i> , 1998	Italy cases: 2569 controls: 3082	raw: H (80th percentile) vs L (20th percentile) 1.0 (0.9-1.1)		no data on cooked tomatoes
Prostate	Key etal, 1997	UK cases: 328 controls: 328	H (>1/day) vs L≤4/week) raw tomatoes:1.06 (0.55-0.62) cooked: 0.92 (0.59-1.42)	NS NS	consumption expressed in frequency. baked beans (cooked in tomato sauce) 0.52 (0.31-0.88) trend P=0.075
	Tzonou <i>et al.</i> , 1999	Greece cases: 320 controls 246	raw tomatoes: NS; cooked for an increment of 2/week to 4/week 0.85 (0.75-0.97)	0.14 0.003	
	Jain <i>et al.</i> 1999	Canada cases 617 controls 636	H(>109.6/day) vs L<9.3/day) 0.64 (0.45-0.91)		tomatoes + tomato soup + sauce
	Cohen <i>et al.</i> , 2000	USA cases : 628 controls: 602	H(≥3/week) vs L (< 1) 0.73 (0.48-1.10)	0.13	raw tomatoes: NS; cooked
	Norrish <i>et al.</i> , 2000	New Zealand cases: 317 controls: 480	H(≥64.2/day) vs L (< 18.7) 0.82 (0.53-1.26)	NS	raw tomatoes: NS; raw +cooked+transformed
Kidney	Yuan <i>et al.</i> , 1998	USA cases: 1204 controls 1204	H (>1/day)vsL(<3/week) 0.93 (0.71-1.22)		tomatoes + juice + soup + pasta H (3-5/week) 0.74 (0.56-0.99)

Table 9: Characteristics and results of recent case-control studies on fruit and vegetables and cancers

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Mouth and pharynx	Franceschi <i>et al.</i> , 1999	Italy cases: 598 control: 1491	raw vegetables: H (>31.1 g/day) vs L (≤8.): 0.29 (0.15-0.56) cooked: H (>4.5 /week) vs L (1.5 /week) : 0.5 (0.3-0.7)	<0.01 <0.01	
	Bosetti <i>et al.</i> , 2000	Italy and Switzerland female cases 195 female control: 1113	green vegetables H(frequency) vs L: 0.25 (0.15-0.44) fresh fruit H (frequency) vs L: 0.58 (0.37-0.89)	<0.0001 0.02	
Oesophagus	Levi <i>et al.</i> , 2000	Switzerland cases:101 controls: 327	raw and cook vegetables: H(9.5/week) vs L(<5.5): 0.14 (0.1-0.4). fruits(other than citrus) H(11.3/week) vs L(<5.2) 0.20 (0.1-0.4)	<0.001 <0.001	citrus OR = other fruits but with 5 times less quantity
Larynx	De Stefani <i>et al.</i> , 2000	Uruguay cases : 148 controls :444	H (≥320.7/day) vs L (≤143.0) : 0.30 (0.15-0.59)	<0.001	cooked vegetables: 0.96 (0.50-1.84)
Stomach	Ji <i>et al.</i> , 1998	China cases: 1124 control: 1451	H (≥9servings/day) vs L (≤5) 0.4 (0.3-0.5)	<0.0001	after subgroups, only yellow- green vegetables:0.5 (0.4-0.7) T: 0.0001
	Ekström <i>et al.</i> , 1998	Sweden cases: 567 controls: 1165	H(> 2/day) vs L(≤5/week) 0.5 (0.3-1.1) cardia 0.7 (0.5-1.0) non cardia	0.05 0.02	
Lung	Nyberg <i>et al.</i> , 1998	Sweden cases: 124 controls: 235	fruits except citurs H(daily) vs L (2-4 /week) 0.49 (0.25-0.94)	0.03	expressed in consumption frequency. tomatoes: 0.79 (0.43-1.46) trend: 0,4
	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls :540	total vegetables: H(>2/day)vs L<1/day) 0.48 (0.34-0.66) total fruits: H(>8/week)vs L (<4/week)0.52 (0.37-0.73)	<0.001 <0.001	
	Brennan <i>et al.</i> 2000	multicentric European cases :256 controls : 599	fresh vegetables:H (daily) vs L(≤1/week) 0.5 (0.3-0.7) adenocarcinoma	<0.05	in non smokers, OR for squamous cell and small cell carcinomas NS fruit:NS
Colo-rectal adenoma	Witte <i>et al.</i> , 1996	USA cases: 519	vegetables H (45.5 /week) vs L (9 /week)	<0.001	high carotenoid vegetables, cruciferous, vitamin C rich fruit

		controls: 556	0.47 (0.29-0.76)		significant OR <1
Colon, Rectum	Franceschi <i>et al.</i> , 1997	Italy cases: 1953 controls: 4154	vegetables H (>18.1 /week) vs L (<8.4/week) 0.57 (0.47-0.69) fruits not agrumes H (>19 /week) vs L (<7.2) 0.72 (0.60-0.87)	<0.01	raw vegetables (salad, carrots) cooked vegetables (peas and beans, greens)
Breast	Longnecker <i>et al.</i> , 1997	USA cases: 3543 control: 9406	carrots and spinach raw and cooked H (>2/ week) vs L (<1/ month) 0.56 (0.34-0.91)	0.0001	each one protective to a lesser extent no effect of supplements of carotene and vit. A
	Favero <i>et al.</i> , 1998	Italy cases: 2569 controls: 2588	raw vegetables H (>12.5 /week) vs L (4.9) 0.73 (0.6-0.9)	<0.01	
	Ronco <i>et al.</i> , 1999	Uruguay cases: 400 controls 405	H(<3/day) vs L(<2) 0.42 (0.26-0.66)	0.005	mainly from vegetables and green leafy ones: H (>4/week) vs L(≤1) 0.36 (0.23-0.55)
Prostate	Deneo-Pelligrini <i>et al.</i> , 1998	Uruguay cases: 175 controls: 233	H (≥3/day) vs L (<2) 0.5 (0.3-0.9)	0.04	mainly due to vegetables
	Jain <i>et al.</i> , 1999	Canada cases: 617 controls: 636	H(>72.7/day) vs L<24) 0.54 (0.40-0.71)		other vegetables and fruits: NS
	Cohen <i>et al.</i> , 2000	USA cases: 628 controls: 602	vegetables H(≥28 servings/week)vs L (<14/week) 0.65(0.45-0.94)	0.01	cruciferous H(≥3 servings/week)vs L (<1/week) 0.54 (0.38-0.76) trend 0.1 Fruit NS
	Norrish <i>et al.</i> , 2000	New Zealand cases: 317 controls: 480	green leafy vegetables H(>88.7g/day)vs L (<29.2) 0.91 (0.60-1.37)	NS	cruciferous+ spinach+ green salad
Kidney	Yuan <i>et al.</i> , 1998	USA cases: 1204 controls: 1204	dark green and cruciferous veg H (≥5/week) vs L (<1/month) 0.5 (0.3-0.9)		

Table 10: Characteristics and results of recent prospective studies on fruit and vegetables and cancers

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Stomach	Galanis <i>et al.</i> , 1998	Hawai Japanese 108/11907	H (<1/day) vs L (≥2/day) 0.4 (0.2-0.8)	0.02	better in men than in women
	Terry <i>et al.</i> , 1998	Sweden, 116/11500	L vs H: 5.5 (1.7-18.3)	<0.05	wide CI. tertiles defined as high, moderate, small, none
	Botterweck <i>et al.</i> , 1998	Netherlands 310/3500 (subcohort)	H (374g/day) vs L (250g/day) 0.72 (0.48-1.10)	0.14	0.49 (0.20-1.18) on first year cases and precancer disorders vegetables only little variation in intake
Lung	Ocké <i>et al.</i> , 1997	Netherlands 19years; 54/561	Fruit: L (<107g/day) vs H (>166g/day) 1.92 (1.04-3.55)	0.03	men Stability of consumption of Fruit: 2.52 (1.15-5.57) Vegetables: NS
	Knekt <i>et al.</i> , 1999	Finland 25years; 138/4545	H vs L (not defined) 0.60 (0.38-0.965)	0.02	Fruit 0.58 (0.37-0.93) p:0.013. root vegetables 0.56 (0.36-0.88) p:0.03
	Voorrips <i>et al.</i> 2000	Netherlands 6.3years; 1010/2953 (subcohort)	H (554g/day) vs L (191g/day) 0.7 (0.5-1.0)	<0.0001	mainly due to vegetables
Breast	Verhoeven <i>et al.</i> , 1997	Netherlands 4years 650/1812 (subcohort)	fruit H (343.1g/day) vs L (64.9g/day) 0.76 (0.54-1.08)	0.10	
	Zhang <i>et al.</i> , 1999	USA 14 years, 784/83234	vegetables H (≥5 servings) vs L (<2 servings) 0.64 (0.43-0.95)	0.10	the OR is for premenopausal cancers
Ovary	Kushi <i>et al.</i> 1999	USA 9years; 139/29083	green leafy vegetables: H (>6 servings/week) vs L (<2) 0.44 (0.25-0.79)	0.01	
Bladder	Michaud <i>et al.</i> , 1999	USA 10 years; 252/47909	H (>8 servings/day) vs L (<3.5) 0.72 (0.47-1.09)	0.09	cruciferous 0.49 (0.32-0.75) trend:0.008
	Nagano <i>et al.</i> , 2000	Japan 20 years; 114/38540	H (>5/week) vs L (≤1) 0.54 (0.39-0.94)	0.02	green-yellow vegetables

1-2-3 Lycopene (Tables 11-14)

Comments on tables 11-14

All the studies (case-control, prospective, based on dietary intake or tissue concentration assessment) showed no relationship between lycopene and various cancer sites with the noticeable exception of the prospective Physicians Health Study which showed a decreased OR in prostate cancer patients with a high plasma level of lycopene, significant only in aggressive tumors. This effect was found in non β -carotene supplemented subjects, although the OR was decreased in β -carotene supplemented subjects with low lycopene plasma levels. Because of this peculiar finding the authors remained very cautious in their conclusions and called for more studies. The study on adipose tissue from breast cancer patients (Zhang *et al.*, 1997) showed a decreased risk with higher than median lycopene concentrations. This was also shown for β -carotene (Table 16). However, this study is limited by the small sample size (46) and by data collection in 2 one-year apart batches. One study from Japan (Nagata *et al.*, 1999) based on plasma lycopene concentration suggest an inverse relationship between lycopene levels and cervical dysplasia, however the authors acknowledge the fact that low levels of carotenoids might be a consequence and not a cause of the cervical dysplasia.

Altogether, these results are disappointing and there is no evidence that improved food composition tables allowed to unravel a protective effect of lycopene on cancers. Because antioxidants and especially carotenoids appeared related to cervical cancer, it might be interesting to look at lycopene relationship with this cancer in larger and better studies than the two reported so far (Palan *et al.*, 1996; Kanetsky *et al.*, 1998).

Comparison between tables 8 and 11-14

This comparison suggests that lycopene is not the micronutrient responsible for the relationship between tomato and lung cancer.

Table 11: Lycopene and cancers. Recent case-control studies base on dietary intake ($\mu\text{g}/\text{day}$)

Cancer sites	Authors and year	Country Design	OR(CI)	Trend	Remarks
Stomach	Garcia-Closas <i>et al.</i> , 1999	Spain cases: 354; controls: 354	H (≥ 1209) vs L (≤ 6) OR: 1.55 (0.91-2.64)	0.9	
Lung	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls : 540	H (≥ 2056) vs L (< 916) OR: 0.83 (0.56-1.21)	0.18	
Colon, Rectum	La Vecchia <i>et al.</i> , 1997	Italy cases: 1953; controls: 4154	H (≥ 11721) vs L (< 5411) OR= 1.02 (0.8-1.2)	0.98	Continuous OR: 1.04 (0.9-1.1)
	Slattery <i>et al.</i> , 2000	USA cases: 1993 controls: 2410	H(med:13072) vs L(1017) 0.96 (0.79-1.19)	0.66	
Breast	La Vecchia <i>et al.</i> , 1998	Italy cases: 2569; controls: 2588	H (≥ 8541) vs L (< 3678) OR= 1.16 (0.95-1.40)	0.42	
	Ronco <i>et al.</i> , 1999	Uruguay cases: 400 controls: 405	H (≥ 4296) vs L (< 2286) OR= 0.30 (0.19-0.47)	< 0.001	the effect of lycopene appears independent from the effect of vegetables
Prostate	Deneo-Pelligrini <i>et al.</i> , 1998	Uruguay cases: 175 controls: 233	H (≥ 3301) vs L (≤ 1300) OR= 1.2 (0.7-2.2)	0.90	
	Jain <i>et al.</i> , 1999	Canada cases: 617 controls: 636	H (> 12681) vs L (< 2103) 1.01 (0.76-1.35)	ND	
	Cohen <i>et al.</i> , 2000	USA cases : 628 controls: 602	H (≥ 9900) vs L (< 4900) 0.89 (0.60-1.31)	0.13	
	Norrish <i>et al.</i> , 2000	New Zealand cases: 317 controls: 480	H (> 1.994) vs L (< 662) 0.76 (0.50-1.17)	0.30	
Bladder	Garcia <i>et al.</i> , 1999	Spain cases: 497 P-controls: 547 H-controls 566	H(not given) vs L(id) 0.80 (0.5-1.2)	0.28	
Kidney	Yuan <i>et al.</i> , 1998	USA cases: 1204 controls 1204	H (≥ 4.125) vs L (≤ 1.174) 0.98 (0.75-1.30)	0.95	

Table 12: Lycopene and cancers. Recent case-control studies base on plasma level ($\mu\text{mol/l}$)

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Colorectal polyps	Shikany <i>et al.</i> , 1997	USA cases: 472; controls: 502	H (>0.906) vs L (<0.425) OR= 1.18 (0.82-1.71)	0.38	
Breast	Zhang <i>et al.</i> , 1997	USA cases: 46 controls: 63	inbreast fat H (>median. not defined) vs L (< median): OR: 0.32 (0.11-0.94)	-	different batches collected 1 year apart no correlation with intake
Cervical dysplasia	Nagata <i>et al.</i> ,1999	Japan cases: 156 controls: 156	H (>0.9736) vs L (<0.307) OR=0.28(0.08-1.01)	0.049	adjustment for papilloma virus and smoking

Table 13: Lycopene and cancers. Recent prospective studies based on dietary intake ($\mu\text{g}/\text{day}$)

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Lung	Knekt <i>et al.</i> , 1999	Finland 138/4545 (25)	H (not defined) vs L (not defined) OR: 1.00 (0.67-1.50)	0.77	smokers (90) mean intake (734): OR: 1.26 (0.81-1.96) non-smokers (28) mean intake 701; OR: 0.46 (0.16-1.33)
Breast	Zhang <i>et al.</i> , 1999	USA 2697/83234 (18 years)	H (12688) vs L (1520) pre-menop OR 1.10 (0.87-1.38) post-menop OR 1.02 (0.88-1.18)	0.34 0.97	

Table 14: Lycopene and cancers. Recent prospective studies based on blood levels ($\mu\text{M}/\text{l}$)

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Lung	Comstock <i>et al.</i> , 1997	USA cohort of blood donors (8) cases 258; selection of matched controls 515	(H vs L, not defined) 1.01	0.99	
Breast	Dorgan <i>et al.</i> , 1998	USA cohort of blood donors (9,5) cases 105; selection of matched controls 203	H (0.51-1.75) vs L (≤ 0.22) 0.5 (0.2-1.2)	0.97	non smokers: (86) 0.4 (0.2-1.1) trend: 0.06 restricted to 60 cases appearing after 2 years:0.4 (0.1-1.1) trend 0.02
Prostate	Gann <i>et al.</i> , 1999	USA Physician's Health Study cases 578; matched controls 1294	H (cut off point:1.08) vs L (0.487) 0.75 (0.54-1.06)	0.12	agressive cases only (259) 0.56 (0.34-0.92) trend: 0.05

1-2-4 Carotenoids (Table 15-17)

Comments on Table 15-17

The recent case-control and prospective studies totally agreed with the previous results on the risk reduction of stomach and lung cancers conferred by fruit and vegetables intake (to which we can add the effect on mouth and pharynx cancer Bosetti *et al.*, 2000). A possible interaction (or confusion) with vitamin C should be noted. For colon-rectum, the new results were inconsistent as stated in the reference books with only one case-control study based on dietary intake from Italy showing a significant risk reduction. Ten new studies were reported for breast cancers: three case-control studies conducted in Mediterranean countries showed significant protection, one in Uruguay showed a protection by α -carotene, whereas the prospective studies conducted in the USA and Netherlands were non significant, although the level of consumption appears similar between Italy and USA. For studies based on tissue concentration, only a small case-control study suggested that β -carotene concentration was lower in breast cancer patient's fat than in fat from normal control. A large transversal multicentric study (Germany, Netherlands, Switzerland, Northern Ireland and Spain) and a prospective one showed no relationship between breast cancer and β -carotene tissue concentration (van't Veer *et al.*, 1996). Thus, positive results solely come from Mediterranean countries Italy and Greece. Reports of a protective effect of β -carotene for endometrium and thyroid cancers also came from Italy. Several questions arise from this observation:

A methodological bias is not likely since methods were not the same in Greece and Italy, and not the same for breast, endometrium and thyroid cancers.

Are these results an indication of a protective common factor in the Mediterranean diet, which is not necessarily carotenoids, but present together with carotenoids in the food habits?

Comparison between tables 8, 9 and 15-18

The main observation of this comparison is that β -carotene can in part mediate the protective effect of tomatoes and other fruit and vegetables for lung and stomach cancers. However a synergic or additive interaction with other compounds, among them vitamin C is strongly suggested.

Table 15: Carotenoids and cancers. Recent case-control studies based on dietary intake

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Salivary gland	Horn-Ross <i>et al.</i> , 1997	USA cases: 133; controls: 191	carotene H (>3.9mg/day) vs L (≤2.1 mg/day) 0.54 (0.29-0.99)	0.05	becomes NS after adjustment for vitamin C
Mouth and pharynx	Bosetti <i>et al.</i> , 2000	Italy and Switzerland female cases: 195 controls: 1113	β-carotene H vs L (not defined) 0.54 (0.34-0.86)	<0.01	
Oesophagus	Terry <i>et al.</i> , 2000	Sweden cases:185 adenocarc.; 165:squamous cell carc.; controls: 815	H (>5mg/day) vs L (≤0.7mg/day) 0,6 (0.4-1.0) for squamous cell carc., 0.5 (0.3-0.8) for adenocarc.	0.05 0.0005	vitamin C+E+b-carotene: squamous id; oesophagus adenocarc.: OR: 0.5 (0.3-0.9) trend: 0.009 for
Stomach	Ji <i>et al.</i> , 1998	China cases: 1124 controls: 1451	M:H (≥1.5 mg/day) vs L (≤0.74) 0.4 (0.3-0.6); F: H (1.3mg/day) vs L (0.66) 0.7 (0.5-1.1)	M <0.0001 F: 0.02	adjustment for alcohol and tobacco only in men
	Kaaks <i>et al.</i> , 1998	Belgium cases: 301 controls: 2851	β-carotene H vs L (not defined) 0.50 P<0.05	<0.001	
	Garcia-Closas <i>et al.</i> , 1999	Spain cases:354 controls: 354	β-carotene H (2.75 mg/day) vs L (0.55 mg/day) 0.83 (0.29-2.35)	0.92	kaempferol was found protective 0.48 (0.26-0.88) trend 0.04
	Ekström <i>et al.</i> , 2000	Sweden cases: 567 controls: 542	β-carotene per 3mg/day increment 0.5 (0.3-0.8)	ND	non-cardia in smokers and/or <i>H Pylori</i> ; NS in cardia cancers, no smoking and/or <i>H Pylori</i> - subjects
Lung	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls : 540	β-carotene H (≥5.86 mg/day) vs L(<1.94) 0.42 (0.28-0.63)	<0.001	risk reduction by all carotenoids except lycopene
	Brennan <i>et al.</i> , 2000	multicentric European cases :256 controls : 599	carotenoids: H(not given) vs L(id) 0.8 (0.6-1.0)	0.22	
Colon, Rectum	La Vecchia <i>et al.</i> , 1997	Italy cases: 1953 controls: 4154	Carotenoids H (≥7.7mg/day) vs L (3.6-4.9mg/day) 0.63 (0.5-0.8)	<0.001	Continuous OR: 0,87 (0,8-1.0)
	Ghadirian <i>et al.</i> , 1997	Canada cases: 402; controls: 668	β-carotene H vs L (not defined) 0.72 (0.49-1.06)	-	
	Slattery <i>et al.</i> , 2000	USA cases: 1993 controls: 2410	α-carotene: H (med: 2636μg/day) vs L (165μg/day) 0.88 (0.71-1.09)	0.58	β-carotene: 1.18 NS

	Negri <i>et al.</i> , 1996-a	Italy cases: 2569 controls: 2588	β -carotene H (>5.8mg/day) vs L (\leq 2.8mg/day) 0.74 (0.6-0.9)	<0.08	Mutual adjustment on other micronut: 0,84 (0,7-1,0) trend <0,05
	La Vecchia <i>et al.</i> , 1998	Italy cases: 2569 controls: 2588	β -carotene H (\geq 6.6mg/day) vs L (<3.0mg/day) 0.68 (0.56-0.82)	<0.001	α -carotene H (>1.2 mg/day) vs L (<0.3) 0,58 (0.48-0.70) trend <0.001
Breast	Ronco <i>et al.</i> , 1999	Uruguay cases: 400 controls 405	α -carotene: H (med: 487;5 μ g/day) vs L 24.75) 0.52 (0.341-0.80)		β -cryptoxanthine id, β -carotene and lutein NS
	Bohlke <i>et al.</i> , 1999	Greece cases:820 controls: 1548	β -carotene H (>8.3mg/day) vs L (3.8mg/day) 0.67 (0.49-0.91)	0.005	preM: 0.36 (0.21-0.61) trend:0.0001 postM: NS
Endometrium	Negri <i>et al.</i> , 1996-b	Italy cases: 368; controls: 713	β -carotene H (>5.5mg day) vs L (2.9mg/day) 0.5	<0.01	no CI given 50 food items
Prostate	Key <i>et al.</i> , 1997	UK cases: 328; controls; 328	carotene H (\geq 3.48mg/day) vs L (<2.65mg/day) 0.83 (0.57-1.21)	0.35	
Prostate	Deneo- Pelligrini <i>et al.</i> , 1998	Uruguay cases: 175 controls: 233	α -carotene H (\geq 601 μ g/day) vs L (<109 μ g/day) 0.9 (0.5-1.6)	0.40	β -carotene OR=1
	Jain <i>et al.</i> 1999	Canada cases 617 controls 636	H (\geq 7.8mg/day) vs L (<3.0mg/day) 1.06 (0.74-1.42)	ND	α -carotene: id
	Cohen <i>et al.</i> , 2000	USA cases : 628 controls: 602	β -carotene H (\geq 4.4mg/day) vs L (<2.2mg/day) 0.81 (0.55-1.21)	0.95	α -carotene: id
	Norrish <i>et al.</i> , 2000	New Zealand cases: 317 controls: 480	β -carotene H (>6.0/day) vs L (<2.5mg/day) 1.09 (0.72-1.64)	0.24	
Bladder	Garcia <i>et al.</i> , 1999	Spain cases: 497 P-controls: 547 H-controls 566	α -carotene H(not given) vs L (<id) 0.87 (0.5-1.5)	0.80	β -carotene 1.62 (0.8-3.2)
Kidney	Yuan <i>et al.</i> , 1998	USA cases: 1204 controls 1204	α -carotene H(\geq 1442 μ g/day) vs L (<447 μ g/day) 0.61 (0.45-0.82)	<0.001	β -carotene and lutein comparable
Thyroid	d'Avanzo <i>et al.</i> , 1997	Italy cases: 399; controls: 617	β -carotene H (\geq 5.8mg/day) vs L (<3.1mg/day) 0.58 (0.4-0.9)	<0.05	29 food items

Table 16: Carotenoids and cancers. Recent case-control studies based on tissue levels

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Colorectal polyps	Shikany <i>et al.</i> , 1997	USA cases: 472 controls: 502	β -carotene in plasma ($\mu\text{mol/L}$) H(>0.486) vs L (<0.186) OR= 1.05 (0.712-1.57)	0.89	all other carotenoids NS after adjustment on fruit and vegetables
Breast	van't Veer <i>et al.</i> , 1996	5 European countries post menopause cases: 347 controls: 374	β -carotene, adipose tissue($\mu\text{g/g}$) H(1,33 vs L 0,69): OR: 0,74 (0,45-1,23)	0.31	Euramic study no interaction no effect of composite antioxidant score
	Zhang <i>et al.</i> , 1997	USA cases: 46 controls: 63	β -carotene in breast fat H (>median, not defined) vs L (< median): OR: 0,30 (0,11-0,85)	-	different batches collected 1 year apart no correlation with intake
Cervix dysplasia	Nagata <i>et al.</i> , 1999	Japan cases : 156 controls : 156	α -carotene in plasma ($\mu\text{mol/L}$) H (0.256) vs L(0.095) 0.16 (0.04-0.62)	0.01	β -carotene (2.1 vs 0.6) OR 0.65 (0.22-1.92) trend NS

Table 17: Carotenoids and cancers. Recent prospective studies based on dietary intake

Cancer sites	Authors and year	Country Design	OR (CI)	Trend	Remarks
Lung	Ocké <i>et al.</i> , 1997	Netherlands prospective (19) 54/561 men	≤33rd vs >33rd percentile of stable β-carotene intake 2,11 (1,02-4,38)	-	NS for the entire sample
	Yong <i>et al.</i> , 1997	USA Prospective (19) 248/10068	Carotenoids intake H (2 290 IU) vs L (<206,2 IU) 0,74 (0,52-1,06)	0.14	+vit C +E .0.32 (0.14-0.74) trend 0.0004; +vit C: 0.41(0.24-0.72) trend: 0.0003; +vit E 0.62(0.36-1.08) trend: 0.04
	Knekt <i>et al.</i> , 1999	Finland prospective(25) 138/4545	α-carotene intake H (not defined) vs L (26µg/day) 0,61 (0,39-0,95)	0.10	β-carotene NS smokers vs non-smokers NS. lycopene no effect
Breast	Kushi <i>et al.</i> , 1996	USA, postM prospective (6) 879/34387	Carotenoids intake H vs L (not defined) 0,88 (0,70-1,12)	0.98	OR=1 in women non supplemented in vitamins A, C and E
	Verhoeven <i>et al.</i> , 1997	Netherlands prospective (4,3) 650/1812 (subcohort)	β-carotene (mg/day)H-0.7 vs L-0.2) 1.01 (0.72-1.42)	0.96	
	Zhang <i>et al.</i> , 1999-a	USA 2697/83234 (18 years)	β-carotene (mg/day H-7.6 vs L-1.7) α-carotene (1.5vs0.2) both OR 0.84 (0.67-1.05) β- cryptoxanthine (0.7vs 0.02) 0.89 (0.70-1.13) luteine/zea (8.8vs1.4) 0.79 (0.63-0.99)	NS NS NS NS 0.04	premenopausal cases only effective in post-menopausal when ERT and in women with family history of breast cancer
	Jumaan <i>et al.</i> , 1999	Sweden	β- carotene (units not precised in the report H-4.09-16.9) vs L(0.16-1.91) 0.41 (0.23-0.72)	0.05	OR appears to decrease with time before dietary intake and cancer diagnosis (latency or memory bias?)

Table 18: Carotenoids and cancers. Recent prospective studies based on blood levels

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Stomach	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 24/2974	carotene plasma conc. L (<0.23µm/l) vs H (≥0.23µm/l) 3.30 (1.42-7.70)		continuous covariates, first 2 years of follow up excluded
Lung	Eichholzer <i>et al.</i> , 1996	Switzerland prospective(17) 87/2974	carotene plasma conc. L (<0.23µm/l) vs H (≥0.23µm/l) 1.90 (1.18-3.07)		continuous covariates, first 2 years of follow up excluded
	Comstock <i>et al.</i> , 1997	USA cohort study on blood donors (8 year) cases:258. selection of matched controls: 515	carotenoids in blood (H vs L, not defined) β-carotene: 0.44 α-carotene: 0.48 cryptoxanthine: 0.2 lutein/zeaxanthine: 0.41	0.002 0.01 <0.001 <0.001	CI not given
Colon, Rectum	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 21/2974	carotene plasma conc. L (<0.23µm/l) vs H (≥0.23µm/l) 1.33 (0.47-3.80)		continuous covariates, first 2 years of follow up excluded
Prostate	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 29/2974	β-carotene plasma conc. L (<0.23µm/l) vs H (≥0.23µm/l) 1.03 (0.43-2.47)		continuous covariates, first 2 years of follow up excluded
Breast	Dorgan <i>et al.</i> , 1998	USA prospective (9,5) 105/7224	β-carotene, plasma levels H (0.69-2.20µm/l) vs L (≤0.29µm/l) 1.1 (0.5-2.4)	0.97	

1-2-5 Vitamin C (Tables 19-21)

Comments on Table 19-21

Except for the case-control study on lung cancer in Uruguay (De Stefani *et al.*, 1999) and the prospective study of Eicholzer *et al.* (1996), only 24 cases with stomach cancer, recent case-control prospective studies confirmed the conclusion of the three reference books on the risk reduction conferred by vitamin C for cancers of the upper aero-digestive tract including stomach, lung and respiratory tract cancers. But association for a greater effect with other antioxidants was often reported, emphasising again the importance of association of antioxidants. For colon cancer, we remain with insufficient and/or inconsistent data. For breast cancer, results were borderline in two Mediterranean case-control studies and non-significant in two prospective North-American and North-European studies. Again the Uruguayan results Ronco *et al.*, 1999; Deneo-Pelligrini *et al.*, 1999 are at odds with other studies on breast cancer and prostate cancer. It might be that vitamin C is confounded by another microconstituent of fruit and vegetables.

Comparison between tables 9 and 19-21 (Same comments as for tables 9 and 15-18).

Table 19: Vitamin C and cancers. Recent case control studies on dietary intake

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Salivary gland	Horn-Ross <i>et al.</i> , 1997	USA cases: 133 controls: 184	H (>200mg/day) vs L (\leq 100mg/day) 0.40 (0.22-0.70)	0.001	vitamin C assessment included supplements
Oesophagus	Terry <i>et al.</i> , 2000	Sweden cases:185 adenocarc.; 165:squamous cell carc.; controls: 815	H (>88mg/day) vs L (\leq 29mg/day) 0.6 (0.4-1.0) for squamous cell carc., NS others	0.06	vitamin C+E+ β carotene: squamous id; oesophagus adenocarc.: OR: 0.5 (0.3-0.9) trend: 0.009 for
Stomach	Ji <i>et al.</i> , 1998	China cases: 1124 controls: 1451	M: (H \geq 86.1) vs L \leq 48.7) 0.5 (0.3-0.7) F: (H \geq 77.3 vs L \leq 46.8): 0.7 (0.5-1.1)	M<0.0001 F: 0.40	adjustment for alcohol and tobacco only in men
	Kaaks <i>et al.</i> , 1998	Belgium cases: 301 control: 2851	H vs L (not defined) 0.43 P<0.05	<0.001	
	Ekström <i>et al.</i> , 2000	Sweden cases: 567 controls: 542	50mg/day increment 0.5 (0.3-0.8) non-cardia in smokers and/or <i>h pylori</i> +;	ND	NS in cardia cancers, no smoking and/or <i>h pylori</i> - subjects
Lung	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls : 540	H (\geq 176.2mg/day) vs L (<90.50mg/day) 1.03 (0.70-1.52)	0.84	
Colon, Rectum	La Vecchia <i>et al.</i> , 1997	Italy cases: 1953 controls: 4154	H(\geq 313mg/day) vs L (140- 188mg/day) 0.73 (0.6-0.9)	<0.01	Continuous OR: 0.86 (0.8-1)
Breast	Negri <i>et al.</i> , 1996-a	Italy cases: 2569 controls: 2588	H (>182mg/day vs L (<81mg/day) 0.81 (0.7-1.0)	NS	continuous 0.92 (0.85-0.99).
	Ronco <i>et al.</i> , 1999	Uruguay cases: 400 controls 405	H (>124.9mg/day vs L (<71.3) 0.45 (0.29-0.69)	0.001	
	Bohlke <i>et al.</i> , 1999	Greece cases:820 controls: 1548	H (\geq 343.1mg/day vs L(\leq 142.9mg/day) 0.67 (0.49-0.91)	0.005	preM: 0.36 (0.21-0.61) trend: 0.0001; NS after mutual adjustm. post M: NS
Prostate	Deneo-Pelligrini	Uruguay	H (\geq 161.91mg/day vs L(\leq 85.8)	0.008	

	<i>et al.</i> , 1998	cases: 175 controls: 233	0.4 (0.2-0.8)		
	Jain <i>et al.</i> , 1999	Canada cases 617 controls 636	H (243.71mg/day vs L(<121.8) 0.75 (0.50-1.22)		
	Cohen <i>et al.</i> , 2000	USA cases : 628 controls: 602	H (≥150mg/day vs L(<70) 0.75 (0.50-1.11)	0.13	
Kidney	Yuan <i>et al.</i> , 1998	USA cases: 1204 controls: 1204	H (≥178mg/day vs L(≤61) 0.76 (0.56-1.02)	0.06	
Thyroid	d'Avanzo <i>et al.</i> , 1997	Italy cases: 399 controls: 617	H(≥225mg/day) vs L (<113mg/day) 0.72 (0.5-1.1)	NS	29 food items

Table 20: Vitamin C and cancers. Recent prospective studies on dietary intake

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Lung	Ocké <i>et al.</i> , 1997	Netherlands prospective men (19) 54/561	L (<80mg/day) vs H (>102mg/day) 2.16 (1.14-4.09)	0.02	
	Yong <i>et al.</i> , 1997	USA Prospective (19) 248/10068	H (>113.055mg/day) vs L (<23.07mg/day) 0.66 (0.45-0.96)	0.01	+vit E +carot: 0.32 (0.14-0.74) trend: 0.0004 +vitE: 0.40 (0.20-0.80) trend: 0.0003; +carot: 0.41 (0.24-0.72) trend: 0.04
Breast	Kushi <i>et al.</i> , 1996	USA prospective (6) 879/34387 post-menopause	H(≥392mg/day) vs L(<112mg/day) 0.87 (0.70-1.08)	0,88	OR=1 in women non supplemented in vitamins A, C and E
	Verhoeven <i>et al.</i> , 1997	Netherlands prospective (4) 650/1812 subcohort	H(165.3mg/day) vs L(58.6mg/day) 0.77 (0.55-1.08)	0,08	

Table 21: Vitamin C and cancers. Recent prospectives studies on tissue concentrations

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Stomach	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 24/2974	plasma conc. L (<22.7µm/l) vs H (>22.7µm/l) 0.97 (0.44-2.14)	-	continuous covariates, first 2 years of follow up excluded
Lung	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 87/2974	plasma conc. L (<22.7µm/l) vs H (>22.7µm/l) 1.82 (0.86-3.85)	-	associated with low vitamin E: 3.70 (1.61-8.52)
Colon	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 21/2974	plasma conc. L (<22.7µm/l) vs H (>22.7µm/l). L vs H 0.90 (0.34-2.34)	-	continuous covariates, first 2 years of follow up excluded

1-2-6 Vitamin E (Tables 22-25)

Comments on tables 22-25

For stomach cancer the results are inconsistent, with the Chinese case-control study based on dietary intake showing a strong reduction in risk and the European prospective study based on plasma levels (but only 28 cases) showing a non-significant increase in risk. This result raises the attention on the different behaviours in food intake among countries and on the difficult interpretation of studies with different designs. The recent studies tend to support a possible role of vitamin E in risk reduction of lung cancer, only in association with vitamin C and/or carotenoids (Berry *et al.*, 1999). For colon cancer, the case-control studies showed a tendency towards a reduction of risk, which was non significant in the prospective study. In the Canadian study the intake appeared more protective than the supplements. For breast cancer, there was a non significant increase of risk in prospective studies conducted in Northern countries but a tendency to a risk reduction in the case-control studies from the Mediterranean countries and Uruguay. This effect decreased or became NS with mutual adjustment for other antioxidants. A decreased risk was also found in the Italian study for high levels of seed oil intake, which is the main source of vitamin E. Therefore, it is difficult to attribute a specific effect to one of these nutrients. Thus, the results from Mediterranean studies were intriguing but underlined the difficulty of inferring the effect of a nutrient from food intake, and even from food habits as a whole. Two case-control studies of the 3 recently reported on dietary vitamin E intake and the prospective studies in Switzerland (Eicholzer *et al.*, 1996) and USA (the Physicians Health Study, Gann *et al.*, 1999) based on plasma concentration indicated an effect on prostate cancer incidence, to be mentioned in spite of the small number of cases and the wide CI, in Switzerland and the borderline significant results in the USA and in the Uruguayan case-control study, because of the results of ATBC study (See below).

Table 22: Vitamin E and cancers. Recent case control studies on dietary intake

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Salivary gland	Horn-Ross <i>et al.</i> , 1997	USA cases: 133 controls: 184	H (>14mg/day) vs L (≤7mg/day) 0.69 (0.38-1.2)	0.22	becomes 1.2 (0.58-2.3) after adjustment for vitamin C
Oesophagus	Terry <i>et al.</i> , 2000	Sweden cases:185 adenocarc.; 165:squamous cell carc.; controls: 815	H (>6.8mg/day) vs L (≤5.5mg/day) 0.5 (0.2-1.0) for squamous cell carc.; NS others	0.09	vitamin C+E+β-carotene: squamous id; oesophagus adenocarc.: OR: 0.5 (0.3-0.9) trend: 0.009 for
Stomach	Ji <i>et al.</i> , 1998	China cases: 1124 controls: 1451	M: H (≥31.7mg/day) vs L (≤19.7) 0.5 (0.3-0.7) F:H (≥28.8mg/day) vsL (≤17.2mg/day) 0.5 (0.3-0.8)	M<0.0001 F: 0.0002	adjustment for alcohol and tobacco only in men
	Ekström <i>et al.</i> , 2000	Sweden cases: 567 controls: 542	per an increment of 8mg/day 0.5 (0.3-0.8) in non cardia smokers and <i>h pylori</i> +	ND	NS in non smokers and <i>h pylori</i> -, cardi and non cardia 0.3 (0.1-1.0) in cardia <i>h pylori</i> +
Lung	De Stefani <i>et al.</i> , 1999	Uruguay cases : 541 controls : 540	H (≥8.5mg/day) vs L (<4.8) 0.50 (0,34-0,74)	<0.001	
Colon, Rectum	La Vecchia <i>et al.</i> , 1997	Italy cases: 1953 controls: 4154	H (≥18.42 mg/day) vs L (9.72-12-31) 0.45 (0.4-0.6)	<0.001	Continuous OR: 0.65 (0.6-0.7)
	Ghadirian <i>et al.</i> , 1997	Canada cases: 402 controls: 668	H vs L (not defined) 0.53 (0.26-0.78)	-	α-tocopherol (supplements?) 0.63 (0.43-0.94)
Breast	Negri <i>et al.</i> , 1996-a	Italy cases: 2569 controls: 2588	H (>13.43mg/day) vs L (≤7.21mg/day) 0.69 (0.6-0.8)	<0.01	continuous 0.84 (0.78-0.91). Mutual adjustment: continuous:0.86 (0.79-0.94)
	Ronco <i>et al.</i> , 1999	Uruguay cases: 400 controls: 405	H (>9.1 mg /day) vs L (<4.7) 0.40 (0.26-0.62)	<0.001	
	Bohlke <i>et al.</i> ,	Greece	H(>8.6IU/day)	0.04	preM: 0.50 (0.25-1.02)

	1999	cases: 820 controls: 1548	vs L (≤ 5.2 IU/day) 0.71 (0.48-1.05)		trend: 0.03; postM: NS NS after mutual adjustm.
Prostate	Tzonou <i>et al.</i> , 1999	Greece cases: 320 controls: 246	increment of 1SD (not given) 0.53 (0.30-0.94)	-	
	Deneo- Pelligrini <i>et al.</i> , 1998	Uruguay cases: 175 controls: 233	H (≥ 7.9 mg/day) vs L (59mg/day) 0.6-(0.3-1.1)	0.15	
	Jain <i>et al.</i> , 1999	Canada cases: 617 controls: 636	H (> 37.25 mg/day) vs L (< 17.7) 1.12 (0.81-1.55)		
Thyroid	d'Avanzo <i>et al.</i> , 1997	Italy cases: 399 controls: 617	H (≥ 15 mg/day) vs L (< 9 mg/day) 0.67 (0.4-1)	NS	29 food items

Table 23: Vitamin E and cancers. Recent case-control study on adipose tissue concentrations

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Breast	van't Veer <i>et al.</i> , 1996	Europe postM cases: 347 controls: 374	adipose tissue H (350mg/g) vs L (231mg/g) 1.15 (0.75-1.77)	0.31	Euramic: 5 centers no interaction; composite antiox. score: 1.54 (0.94-2.52)
Cancer dysplasia	Nagata <i>et al.</i> , 1999	Japan cases : 156 controls : 156	H (20.8 μ mol/l) vs L (13.8) 0.85 (0.28-2.61)	0.80	

Table 24: Vitamin E and cancers. Recent prospective studies on dietary intakes

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Lung	Ocké <i>et al.</i> , 1997	Netherlands prospective(19) 54/561 men	L (<15.3mg/day) vs H(>19.8mg/day) 1.47 (0.66-3.17)	0.34	
	Yong <i>et al.</i> , 1997	USA prospective (19) 248/10068	H (>6.71mg/day) vs L (<3.69mg/day) 0.88 (0.62-1.25)	0.30	+vit C +carot.0.32 (0.14-0.74) trend: 0.0004 +vitC: 0.40(0.20-0.80) trend: 0.0003; +carot: 0.62(0.36-1.08) trend: 0.04
	Woodson <i>et al.</i> , 1999	Finland (7.7) 1144/29102	H (>14.2mg/day) vs L (<9.34) 0.80 (0.66-0.97)	0.2	+ suppl: 0.77 (0.64-0.93) trend:0.01
Breast	Kushi <i>et al.</i> , 1996	USA post-M prospective (6) 879/34387	H (≥35.66mg/day) vs L (<5.66 mg/day) 1.05 (0.83-1.33)	0.85	id in women non supplemented with vitamins A, C and E
	Verhoeven <i>et al.</i> , 1997	Netherlands prospective (4) 650/1812 (subc.)	H (19.82mg/day) vs L (5.96mg/day) 1.25 (0.85-1.85)	0.37	

Table 25: Vitamin E and cancers. Recent prospective studies on tissue concentrations

Cancer sites	Authors and year	Country Design	OR	Trend	Remarks
Stomach	Eichholzer <i>et al.</i> , 1996	Switzerland prospective(17) 24/2974	plasma conc. L (<30.02µm/l) vs H (>30.02µm/l) 0.52 (0.20-1.34)		continuous covariates, first 2 years of follow up excluded
Lung	Eichholzer <i>et al.</i> , 1996	Switzerland prospective(17) 83/2974	plasma conc. L (<30.02µm/l) vs H (>30.02µm/l) 1.03 (0.60-1.78)		associated with low vitamin C: 3.76 (1.63-8.71) NS continuous variables
	Woodson <i>et al.</i> , 1999	Finland (7.7) 1144/29102	plasma conc. H (<13.5mg/l) vs L (>10mg/l) 0.81 (0.67-0.97)	0.09	adj for serum cholesterol
Colon	Eichholzer <i>et al.</i> , 1996	Switzerland prospective(17) 21/2974	plasma conc. L (<30.02µm/l) vs H (>30.02µm/l) 1.33 (0.47-3.80)		continuous covariates, first 2 years of follow up excluded
Prostate	Eichholzer <i>et al.</i> , 1996	Switzerland prospective (17) 29/2974	plasma conc. L (<30.02µm/l) vs H (>30.02µm/l) + smokers:19.89 (3.6-109-8) non smokers: 5.66 (0.88-36.34)		continuous covariates, first 2 years of follow up excluded: 8.34 (1.01-68.7) 3.07 (0.55-17.3)
	Gann <i>et al.</i> , 1999	USA Physician's Health Study cases: 578; matched controls: 1294	H(cut off point:33.5) vs L (19.9) 1.06 (0.76-1.48)	NS	agressive cases only (259) 0.64 (0.38-1.07) trend: 0.11 agressive cases current/ex-smokers 0.51 (0.26-0.98)

1-3 -MAJOR INTERVENTION TRIALS

We are considering in this paragraph the intervention trials with an epidemiological dimension. Most of them used cancer incidence or mortality as an end-point, a few used a precancerous state (oesophageal dysplasia or colo-rectum adenomas). The characteristics of these intervention trials are given in table 25, but the results will be given in the text for each study as follows.

1-3-1 The Linxian studies

This study, conducted in a region of China where vitamin and mineral deficiencies are frequent, was characterized by a high incidence of gastric and oesophagus cancers. Four treatments (A, B, C, D,) were included in the design of the study. Only C and D regimens are relevant to our interest in this report. Only D treatment in Linxian 1 study, the main study, resulted in a significant but weak reduction of total mortality (0.91; CI:0.84-0.99), mainly due to lower cancer rates and especially stomach cancer, both cardia and non cardia tumors (0.79; CI:0.64-0.99). The patterns of cancer incidence are comparable but are only borderline significant. No effect of the other treatments was noted. The Linxian dysplasia trial (Linxian 2 and 3) had a more complex supplementation and addressed a subsample of the general study, subjects with various grades of oesophageal dysplasia. The all causes- and oesophageal/gastric cardia cancers- mortality was not significantly lower in the treated group. Cumulative cancer incidence rates were nearly the same in the treated as in the placebo group. However, in the Linxian 3, the treated group showed a significant tendency to dysplasia reversion as the OR of not having dysplasia during the follow-up was 1.23 (1.08-1.37) in the treated group compared to the placebo group.

1-3-2 The ATBC study.

At the end of the follow-up the relative risk for lung cancer was 1.2 (1.0-1.3) among the people receiving β -carotene and 0.99 (0.87-1.1) among the people receiving α -tocopherol. After 7.5 years the group receiving β -carotene had a 16% higher cumulative incidence of lung cancer than those not given β -carotene. With regard to prostate cancer, there was also a 35% higher cumulative incidence in β -carotene supplemented group, significant for the clinical tumors (stage II to IV), whereas, vitamin E decreased significantly the incidence by 40% (Heinonen *et al.*, 1998). Neither agent had a statistically significant effect on latent cancers (stage 0-I).

1-3-3 The CARET study

The relative risk for cancer incidence in the treated group was 1.3 (1.0-1.6), and the mortality for the whole cohort was 1.5 (1.1-2.0). A subsequent analysis showed that the risk was concentrated in the asbestos exposed group (1.8; CI:1.2-1.8), current smokers group (1.4; CI: 1.0-1.9), and former smokers exposed to asbestos (2.3; CI:1.2-4.7), whereas former smokers at base-line not exposed to asbestos showed no excess risk.

1-3-4 The Physician Health Study

There were virtually no differences in the overall incidence of malignant neoplasms (0.98; CI:0.91-1.06), or in the overall mortality. There were also no difference according to the type of cancer or tobacco use. It is to be noted that there was a lower OR for prostate cancer in cases supplemented with β -carotene who displayed a low level of lycopene than in the placebo group with the same lycopene plasma concentration (Gann *et al.*, 1999).

1-3-5.The Women's Health Study

There were virtually no differences in the overall incidence of malignant neoplasms (1.03; CI: 0.82-1.28), or in the overall mortality (1.11; CI: 0.67-1.85). There were too few smokers to conduct meaningful analysis for site specific cancers but the OR was non significant for all-cancer sites in smokers (Lee *et al.*, 1999)

1-3-6 The Polyp Prevention Study

There was no evidence that either β -carotene (1.01; CI:0.85-1.2) or vitamins C and E (1.08; CI:0.91-1.29) reduced the incidence of adenomas. For one or more adenomas developed in the left colorectum, the OR was 1.16 (0.88-1.52) for β -carotene supplementation and 0.88 (0.67-1.15) for vitamins C and E supplementation.

1-3-7 The Australian Polyp Prevention Study

There was no evidence of a reduction in incidence in the group receiving β -carotene (1.3; CI:0.8-2.2). In the supplemented group the adenomas tended to be larger than 1cm, but this increase in risk was also not significant.

1-3-8 The Skin Prevention Study

There was no effect of β -carotene supplementation on the development of new skin cancer in any subgroup (sex, age, smoking, skin type, number of previous skin cancers). The relative risk for all deaths from cancers was 0.83 (0.54-1.3) in the treated group and the OR for skin cancer was 1.4 (0.99-2.1) among the smokers

Table 26: Intervention studies

Authors Country	Subjects	Treatment	Follow-up	Endpoints
LINXIAN 1 China Blot <i>et al.</i> , 1993	29584 general population in Linxian compliance 93%	C: vit C+ molybdenum 120mg+30µg D: β-carot+vit E+Se 15mg+30mg+50mg Plasma level 1.5 µmol/L (14.5x)	5 years and 3 months	Mortality all causes, all cancers
LINXIAN 2 China Li <i>et al.</i> , 1993	3318 subjects from the above cohort with oesophagus dysplasia	14 vitamins + 12 minerals at 2-3 x RDA among them β-carotene, (15mg/day), vit A, E, 60 IU, C, 180mg, B1,B2,B6, B12, folic ac, biotin, Ca, Se, Mb, Zn, Mn, I, Cu, Fe.	6 years	oesophagus/gastric cardia cancer mortality and incidence
LINXIAN 3 China Mark <i>et al.</i> , 1994	same study as above	id	6 years	oesophagus dysplasia reversion
ATBC Finland The ATBC cancer prevention study group, 1994	29000 intense smokers compliance 93% drop out 31%	1-β-carot. 20mg/day plasma level: 5.3 µmol/L (17.6x) 2-vit. E 50mg/day plasma level: 42.5 µmol/L (1.5x) 3-1+2	5 to 8 years	lung and other cancers incidence
CARET USA Omenn <i>et al.</i> , 1996-b	18314 smokers, ex- smokers, asbestos exposed compliance 93% drop out 35%	β-carot. 30mg/day + 25000 UI vit. A plasma level: 3.6 µmol/L (12.4x)	Mean:4 years	and other cancers incidence
PHYSICIANS' HEALTH STUDY USA Hennekens <i>et al.</i> , 1996	22071 physicians compliance 78% drop out 20%	β-carot. 50mg/day plasma level: 1.7 µmol/L (4x)	Mean: 12 years	lung cancer (and CVD) incidence
THE WOMEN'S HEALTH STUDY Lee <i>et al.</i> , 1999	39876 women aged ≥45	50mg on alternate days for 2.1 years plasma level:0.70 mg/dL vs 0.20 compliance 87%	4.1 year	cancer (and CVD) incidence
THE POLYP PREVENTION STUDY USA Greenberg <i>et al.</i> 1994,	864 subjects with sporadic adenomas removed in the previous 3 months compliance 82% drop out 13%	1-β-carotene 25mg/day plasma level 1.1 µmol/L (2.7x) 2-vitamin C 1g/day + vit. E 400mg/day plasma level 30.6 µmol/L (1.4x) 3-1+2	4 years	new adenomas
THE AUSTRALIAN POLYP PREVENTION STUDY Australie Mac Lennan <i>et al.</i> 1995,	390 subjects with >1 polyp removed at day 0 drop out 28%	β-carotene 20mg no information on plasma levels	24 et 48 months	reoccurrence of small and large adenomas
SKIN CANCER PREVENTION STUDY (SCPS) Greenberg <i>et al.</i> , 1990	1720 subjects with skin cancer recently removed compliance 80%	β-carot. 50mg/day plasma level 3.3 µmol/L (10x)	Mean: 4.3	secondary non- melanoma skin cancer

Comments on the results of the intervention trials

With the exception of a protective effect of β -carotene at 15mg in combination with vitamin E at 30mg and Se at 50 μ g against gastric cancer in the main Linxian trial, which was borderline significance and not confirmed in the ATBC study, none of the studies provided evidence of a protective effect of β -carotene, alone or in combination with vitamin E or retinol. This is also true for studies on colorectal polyps and adenomas. However, the overall mortality, cancer mortality (SCPS) and lung cancer incidence (ATBC, CARET) was lower in people with higher baseline β -carotene plasma concentrations in agreement with the results of the observational studies.

How to explain this failure? With regard to the absence of effect, an easy explanation might be that β -carotene is the marker of other(s) beneficial compounds found in fruit and vegetables together with β -carotene. However this is not sufficient to understand the observed increase in risk. It is worthwhile to underline that this adverse effect occurred in subjects at risk (tobacco use, asbestos exposure, colo-rectal adenomas, etc.). Therefore, β -carotene might interfere with carcinogenicity at a stage where antioxidant properties might enhance cell proliferation (Gerber, 1996). β -carotene might act in some situations (high oxygen pressure, exposure to NO₂) as pro-oxidant, capable of damaging DNA or to act on cell signal transduction at the promotion phase (Carotenoids, IARC, 1998). This would explain the increase in risk in smokers (ATBC, CARET), and the absence of effects in cohorts with few smokers (The Physician Health's study and the Women Health's study). Other explanations include the very high dosage used and the possible necessity to combine several protective micro-compounds.

Vitamin E significantly lowered the prostate cancer mortality by 41%, and the incidence of advanced tumors by 40% (Heinonen *et al.*, 1998). It was without effect on latent cancer, and on the time-lag between diagnosis and death. There was no relationship between prostate cancer incidence and mortality with vitamin E intake and plasma levels at baseline. Indeed, this result has to be confirmed but, the results of the studies reported by Eichholzer *et al.* (1996) and Gann *et al.*, (1999) are in line with these results although they were only significant in the smoker groups (Table 25).

1-4 SYNTHETIC CONCLUSION

The conclusions of the reference books (CNERNA, 1996; WCRF, 1997; COMA, 1998) on the protective effect of **tomatoes on cancers of upper aero-digestive tract, as well as lung and stomach** are supported by the recent studies. Whether the tomatoes are raw, cooked or processed was not precised in these studies. These cancers are those which are presenting a lower incidence in subjects with high intake, or high plasma concentration of common antioxidants, namely β -carotene and vitamin C (see tables 5, 6, 14-20). However, **lycopene**, the specific carotenoid of tomato, which is a powerful antioxidant (see group one) was not associated to these cancer sites (upper aero-digestive tract, plus lung and stomach) in six studies out of 8 (significantly inversely associated with oesophagus and mouth and pharynx cancers). Although the difficulty in assessing lycopene intake and plasma concentrations has to be kept in mind, these observations suggest that it is not lycopene which is responsible for the protective effect of tomato against upper aero-digestive tract, as well as lung and stomach cancers, but the **other compounds present in tomatoes, β -carotene and vitamin C, and possibly phenolics**. These compounds might act additively or synergistically (see table 16, Yong *et al.*, 1997). Thus, it is the food as a whole which is most likely to be protective, and this may be extended to food habits or typology.

So far, no effect of tomatoes on other cancers has been demonstrated except for the suggestion of a protective effect of **cooked tomatoes** against prostate cancer (Giovanucci *et al.*, 1995, Key *et al.*, 1997; Tzonou *et al.*, 1999). This suggests a role for **lycopene** since cooking and processing tomatoes concentrates lycopene and makes it more absorbable, whereas vitamin C is decreased in cooked and processed tomatoes. Again, in five studies investigating the relationship of lycopene and prostate cancer, two, the Health Professional Study and the Physicians Health Study (Giovanucci *et al.*, 1995; Gann *et al.*, 1999), showed a risk reduction for prostate cancer, especially in aggressive cases. However, vitamin E has been shown protective against prostate cancer in a case-control study (Tzonou *et al.*, 1999, see Table 22), in two prospective studies based on plasma concentrations, especially in smokers (Eicholzer *et al.*, 1996; Gann *et al.*, 1999, see table 25) and in an intervention trial (Heinonen *et al.*, 1998). Vitamin E present in tomato seeds is generally kept out from the edible part or excreted, but vitamin E-rich vegetable oil is often used to prepare canned processed tomato sauce and home cooked tomatoes. Thus, a confounding might occur, since

this tomato sauce-linked vitamin E intake might not be recorded in most studies, and lycopene might be a marker of this intake. Another confounding factor might be fructose, concentrated by cooking, and recently proposed as part of a protective mechanism for prostate cancer, via vitamin D and phosphates (Giovanucci *et al.*, 1999). Current results of epidemiological studies cannot solve this problem.

Part 2: Tomatoes and tomato products, lycopene, other carotenoids, vitamins C and E and CVD

2-1 Fruit and Vegetables, Tomatoes

The studies on fruit and vegetables intake and relative risk of cardiovascular disease have been comprehensively reviewed by Ness and Powles (1997) and Law and Morris (1998). The review by Ness and Powles summarized the data from 1966 until 1995. All studies (i.e. ecological, case-control, cohort with either fruit and vegetables consumption or proxy nutrients) were recorded and no attempt was made to evaluate the data critically. Ness and Powles concluded that all the data are consistent with a strong protective effect against stroke and a weaker protective effect against coronary heart disease. Concerning the studies of dietary intake, only one included tomato (Gaziano *et al.*, 1995). In this study, significant correlations were obtained for carrots/squash and salads/green vegetables, but no correlation was obtained for tomatoes. The subsequent review of Law and Morris (1998) made a more critical analysis with the objective of quantifying the relationship between fruit and vegetable consumption and the incidence of ischaemic heart disease (including coronary heart disease, myocardial ischaemic and myocardial infarction). The main outcome measures were risk of ischaemic heart disease at the 90th centile of consumption and at the 10th. Their conclusion was that the risk of ischaemic heart disease is about 15% lower at the 90th than the 10th centile of fruit and vegetables consumption, which is equivalent to about a four-fold difference in fruit consumption and a doubling of vegetables consumption. However, there was no clear correlation with the antioxidant components of fruit and vegetables. Our analysis took into account both of these reviews and a summary of the most relevant studies is given in table 27. Among the studies on fruit and vegetables, none mentioned tomatoes. There are no randomized, intervention studies either for fruit and vegetables or for tomatoes. No case-control studies were found with subjects free from disease. However, two small case studies involve relatively small numbers (287 women in the Italian study by Gramenzi *et al.*, 1990

and 154 cases in the Bulgarian study by Georgieva *et al.*, 1995) with patients already suffering from either myocardial infarction (MI) or coronary heart disease (CHD) may be reported. In both studies, an inverse association was found for consumption of fruit and vegetables and risk of acute MI or CHD. All prospective studies are based on dietary intake estimated either by food frequency questionnaire or by recall. In two studies, protective effects were found for fruit and vegetables against stroke (Gillmann *et al.*, 1995; Manson *et al.*, 1994) and four studies showed protection either against CHD or total circulatory disease (Knekt *et al.*, 1994, 1996; Gaziano *et al.*, 1995; Sahyoun *et al.*, 1996). The protection is higher from vegetables than from fruit. Insufficient studies have been done to conclude on which type of vegetable or which component is protective. However, Knekt *et al.*, (1996) found a protection of flavonoid-rich fruit and vegetables (i.e. apples, onions) and the protection found by Gaziano *et al.*, (1995) was from carotene-rich fruit and vegetables.

2-2 Lycopene and other Carotenoids (Tables 28-30)

As part of a European multicentre case-control study on antioxidants (EURAMIC), carotenoids were measured in adipose tissue in 683 individuals with non fatal myocardial infarction (MI) and 727 controls (Kardinaal *et al.*, 1993). A borderline significant decreased risk of intima media thickness (OR: 0.81, CI: 0.60-1.08) was observed in the ARIC study (231 cases , 231 controls) based on lycopene plasma concentration after adjustment for all confusion factors (Iribaren *et al.*, 1997). An inverse association was found between carotenoids and MI. After adjustment for age, body mass index, socioeconomic status, smoking, hypertension, and maternal and paternal history of disease, lycopene remained independently protective, with an odd ratio of 0.52 for the contrast of the 10th and 90th percentiles (95% confidence interval 0.33-0.82, P = 0.005) (Kohlmeier *et al.*, 1997). Lycopene, or some substance highly correlated which is in a common food source, may contribute to the protective effect of vegetable consumption on myocardial infarction risk.

In contrast, in the Street *et al.*,’s study, (1994), based on the determination of carotenoids concentrations in the sera of patients with MI and controls, no association was observed with lycopene level. There was a significantly increasing risk for subsequent myocardial infarction with decreasing levels of β -carotene (P value for trend, 0.02) and a suggestive trend with decreasing levels of lutein (P= 0.09). In addition, low serum levels of carotenoids were associated with an increased risk of subsequent myocardial infarction among smokers. An ecological study (Bobak *et al.*, 1999) based on biochemical measurements of cardiovascular

risk factors and of carotenoids in 3 populations differing by coronary heart disease mortality might be noted. All carotenoids, including lycopene were inversely related to the coronary heart disease mortality rates. And in the Rotterdam study, high β -carotene intake halved the risk of MI (Klipstein-Grobush *et al.*, 1999).

Table 27 Prospective studies on fruit and vegetable dietary intake

Authors	country n cases M or F, age (Follow-up duration)	outcome	exposure measurement	Results OR, comparison group, CI, p trend	remarks
Knekt <i>et al.</i> , (1994)	Finland 5133 M+F; 30 - 69 14 years	CHD	Dietary history method of food consumption	OR T3/1 M vegetables: 0.66 (0.46-0.96) Trend: P=0.02), W: NS M +W fruit NS	Protective effect for men. Similar effects for women but not significant.
Knekt <i>et al.</i> , (1996)	Finland follow-up study 26 years	total and coronary mortality	dietary intake of flavonoid-rich foods (onions, apples)	OR Q4/1 0.57 (0.36-0.91), W apples 0.81 (0.61-1.09), M apples 0.50 (0.3 - 0.82), W onions 0.74 (0.53-1.02), M onions	Inverse association for flavonoid-rich F&V
Gillmann <i>et al.</i> , (1995)	Framingham, USA 832 M; 45 - 65 20 years	stroke	24h recall servings F&V/ day	OR T5/1 of servings/day 0.78 (0.62-0.98) for each increase of 3 servings/day	Inverse association of F&V and stroke in men.
Manson <i>et al.</i> , (1994)	USA Nurses 87 245 F; 34 - 59 8 years	stroke	FFQ	OR Q5/1 5+ / week vs <1 mth. 0.74 (CI not mentioned) all veget. trend: P=0.03 0.32 carrots 0.57 spinach	inverse association for vegetables, null for fruit
Gaziano <i>et al.</i> , (1995)	Massachusetts, USA 1299 elderly M + F; >66 4.75 years	total circulatory disease	43 category FFQ	OR Q4/1 0.54 (0.34-0.86), p=0.04 CVD 0.25 (0.09-0.67), p=0.002 MI	inverse association for carotene-rich fruit and vegetables
Vollset, Bjelkie (1983)	Norwegian postal survey 16 713 M+F, 45-74 11.5 years	stroke	Postal dietary survey	T3/1 CVD death OR=0.67 (0.52-0.87)	Protective effect of potatoes, vegetables, fruits
Sahyoun <i>et al.</i> , (1996)	USA 747elderly M+F; >60 9 - 12 years	CHD	3day food record	Inverse association between vegetable intake and coronary mortality (P for trend = 0.04)	Inverse association for vit C (see below)

Table 28 Case-control and prospective studies based on carotenoids serum or adipose-tissue levels

First Author (year)	Country n case (control) M or F age	outcome	exposure measurement	results OR and OR p trend	Remarks
Kardinaal <i>et al.</i> , (1993)	EURAMIC (multicentric study) 683 (727)		Adipose β car 0.35 μ g/g in case 0.42 μ g/g in controls	L vs H: OR=1.78 (1.17-2.71) P trend 0.001	Association strongest among current & ex-smokers
Morris <i>et al.</i> , (1994)	LRC-CPPT M 266 (1 899) 40-59y	Fatal CVD, non-fatal MI	serum carotenoids <2.3 vs >3.2 μ M/l	OR= 0.64 (0.44-0.92), 0.28 (0.11-0.73) for never smokers	13 years plasma β car and vit E inv. related to carotid artery wall thickness
Street <i>et al.</i> , (1994)	USA 125 (25 802) 35-65y	IMI	plasma β car	OR= 0.45(0.22-0.90) P trend 0.03	7-14y
Sahyoun <i>et al.</i> , (1996)	USA (747)	ACVD death	plasma	OR= NS trend NS	
Iribarren <i>et al.</i> , (1997)	Minnesota (261)	LDL oxidation IMT	serum carotenoids and vitamins	OR =0.75 (0.59-0.94) β crypto OR = 0.76 (0.59-0.95) lut+zea OR=0.81 (0.60-1.08) for lycopene	β car, lut and zea were inversely correlated to atherosclerosis
Kohlmeier <i>et al.</i> , (1997)	EURAMIC 10 european countries	MI	adipose tissue	OR=0.52 (0.33-0.82) for lyc	α and β car eliminated for associations β car

Table 29: Prospective study based on dietary intake of carotenoids

First Author (year)	Country n case (control) M or F age	outcome	exposure measurement	results OR and p trend	Remarks
Manson <i>et al.</i> , (1991)	Nurses' Health 552 (671 185) 150 deaths 437 MI	Incident CHD	Food frequency questionnaires	OR=0.78 (non mentionned) P trend 0.02	8 years 22% reduction for HQ
Rimm <i>et al.</i> , (1993)	Health professionals 667 (39 910)	Incident CHD	Food frequency questionnaire	OR=0.71 (0.53-0.87) 0.30 (0.11-.82) for smokers	4 years
Knekt <i>et al.</i> , (1994)	Finland 244 (5133) 186M, 58F 30-69 y free from heart disease	Fatal CHD	M \geq 258 vs \leq 147 mg F \geq 383vs \leq 182 mg)	For M, OR= 1.02 (0.70-1.48) For F, OR = 0.62 (0.30-1.29)	143 years inverse association role of other constituents (flavonoids?)
Pandey <i>et al.</i> , (1995)	Western Electric Company study II, Ch (middle-aged men) 231 (1 556)	CHD mortality	LT 2.3 mg β car /d HT 5.3 mg β car /d	OR= 0.70 (0.49-0.98)	20 years
Gaziano <i>et al.</i> , (1995)	USA 161 (1 299) elderly residents) 48 MI 113 due to other CV causes	MI and CVD death	LQ 0 to .8 HQ \geq 2.05 servings/d of foods high in carotenoid content	For CHD death: OR=0.54 (0.34 to 0.86) p trend 0.004 For MI: OR= 0.25 (0.09 to 0.67) P trend 0.002	4.75 years tomatoes NS, significant effect of carrots/and or squash (OR 0.40) salads and/or green vegetables (OR 0.49), broccoli and/or brussel sprouts (OR 0.29, NS)
Sahyoun <i>et al.</i> , (1996)	USA (747)	CVD death	3 day food record	OR = 0.64 for high intake of all vegetables OR = 0.49 for dark/green vegetables OR = 0.61	12 years role of folate in high quantities in foods rich in carotenoids
Klipstein-Grobush <i>et al.</i> , (1999)	The Rotterdam study Netherlands 124 (4802 elderly, aged 55-95)	MI	Food frequency questionnaire	OR= 0.55 (0.34 to 0.83) P trend 0.013	
Yochum <i>et al.</i> , (2000)	The Iowa Women's Health Study 34492 post-menopausal women baseline 1986	stroke	Dietary intakes + supplements by Willett FFQ	H(1.7mg/day) vs L(0.325) 0.80 (0.45-1.40) trend NS	also NS when food intake alone

Table 30: Human intervention studies on supplementation of carotenoids

Study , First Author (year)	Country n case (control) M or F age	outcome	supplement	Results OR	Remarks
Linxian Blot <i>et al.</i> , (1993)	(523) 29584 M and F rural 40-69 y	Cerebrovascu- lar death	15 mg/d β car 30 mg/d α toco 50 μ g Se	OR=0.90 (0.76- 1.07)	5.25 years
ATBC cancer prevention study group (1994)	1 473 (29 133) M smokers; 50-69 y	IHD, stroke	20 mg β car/d	11 % increase in IHD mortality, 20% increase in stroke mortality	8 years increase in treated group no effect on angina
ATBC Study Rapola <i>et al.</i> , (1997)	Sub group (424) 1862 with a previous MI (39 non-fatal MI and 74 fatal CHD) 461 β car	MI CHD Death	20 mg/d β car 50 mg/d β car+ α toco	OR = 0.67 MI OR = 1.75 death for β car (1.16- 2.64) OR = 1.58 death for β car+ α toco (1.05-2.40)	5.3 years Supplements of α -toco and β car were not recommended
CARET Omenn <i>et al.</i> , (1996)	US 18314 M and F 45-69 y, smokers former smokers workers exposed to asbestos	CVD Death	30 mg β car/d +25 000 IU retinol	OR = 1.26 (0.99- 1.61)	4 years Supplement No beneficial effect Adverse effect

2-3 Antioxidant Vitamins

The antioxidant vitamins have long been considered as being some of the most important functional components of fruit and vegetables. However, the epidemiological evidence to support this hypothesis is somewhat controversial, especially for vitamin C. (Tangney *et al.*, 1997; Faggiotto *et al.*, 1998; Ness and Powles, 1997; Law and Morris, 1998).

2-3-1 Vitamin C

Overall, the data are inconclusive for a protective effect of vitamin C against atherosclerotic disease (Ness and Powles, 1996; Jacob, 1998) although limited data is consistent with vitamin C being protective against stroke (Ness *et al.*, 1996). Prospective studies where dietary intakes or plasma levels of vitamin C have been used as proxy nutrients for indicators of fruit and vegetables intake are summarized in tables 31-32. Studies using serum levels are more reliable than those using dietary intake. There are strong indicators that low serum vitamin C levels are a high risk factor for development of coronary disease (Vita *et al.*, 1998; Nyssonen *et al.*, 1997; Gale *et al.*, 1995; Eichholzer *et al.*, 1992). The dietary studies, although rather inconclusive, are in agreement with the serum studies in the NHANES II follow-up study (Simon *et al.*, 1998), the increase in CHD prevalence was mainly found among individuals with low to normal vitamin C blood levels. The Finnish study by Nyssonen *et al.*, (1997) showed significant increased risk in vitamin C deficient Finnish men but no difference in normal to high vitamin C levels. These findings may explain why there were insignificant effects of vitamin C intake in well-nourished populations such as the Iowa postmenopausal women, Nurses Health and the Health Professionals studies (Kushi *et al.*, 1996-b; Stampfer *et al.*, 1993; Rimm *et al.*, 1993). The NHANES I Epidemiologic follow-up study found that individuals with the highest intakes of vitamin C had a 25-50% reduction in cardiovascular mortality (Enstrom *et al.*, 1992). Dietary vitamin C was also associated with a decreased risk of CHD death among Finnish women (Knekt *et al.*, 1994) but not in the Rotterdam study (Klipstein-Grobush *et al.*, 1999). There is no published clinical trial examining the independent effect of vitamin C on CHD.

Table 31: Prospective studies based on serum vit C levels

Authors	country n cases M or F, age (Follow-up)	outcome	exposure measurement	Results OR, comparison group, CI, P trend	remarks
Vita <i>et al.</i> , (1998) case study	Country ? 149 cases M + F Age ?	Unstable coronary syndrome	12 plasma markers of AO/ox status, correlation with atherosclerosis	Low vit C plasma levels were significant predictors of unstable coronary syndrome OR=0.59 (0.40-0.89), p=0.01	Antioxidants may slow down progression of atherosclerosis
Simon <i>et al.</i> , (1998)	NHANES II 6624 US adults M+F, 40-74	CHD Stroke Peripheral vascular disease	Serum vit C	H vs L CHD : 0.73 (0.59-0.90) Stroke: 0.74 (0.56-0.97)	the effect of vitamin C is observed only at saturated levels of plasma vit C; No effect on peripheral vascular disease
Sahyoun <i>et al.</i> , (1996)	Massachusetts 747 Elderly M+F, >60 9-12 years	CHD Total mortality	Plasma vit C (together with vit E and carotenoids)	T5/1 plasma vit C OR=0.54 (0.32-0.90)	High plasma vit C levels may protect against early mortality and mortality from CHD
Nyssonen <i>et al.</i> , (1997)	Finland 1606 M, 42-60 5 years	MI	Plasma vit C	Low vs high serum vit C: Adjusted OR=2.5 (1.3-5.2),	Vit C deficiency (low serum levels) is a risk factor for CHD. Normal to high plasma vit C showed no difference in risk
Gale <i>et al.</i> , (1995)	British elderly 730 M+F, > 65	CHD stroke	Plasma vit C +	No association Check for plasma values	Vit C conc is strongly related to subsequent risk of death from stroke but not from CHD

Table 32 Prospective studies based on dietary estimation of vit C

Authors	country n cases,sex, age (Follow-up)	outcome	exposure measurement	Results OR, comparison group, CI, p trend	remarks
Kushi <i>et al.</i> , (1996)	USA 34 486 PM-W, 55-697 7 years	CHD	127 item FFQ	No association	vit C intake, no association; vit E significant inverse relation
Sahyoun <i>et al.</i> , (1996)	USA Elderly 747 M+F, >60	CHD	3 day record	T5/1 vit C intake OR=0.38 (0.19-0.75)	High intake of C may protect against mortality from CHD
Pandey <i>et al.</i> , (1995)	USA 1556 M, 40-55 24 year complete	CHD	Dietary history X2 (1 year apart) n vit C and carotenoid-rich foods	T3/1 in vit C and β -car index Adjusted OR=0.70 (CI=0.49-0.98)	
Gale <i>et al.</i> , (1995)	UK elderly 730, M+F, 65+ 20 years	CHD stroke	7-day weighed record	No association T3/1 vit C Adjusted OR=0.5 (CI:0.3-0.8)	
Knekt <i>et al.</i> , (1994)	Finland 5133 M, 30-69 14 years	CHD mortality	Dietary history	T3/1 OR=0.49 (0.24-0.98) women OR = 1 (0.68-1.45) men	Inverse association between dietary intake of vit C and CHD mortality for women, not men
Fehily <i>et al.</i> , (1993)	Wales 2512 M, 45-59 5 years	CHD (25% had IHD at baseline)	FFQ	T1/5 OR of IHD event = 1.6 in lowest quintile	Trend of increasing IHD risk with decreasing vit C intake
Rimm <i>et al.</i> , (1993)	USA Health Professionals, 39 910 M, 40-75 4 years	CHD	FFQ, 131 items	No association	Probably had sufficient vit C in the diet
Manson <i>et al.</i> , (1992) check	USA Nurses 87 245 F, 34-59 8 years	CHD	FFQ	Q5/1 OR= 0.80 (0.58-1.10)	
Enstrom <i>et al.</i> , (1992)	USA NHANES I 11 348 M+F	All CVD disease	24h recall and FFQ intake from food or supplements	Standardized mortality ratio M=0.58 (0.41-0.78) F=0.75 (0.55-0.99)	Comparison is made to US whites with a SMR of 1.0

Klipstein-Grobush <i>et al.</i> , (1999)	The Rotterdam study, Netherlands 124 (4802 elderly, aged 55-95)	MI	FFQ	OR= 1.05 (0.65 to 1.67) P trend NS	
Yochum <i>et al.</i> , (2000)	The Iowa Women's Health Study 34492 post-menopausal women baseline 1986	stroke	Dietary intakes + supplements by Willett FFQ	H(678 mg/day) vs L(82.4) 1.23 (0.76-1.23) trend NS	also NS when food and supplement intake

2-3-2 Vitamin E

Vitamin E is not judged to be a suitable proxy nutrient for fruit and vegetables intake. Several groups have measured serum vitamin E levels in patients with CVD but there was no consistent relationship with the presence or absence of disease (Tangney, 1997; Law and Morris, 1998). The cohort studies involving dietary consumption of vitamin E either by food or supplements are summarized in Table 33. The Nurses Health Study (Stampfer *et al.*, 1993) showed a significant reduction of non-fatal and fatal myocardial infarction in nurses regularly taking vitamin E from dietary sources and supplements. Similar risk reductions were observed in the Health Professionals Follow-up Study (Rimm *et al.*, 1993) as an inverse correlation between vit E and β -carotene intake and relative risk of CHD was found. In the Finnish study (Knekt *et al.*, 1994), significant coronary protection was observed only for women consuming high amounts of vitamin E, but not in the Rotterdam study (Klipstein-Grobusch *et al.*, 1999). In the Iowa Women's Health study (Kushi *et al.*, 1996-b), the reduction in risk from coronary disease was in the group not taking supplements.

Three large intervention trials of vitamin E have been published (The ATBC study prevention group, 1994; Blot *et al.*, 1993; Stephens *et al.*, 1996) and summarized in Table 34. In the ATBC follow-up study, supplements of vit E (50 mg/day, a relatively low dose) had no effect on cardiovascular deaths after 5-8 years of follow-up. In the Cambridge Heart Antioxidant Study (CHAOS) of patients with evidence of angiogenic heart disease, (Stephens *et al.*, 1996), a large and significant reduction of nonfatal myocardial infarction was reported in the vitamin E group (400 or 800 IU/day) compared to the placebo. No effect was seen on CVD death or total mortality. The recent GISSI studies (GISSI-Prevenzione Investigators, 1999 and HOPES, 2000) did not show a significant benefit of a supplementation of 300mg and 400 IU/day on overall survival. The Chinese Cancer Prevention Study (Blot *et al.*, 1993) tested combinations of 9 different micronutrients including vitamins A, C, E, beta-carotene. Although statistically significant reductions in total mortality of 9% to 13% were seen, it could not be attributed to any single nutrient.

Table 33 Prospective studies on dietary intake of vitamin E

Authors (year)	country n cases M or F, age (Follow-up duration)	outcome	exposure measurement	OR, comparison group, CI, P trend	Remarks
Kushi <i>et al.</i> , (1996) Iowa women's health study	Iowa PM women 34 486 F, 55-69 7 yr	Deaths from CHD	Dietary intakes + supplements by Willett FFQ	T5/1 OR=0.38.(0.18-0.80) (food) OR=1.09 (0.67-1.77) (suppl) OR=0.96 (0.62-1.51) (food + suppl)	In non-supplement users, vit E intakes inversely related to CHD deaths; overall trend, P<0.008
Knekt <i>et al.</i> , (1994) Finnish mobile clinic study	Finland 2748 M, 30-69 2385 F, 30-69	Incidence of CHD deaths	Intakes by FFQ	In women only: OR=0.35 (0.14-0.88)	In women, a significant inverse association between vit E and CHD mortality
Stampfer <i>et al.</i> , (1993) Nurses Health Study	US nurses 87 245 F, 34-59 8 yr	Non-fatal MI Deaths from CHD	Dietary intake by Willett FFQ	T5/1 OR=0.66 (0.50-0.87) For greater than 2 yr use of suppl: OR = 0.59 (0.38-0.91)	
Rimm <i>et al.</i> , (1993) Male Health Professionals Study	Male health professionals 39 910 M, 40-75 4 yr	Non-fatal MI Deaths from CHD	Dietary intake by Willett FFQ	T5/1 OR for <60 vs >7.5 IU vit E = 0.64 (0.49-0.83)	
Klipstein-Grobusch <i>et al.</i> (1999) The Rotterdam study	Netherlands 124 (4802 elderly, aged 55-95)	MI (elderly)	FFQ	OR= 121 (0.75 to 1.98) P trend NS	
Yochum <i>et al.</i> (2000) The Iowa Women's Health Study	34492 post-menopausal women baseline 1986,	stroke	Dietary intakes + supplements by Willett FFQ	H(238mg/day) vs L(4.9) 0.91 (0.55-1.52) trend NS	also NS when food intake alone but mayonnaise: 0.56 (0.29-1.11)

Table 34 Large (>1000) randomized clinical trials on vitamin E with and cardiovascular diseases

Authors (year)	country, cases, M or F, age (Follow-up duration)	outcome	treatment	OR, comparison group, CI, p trend	Remarks
(ATBC, 1994) <i>Virtamo et al.</i> , (1998)	ATBC, Finland 29 133 M smokers, 50-59 6.1 yr	Total mortality from CVD	50 mg a-tocopherol acetate, 20 mg beta-carotene	OR=0.90 (0.75-1.08)	Marginal effect of suppl. in vit E on CVD in male smokers
<i>Blot et al.</i> , (1993)	Chinese Cancer Prevention study 29 584	Total mortality	30 mg vit E suppl + 15mg β -carotene 50 μ g Se	OR= 0.90 (0.76-1.07)	
<i>Stephens et al.</i> , (1996)	CHAOS, UK 2002 patients with angiogenic evidence of CVD, M+F 2 yr	Nonfatal MI and deaths from CHD	400 or 800 IU vit E or placebo	OR=0.53 (0.34-0.83) p 0.005	Significant reduction in major CV events: no effect on CV deaths and all-cause mortality
GISSI Prev Invest, 1999	Italy, 11234 patients with recent MI, 3.5 years	death, non fatal MI, stroke	300mg vit E/ placebo	CV death: 0.94 (0.81-1.10)	n-3 PUFA 0.78 (0.65-0.92)
HOPES, 2000 The HOPES investigators, 2000	Canada, 2545 W, 6996 M high risk 4.5 years	MI, stroke, dath CVD	400IU/day	1.05 (0.95-1.16) no trend	

2-4 CONCLUSION

Fruit and vegetables, including tomatoes, appear protective against CVD. Studies are convincing. If we considered lycopene, studies are insufficient to conclude that lycopene is protective. For the other antioxidants results are not consistent, and supplements in micro-constituents at high dosage are certainly not recommended. In addition, other micro-constituents, present in tomatoes, especially phenolic compounds and folic acid, could also contribute to their protective effect against CVD.

The decrease of CVD risk is certainly due to a synergistic effect of the different micro-constituents present in tomatoes, including lycopene. If lycopene is a necessary part of the synergistic protective action together with the other nutrients of fruit and vegetables, tomatoes, and essentially processed tomatoes, are the unique source of it. Further studies are needed to demonstrate such a synergistic effect.

Part 3: Report on the relationship between tomatoes and their constituents (carotenoids, vitamin C and vitamin E), and lung function

There are no specific studies on tomatoes and lycopene. Therefore we report on antioxidants present in tomatoes. Several experimental animal studies have shown that exposure of lungs to oxidative stress could be positively influenced by antioxidants (Matsui *et al.*, 1991; Chow *et al.*, 1984; Calabrese *et al.*, 1985). This has also been shown in humans (Mohan *et al.*, 1989; Mudway, *et al.*, 1996). Fresh fruit intake assessed in 2650 children by a food frequency questionnaire has been positively associated with forced expiratory volume (Cook *et al.*, 1997). No specific micronutrient was found responsible for this effect. In a study conducted in China (Hu *et al.*, 1998) an increase in 100mg/day in vitamin C was significantly associated with an increase of 21.6ml of forced expiratory volume.

The research group of Dr Mariette Gerber (Groupe d'Epidémiologie Métabolique, GEM) reported several studies on the effect of photooxidants on plasma carotenoids, namely α and β -carotene (Bernard *et al.*, 1998; Saintot *et al.* 1999). In non-smokers, ozone exposure was a significant determinant of blood α and β -carotene. The GEM participated also in an intervention trial conducted in Mexico city showing that lung function of people exposed to ozone was less damaged when they were supplemented by β -carotene, vitamin E (75mg) and

vitamin C (650mg) (Romieu *et al.*, 1998). It was a double blind cross-over trial (2 periods of 10 and 7 weeks, respectively) on 42 shoe cleaners. Two other small intervention trials have been conducted in the Netherlands showing the same type of results with beginners cyclists supplemented with β -carotene (15mg), vitamin E (75mg) and vitamin C (650mg) once a day for 3 months in the first trial (Grievink *et al.*, 1998), and vitamin E (100mg) and vitamin C (500mg) daily for 15 weeks for the second one (Grievink *et al.*, 1999). These results are in line with a large recently reported study on antioxidant and pulmonary function (Hu and Cassano, 2000) which showed that an 1 SD-increased intake of each antioxidant, β -carotene, vitamins C and E, was significantly associated with a better pulmonary function.

Table 35: Studies on lung functions and antioxidants

Authors	Design	Supplements	Treatment effect difference (regression coefficients)
Romieu <i>et al.</i> , 1998	42 shoe cleaners Mexico city double blind cross-over trial	β -carotene (15mg), +vitamin E (75mg) +vitamin C (650mg) phase 1: 10 weeks phase2: 7 weeks	FVC: 1.29 p<0.01 FEV ₁ :1.65 p<0.01
Grievink <i>et al.</i> , 1998	23 bicyclists The Netherlands measurements after training session summer, rural area no placebo	β -carotene (15mg), +vitamin E (75mg) +vitamin C (650mg) 70 days	FVC: 2.08 (1.31-2.85) FEV ₁ :1.66 (0.62-2.70)
Grievink <i>et al.</i> , 1999	38 bicyclists The Netherlands measurements after training session summer, rural area placebo group	vitamin E (100mg) vitamin C (500mg) 15 weeks	

FVC: forced vital capacity; FEV₁: forced expiratory volume in 1sec

All the studies quoted above, showing effects on antioxidants (Bernard 1998, Saintot 1999) and lung function (Romieu *et al.*, 1998; Grievink *et al.*, 1998 and 1999) are still preliminary and require further investigation but the consistency of these limited results, their biological plausibility, the existence of relevant experimental studies, are supporting a relationship between carotenoids, vitamins C and E and lung function, which might be protective by nature.

Part 4: Tomatoes and tomato products, lycopene, other carotenoids, vitamins C and E and age-related eye diseases.

Cataract is one of the major causes of blindness throughout the world and is particularly important in developing countries (Schwab, 1990). The cataract can be defined as dysfunctions of the eye lens, which collect and focus light on the retina, leading to its opacification. Besides metabolic disorders, opacification is usually associated with old age. Another age-related eye disease is the macular degeneration. It affects a region situated in the middle of the retina called fovea leading to a loss of visual acuity. Delaying the appearance of these disease in old people would enhance the quality of life by decreasing the disability and reducing its costs estimated to 5 billion \$ in the United States. The biological mechanisms for both disease involve oxidative processes resulting from chronically exposure of eyes to light and oxygen and leading to damages of lens proteins which can then aggregate and precipitate (Taylor *et al.*, 1993) or to accumulation of oxidised polyunsaturated fatty acyls which are very high in the retina membranes (Noell, 1980) and of lipofuscin, a complex made by oxidised products (Iwasaki and Inomata. 1988). In the following tables, epidemiological studies on the prevalence of antioxidants including vitamin C, vitamin E and carotenoids on cataract and age related macular degeneration will be summarised.

4-1 Tomatoes, Fruit and other Vegetables and Cataract.

Studies were carried out by Jacques and Chylack (1991) on only 77 subjects with senile cataract and 35 controls. A food frequency questionnaire was used to estimate dietary intake for the one year period preceding entry into the study. Subjects who consumed fewer than 3.5 servings of fruit or vegetables per day had an increased risk of both cortical (OR = 5.0, $p < 0.05$) and posterior subcapsular cataract (OR = 12.9, $p < 0.01$). Another recent case-control study from Tavani *et al.* (1996) established the relationship between cataract extraction and food intake including spinach, cruciferae, citrus fruit and tomatoes. For the latter, a significant inverse trend in risk was found (OR = 0.5, $p < 0.01$). This data is in contrast with that from Mares-Perlman *et al.* (1995) describing the tomatoes and the derived products as a potential risk factor for nuclear sclerosis in the Beaver Dam eye study. The latest data published on Health Professionals Follow-up Study by Brown *et al.* (1999), showed no significant decrease of relative risk of cataract extraction in men consuming tomatoes > 2 times per week (OR = 0.91; CI:0.68-1.21). No decreased relative risk was found for tomato sauce (OR = 0.89,

CI:0.68-1.16), (Table 36), but a decreasing trend with higher consumption was borderline significant ($p=0.07$).

4.2 Lycopene

Serum lycopene was found not to be significantly associated with decreased risk of cataract (OR = 1.1, CI 0.5-2.6; Lyle, B.J. *et al.* 1999). Furthermore, high intake of lycopene was related to more severe nuclear sclerosis (Mares-Perlman *et al.*, 1995) attaining statistical significance in women (OR = 1.49, CI:1.01 - 2.19, $p < 0.05$). But some critics were brought up such as the non-adjustment on energy, the only use of multivariate analysis. Indeed, in a follow-up of the same study, such an association was not found, but the protective effect of lutein remained (Lyle *et al.*, 1999). Significant protective effects of lycopene were neither detected for cataract extraction on Health Professional Follow-up Study (Brown *et al.*, 1999) and Nurses' Health Study (Chasan-Taber *et al.*, 1999). However, in the former study a significant decreasing trend ($p= 0.05$) of nuclear cataract has been shown in men with increasing lycopene intake (Table 37).

Table 36 Tomatoes & tomato products and cataract

Authors, year and number of subjects	Odds ratio and CI	Comments
Mares-Perlman <i>et al.</i> , 1995 (n = 1 919) Beaver Dam Eye Study; 1988 - 1990	severe nuclear sclerosis tomatoes and tomato juice men high (3.4) vs low (0.2) intake frequency 1.07 (0.70 - 1.65) women high (3.7) vs low (0.4) intake frequency 1.12 (0.76 - 1.64) pasta with tomato sauce women (43 - 84 years); n = 1060 men (43 - 84 years); n = 859 high (1.0) vs low (0) intake frequency 1.70 (1.14 - 2.52) p=0.01 high (1.0) vs low (0) intake frequency 1.25 (0.81 - 1.94) ns pizza women (43 - 84 years); n = 1060 men (43 - 84 years); n = 859 high (1.0) vs low (0) intake frequency 1.22 (0.83 - 1.78) high (1.0) vs low (0) intake frequency 1.48 (0.95 - 2.3)	age: 43 - 84 years
Tavani <i>et al.</i> , 1996 (n = 913) case-control study	cataract extraction tomatoes high (>3) vs low (<1) portions per week 0.5 (0.4 - 0.8) p<0.01	multivariate odd ratio.
Brown <i>et al.</i> , 1999 (n = 36 664) Health Professionals Follow-up Study; men (45-75 y)	tomatoes high (>2 times per week) vs low (<1 time per month) 0.91 (0.68 - 1.21) tomato sauce high (>2 times per week) vs low (<1 time per month) 0.89 (0.68 - 1.16) p=0.07	multivariate odd ratio. prospective study

Table 37 Lycopene and cataract

Authors, year and number of subjects	Odds ratio and CI	Comments
Mares-Perlman <i>et al.</i> , 1995 (n = 1919) Beaver Dam Eye Study; 1988 - 1990 n = 1060	nuclear scleriosis Lycopene intake , low (66 µg) vs high (203 µg); 1.49 (1.01-2.19)	women (43 - 84 years) men NS
Brown <i>et al.</i> , 1999 (n = 36 664) Health Professionnals Follow-up Study; men (45-75 y);	cataract extraction Lycopene intake, low (3413 µg) vs high (18 901); 1.10 (0.88 - 1.36)	multivariate relative risk.
Chasan-Taber <i>et al.</i> , 1999 (n = 77 466) Nurses' Health Study; women (45 - 71 years)	cataract extraction Lycopene intake, low (3592 µg) vs high (15 839); 1.01 (0.83 - 1.24)	multivariate relative risk
Lyle <i>et al.</i> , 1999 (n = 400) Beaver Dam Eye Study (1993-1995); men & women (50 - 86 years)	nuclear cataract Serum lycopene , low (0.29 µmol/L) vs high (0.73); 1.10 (0.5 - 2.6)	multivariate odds ratio.

4.3 Carotenoids

4.3.1 Cataract (Table 38)

Among the four studies explored, only one indicated that persons with plasma total carotenoids higher than 3 $\mu\text{mol/L}$ had less than one fifth the prevalence of cataract when compared with those with low concentrations ($<1.7 \mu\text{mol/L}$). Very recent studies (Teikari *et al.* 1998; Brown *et al.* 1999, Chasan-Taber *et al.* 1999) confirmed previous observations (Tavani *et al.*, 1996) that β -carotene had no effect on cataract. It can be noticed that spinach had no effect either whereas the study of Tavani *et al.*(1996) found a significant decreased risk for cataract extraction (OR = 0.6, ; CI : 0.4 - 0.9 ; $p < 0.05$) with consumption of spinach and similarly, less severe nuclear sclerosis was found in women consuming high level of spinach (Mares-Perlman *et al.*, 1995). This may open to the question of what components of fruit and vegetables may have the protective effect previously shown on cataract. In the ATBC study, there was no beneficial effect of supplementation with β -carotene on the number of cataract extraction (Teikari *et al.*, 1998).

4.3.2 AMD (Table 39)

The only two studies reported showed some association between plasma carotenoid contents and neovascular AMD. It can be noticed that β -carotene also induced a 40% decrease of prevalence of AMD. Thus, this latter carotenoid may present a differential protective effect on macula and lens. As expected, lutein and zeaxanthin, highly present in the macula, also had the strongest protective effect against AMD. No information was available on the potential effect of lycopene or more generally of fruit and vegetables. In the ATBC study, there was no beneficial effect of supplementation with β -carotene on the incidence of AMD (Teikari *et al.*, 1998).

Table 38 Carotenoids and cataract

Authors, year and number of subjects	Odds ratio and CI	Comments
Jacques and Chylak, 1991 (n = 77)	high (>3.3 $\mu\text{mol/L}$) vs low plasma total carotenoids (< 1.7 $\mu\text{mol/L}$) 0.18 (0.03 - 1.03)	no effect of β carotene
Hankinson <i>et al.</i> , 1992 (n = 50 828)	cataract surgery High (14 558 IU/d) vs low (2935 IU/d) of carotenes intake 0.73 (0.55 - 0.97)	no association with carrots associated with low spinach intakes
Vitale <i>et al.</i> , 1994 (n = 671)	high (>0.88 $\mu\text{mol/L}$) vs low plasma β -carotene (< 0.33 $\mu\text{mol/L}$) nuclear cataract 1.57 (0.84 - 2.93) cortical cataract 0.72 (0.37 - 1.42)	adjusted on age, sex and diabetes
Teikari <i>et al.</i> , 1998 (n = 28 934) (50 - 69 years); intervention followed for 5 to 8 years	advanced cataract β -carotene supplementation vs placebo; 0.97 (0.79 - 1.19)	male smokers
Brown <i>et al.</i> , 1999 (n = 36 664) Health Professionals Follow-up Study; men (45-75 y);	carotene supplements low (3777 IU) vs high (18499) cataract extraction 0.85 (0.68 - 1.07)	multivariate relative risk. no effect of β -carotene
Lyle <i>et al.</i> , 1999a (n=1349) Beaver Dam Eye Study	high intake (1.245mg/kcal) vs low (0.3) 0.5 (0.3-0.8) trend 0.002	all carotenoids NS excepted lutein
Lyle <i>et al.</i> , 1999b (n=400) Beaver Dam Eye Study	all carotenoids plasma levels NS 0.3 (0.1-1.2) trend 0.13	lutein borderline in >65 years
Chasan-Taber <i>et al.</i> , 1999 (n = 77 466) Nurses' Health Study; women (45 - 71 years)	carotene supplements low (2944 IU) vs high (14 583); 0.85 (0.72 - 1.00) ns	multivariate relative risk. cataract extraction; no effect of β -carotene

Table 39 Age related macular degeneration

Authors, year and number of subjects	Odds ratio and CI	Comments
Carotenoids		
Eye Disease Cas-Control Study, 1993, (n = 390)	neovascular AMD low group <0.34 µmol/L and high group > 0.88 µmol/L blood b-carotene 0.3 (0.2 - 0.6)	Lut-zea, β-car, sum of all, same odds ratio
West <i>et al.</i> , 1994, (n = 226)	β-carotene in the blood High (>0.88 µmol/l) vs low group (< 0.34 µmol/l) 0.62 (0.36 - 1.07)	
Vitamin C		
Eye Disease Cas-Control Study, 1993, (n = 390)	Middle vs low group low group <39.8 µmol/L and high group > 90.8 µmol/L. 0.60 (0.4 - 0.9)	
West <i>et al.</i> , 1994, (n = 226)	High (>82 µmol/l) vs low group (< 59.6 µmol/l) 0.55 (0.28 - 1.08)	
Vitamin E		
Eye Disease Cas-Control Study, 1993, (n = 390)	Middle vs low group (no improvement in high group) low group <24.5µmol/L and high group > 43.4 µmol/L. 0.6 (0.4 - 0.9)	
West <i>et al.</i> ; 1994, (n = 226)	High (>82 µmol/l) vs low group (< 18.6 µmol/l) 0.43 (0.25 - 0.73)	

4.4 Vitamin C

4.4.1 Cataract (Table 40)

Among the eight studies published between 1989 and 1996, three studies based on the plasma vitamin C level found no effect or a non significant effect on nuclear or cortical and early cataract. Taking into account vitamin C intake, three studies showed a significant lower risk in persons consuming more than 290 mg/day. Three other studies found no changes even if in one of these studies, adjustment on women taking vitamin C for more than 10 years showed a low relative risk (OR: 0.27 CI 0.11-0.67). Vitamin C is normally present at relatively high concentration in the lens and the aqueous humor of diurnal mammals including humans (about 1 mmol/l and 1.2 mmol/kg wet weight in humor and lens of humans respectively (Varma, 1991). It has been noticed that aqueous humor vitamin C in patients with cortical cataract was fifty percent lower than those with nuclear cataract (Varma, 1991). In an investigation comparing the self reported consumption of vitamin supplements by 175 patients and a same number of matched controls, it was reported that individuals from the control group took significantly more vitamin C ($p = 0.01$) and vitamin E supplements ($p = 0.004$). In the follow-up of the Beaver Dam Eye Study (Lyle *et al.*, 1999) smokers and subjects with hypertension showed a reduced risk of nuclear cataract (Table 39).

4.4.2 AMD (Table 41)

Two studies indicated that a blood vitamin C concentration of 80 $\mu\text{mol/L}$ led to a protective effect against AMD (OR = 0.55 CI 0.3-1.1 and 0.60 CI 0.4-0.9). One of these studies also indicated that for the highest values there was no better protection and perhaps even less protection.

Table 40 Vitamin C and cataract

Authors, year and number of subjects	Odds ratio and CI	Comments
Jacques and Chylak, 1991 (n = 112)	high (>90 µmol/L) vs low plasma vitamin c (< 40 µmol/L) 0.29 (0.06 - 1.32); ns	after adjustment on age, sex, race and history of diabetes
Jacques and Chylak, 1991 (n = 112)	early cataract higher (> 490 mg/d) vs lower vitamin C intake (< 125 mg/d); 0.25 (0.06 - 1.29); ns	
Robertson <i>et al.</i> , 1989 (n = 304)	advanced cataract higher (> 300 mg/d) vs no vitamin C intake as supplements; 0.30 (0.24 - 0.77); P<0.05	age and sex matched individuals
Leske <i>et al.</i> , 1991 (n = 1380)	nuclear cataract Vitamin C intake at the highest 20% vs the lowest one 0.48 (0.24 - 0.99); P<0.05	age and sex matched individuals;
Hankinson <i>et al.</i> , 1992 (n = 50 828)	higher (> 705 mg/d) vs lower vitamin C intake (< 70 mg/d) 0.98 (0.72 - 1.32)	Women after adjustment for confounders: age, diabetes, smoking and energy intake
Hankinson <i>et al.</i> , 1992, (n = 50 828)	0.55 (0.32 - 0.96)	women consumed vit. C for >10 years
Hankinson <i>et al.</i> , 1994, unpublished	advanced opacities higher (> 294 mg/d; n= 165) vs lower vitamin C intake (< 77 mg/d; n= 136); 0.27 (0.11 - 0.67)	
Mares-Perlman <i>et al.</i> , 1994, (n = 1980)	nuclear cataract 0.7 (0.5 - 1.0) cortical cataract 1.8 (1.2 - 2.9)	past use of supplements containing vit. C; age, sex smoking & alcohol consumption
Mohan <i>et al.</i> , 1989 (n = 1990)	mixed cataract 1.87 (1.29 - 2.39)	plasma vit C concentration
Vitale <i>et al.</i> , 1994 (n = 671)	nuclear cataract 1.31 (0.61 - 2.39) high (>80 µmol/L) vs low plasma Vit. C (< 60 µmol/L) cortical cataract 1.01 (0.45 - 2.26)	age, sex and diabetes
Lyle <i>et al.</i> , 1999a (n=1349) Beaver Dam Eye Study	high intake (281mg/kcal) vs low 27) 0.8 (0.5-1.4) trendNS	smokers 0.3 (0.1-0.8) trend 0.01 hypertension: 0.3 (0.1-0.8) trend 0.02

4.5 Vitamin E

4.5.1 Cataract (Table 41).

Five studies based on plasma vitamin E level found that a level higher than 30 $\mu\text{mol/L}$ reduced cataract risk by about 50 percent. These studies did not correlate with the amount of vitamin E intake since four among seven studies found no effect whereas in one of the two other studies, the effect was not significant. Lower risks of cataract extraction (OR = 0.5, $P < 0.05$) for patients in the highest quintile of vitamin E intake compared to the lowest one was found by Tavani *et al.* (1996). It can also be noticed that the two larger studies, with more than 25 000 people, and showing no effect were made on either women only and on male smokers (Hankinson, 1992 ; Teikari, 1998). Vitamin E in experimental light-induced lens oxidation protects against lipid oxidation (Varma *et al.*, 1982). Using spontaneously developing cataract in Emory mice, Varma *et al.*, (1991) also demonstrated that vitamins E and C treatment of deficient mice resulted in either delayed and increased frequency of cataract. According to Robertson *et al.* (1991) the strength of the epidemiologic evidence for the vitamin/ataract association is good for the strength of association, time sequence and biological plausibility and fair for dose-response relationship and for consistency and specificity of association.

In the ATBC study, there was no beneficial effect of supplementation with α -tocopherol on the cataract extraction (Teikari *et al.*, 1998)

4.5.2 AMD (Table 39)

The two studies reported found that high plasma content of vitamin E reduced AMD risk. It can be noticed that with a cut-off of plasma level higher than 80 $\mu\text{mol/L}$ the relative risk is lower than that obtained with a cut-off of 43 $\mu\text{mol/L}$ (0.43 vs 0.60). Actually, the protective effect of vitamin E was established in studies using monkeys fed vitamin E deficient diet which developed degenerative changes in the macular region of the retina (Hayes, 1974). In the ATBC study, there was no beneficial effect of supplementation with α -ocopherol on the incidence of AMD (Teikari *et al.*, 1998).

Table 41 Vitamin E and cataract

Authors, year and number of subjects	Odds ratio and CI	Comments
Knekt <i>et al.</i> , 1992 (n = 141)	advanced cataract serum vit. E above and under 20 µmol/L; 0.53 (0.24 - 1.1)	case control matched for age, sex and municipality.
Vitale <i>et al.</i> , 1994 (n = 671)	advanced cataract plasma vit. E > 29.7 vs < 18.6 µmol/L; 0.52 (0.27 - 0.99)	
Jacques and Chylak, 1991 (n = 112)	posterior subcapsular cataract plasma vit. E > 35 vs < 21 µmol/L; 0.33 (0.03 - 4.13) early cataract plasma vit. E > 35 vs < 21 µmol/L; 0.83 (0.20 - 3.40) cortical cataract plasma vit. E > 35 vs < 21 µmol/L; 0.84 (0.20 - 3.60)	
Leske <i>et al.</i> , 1998 (n = 764)	Nuclear opacification group with higher plasma vit. E.; 0.58 (0.36 - 0.94)	
Robertson <i>et al.</i> , 1989 (n = 304)	advanced cataract no supplement vs intake > 400 IU/d; 0.44 (0.24 - 0.77)	
Jacques and Chylak, 1991 (n = 112)	higher (> 35.7 mg/d) vs lower vitamin E intake (< 8.4 mg/d) 0.45 (0.12 - 1.79); ns	
Leske <i>et al.</i> , 1991 (n = 1380)	cortical cataract 0.59 (0.36 - 0.97) mixed cataract 0.58 (0.37 - 0.93)	highest quintile vs lowest one. highest quintile vs lowest one.
Mares-Perlman <i>et al.</i> , 1994, (n = 1980)	nuclear cataract use of vitamin E supplements; 0.9 (0.6 - 1.5) cortical cataract 1.2 (0.6 - 2.3)	
Hankinson <i>et al.</i> , 1992 (n = 50 828)	higher (> 210 mg/d) vs lower vitamin E intake (< 3.3 mg/d) 0.96 (0.72 - 1.29)	Women after adjustment for confounders: age, diabetes, smoking and energy intake)
Leske <i>et al.</i> , 1998 (n = 764) longitudinal study; nutritional data and yearly follow-up visits	Nuclear opacification 0.43 (0.19 - 0.99)	group using vit. E supplement.
Teikari <i>et al.</i> , 1998 (n = 28 934) (50 - 69 years); intervention followed for 5 to 8 years	advanced cataract 0.91 (0.74 - 1.11)	Vit. E supplementation vs placebo; male smokers;
Lyle <i>et al.</i> , 1999a (n=1349) Beaver Dam Eye Study	high intake (28.3mg/kcal) vs low 3.7) 0.8 (0.4-1.4) trend 0.08	hypertension: 0.4 (0.2-1.1) trend 0.02
Lyle <i>et al.</i> , 1999b (n=400) Beaver Dam Eye Study	high plasma concentration (5.6mmol/mmol cholesterol) vs low (3.2); 0.5 (0.2-1.1) trend 0.07	

4.6 Conclusions

The concluding remarks are related to cataract because too few studies report on AMD to propose any conclusion on this condition.

Whether tomatoes may protect against cataract remain an opened question. One study clearly showed a highly significant decreased risk in Italians consuming more than 3 servings per week (Tavani *et al.* 1996). Two other studies from North America did not show any effect of tomatoes consumption on cataract extraction. (Mares-Perlman *et al.*, 1995, Brown *et al.* 1999). It can be noticed that age-adjusted relative risk for cataract decreased significantly with increasing tomato sauce intake. The geographic differences suggest that other foods from the diet may interfere on the development of cataract, a multifactorial disease.

For lycopene, the available studies based either on intake estimation (Brown *et al.*, 1999, Chasan-Taber *et al.*, 1999; Lyle *et al.*, 1999a) or on serum concentration (Lyle *et al.*, 1999b) do not show significantly decreased OR for cataract extraction, but a borderline significant trend of reduced risk of nuclear cataract was observed with high lycopene intake.

Together these data indicate that tomatoes, tomato products and lycopene may have only minor effect on cataract appearance, in contrast with other foods and components such as spinach and lutein for which consistent epidemiological data have been shown. The possible benefit of eating tomatoes and tomato products, associated with other fruits and vegetables, may result from other components than lycopene, vitamin C and vitamin E for which protective effects were found including, and possibly also from the synergy of all components including β -carotene and folates.

Part 5: Tomatoes and tomato products, lycopene, other carotenoids, vitamins C and E and ageing

There are no specific studies on tomatoes and lycopene. Therefore we report on antioxidants present in tomatoes. Eventhough, there are few studies (8) reporting on this topic, among them, 5 observational studies deserved to be reported in details. They are based on antioxidant intake (4) or plasma concentrations (1) in 5 cohorts (Australia, The Netherlands, UK and USA), one is really prospective, and the four others are cross-sectionnall at the beginning of the study.

In one American study (Mendelshon *et al.*, 1998), cognitive function (15 tests measuring performance in various domains known to be affected in dementia) was related to the self-reported use of supplements (defined as any over-the-counter medication containing vitamin A, C or E, or β -carotene, or Zn or Se) in 1059 rural non-institutionalised subjects. After adjustment for confounders, there were no significant differences in cognitive test performance between antioxidant users and non-users. The other American study (Perkins *et al.*, 1999) explored memory performance in an elderly multiethnic population sample (4 809 >60 years old). The lipophile plasma antioxidants were expressed as ratios of cholesterol. A low ratio vitamin E/cholesterol (< 4.8) was associated with an increased OR: 2.09 (1.02-4.26), whereas there was no association with plasma vitamin C, nor β -carotene/cholesterol ratio. In the Rotterdam study, the data were derived from 5182 community participants (Jama *et al.*, 1996). Cognitive function was tested with the 30-point Mini-mental State Examination at baseline when dietary intake was assessed through a 170 item semi-quantitative validated food questionnaire, which was checked and verified at the visit to the research center. After controlling for confounders, the OR for cognitive impairment was 1.90 (CI: 1.17-3.08; trend <0.04) for a β -carotene intake < 0.9mg/day compared to ≥ 2.1 mg/day (this category included supplement users). There was no relationship with vitamins C and E. The study in UK (Gale *et al.*, 1996) analyzed altogether the relationship between mortality, cognitive impairment (measured by the Hodkinson mental test) and vitamin C intake (food record over a week) on 921 men and women. The OR for cognitive impairment was 1.7 (1.2-2.4) for an intake ≤ 27.9 mg/day compared to an intake > 44.9mg/day, after adjustment for all confounding factors. The authors quoted two previous studies showing comparable results. It should be noted that in this study vitamin C intake was very low, whereas in the Rotterdam study (Jama *et al.*, 1996), the vitamin C intake is quite normal (from < 77mg/day to ≥ 160 mg/day).

The Australian prospective study (Paleologos *et al.*, 1998) corroborated the British study. In 117 subjects recruited in a retirement community, vitamin C intake was assessed with a self-administered food frequency questionnaire, and validated four weeks later by a repeat food frequency questionnaire. Assessment of cognitive function was done four years later by the Mini-mental State Examination. The OR for cognitive impairment was 0.32 (CI: 0.18- 0.88; trend $p=0.05$) for 706mg/day versus < 62mg/day.

It is only because they involved carotenoid serum levels that the two following studies are mentioned. They are small studies, 61 Parkinson disease patients and their spouses as controls, and 88 subjects, respectively. Besides, the statistics are poor. In the first one (Jimenez-Jimenez *et al.*, 1993), β -carotene, α -carotene and lycopene were measured and there was no significant difference between Parkinson patients and controls. In the second one (Snowdon *et al.*, 1996), all carotenoids were measured in plasma of nuns and analysed in relation to dependence in self-care. Lycopene was inversely correlated to the number of dependencies (as was cholesterol; there is no adjustment between the two variables). The plausibility of this finding is weak.

Finally an intervention study has been conducted on 341 patients suffering a moderate Alzheimer's disease for two years (Sano *et al.*, 1997). The end-point was the time-lag before appearance of one event (death, severe dementia, loss of self-care capacity necessary institutionalisation). The treatment was either vitamin E (2000 IU/day), or selegiline or both. In vitamin E treated patients the risk for an event to occur was 53% lower than in the placebo group, and was lower than in patients treated with selegiline or the combined treatment.

Conclusion

There was no study conducted on the effect of tomatoes, or even fruit and vegetables. Further research is needed to confirm the results on vitamin C and E. Nutritional intervention studies would also be interesting to implement. A food approach, instead of the nutrient approach might be more informative. The necessity of collaborative studies between neurologists, biologists and epidemiologists specialised in nutrition and ageing is mandatory.

Part 6: Other microconstituents of tomatoes

We evoked the possible role of other micro-compounds (phenolics compounds, potassium and folates) of the tomatoes that could be responsible at least in part of its beneficial health effect several times in this report. To achieve this report we decided to include the few important studies related to these compounds.

Table 42 Micro-compounds content of tomatoes and other vegetables (carotenoids, folates, quercetine and lignans: $\mu\text{g}/100\text{g}$; Vitamins C and E,: $\text{mg}/100$)

	lycop.	β -car	α -car	β -crypt	lutein	vit C	vit E	folat.	querc.	lignans*
tomatoes var min-max	2116 – 62273	393- 494	-	-	44-72	18	1	20	700	21
carrots raw cooked	-	6628- 8162	2895- 3245	-	288- 273	4 2	0.4 6 0.5	10 22	-	346
broccoli raw cooked	-	414 450	-	-	1108 1043	11 0 60	1 1.1	110 73	1800	226
cauliflower rawcooked	-	2 7	-	-	4 15	50 47	0.1 7 0.1 1	83 59	50 (K 100)	145
garlic raw cooked	-	51 99	-	-	76 171	30	tr	3	-	407
oranges- tangerines	-	48 213	0-13	448 843	0 68	53 37	0.2 4 0.2 4	30 33	-	39
apple	-	20	-	8	6	5	0.5	13	2250	35
onions raw cooked	-	1 3	-	-	2 5	7 5	0.1 4 0.1	20	44 550	112

(-) no or negligible content

*no precision whether found in raw or cooked vegetables

** kaempferol

Sources: Favier J-C *et al.*, 1995 ; Olmedilla B *et al.*, 1996 ; Salvini S *et al.*, 1998 ; Pillow PC *et al.*, 1999.

6-1 Cancers

6-1-1 Phenolics

Many phenolics are present in tomatoes in total quantities from 85 to 130mg/100g (Léger and Amiot, 2000), essentially caffeic, ferulic, chlorogenic acids and the flavonols, naringenin, quercetin and kaempferol. Lung cancer has been the most often investigated with regard to the flavonols, naringenin, quercetin and kaempferol (Knekt *et al.*, 1997; le Marchand *et al.*, 2000). But the food sources were not tomatoes.

Quercetin and kaempferol were shown also to decrease gastric cancer, significantly only for kaempferol: OR=0.48; CI 0.29-0.88, trend, $p=0.04$, but were without effect against bladder cancer.

tomatoes	100	8.3	-	-	32.3	10.2	5.8	3.8	4.8	4.6
raw	7	3.0			18.6	7.7	4	3	3.3	3.2
cooked	93	5.3			13.7	2.5	1.8	0.8	1.5	1.4
carrots:		36.5	99.6	-	41.2	-	-	1.3	-	18.5
raw		9.7	9.7		13.3			0.5		12.8
cooked		26.8	28.5		28.0			0.8		5.7
cauliflower		-	-	-	13.0	4.0	-	2.4	0.09	7.1
cooked										
garlic		-	-	-	3.5	-	-	-	-	4.2
raw-cooked										
oranges- tangerines		-	-	98.3	9.8	15.0	-	-3.2	-	4.0
apple		-	-	1.7	2.0	21.8	1.6	2.5	9.9	5.0
onions raw- cooked		-	-	-	-	-	-	-	14	1.2

(-) no or negligible content

Sources: Gerber *et al.*, 1999 ; Holdsworth *et al.*, 2000; Gerber *et al.*, 2000; Scali *et al.*, in press.

Part 7: General Conclusion

The achievement of the state of art related to tomato, lycopene and health was necessary to clarify the scientific message on the topic. But it provides another information relevant to the epistemology of sciences, it is that, to some extent, the model of study governs the type of knowledge.

Coronary heart disease is the major pathology in the cardiology field with a moderate lag time between the notice of the risk factors and the disease and between the beginning of a treatment and the effect of this treatment. The effect is readily measured on markers. Contrarily, latency is a characteristic of carcinogenesis. There are no markers for cancers. Thus cardio-vascular studies are shorter and easier to evaluate than cancer studies which cover all potential risk factors on a long period of time, therefore, less studies are necessary to come to a conclusion.

Because mechanistic explanations of cardio-vascular disease involved oxidation of the LDL-particle, most of the epidemiological studies focused on the relationship between the disease and specific antioxidant nutrients, instead of food. The same is true for lung function with the studies of oxidative stress on bronchocytes, for cataract and macular degeneration with the description of damaged lens proteins and oxidised compounds (polyunsaturated fatty acyls and lipofuscin) in the retina following chronic exposure to light and oxygen and for ageing

with the evaluation of cognitive functions. Contrarily, the biochemical compounds or gene regulation effects from nutrients are not completely unravelled leading to speculative mechanistic explanations in cancer field. This explain why most of the cardiovascular disease, lung function and age-related eye pathology studies focused on antioxidants and not on other nutrients of food, and why cancer studies covered both food and nutrients.

Both the number of the studies and the consistency of their results permit to say that eating tomatoes daily reduces the risk of developing a cancer of upper aero-digestive tract, stomach, and lung. For all other cancers results are insufficient. Most of the time studies do not differentiate between raw and cooked tomatoes. But, if confirmed, the decreased risk for prostate cancer is mainly related to cooked tomatoes.

In other disease, for the reasons presented above, there are insufficient data to fix a specific role to tomatoes. Tomato is a very special plant fruit, rich in many nutrients and non-nutrients of interest. Thus it might potentially contribute to reduce most of the chronic degenerative diseases, except perhaps, age-related eye disease. These conditions appear to mainly benefit from lutein, which is only present in tomatoes at low concentrations.

With lycopene, we could expect more data especially in diseases other than cancers which focus on antioxidants, since this property is acknowledged for lycopene (see WG 1). Only one case-control study based on adipose tissue lycopene concentration showed a CHD risk reduction associated with high lycopene concentration, and for cancers, a possible effect is suggested for prostatic cancers, and to a lesser extent for cervix and pancreas cancers.

However, there is only fragmentary food composition data about lycopene, and for a long time analytical biochemistry did not differentiate the major carotenoids in human blood and tissues. Thus most of the studies reported on β -carotene only, explaining the paucity of results. Another point is the bioavailability of lycopene in humans, its transport in plasma and its storage (see WG2). It is known that plasma levels are not good markers of the intake (the correlation between intake and plasma concentrations ranges between 0,11-0,15). Beside, lycopene is stored differently in the various tissues of the human body. Generally the concentration is high in prostate, but there are contradictory results on the levels of malignant prostatic tissues compared to normal prostatic tissues (Clinton *et al.*, 1996; Rao *et al.*, 1999). All this might explain the little agreement between the effect of tomatoes and lycopene, and the paradoxical observation of the EURAMIC study: the Malaga centre showed the lowest lycopene concentration in the adipose tissues of its participants, whereas the MI incidence was the lowest in this centre of the study, compared with the others (Gomez-Aracena, 1997).

Thus, although we have strong evidence with regard to the protective effects of fruit and vegetables, we do not have enough data for tomatoes. Further research could be directed to the re-analysis of available epidemiological studies with regard to tomato intake, interaction among nutrients and estimation of risk associated with the sum of interacting nutrients.

It is obvious that we do not have enough data on lycopene, either bioavailability and tissue targeting in humans, or on relationship to disease. Further research should concentrate on human studies and disease for which there exists some indication, cervix, pancreas, and prostate cancers.

We conducted a literature review on the other components of tomatoes relevant to health, such as folates and phenolics. Tomatoes appear to be a food where most health-related nutrients are present and well balanced. Also, because tomato consumption is large and widespread, it provides a sizable amount of these microcompounds to populations with a tomato intake of 90 to 100g/day. It is possible also that lycopene is a necessary part of the synergistic protective effect of fruit and vegetables and further of Mediterranean diet. In this case, tomatoes, and essentially processed tomatoes, are a unique type of food in this model, since they are the sole source of lycopene.

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The Role of Dietary Tomato Products in Protection Against Oxidative Cellular Injury: A Review of Biomarkers, Mechanisms and Intervention Studies

Working Group 4: Mechanisms and Biomarkers

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Mission Statement:

The brief of WG4 has been to carry out research and collate data to provide supporting mechanistic evidence for the WG3 epidemiological programme, to assess the current state of biomarker research, and to evaluate the role of tomato antioxidants in healthy diets to support the aims of WG1 and WG2 in the optimisation of crop quality, product composition and process control.

Review Structure:

In chapter 1 Edmond Rock introduces the chemistry of oxygen radicals and their role in oxidative stress.

Chapter 2 by Rod Bilton further elaborates the biomarker concept and examines the function of oxygen radicals and 'active species' in oxidative cellular damage.

In chapter 3 Gordon Lowe reviews current mechanistic evidence for the purported protective functions of the major tomato carotenoids against oxidative stress.

Finally in chapter 4 Marisa Porrini and Patrizia Riso present evidence for the protective role of tomato products in healthy diets, in a comprehensive review of recent human dietary intervention studies.

Further support was provided by Mariette Gerber and Elizabeth Offord who supplied additional reference material and critical analysis of the review chapters.

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Introduction

A basic concept on which all nutritional scientists can agree is that the increased consumption of diets rich and varied in fruit and vegetables will improve the health of almost any human population. However, what is by no means clear, despite extensive research in recent years, is the means by which the protective components in these diets mediate their effects against oxidative stress. The plant kingdom was first to evolve a sophisticated defence system to successfully colonise the hostile terrestrial environment, and what omnivorous and herbivorous animals (including humans) have done is to effectively hijack these systems for their own survival. However, it is interesting to note that flavonoids and carotenoids, which have been shown to have excellent anti-oxidant properties *in vitro* are only poorly bio-available compared with vitamins E and C. Moreover, there appears to be no specific uptake and transport system for flavonoids and carotenoids which are absorbed passively in the human GI tract. This is not to say that they may play a crucial role in the GI tract by titrating out reactive oxygen, nitrogen and chlorine species (ROS, RNS and RCS- hereafter referred to globally as RS) and other genotoxins and chelating redox active iron and copper. It has been calculated that daily ROS damage to the colon could be as high as a 10,000 rad equivalent daily radiation dose (Babbs, 1990) in the absence of adequate anti-oxidant protection. Plants are a rich source of both oil soluble and water soluble anti-oxidant pigments and vitamins which play a crucial role both in intermediary metabolism and cellular protective mechanisms in animals. The greatest intellectual challenge in this field of research is to dis-entangle the immensely complex milieu of conflicting and interacting variables that form what constitutes a superficially healthy diet. Superimpose on this human genetic variability and susceptibility to environmental hazards and the problems become truly daunting. Early attempts to spend our way out of these problems through expensive large scale intervention studies have been of dubious value. With hindsight it is easy to see how a basic understanding of biochemistry and physiology led to a misinterpretation of the earlier epidemiological data. Subjects consuming fruit, vegetable and grain-rich diets tend to have higher plasma levels of vitamins C and E, carotenoids and some flavonoids, and on average are at lower risk of developing cancer and heart disease (Gey, 1995). Such data should not, however, be used to infer that the above components are the protective agents, or that they are mediating their beneficial effects via antioxidant mechanisms. In fact, any agent in such protective diets would appear to correlate with decreased disease incidence. The associative evidence comes from a wide range of case-

control, cohort and epidemiological studies. Also analytical and epidemiological studies contribute evidence of potential causal relationships. It is important to note, however, that some of these studies provide evidence more for dietary and lifestyle trends rather than the function of a particular constituent, in disease prevention. It has been shown that the frequency of fruit and vegetable intake increased with increasing physical activity. Conversely, consumption of similar protective diets was lower in subjects who were sedentary, heavy smokers, or heavy drinkers (Serdula et al., 1996). Many studies with animal models and cell tissue culture systems have provided valuable insight into the potential protective role of fruit and vegetables against chronic disease risk in humans, but the relationships are not always clear. The rat model, for example, is difficult to apply where the stomach pH is high and supports an abundant microflora as compared with the human stomach which is very acidic and has a very sparse microflora. Notwithstanding the limitations of animal test systems, well-controlled in vitro and animal experiments provide the most rapid and cost-effective means of generating data to design future human intervention trials. For example, experiments spanning some 50 years have demonstrated the value of caloric restriction in preventing cancer and prolonging life in animal feeding trials. If any phytochemical or dietary regime produces similar results in an animal model, then this would provide powerful evidence to study the same regime in human subjects.

Little is known of how many phytochemicals are processed in vivo, how they are absorbed, metabolised and distributed in the body and whether they even reach the tissues of interest. Moreover, little account is taken of the wide-ranging metabolic capabilities of the human gut microflora which can transform a variety of ingested phytochemicals (Borriello et al., 1984). In the past too much reliance has been placed on the plasma concentrations of dietary components as biomarkers of uptake and effect and the potential of gut-derived biotransformation products has rarely been assessed. Cell culture presents considerable problems, particularly with respect to the study of the role of diet in carcinogenesis. Cell lines, by definition are not representative of cells in healthy tissues which have a finite lifespan, have attained the transformed genotype which confers immortality, and many biochemical features that differ markedly from normal cells. Moreover, traditional cell-culture is carried out under non-physiological conditions at atmospheric pressure (160 ppm O₂) when normally protective antioxidant compounds may exhibit prooxidant activity eg. β -carotene. The ideal test of causality would be to carry out human intervention studies with a disease as an endpoint, but ethical considerations preclude the design of placebo-controlled, randomised trials with multiple doses of different phytochemicals and dietary factors to evaluate disease

outcomes. Problems have also arisen in the parallel and often independent development of free radical and epidemiology research. Large scale intervention trials initiated in the late 1980's to evaluate β -carotene, vitamin E and retinol were seriously compromised when it was found that excessive β -carotene consumed by high risk groups (male Finnish smokers ATBC study) resulted in an 18% increase in cancer incidence compared to placebo group. The effect of supplemental synthetic β -carotene was even more pronounced in the aborted U.S. CARET study where smokers receiving β -carotene and retinol had a 28% increase in the incidence of lung cancer compared to the control group. These were classic mistakes of misinterpreting association for causation and have been the major confounding influences in this field of research. Intervention trials can also miss important factors, if for example supplementation is in a well-nourished rather than a poorly nourished group (Esterbauer et al., 1997). Protective properties might also be missed if the supplementation dose is too small or too large. In light of previous experience future intervention trials should be designed to take account of the behaviour of test compounds in relevant *in vivo* models.

Chapter 1. The Chemistry of Oxidative Stress and its Pathophysiological Consequences

INTRODUCTION

This chapter introduces the concept of oxidative stress, a phenomenon universal to all higher organisms that have evolved to use oxygen as a respiratory substrate. Oxygen, like iron (Bilton, 2000) presents a unique contradiction in its role in aerobic life forms, where it is as fully essential to life as it is highly toxic in the absence of adequate antioxidant protection.

An overview of scientific advances accumulated since 1986 is presented, corresponding to the discovery of superoxide dismutase, to the present. This should help the reader to have milestones on oxidative stress through the concepts defined in it including, free radicals, antioxidants, and their role in the physiology; but also to introduce the analytical methodologies used to identify and quantitate this type of stress.

Oxidative stress : basic concepts

It is important to realise that oxidative stress is an imbalance occurring when the deleterious effects of oxygen metabolites overwhelm the array of body defences. In the following a review will be developed on the metabolism of oxygen, the resulting reactive species, the mechanisms involved in the interactions with biological molecules, the defence systems to neutralise the reactive species and the ways used to measure these processes.

Oxygen chemistry and metabolism

Oxidative stress mainly involves oxygen which is essential for aerobic living organisms. The chemistry of oxygen molecules already indicates a particular distribution of electrons in the external orbital making this molecule unique from the others found in the biosphere. Indeed, the usual electron coupling necessary to stabilise a given molecule does not occur and the stable oxygen molecule possesses two unpaired electrons which render them reactive (Balentine, 1982). This chemical instability may lead to the formation of the reactive oxygen molecule: singlet oxygen generated by absorption of energy (22 kcal from the ground state). On the other hand, other reactions which can be expected from the chemical unstability of oxygen molecule are: i) an intrinsic pairing of these electrons leading to a triplet state, ii) a stabilisation by an external electron transforming oxygen molecule into superoxide anion and iii) a second addition of an electron resulting in the formation of peroxide anion (Halliwell, 1993). All the resulting products are known to be chemically very active. Oxygen is also an

important intermediate for the oxidation-reduction reactions in intermediary metabolism, such as the catabolism of xanthine to uric acid by flavoprotein oxidase. However, under normal conditions, the main site of oxygen metabolism is the mitochondria where most of molecular oxygen is consumed in the respiratory chain (Chance et al., 1979). Oxygen is reduced to water by addition of four electrons, catalysed by the components of the respiratory chain. During these reactions, 1 to 2 percent of oxygen not consumed are liberated as superoxide anion (monovalent reduction) or hydrogen peroxide (divalent reduction). Other sites where reactive metabolites of oxygen can be liberated are the liver and the immune cells. The potential contribution of these sources of reactive oxygen often occurs during conditions such as detoxication of xenobiotics during their metabolism in the liver or during inflammatory processes often underlying pathological situations (Ames et al., 1993; Cannon and Blumberg, 1994). During these processes oxidases with the cofactors [(NAD(P)-H] use oxygen either to metabolise the foreign toxic compounds into less toxic ones, a reaction which results in the production of superoxide anion, or to destroy foreign organisms (bacteria, viruses) with superoxide anion but also with other reactive oxygen species. Through these processes, many compounds are formed, and those with unpaired electrons can be highly reactive free radicals.

Free radicals and reactive oxygen and nitrogen species

A free radical is any molecule containing one or more unpaired electrons and able to have an independent existence (Halliwell, 1994). Thus superoxide anion is one example of an oxygen centred free radical. Others have been identified centred on carbon (CCl_3°) or on sulphur (R-S° , thiyl radical) and in some cases the unpaired electron may be delocalized between 2 atoms such as nitric oxide (NO°) or ascorbyl radical. The reactivity of free radicals is due to their stabilisation by capture of another electron (from non radical or radical species). This leads to 2 consequences: first the life-time of free radicals are very short (10^{-9} to <1 second) and secondly may lead to a chain reaction where such a reaction generates other radicals unless two radicals meet and join their unpaired electrons. Thus besides an initiation reaction leading to the formation of a radical, a propagation reaction and a termination reaction can be distinguished. The radical reactions may also be facilitated by transition metals such as iron (Fe) or copper (Cu) by single electron transfer. From a chemical point of view, the metabolism of oxygen may thus result in the appearance of many reactive products where some of them are directly formed from oxygen as free radicals (superoxide anion) or non radicals (singlet oxygen, ozone). Other species may also be formed which are often more

hazardous for the organisms such as hydroxyl radical, hydroperoxyl radical, hydrogen peroxide, nitric oxide or peroxyxynitrite (Rice-Evans et al., 1994).

The most aggressive free radical is the hydroxyl radical (HO°) because of its reaction with a diffusion-controlled rate with almost every molecule in the living cell. Even though its diffusion is limited to 5-10 molecular diameters, the second-order rate constants is so high (10^9 - $10^{10} \text{ M}^{-1}\text{s}^{-1}$) that every molecule encountered will be attacked. Hydroxyl radicals are generated by three main mechanisms :

-homolytic fission of water molecules by ionising radiation: ultraviolet, X- and δ -rays, microwave (von Sonntag, 1987).

-reaction of transition metals ions with hydrogen peroxide illustrated by the Fenton reaction occurring with iron and efficiently increased by the presence of ascorbate. It can be noticed that superoxide anion is involved in reduction of ferric ion into ferrous ion which then reacts with hydrogen peroxide to generate hydroxyl radical, these reactions are described as Haber-Weiss reaction (Halliwell and Gutteridge, 1990a). In contrast with some pathological conditions such as inflammation and ischemia-reperfusion, transition metals such as iron and copper are not freely available for such a reaction since they are bound to proteins including caeruloplasmin or ferritin (Halliwell and Gutteridge, 1990b).

- Hydroxyl radicals may also be formed during homolytic scission of peroxyxynitrous acid ONOOH (Pryor and Squadrito, 1995) in the complete absence of metal transition ions, but current opinion tends to favour other mechanisms to explain the reactivity of ONOOH , which has a reduction potential of 2.1 vs 2.3 for $\cdot\text{OH}/\text{H}_2\text{O}$.

The hydroperoxyl radical (HOO°) level under physiological condition is low because of the low pKa of 4.5 of that reaction. However in pathological conditions when the pH decreases (ischemia, acidosis) or at the proximity of the inner mitochondrial membrane its production may be increased. Even though little evidence has been shown towards its action *in vivo*, the reactivity can be supposed higher than superoxide anion because of its lipophilicity that render it able to cross and oxidise the membrane lipids and to reach the intracellular compartment (Halliwell and Gutteridge. 1986).

The hydrogen peroxide (H_2O_2) is a non radical oxygen species resulting from divalent reduction of superoxide anion. This reaction can be spontaneous as this species can be detected in water at concentrations of 10^{-5} - 10^{-8} mol/L and in the body its generation is catalysed by oxidases (amine- or urate-oxidases) and more specifically by the superoxide dismutase (see below). Besides of oxidant activity, hydrogen peroxide may also have

physiological role through AP-1 and the resulting gene transcription (Brennan and O'Neill, 1995).

The Nitric oxide (NO°) is an important free radical as it is involved in the physiology particularly through the control of regulation of blood tension (Moncada and Higgs, 1993); it was also defined as endothelium derived relaxing factor (EDRF). NO° can readily react with superoxide anion leading to the formation of peroxynitrite anion (ONOO^-) (Beckman et al., 1994). The latter reaction occurred at near diffusion controlled limits at approximately 3 times the dismutation rate of the anion superoxide by the dismutase. Thus, depending on conditions, each of these radicals can control the action of the others. The resultant peroxynitrite anion can be an aggressive molecule capable of damaging many biologically active molecules. However, it has a pKa of 6.8 such that at physiological pH of about 7, a protonation occurred leading to the formation of peroxynitrous acid (ONOOH). At pH 7.0, this strong oxidant could be broken by homolytic scission into two radicals, hydroxyl radical and nitronium. The labile NO° radical with an estimated half-life of around 1 second may have a longer lifespan in the tissues before reacting with oxygen and water to form nitrites and nitrates. The determination of these latter compounds is an indicative of the formation of NO° . This latter is mainly formed from the metabolism of arginine to citrulline by specific enzymes, the nitric oxide synthases (NOS), existing in different isomer forms which are either constitutive (c-NOS) such as those found in the endothelial cells (e-NOS) and in the brain or inducible (i-NOS) in certain conditions such as those found in the immune cells and in the liver (Snyder and Bredt, 1992).

Oxidants and biological components interactions

The deleterious effects of the reactive oxygen- and nitrogen-species formed in the organism are expressed through the reactions occurring with the other constitutive biological molecules i.e. the lipids, the proteins, and the DNA bases.

Lipid oxidation

The main target of free radicals are the polyunsaturated fatty acids of cell membranes and lipoproteins (Halliwell and Chirico, 1995 ; Rubbo et al., 1994). The major reactive species involved in lipid peroxidation are hydroxyl radical and peroxynitrite. The mechanism underlying the reaction is an hydrogen abstraction from a methylene group of unsaturated fatty acyl chain. The initial lipid radical formed undergoes several fates including a rearrangement of the double bonds to stabilise the oxidised molecule and that results in conjugated diene formation and then a decomposition resulting to the formation of alkanals

(malondialdehyde), alkenals (4-hydroxynonenal) and alkanes (pentane) resulting from decomposition of oxidised arachidonic and linoleic acids. Moreover, under aerobic conditions, such interaction results in the formation of peroxy radicals by reaction with oxygen and the peroxy radicals propagates the radical reaction through attacks of proteins and of the other PUFA in the lipid structure. The propagation reaction may convert hundreds of fatty acids into monohydroperoxides from an initial one. Termination reaction then results from either reaction between 2 radicals stabilised by the assembly of the unpaired electron or the presence of lipophilic compounds able to scavenge and stabilise the unpaired electrons. Such compounds are defined as antioxidants (see below). Actually, the amplification of lipid oxidation also depends on the oxygen supply and unoxidized fatty acyl chain or more precisely on the lipid/protein ratio. Lipid peroxidation leads to damage that may alter the membrane physical (fluidity) and functional properties (transport, membrane permeability, assembly of membrane proteins); such alterations of critical membrane enzyme systems or the destruction of cell membranes invariably lead to cellular injury and cell death. Similarly, the oxidation of lipoproteins may alter their clearance and also may be at the origin of the atherogenic processes through the accumulation of oxidised low density lipoproteins (LDL) into macrophages leading to the formation of foam cells, a first step in the atherogenesis process (Parthasarathy et al., 1999).

Protein oxidation

Many amino acyl constituents of the proteins are critical targets for free radical attacks, primary radicals such as oxygen (Amici et al., 1989) and nitrogen species (Ischiropoulos and Al-Mehdi, 1995) or secondary radicals such as the intermediates of lipid peroxydation. As for membrane lipids, radicals formed at a specific aminoacyl site can rapidly be transferred to other sites within the protein infrastructure. Free radical attack may lead to a loss of protein function (enzymatic activity) when aminoacyls located in the catalytic sites are oxidatively modified (Jacob, 1995). More generally, protein oxidation results in their aggregation and fragmentation and degradation. Overall, protein modifications largely participate to oxidation-induced cell death by loss of ionic homeostasis as a consequence of increased permeability. It should be emphasised that among the ions, calcium plays an important role in the maintain of numerous intracellular functions and initial damage to membrane proteins regulating its fluxes, i.e. Ca-ATPases and Ca channels (Astier et al., 1996) may be exacerbated by activation of phospholipases, proteases and mitochondrial functions.

DNA oxidation

Among the reactive species, hydroxyl radicals are thought to be the major contributor of oxidation of DNA bases because of its high electrophilicity and its production in the vicinity of DNA molecules (Dizdaroglu et al., 1991). Estimates of the daily production of oxidised bases range from 10^4 to 10^6 per cell (Ames and Gold, 1991). It is important to note that daily DNA damage is higher than that of protein. Three mechanisms for interaction between DNA bases and hydroxyl radicals are proposed (Aust and Eveleigh, 1999):

- i) hydrogen abstraction from the deoxyribose sugar ($k=10^9$ M⁻¹s⁻¹) leading to a cleavage of the sugar-phosphate backbone,
- ii) electron transfer,
- iii) addition of OH to the pi-bond (double bonds); the latter gives rise to formation of base radicals having either reducing or oxidising properties.

One of the major products of DNA oxidation is 8-hydroxy-2'-deoxyguanosine reaching between 8 and 128 adduct molecules formed for every 10^6 normal bases in rat liver nuclear and mitochondrial DNA respectively (Richter et al., 1988). Despite the fact that no compelling evidence has been presented that oxidised DNA is directly responsible for cancer, experimental approaches consisting of treatment with certain chemicals (2-nitropropane) or physical agents clearly showed the formation of DNA adducts associated with tumour development (Unemura et al., 1990).

Antioxidants

The chemistry and biochemistry of oxygen as developed above show that highly reactive species may be formed during the metabolism and should normally result in damaged biomolecules which in turn should affect the normal functions of the organism. Fortunately, during evolution as photosynthesising organisms increased the amount of atmosphere oxygen, anaerobic creatures adapted to the metabolic reduction of oxygen to produce fuel molecules (ATP), and developed not only the different metabolic transformations using oxygen (oxidases, hydroxylases, nitric oxide synthase, ..) but also by developing different systems to detoxify the unavoidable toxic by-products and to repair potential damages. The various defences are complementary by acting on different oxidants and in different cellular compartments, in organisms exposed to a 20% oxygen atmosphere. Among these defence systems one can find the antioxidants. An antioxidant has been defined by Halliwell as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell et al., 1995).

Antioxidant systems in aerobic organisms can be divided into those involving protein whose amount and function is regulated genetically and those directly provided by nutrients from foods. Obviously, interactions exist between these antioxidants and combinations may lead to considerably increase the efficiency when compared to a single antioxidant.

Enzymatic antioxidant proteins

Among the antioxidant proteins, three main enzymes form the primary line of defence against the reactive oxygen species i.e. superoxide anion and hydrogen peroxide.

Superoxide dismutase was the first enzyme investigated by Fridovich (1986). This enzyme dismutates the super oxide anion into hydrogen peroxide at rates 10 000 times higher than spontaneous dismutation. It represents the major intracellular antioxidant enzyme in aerobic cells. SOD in the cytoplasm differs by the presence of Cu and Zn in the catalytic site from the one found in the mitochondria characterised by the presence of manganese (Mn) (Fridovich, 1989). Superoxide dismutase exerts antioxidant protection by avoiding the reaction of superoxide anion with biomolecules and also by inhibiting the formation of the cytotoxic peroxynitrite (Radi et al., 1991a).

Glutathione peroxidase is a selenium-containing enzyme which decomposes hydrogen peroxide to water by reducing equivalent amounts of glutathione (GSH) (Jones et al., 1981). It can also decompose other peroxides such as lipid hydroperoxides. The renewal of GSH pool is made by glutathione reductase which reduces GSSG (the oxidised form of GSH) to GSH using NADPH as co-factor. These reactions occurred in the cytoplasm and cytoplasmic GSH/GSSG ratio is an indicator of oxidative stress (Trible and Jones, 1990).

Catalase is a heme containing protein which also decomposes hydrogen peroxide into water and molecular oxygen (Thayer, 1986). Catalase is mainly present in the peroxisomes of nucleated cells and it has been recently detected in heart mitochondria where it is involved in the defence against hydrogen peroxide produced at high levels in the heart mitochondria as compared with other organs (Radi et al., 1991b). The heart catalase becomes effective when the H₂O₂ concentration rises above that which can be effectively cleared by glutathione peroxidase.

Non-enzymatic antioxidant proteins

As a secondary protection, one must consider the proteins involved in the degradation of oxidised biological molecules (nucleases, lipases and proteases) as well in the repairing mechanisms, particularly those involved in DNA (Dempsey and Harrison, 1994) and protein

damages (Grune et al., 1995). Most of these proteins found in the extracellular space are involved in the chelation of transition metals which otherwise may help to produce toxic hydroxyl radicals (see above, Halliwell and Gutteridge, 1990a). The transferrin binds strongly to iron for transport and delivery to cells. The presence of this protein makes the unavailable ferric form of iron for iron-catalysed radical reaction. Only 30 percent of iron-binding sites on the transferrin is occupied in the blood circulation. Caeruloplasmin and albumin are able to chelate copper ions. Caeruloplasmin may also convert ferrous iron into less reactive ferric ones. Copper bound to albumin is still available for generation of radical species in the presence of hydrogen peroxide. However, because of the limited action area of hydroxyl radicals, damage occurs only in the protein which is then rapidly replaced. Thus, albumin is considered as a sacrificial antioxidant. Haptoglobin acts by binding to free haemoglobin blocking the reactivity of iron contained in it. Metallothioneins are generated after the cells are exposed to toxic metal ions to chelate them very efficiently (Chubatsu and Meneghini, 1993).

Non enzymatic non protein antioxidants

In contrast with the primary antioxidant enzymes involved in the detoxification of superoxide anion and hydrogen peroxide, some of the following antioxidants are involved in the detoxification of hydroxyl radicals which are not catalysed by enzymatic systems, and of organic oxy-radicals such as peroxides.

Uric acid and bilirubin (Stoker et al., 1987) are involved in the chelation of metal ions and the protection of the albumin and PUFA respectively. Uric acid can be considered as an important antioxidant in primates and humans (Ames et al., 1981). Indeed in the primates the lack of uricase lead to an accumulation of uric acid reaching a level as high as 300 to 500 μM in the blood. In addition with its chelating property, it was shown that uric acid also inhibits the activity of circulating xanthine oxidase known to be increased in conjunction with high concentrations of purine substrates secondary to a variety of pathological conditions (ischemia injury, thermal injury, virus infection) and used to generate superoxide anions from molecular oxygen.

Glutathione is a tripeptide (γ -glutamyl-cysteinyl-glycine) used by glutathione peroxidases (see above). The detoxication reaction actually results in the formation of the glutathiyl radical (GS°) which is less reactive than the reactive oxygen species. GSH together with SOD is considered as the most important antioxidant in cellular defences. The intracellular concentration of GSH is far greater than reactive oxygen species as it can reach up to 5 to 10 mmol/L. It is noteworthy that other thiol compounds are involved in the control of free

radicals including N-acetylcysteine acting through elevation of intracellular cysteine (hence glutathione) and homocysteine which can be involved in the control of the plasma redox thiol status (Brooks et al., 1991).

Ubiquinone (Coenzyme Q10): this powerful antioxidant is a fat soluble quinone mainly found in the inner membrane of mitochondria. Its level in mitochondrial membrane is 10 times higher than in plasma membrane when compared with another lipid soluble antioxidant such as vitamin E (Takada et al., 1982). It is as efficient as vitamin E in chain-breaking antioxidant reactions. However, it is believed to regenerate vitamin E radical (tocopheroxyl) formed while preventing lipid peroxidation (Frei et al., 1990). The oxidised product of ubiquinone (ubisemiquinone) is reduced back by the electron transport chain of mitochondrial respiratory chain.

Vitamin E provided by the food is a fat soluble antioxidant found in all lipid structures (membranes and HDL/LDL). The plasma level is about 15 $\mu\text{g/mL}$ and up to 7 molecules of vitamin E was found per molecule of LDL (Esterbauer et al., 1989). Vitamin E comprises 8 naturally occurring homologues defined as tocopherols. The most abundant α -tocopherol is a chain breaking agent reacting with lipid oxides and preventing lipid peroxidation (Diplock et al., 1989). Such a reaction leads to the formation of tocopheroxyl radical which, by spreading the energy of the unpaired electron into the entire structure, increases the half life of the radical and hence blocks the reactivity of the captured electron. Tocopheroxyl can react either with another tocopheroxyl or lipid peroxide radical to form stable vitamin E dimer and vitamin E-lipid adduct respectively. The tocopheroxyl radical can also be regenerated to tocopherol either by ubiquinone from inner mitochondrial membrane or by vitamin C from the aqueous compartment of cell cytoplasm (Kagan et al., 1990). This latter reaction is an important way to maintain the level of α -tocopherol in the membrane at the expense of vitamin C.

Vitamin C (or ascorbic acid) is a water soluble compound found at relatively low concentration in the plasma (40-140 $\mu\text{mol/L}$) and can attain millimolar level in tissues. It is essential for humans as it cannot be synthesised and is obtained from the diet (and/or supplement). Besides of several important metabolic roles, ascorbate also has the capacity to scavenge in vivo almost all reactive oxygen and nitrogen radical species (Rose and Bode, 1993). Its antioxidant property results from the formation of semidehydroascorbate which is less reactive than the free radicals scavenged (Bendish et al., 1986). As discussed above, one of the roles of vitamin C is to act synergistically with vitamin E through the reduction of α -

tocopheryl radical (Buettner, 1993). The ascorbyl radical is reduced back to ascorbic acid by enzymatic systems using NADH or GSH as co-factors. The ascorbyl radical may have a strong prooxidant activity in the presence of metal ions leading to the production of superoxide anion and hydrogen peroxide. This property is largely used to induce peroxidation reaction *in vitro* by using ascorbate-iron mixture. Fortunately, in normal physiological conditions, transition metals are sequestered by many proteins as mentioned above.

Carotenoids are coloured pigments found in vegetables such as carrot, spinach or sweet potatoes. Some carotenes (β -carotene) are precursors of vitamin A and many carotenes including β -carotene, lutein and lycopene are chain breaking antioxidants and singlet oxygen quenchers *in vitro* (Palozza et al., 1992). For instance β -carotene may react with peroxy radical (ROO°) to form a carotenoid radical species or a resonance-stabilized carbon-centered radical. This latter stabilisation is authorized by the presence of conjugated double bonds able to dissipate the energy of the unpaired electron. However, the antioxidant activity of carotenoids may become prooxidant depending on the level of oxygen present (Palozza, 1998). For high oxygen tension ($p\text{O}_2$), β -carotene may react with oxygen and transform into a peroxy radical ($\beta\text{Car-OO}^\circ$) capable of acting as prooxidant and undergoing autooxidation. The formation of such radicals have been demonstrated only by indirect *in vitro* observation where β -carotene was used at high concentration and with oxygen tension not likely to occur in body tissues. However, such a mechanism may be a basis for increased lipid oxidised products in liver of mice fed a high level of β -carotene and exposed to methyl mercuric chloride (Andersen and Andersen, 1993) and the prooxidant activity of β -carotene in rats fed vitamin E deficient diet (Lomnitsky et al., 1991). Thus carotene may function as part of the human antioxidant defence system but may also have prooxidant properties. Epidemiologic studies have shown that people having a high intake of carotene rich foods had lower rates of cancer and heart diseases than control populations (Gaziano et al., 1995). However, carotenoids rich plant foods are also rich in other protective components such as vitamin C, folate or fibers which may contribute to that observation. In a recent epidemiological study (Kohlmeier et al., 1997), it was reported that α -carotene, β -carotene and lycopene each appeared to be protective against myocardial infarction risk. Other studies reported possible beneficial effects of lycopene on prostate cancer (Giovanucci et al., 1995) particularly when lycopene was provided through processed tomatoes. Indeed, lycopene is mainly found in tomatoes and tomato products and it accounts for about 50 % of carotenoids in human plasma and is concentrated in the testis (Gerster, 1997). Data on β -carotene are more controversial. A recent placebo-controlled study in women healthy volunteers clearly show that low carotenoid

diet induced a significant increase of lipid oxidised by-products in the plasma suggesting that carotenoids are necessary to prevent lipid peroxidation; moreover, a supplementation with β -carotene normalise plasma malodialdehyde level. (Dixon et al., 1998). Other studies showed that β -carotene supplementation may decrease plasma and tissue vitamin E concentration. The most well known of adverse effects of β -carotene is shown in the ATBC intervention study where supplementation with that carotenoid in Finnish male smokers actually increased, rather than reducing, lung cancer incidence (ATBC, 1994). In the same study, it was also shown that in this male smokers population, β -carotene supplementation also increased coronary heart disease mortality by 11%. One explanation for such an observation is that antioxidant activity of β -carotene may shift into prooxidant one, depending on oxygen tension, concentration and also with other antioxidants (Palozza, 1998). Whether one or more of these conditions exist in smokers has to be established. On the other hand, other large scale studies including the CARET (Omenn et al., 1996) and Physicians' Health Study (Hennekens et al., 1996) did not show any beneficial effect of β -carotene on atherosclerosis or its sequelae. It should be emphasised that in contrast with cholesterol-lowering trials, no individuals were selected on grounds of low antioxidant intakes or high cardiovascular disease susceptibility. Another carotenoid of interest is lutein mainly found in spinach and which accumulates in the lens. High intake of this carotenoid has recently been associated with decreased risk of cataract formation (Brown et al., 1999; Chasan-Taber et al., 1999). The possible biological mechanisms may be the antioxidant activity and light absorption, but no experiments specifically showed the actual mechanisms by which lutein may confer its protective effect on lens opacity.

Other phytomicronutrient antioxidants

Many metabolites found in plants are supposed to act as antioxidant. These include phenols, polyphenols, flavonoids and possibly lignans, all of which are characterised by the presence of one or more hydroxyls conferring either metal chelating or radical scavenging properties (Arora et al., 1998).

Phenolics - Many phenolic compounds exist in plants either in free or conjugated forms (ferulic acid, thymol, ...). Investigations of their antioxidant properties clearly show their ability to inhibit lipid peroxidation of liposomes by scavenging peroxy radicals or to protect deoxyribose against hydroxyl radical attack. However, the beneficial effects of consumption of food-plants high in phenols has not been clearly established and it is difficult to reach high circulating levels of these phenolic compounds by means of diet. However, they may present

an alternative way to prevent food spoilage than the one using synthetic antioxidants (Aeschbach et al., 1994).

Polyphenols and flavonoids - Polyphenols are complex groups of secondary metabolites found in high levels in plants. The proanthocyanidins are known to be formed of flavan-3-ol units and to form complexes with proteins and carbohydrates but their antioxidant activity remains to be further established.

Flavonoids are composed of 6 classes (flavanones, flavones, flavonols, isoflavonoids, anthocyanins and flavans) varying around the heterocyclic oxygen ring (Peterson and Dwyer, 1998). Honey, red wine, onions, apples, citrus, chocolate and sweets that contain some plant constituents have flavonoids. Dietary intake estimates vary from 23 mg/day to 1000 mg/day (Hertog et al., 1993). Absorption and metabolism of flavonoids are not still clearly established. They are metabolised in the liver, small intestine or kidney and by the colonic bacteria. The resulting conjugated and phenolic acids are excreted into bile, urine or feces (Hollman et al., 1995) They are characterised by antioxidant but also antimutagenic activities (Shimoi et al., 1994). Following ingestion of foods containing polyphenols, plasma antioxidant capacity increased and lipoprotein oxidation decreased (Ursini et al., 1999). Flavonoids are reported to scavenge efficiently the reactive oxygen- and nitrogen-species, and also to chelate metal ions. Many studies were done on the structure-antioxidant activity relationships with different structurally related compounds (Van Acker et al., 1996). In vitro studies (Bors et al., 1995) using electron pulse radiolysis and cyclic voltametry approaches, established a positive correlation between inhibition of enzymatic and non enzymatic lipid peroxidation and the oxidation potentials (up to 200 mV) of the flavonoids. For some flavonoids a higher antioxidant effect was observed when used against metal ion-induced peroxidation suggesting that metal chelation plays a major role in the antioxidant capacity of at least some of the flavonoids. The catechol moiety and/or some OH groups positioned at particular positions (positions 3 and 5) give the capacity to chelate metals. Altogether these data indicated that antioxidant activity of the different flavonoids depend on their chemical structure: the pyrogallol group, the carbonyl group in conjugation with particular hydroxyl groups may increase the electron delocalization and thereby stabilising the radical form of the flavonoid, conjugation of the pyran ring found in anthocyanins also increases the stabilisation of the radicals formed, a free hydroxyl group an position C3 may increase the lipophilicity of the compound. Case control studies suggest that flavonoids may reduce cardiovascular risk and stroke (reviewed by Hertog, 1998).

Oxidative stress: pathophysiological consequences

From the basic concepts developed above on free radicals and antioxidants, it can be assumed that both coexist in the organism. The first unavoidably produced from normal metabolism is necessary for physiological functions and the others to protect the basic processes and also to cope with exogenous sources of oxidants mainly pollutants (cigarette smoke, dust and gases resulting from industrial activities) and radiations. The systems involved in the production of free radicals and the antioxidants act synergically to maintain a balance between production and elimination of reactive oxygen and nitrogen species. Oxidative stress can be defined as a stress occurring when an imbalance is produced either by an increase of the amount of free radicals and/or by a deficiency in the defence system. The resulting damage to cell components (membranes, organites, proteins, lipids and DNA) may thus lead to irreversible dysfunction and occurrence of pathologies (Kehrer, 1993). Oxidative stress has thus been hypothesised to be a major contributor to major diseases in humans such as cancer, and cardiovascular diseases (Halliwell and Gutteridge, 1994). On the other hand, measurements of toxic oxidised compounds in the plasma demonstrated that their level is never null and tend to increase with age (Yu, 1996). From this observation, one can suggest that oxidative stress is a continuous phenomenon and demonstrates the imperfection of natural defences including antioxidant and repair systems. Another implication is that oxidative stress also contributes to ageing and the associated degenerative diseases such as cataracts, Alzheimer and Parkinson diseases, and diabetes. Some experimental data will be developed in the following section emphasising the oxidative stress hypothesis in some pathological conditions (atherosclerosis and cancer) and also in more physiological ones including ageing and physical exercise.

Cancer and oxidative stress

Cancer by itself is a multistep and complex process initiated by the transformation of normal cells into malignant ones. The initiation involves modification of DNA bases where free radicals may have a role together with repair processes (Kasai et al., 1986). Thus it was proposed that human cell experiences 10.000 oxidative hits daily and the repair enzymes remove most but not all of them. Moreover, oxidative damages accumulate with age and so increases the risk of cancer during ageing. Damage of DNA just before cell division may result in permanent genetic alteration and cells that divide rapidly are indeed more susceptible to carcinogenesis. Epidemiological studies made on fruit and vegetables (complex foods containing many antioxidant micronutrients) and cancer prevention showed that among 170 observations, 132 studies demonstrated a significant protective effect and only 6 a negative effect (Block et al., 1992). Intervention trials are more controversial: trials in Linxian showed

a 13% decrease of total cancer deaths and another trial showed no effect on oesophageal cancer risk. ATBC (1994) trial in Finland on former smokers showed significant increase of 18% in lung cancer incidence. Finally, a trial in USA fails to show any effect on the occurrence of new colorectal adenomas in patients with a history of past adenomas. Antioxidants may thus protect the cell towards initiation processes and are not thought to influence the development of cancer even though β -carotene was shown to be of benefit in oral leukoplasia, precursor of oral cancer. Beneficial effect of antioxidants may also be derived from other mechanisms such as enhancing the immune function or increasing the gap junctions recently shown for carotenoids.

The association between oxidative stress and cancer was also suggested through earlier correlation between increased iron stores and increased cancer of all organs (Stevens et al., 1988). Similarly, another prooxidant substance, peroxynitrite, was linked to the formation and progression of tumours. Peroxynitrite production was identified in human colon adenomas and carcinoma development related to the induction of nitric oxide synthases (Ambs et al., 1998). Another mechanism by which peroxynitrite may be involved in carcinogenesis is via its inhibitory effect on the p53 gene playing an important role in the cellular response to DNA damage (Ambs et al., 1997). Direct evidence of DNA oxidation and cancer has been shown in animal model (Unemura et al., 1990) by administration of iron chelated to nitrilotriacetate (Fe-NTA) which causes kidney tumours and yielded a significant increase of 8-oxo-dG, the most abundant and easily measured product of DNA oxidation.

Atherosclerosis and oxidative stress

It is now recognised that low density lipoproteins (LDL) play a significant pathogenic role in atherosclerosis, a chronic disease, at the origin of angina pectoris, myocardial infarction and ischaemic stroke (Parthasarathy et al., 1998). Atherosclerosis is the principal cause of death in Western countries (>40% of all death). The atherogenic effects of oxidised LDL derived from the oxidised lipid components which are able to induce adhesion molecules from monocytes at the surface of endothelial cell surface and to modulate the chemotactic processes, cell proliferation and are at the origin of lipid-laden foam cells, a first step of development of the atherosclerotic lesion (Parthasarathy et al., 1999). Involvement of oxidative stress in the development of atherosclerosis has been illustrated by the use of antioxidants that inhibit atherogenesis. This protective effect can be explained by 2 mechanisms: a specific action on LDL particles (exerted by vitamin E and ubiquinone) and the action at the tissue and cell levels resulting in an increased uptake of antioxidants by vascular cells and increased cellular antioxidant status which may result in a decrease of reactive oxygen species and in turn less

cell-mediated LDL oxidation (Frei, 1999). Work done by Frei et al. clearly demonstrated that plasma antioxidants (mainly vitamin C) are able to protect LDL oxidation induced by many different oxidising conditions and that vitamin C is also able to protect isolated LDL particles against metal ion-dependent and -independent oxidative modification. These in vitro studies agreed well with epidemiological studies showing a negative correlation between the presence of antioxidants in the plasma and the incidence of coronary heart diseases (Gey et al., 1993); other data from ongoing studies suggest that protective effect of vitamin E could be effective only in users of supplements. Data on EURAMIC study (Kardinaal et al., 1993) however, showed that low adipose tissue β -carotene concentrations were associated with significantly increased risk of myocardial infarction. Interestingly, in that study myocardial infarction was mainly confined to smokers and similar data were also obtained on the protective effect of β -carotene in health professional studies and showing 22 % reduction of coronary risk in the individuals from the top fifth of β -carotene intake. Concerning the low incidence of cardiovascular disease in individuals consuming the « Mediterranean diet » antioxidants, polyphenolic substances but also mono unsaturated fatty acids may contribute to protective effects. Concerning flavonoids, a recent paper reviewed the data obtained on flavonoids from different foods on coronary heart disease (Lairon and Amiot, 1999). Based on epidemiological studies on protective effect of red wine and tea consumption, many intervention studies were designed to confirm the effect of flavonoids on cardiovascular risk in humans. Altogether, some of these studies on tea and onion or parsley failed to show any protective effect, whereas others using red grape juice, red wine and beer effectively showed LDL protection against oxidation, tendency to increase HDL cholesterol, rise of plasma antioxidant capacity and plasma vitamin E levels. The authors proposed that such a discrepancy may result from factors such as the initial physiological state on the volunteers, the composition and the amount of foodstuffs ingested during supplementation period or the duration of the experimental period. Other factors are the lack of knowledge on the bioavailability and metabolism of flavonoids contained in the foods tested.

Ageing

As mentioned above, oxidised by-products are always found in the circulation and thus non specific oxidative damage may be at the origin of ageing processes as stated by Harman (1956) some 40 years ago. Whether oxidative stress is a marker or causal agent in ageing is a question recently addressed by Fukagawa (1999) and the following will be based mainly on what he developed in that review. Undoubtedly physiological functions decline inevitably with age, these adverse changes finally leading to death. Ageing is also associated with

diseases occurring with high prevalence and designated degenerative diseases including cardiovascular diseases and cancers but also osteoporosis, cataracts and neurodegeneration. Because of the expected increase of aged people in western countries, many investigations attempt to increase the half-life as well as the maximum life span which may have important implications in terms of the social and economic points of view for our societies. In humans, the role of oxidative stress in ageing was suggested by the increase, with age of oxidised DNA bases, oxidised lipids and oxidised proteins (Meccoci et al., 1999). The role of oxidative stress in ageing was better assessed by increasing superoxide and catalase expression in drosophila (Orr and Sohal, 1994). These transgenic flies had live 13% longer than controls. In humans, the role of oxidative stress in the pathogenicity of degenerative diseases is recognised but it is not known whether reactive oxygen species are primary or secondary effectors (Markesbery, 1997). Many studies focused on mitochondria which are the greatest source of reactive oxygen species and which also show age-associated oxidative damage (in lipids, proteins and DNA) and DNA mutations (Wei, 1998) and thus may be at the origin of neuro degenerative diseases. However, recent observation by Urano et al. (1998) on mitochondrial function in Parkinson's disease concluded that similar changes were observed in control that can be attenuated by vitamin E. Another important area of research on ageing is the effect of caloric restriction known to retard ageing. Recent studies also demonstrated that caloric restriction also considerably attenuates the oxidative stress evidenced by a decrease of oxidised tyrosine, amelioration of age-associated increase of cytokines; prevention of mitochondrial deletions in the liver or reduction of protein and lipid oxidative damage (Byung, 1996). From these latter observations questions arise as to whether supplemental antioxidants slow the ageing process. The high complexity of the ageing process led the gerontologists to consider that it is part of the pathological process. The data obtained on antioxidant supplementation trials already show a positive relation with age-related chronic diseases such as cancer, heart diseases or diabetes. These data provide evidence that antioxidants may attenuate the pathological process. Other studies on different species (fruit flies, nematodes, and rats) also showed that antioxidants significantly extend of median life spans and some of maximum life span. Unfortunately, in these latter studies, no physiological parameters were measured. One study on mouse supplementation with mercaptoethanol (Heidrick et al., 1984) demonstrates that median and maximum life spans are increased by 15% together with improved immune function, a functional age-related biomarker. Interestingly, vitamin E is now recognised to act as an immune stimulant (Meydani et al., 1995).

Exercise and oxidative stress

An elevated metabolic rate as a result of exercise can dramatically increase oxygen consumption and hence reactive oxygen species that may lead to oxidative stress. This is particularly shown during unaccustomed and strenuous exercise (Sen, 1995). Using animal models it has been shown that strenuous in vivo exercise indeed enhances reactive oxygen species production detected in muscle homogenates and detected by electron spin resonance techniques (Davies et al., 1982; Kumar et al., 1992) or a fluorimetric method using dichlorofluorescein (Bejma and Ji, 1999). As stated before, most of the oxygen is metabolised in the mitochondria and during its reduction a certain amount of oxygen not consumed is liberated as reactive oxygen species. During maximal exercise, whole-body oxygen consumption may increase up to 20 fold whereas in muscle fibre may be up to 100 fold. One may expect a proportional increase of reactive oxygen species. However, no clear data demonstrated such an increase and some even showed that this may not be the case as isolated mitochondria reactive oxygen species production from exercised muscle did not differ from that of rested one (Bejma and Ji, 1999). The mitochondrial theory is indirectly supported by the increase of mitochondrial lipid peroxidation (Ji et al., 1988), decreased GSH redox status in muscle (Leichtweis et al., 1997) and training adaptation of mitochondrial antioxidant enzymes (Higuchi et al., 1985). Another source of free radicals is the one produced by xanthine oxidase during conversion of hypoxanthine to xanthine and uric acid. Such a process has been described during ischaemia-reperfusion of tissues. After intense muscular contraction, accumulation of uric acid strongly support the activation of xanthine oxidase which is found increased 10 fold in the plasma. Whether such an activation normally occurred during aerobic exercise remains to be demonstrated. Another potential source of free radicals is the activation of neutrophils during the inflammatory response following muscle damage arising from oxidative stress or simply mechanical forces (Ji, 1999). The occurrence of oxidative stress after exercise may result from the activation of immune cells. It was shown for instance that phagocytosis and superoxide anion production by neutrophils were increased at 24 hours postexercise. This long delay together with the time necessary for neutrophils to infiltrate the tissue suggest that immune cells are not a primary source of free radicals but they serve as an important secondary source of free radical production during endurance exertion and also during recovery. The role of antioxidants is first investigated on rats fed vitamin E deficient diet and it was shown that endurance performance decreased and lipid peroxidation increased. Interestingly, an acute bout of exercise in contrast to chronic exercise did not decrease tissue vitamin E level, indicating that physiological levels of vitamin E are

adequate but that the protective margin is relatively small. In humans, it was shown that dietary supplementation with vitamin E increased tissue resistance to exercise-induced lipid peroxidation (Kanter et al., 1993). No data on specific oxidative markers were obtained with vitamin C supplementation. GSH deficiency has been associated with decreased GSH status and increased lipid peroxidation after exercise. GSH and N-acetyl-cysteine supplementation may improve endurance and decrease lipid peroxidation. Finally, studies on antioxidant enzymes clearly showed an adaptation of these latter to exercise. This is particularly true for superoxide dismutase which has been shown to increase after an acute bout of exercise (Lawler et al., 1993). This activation was proposed to result from increased superoxide anion production during exercise. These relatively early studies now opened to the concept that reactive oxygen species may have an essential role in signal transduction at the cell level :

- through a chemical recognition system involving their ability to interact and covalently modify selected targets of protein components, thiol groups of cysteine residues or heme iron (Lander, 1997)
- by regulation of the nuclear transcription factors sensitive to redox status of cells, including AP-1 and NF-kB (Flohé et al., 1997).

Antioxidant status

The balance between antioxidants and prooxidants in living organism may be defined as antioxidant status. This balance is dynamic and slightly in favour of oxidation since DNA, lipid and protein oxidation is ongoing throughout life. The body's antioxidant including repair systems are adapted to this imbalance. Antioxidant status is multiparametric and depends on genetics, physiological state but also on environment and diet. Oxidative stress thus represents a more serious imbalance where the primary line of defence represented by the enzymatic systems that are overwhelmed leading to the use of a secondary line of antioxidants including vitamins, glutathione and carotenoids. Depletion of such antioxidants should raise the level of prooxidants including reactive oxygen and nitrogen species. As a consequence, oxidative stress ultimately leads to damage of lipids, proteins, carbohydrates and DNA bases. An evaluation of antioxidant status may consist of determination of components belonging either to the primary line of defence (i.e. enzymes) and/or the secondary line of defence (other proteins and low-molecular weight compounds) and/or the oxidised biomolecules. However, the main interest of antioxidant status determination is to link this status to pathological conditions such as cancer or cardiovascular diseases. One main difficulty to assess such a relationship is to identify early biomarkers which can surrogate for pathophysiological

changes usually occurring with more or less long delay. It should be emphasized that such biomarkers are very useful to investigate the interactions of diet components on antioxidant status in short-term experiments. Thus, based on assumptions that oxidised DNA and lipoproteins are respectively involved in cancer and cardiovascular diseases, measurement of oxidised DNA bases and level of peroxidised lipids in LDL were used as surrogate biomarkers for the latter diseases. The methods and the limits of these biomarkers were recently discussed by Halliwell (1999). Other parameters are now considered to evaluate antioxidant status. The latest investigations were designed to be used for human studies implying minimally invasive methods and focused mainly on products resulting from free radicals as biomarkers of free radical damage (De Zwart et al., 1999) and on the determination of plasma antioxidant capacity (Prior and Cao, 1999).

Among the products resulting from free radical damage, the most representatives of lipid peroxidation products are exhaled pentanes, plasma and urine isoprostanes and aldehydic products. Hydroxylated DNA products (mainly Hydroxylated guanosine, 8-OH-dG) and carbonyls groups are the most usual markers for DNA and protein damage, respectively.

More recently, a concept of antioxidant capacity has been developed and can be defined as an individual measure reflecting the sum of available endogenous and exogenous defence mechanisms which ensure the oxidative balance (Fürst, 1996). Measurement of antioxidant capacity is mostly developed in the plasma assuming as representative of *in vivo* whole-body antioxidants. Such a measurement avoids the measurement of each antioxidant separately and takes into account the possible interactions between them. The methodology consists on using a prooxidant, generally a free radical, and an oxidizable substrate. The prooxidant induces oxidative damage to the substrate which is inhibited by the antioxidants contained in the biological samples to be tested. In these inhibition methods many techniques were developed and the most usual one is oxygen radical absorbance capacity also known as ORAC assay (Cao et al., 1993). It uses phycoerythrin as oxidizable protein substrate and AAPH (an azo compound) as a peroxy radical generator or copper-hydrogen peroxide as hydroxyl generator. Protein oxidation induced a quenching of fluorescence which is retarded by the antioxidants present in the tested samples. The quantitation is made by area-under-curve determination after completion of the reaction. A single value is thus determined for both inhibition percentage and length of inhibition time (the lag time during which the antioxidants from the sample protected oxidation of the protein). The ORAC assay has been used in several laboratories to determine the antioxidant of biological samples but also for pure compounds (melatonin, flavonoids) and complex matrices (tea, fruits and vegetables, and tissues). Other

usual antioxidant capacity assays are FRAP, TEAC and TRAP assays where the oxidants used are not necessarily prooxidants. Trolox equivalent antioxidant capacity (TEAC) assay (Miller et al., 1993) is based on the inhibition of the absorbance of the quite stable radical cation $ABTS^{\circ+}$ of ABTS with ferrylmyoglobin radical species generated by the activation of metmyoglobin with hydrogen peroxide. The assay measures the ability of antioxidants of given samples to reduce ABTS radical and compared with a reference, i.e. the Trolox, an hydrosoluble form of α -tocopherol. Ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) measures the ability of biological samples to reduce the ferric-tripyridyltriazine (Fe^{3+} -TPZ) into Fe^{2+} -TPZ reflecting the ability of the antioxidants within the sample to reduce the reactive species. This method is not suitable for glutathione determination and was weakly correlated with ORAC assay.

Total Radical-Trapping Antioxidant Parameter (TRAP) is a fluorimetric assay (Ghiselli et al., 1995) that monitors the rate of peroxidation induced by thermal decomposition of 2,2-diazobis-(2-amidinopropane)dihydrochloride through the loss of fluorescence of the protein R-phycoerythrin. Also in this assay the lag phase induced by plasma antioxidants is compared with that induced by Trolox.

Chapter 2. The Biomarker Concept: Indicators of Cellular Injury and Protection

Biomarkers

An ideal biomarker should give an unambiguous measure of change in a biological system that is being subject to insult or treatment with agents intended to reduce environmentally induced damage (Greim et al., 1995).

Biomarkers can be divided into 3 categories: of exposure, effect and susceptibility.

Types of monitoring:

1. Environmental monitoring- biomarkers of **potential exposure**.
2. Biological monitoring- biomarkers of exposure ie. **internal dose**, and effects at target sites.
3. Biological effect monitoring- biomarkers of **effect**.
4. Health surveillance- biomarkers of effect and **medical** examination.

Biomarkers of Oxidative Stress

Table 1. A partial list of disease and degenerative states where RS are implicated in the initiation and/or potentiation of the conditions

Ageing	Accumulated tissue damage Alzheimers disease Parkinson's disease Macular degeneration
Alcoholism	
Amylotrophic lateral sclerosis	
Blood	Chronic granulomatous disease Iron overload disorders
Brain	Anoxia Aluminium toxicity Neuronal lipofuscinosis Multiple sclerosis

	<p>Down's syndrome</p> <p>Vitamin E deficiency</p> <p>Hyperbaric oxygen damage in premature infants</p> <p>Hypertensive cerebrovascular injury</p> <p>Post-traumatic injury</p> <p>Accident/surgery sequelae</p> <p>Stroke sequelae</p>
Cancer	<p>GI cancers</p> <p>Hepatocarcinoma</p> <p>Pulmonary carcinomas</p>
Cardiovasculature	<p>Atherosclerosis</p> <p>Cardiovascular</p> <p>Heart disease</p> <p>Cardiomyopathy</p> <p>Keshan disease</p> <p>Adriamycin</p> <p>Cardiomyopathy</p>
Gastrointestinal tract	<p>Crohn's disease</p> <p>Ulcerative colitis</p> <p>Iron overdose</p> <p>Endotoxin insult</p> <p>Combination ascorbate/Fe therapy</p> <p>Pancreatitis</p> <p>Diabetes mellitus</p>
Eye	<p>Photic retinopathy</p> <p>Occular haemorrhage</p>

	Cataractogenesis Retinal degeneration Premature retinopathy
Inflammatory immune/ autoimmune conditions	Rheumatoid arthritis Glomerular nephritis HIV/Aids Vasculitis Autoimmune nephrotic syndromes
Ischaemia/Reperfusion	Myocardial infarction Organ transplantation Circulatory obstruction in crash victims Thrombophlebitis
Kidney	Fe-mediated nephrotoxicity Aminoglycoside toxicity
Lung	Bronchial dysplasia Mineral dust pneumoconiosis Hypoxia Cigarette smoke Emphysema ARDS (adult respiratory distress syndrome)

Table 1., although not an exhaustive list, gives an indication of the importance of free radicals in the aetiology of many degenerative conditions and disease states, and highlights the need for more rigorously conducted experimental dietary studies in humans, where the chosen biomarkers give an unequivocal measure of intermediate end-points related to disease risk.

The use of biological markers of exposure to oxidative stress has increased dramatically over the last few years and together with genetic screening, these approaches could yield more clinically relevant data. The function of dietary anti-oxidants has been discussed recently in several excellent reviews (Hornstra et al., 1998; Diplock et al., 1999; DeZwart et al., 1998; Halliwell, 1999; Hughes, 1999)

Table 2. Putative antioxidants in Tomatoes and their cooked products.

Candidate biomarkers for dietary protection against oxidative stress.

PUTATIVE ANTIOXIDANT	PROPERTIES
Vitamin C	Essential in humans where it performs many functions in addition to its crucial antioxidant role. Has pro-oxidant activity in vitro in the presence of Fe ³⁺ or Cu ²⁺ (Woods et al., 1997)
Lycopene , β-carotene and lutein.	Adequate, dietary-derived body levels associated with reduced risk of cancer and cardiovascular disease, particularly in smokers (Riemersma, 1994). High body levels associated with pure β-carotene supplementation in smokers yielded deleterious effects (ATBC and CARET trials). Non-antioxidant properties may be equally or more important in affecting cell-cell communication (Bertram, 1993), conversion to retinoids (Nikawa et al., 1995), and in modulating membrane fluidity (Havaux, 1998)
Vitamin E	Added as a stabiliser to cooked tomato products, this essential antioxidant protects against heart disease and severe

	deficiency causes neurodegeneration (Traber et al., 1987). In the absence of regenerating antioxidants such as vitamin C and glutathione this vitamin can behave as a pro-oxidant (Stocker, 1999).
Polyphenols and flavonoids quercetin, kaempferol chlorogenic acid and p-coumaric acid	In vitro studies indicate potent antioxidant, metal-chelating, and RS scavenging properties of these plant products (Salah et al., 1995). Can act as pro-oxidants in the presence of Fe ³⁺ or Cu ²⁺ ions (Rahman et al., 1989). Sparse data on absorption and bioavailability but uptake from tea and wine supports a role for these compounds in the protective function of these beverages, and other sources like tomatoes. (Weisburger, 1995; Goldberg, 1995).

Biomarkers of oxidative stress and injury.

The perceived wisdom that antioxidants protect against cancer and cardiovascular diseases is based on the well demonstrated fact that free radicals and RS are constantly produced in the human body. Their removal by endogenous mechanisms and dietary antioxidants is never complete, nor is the repair of damaged cellular components. This supports the notion that these diseases are largely the consequence of long-term exposure to RS which are major contributors to the age-related development of these conditions. It then follows that biomarkers of oxidative damage should be significant indicators of intermediate risk in both conditions. It is well established that free radical damage to LDL-lipids is associated with the development of atherosclerosis, which is a risk factor of myocardial infarction, stroke, and general vascular disease (Steinberg and Lewis, 1997). End products of lipid peroxidation can bind to DNA to yield mutagenic lesions (El Ghissassi et al., 1995), and several RS can react directly with DNA to cause oxidative mutations (Kasai, 1997). It should be noted that radical oxidation of proteins may play a crucial role in the progression of many diseases. However,

the current methodologies for analysing oxidative protein damage *in vivo* have not reached a sufficient degree of sophistication for universal use (Dean et al., 1998).

In this report we wish to make the distinction between biomarkers of cellular insult/protection measured as simple chemical end points (8-oxoG, o-OH Tyr, MDA, HNE etc.) and the more complex biochemical, genetic and histological changes associated with oxidative stress and injury. Chemical biomarkers will be affected to a greater extent than biological end points by the often complex multi-step work up procedures. Biological end points usually involve direct measurement of relevant parameters (gene expression, chromosome damage, tumour regression and ultra-sound plaque measurement etc.) and are perhaps less subject to artifactual variation, and may be more relevant indicators of disease progression. An ideal biomarker should possess the following properties:

1. Highly specific for the process under scrutiny.
2. Identifiable at an early stage in the investigation.
3. Simple and reproducible method(s) of analysis.
4. Obtainable by non or minimally invasive procedures (urine, blood).
5. Low background contamination.
6. Unequivocal response of biomarker to exposure.
7. Proven relationship between the biomarker and modulation in damage/protection.

Chemical Biomarkers

Biomarkers of Lipid Peroxidation.

Development of accurate methods for measuring *in vivo* lipid peroxidation has been even more difficult than developing optimum methods for detection of oxidative DNA damage. The major reason for this is that LDL peroxidation that contributes to atherosclerosis occurs within the vessel wall not in the peripheral circulation (Steinberg and Lewis, 1997). However, since minimally oxidised LDL can escape recognition by the macrophages, and re-enter the circulation, the measurement of ex-vivo LDL peroxidation may be a potentially useful biomarker. It should also be noted that ex-vivo LDL peroxidation studies will not reflect the potential protective role of other antioxidant substances such as vitamin C that may be lost during the LDL isolation procedure. Lipid peroxidation is commonly initiated using Cu^{2+} ion. Although there is evidence to suggest that copper plays a role in lipid peroxidation (Ferns et al., 1997) there is also data indicating that RS can also be involved (Luoma et al., 1998), and the oxidative mechanism may differ between lesions. The demonstration of antioxidant

protection against lipid peroxidation *in vitro* suggests but does not prove the capacity to protect *in vivo*. As a measurement of lipid peroxidation in humans the much used TBARS assay is now largely discredited due to the confounding influence of diet, (Brown et al., 1995) and formation of arachidonic acid metabolic products can be misinterpreted (Stocker, 1999). Also lipid peroxides and their decomposition products including MDA can be absorbed from the diet and aldehydes re-excreted in the urine (Grooveld et al., 1998; Aw, 1998). A more reliable biological biomarker for LDL oxidation *in vivo* may be an assay of circulating antibodies against oxidised LDL (Yla-Herttualla, 1998).

Isoprostanes - The F₂ isoprostanes are specific peroxidation products arising from arachidonic acid residues in lipids (Pratico et al., 1997) and offer promise as specific biomarkers of lipid peroxidation in the human body. Mean levels are raised in conditions associated with oxidative stress (Mallat et al., 1998) and steady state levels in human plasma can be readily detected by sensitive mass spectrometric techniques. Urinary detection of isoprostanes may be a useful non invasive biomarker for whole body lipid peroxidation (Basu, 1998). However, the primary source from which the unmetabolised F₂ isoprostanes in urine originate is not known. They may originate from plasma via filtration in the kidney from formation in the kidney or from a combination of both. Further, a lack of validated assays for isoprostanes other than those based on mass spectrometry has still reduced its general applicability as a potential biomarker. However, several immunological assays have been developed to detect non cyclo-oxygenase derived isoprostane F₂ (Wang et al., 1995).

Breath Alkanes - As common end-products of lipid peroxidation the alkanes present superficially attractive candidates as non-invasive biomarkers for the process and its involvement in disease states.

Ethane and pentane have been commonly used to assess lipid peroxidation in both *in vivo* and *in vitro* studies following the pioneering studies of Riely et al. (1974). Improvements in analytical procedures enabled the measurement of other volatile alkanes such as ethane, propane, n-butane, iso-pentane and iso-butane (Frank et al., 1980). Feeding studies with rats yielded a relationship between the mono and poly unsaturated fatty acids (PUFA) ingested and the alkanes identified in the breath samples. Oxidation of ω -3 PUFA increased ethane excretion, while ω -4 PUFA, ω -6 PUFA, and ω -7 PUFA oxidation yielded increased excretion of propane, pentane, and hexane respectively (Kivits et al., 1981). The PUFA content of liver depends closely on the nature and quantity of PUFA in the diet, and in turn the exhaled alkanes in rats subject to oxidative stress corresponded well with the composition of the liver phospholipids in terms of the fatty acid profile. ω -3 and ω -6 PUFAs are the most abundant

PUFAs in animal and human cell membranes, making ethane and pentane the biomarkers of choice for measuring membrane lipid peroxidation. Numerous studies have been carried out which demonstrated increased alkane exhalation as a result of diseases or disorders which have oxidative stress in their aetiologies, and correlate with many dietary factors and toxicological agents (de Zwart et al., 1999). Although this technique has been widely used over the last decade, there are practical limitations to the methods used. There are significant background levels of both pentane and isoprene in human breath. Pentane is accepted as a measure of lipid peroxidation but the background source of isoprene is, as yet, unknown. Also, because of the similar boiling points the 2 compounds are very difficult to separate (Springfield and Levitt, 1994). However, a new GC method has been developed which can resolve pentane and isoprene (Mendis et al., 1994). It is tempting to speculate on the origin of isoprene and this author would like to suggest that dietary carotenoids might be the source following radical attack and cleavage. If this were to be the case then we have a valuable new biomarker with which to follow the fate of plasma/tissue carotenoids in response to oxidative stress.

Biomarker of Oxidative of DNA Damage

Can we use variation in the steady-state levels of DNA damage as an intermediate measure of potential cancer risk?

RS can attack most biological molecules, but with respect to cancer and ageing DNA is proposed as the major target in the absence of adequate data on the oxidative damage to proteins, which may, in the future be found to have a significant role in controlling the fidelity of polymerases, repair enzymes and gene expression (Dean, 1998).

Several reactive oxygen species can attack DNA directly to create mutagenic oxidation products (Halliwell, 1998). This may contribute significantly to age related development of cancer, therefore, a decrease in such damage should decrease the risk of cancer development. A note of caution, when considering mechanisms of protection is that one must consider that an agent decreasing steady state levels of oxidative DNA damage *in vivo* may do so either by decreasing the rate of oxidative DNA damage and/or by increasing the rates of repair of such damage (Halliwell, 1999).

RS can cause the formation of DNA-protein crosslinks, damage to the sugar phosphate backbone and many specific chemical modifications of the purine and pyrimidine bases. Oxidative base modification can cause mutations, whereas oxidation of the sugar moieties induces base release or DNA strand breaks which can be conveniently analysed in the Comet assay (see later). During the repair process endonucleases and glycosylases excise

oligonucleotides and bases respectively. Bases are excreted directly into the urine whereas the oligonucleotides are further hydrolysed to nucleosides before excretion into the urine. The presence of background levels of a wide variety of oxidised nucleosides in urine indicates that DNA oxidation by RS is occurring constantly under non-pathological conditions. In principle the best analytical approach would be to measure a wide range of DNA base damage products by mass spectrometry to obtain a comprehensive and rigorous identification of the products. However, a major problem with the use of GC-MS is the formation of artefacts by the acidic hydrolysis and derivatisation procedures required by the conventional process (Dizdaroglu, 1998). An obvious choice for non-destructive analysis of products would be LC-MS, but this technique is still in the development phase for DNA oxidation products. Since the development of a simple electrochemical detection method linked to HPLC (Floyd et al., 1986) urinary 8OHdG has become the most widely analysed chemical biomarker for oxidative DNA damage, reviewed in Loft et al. (1998). Limitations of the use of 8OHdG are:

1. It is not a quantitative marker for the oxidative damage to DNA.
2. It is only a minor product of the attack on DNA by reactive nitrogen and chlorine species.
3. The redox state of the cell will effect the ratio of 8OHdG to FAPyG, as will the presence of transition metals (Dizdaroglu, 1998). Theoretically one can measure a wide range of base damage products by mass spectrometry, this would also measure the attack on DNA by reactive nitrogen and chlorine species and would enable a rigorous identification of products, since techniques to overcome the problems of artifactual hydrolysis and derivatisation for GC-MS have recently been overcome.

Biological markers of DNA damage and cancer development

The current most important barrier to progress in the search for cancer biomarkers is the lack of intermediate biomarkers for the major cancers of the lung, breast and pancreas. Significant intermediate biomarkers of full-blown oral and cervical cancers have been identified as the hyperplastic or dysplastic lesions of oral leukoplakia and cervical dysplasia, which identify individuals at high risk for development of these diseases. Fortunately these conditions are reversible by appropriate interventions.

Membrane Damage: a prelude to DNA damage

LDH release is a marker for gross membrane damage resulting in a loss of membrane integrity, it is suggested that over 10% LDH release leads to irreversible changes and cell death (Miyashita et al., 1997). Trypan blue uptake is a measure of membrane

permeabilisation/damage resulting in cell death. Ethidium bromide (EtBr) and acridine orange can be used in conjunction to demonstrate apoptotic as well as cytotoxic events via cellular uptake as a result of membrane damage. EtBr has been used to demonstrate a link between membrane and DNA damage by ROS which was modulated by β -carotene and lycopene (Lowe et al., 1999).

DNA Damage

Intervention studies where DNA damage is an intermediate biomarker can employ gross changes in DNA as an indicator of disease progression.

Comet Assay - The comet assay measures single and double strand breaks, by a single cell gel electrophoresis technique and is a useful indicator of oxidative DNA damage (Gedik et al., 1998). The traditional comet assay has been used in my laboratory to detect DNA strand breaks in HT29 cells challenged with xanthine-xanthine oxidase. Subsequent incubations with β -carotene and lycopene reduced DNA damage at low to physiological levels, but showed a progressive loss of protection with increasing carotenoid concentration (Lowe et al., 1999). The method can be further refined to measure oxidised bases by incubating the ROS stressed cells with endonuclease III and formamidopyrimidine glycosylase for detection of oxidised pyrimidines and purines respectively (Kruszewski et al., 1998). Supplementation with vitamin C, E and β -carotene has been shown to reduce lymphocyte DNA damage in a smoking intervention trial using the modified comet assay (Duthie et al., 1996). Lymphocytes from the supplemented subjects were also resistant to *in vitro* strand breakage induced by H_2O_2 .

A challenging future development of this sensitive method is the application of fluorescent *in-situ* hybridisation (FISH) to investigate the integrity of specific genes within the damaged DNA (McKelvey-Martin et al., 1998).

Micronucleus Assay (MN) - Similar to the comet assay this cytogenetic technique can be used as an intermediate end point in *in vivo* studies. Several laboratories have demonstrated protective effects of β -carotene against X and γ -ray induced MN formation in mice and isolated human lymphocytes (Umegaki et al., 1997. Konopacka et al., 1998) β -carotene was shown to have no effect on spontaneous chromosomal damage in peripheral blood lymphocytes from 30 healthy non smoking human donors, using the MN assay (Odagiri and Uchida, 1998). Xue et al. (1998) revealed interesting differences in MN frequency depending on the carotenoid isomers used, the all *trans* isomer appeared to increase MN formation

whereas natural β -carotene oil (approximately 1 : 1 all *trans* : 9 *cis*) appeared to confer protection. To date there have been no MN studies involving lycopene.

Sister Chromatid Exchange (SCE) - With respect to β -carotene and lycopene the literature involving SCE is sparse or non-existent. Vitamin C and β -carotene have been shown to inhibit H_2O_2 induced SCEs but increase those induced by H_2O_2 and bleomycin (Cozzi et al., 1997). Using a murine bone marrow model β -carotene was found to increase the frequency of SCEs induced by γ -irradiation (0.62Gy) (Morales-Ramirez et al., 1998).

Estimation of *In-situ* Oxidative DNA Damage - A recently developed assay based on the direct binding of a fluorescent probe to the DNA adduct 8oxo guanine has just become available. The probe is applied directly to test cells in an immunohistochemical procedure that has many advantages over current methods and can be adapted to standard fluorescent cell sorter protocols and multi-well plate readers (Struthers et al., 1998).

Chapter 3. Tomato Carotenoids: A Mechanistic Approach to Redox Functions

INTRODUCTION

The tomato contains a complex mixture of nutrients and micronutrients, which interact during digestion and uptake to confer protection to wide variety of tissues, following toxic insults. Attempts to isolate and study the individual components have yet to unequivocally support the apparent benefits of consuming a Mediterranean type diet. The clear health benefits of fruit and vegetable consumption conceal a complex mass of interacting variables. A simplified understanding of these interactions forms the basis of the network philosophy. Although this network focuses on lycopene as the most important tomato component we must be receptive to potentially valuable synergies that may exist between lycopene and the other antioxidants or protective components such as vitamin C, vitamin E, polyphenols, flavonoids and folic acid.

Future studies must seek to establish a unifying theme for the role of dietary protective agents.

MECHANISMS

Gap junctions

Gap junctions are small, narrow hydrophilic pores connecting the cytosol of adjacent cells. These pores allow the intracellular transport of small molecules (<1000 Da). This enables the transfer of nutrients and ions, the transfer of electrical signals and the promotion of effective cell signalling in tissues. The pores close in response to a decrease in pH and a rise in intracellular calcium. Tumour promoters such as the phorbol esters activate protein kinase C and this subsequently causes a decrease in the expression of GAP junctions. In contrast carotenoids and retinoids induce the expression of gap junctions.

Carotenoids and their derivatives vary in size and structure, and this is reflected in their ability to enhance GAP junction expression. It has been noted that natural and synthetic carotenoids bearing a five or six-membered ring system promote an increase in the expression of GAP junctions, whereas open chain polyene compounds had no significant effect upon GAP junction expression (Stahl & Sies, 1998).

The most widely studied six-membered ring carotenoid is β -carotene. This compound can be cleaved by the enzyme 15,15'-dioxygenase to yield retinal. Retinal can be further metabolised to yield retinoic acid. Intracellular receptors (RARX) for retinoic acid and associated products, have been identified. Association of retinoic acid or its analogues to the receptor

promote an up-regulation of the GAP junctions. This mechanism may account for some of the noted increase in GAP, but it is not the whole story.

When lycopene, present in serum or LDL is examined by HPLC, several isomers are present. The majority of the lycopene in tomatoes is in the all-trans state, while that isolated from plasma consists of both trans and cis-isomers. One suggested mechanism of lycopene activity is its oxidation or cleavage to generate compounds of biological activity.

Khachik (1997) identified two oxidative metabolites of lycopene with 5 membered ring end groups. These compounds were identified in both human milk and serum. The metabolites were identified as a pair of 2,6-cyclolycopene-1,5-diols. At present it is not certain if these components arose by in-vivo oxidation of lycopene or if they are of dietary origin. However the biological activity of these compounds is unknown at this present time.

As previously stated β -Carotene has provitamin A activity, and this is synthesised by enzymatic cleavage at the central double bond of carotene. This conversion occurs in the intestinal cells. Another mechanism of that may yield retinoids is eccentric cleavage, at random positions along β -carotene. The autoxidation of β -carotene results in eccentric cleavage of the molecule producing retinal and β -apo-carotenals. This mechanism would suggest that non-provitamin A carotenoids such as lycopene can be cleaved to retinoid like fragments under oxidative conditions in the tissues. In vitro oxidation (Nagao 1999) of lycopene produced γ -retinoic acid, γ -retinal and a series of apo-lycopenals was also identified. Stahl (2000) went one stage further and proposed a biological function for a potential metabolite of eccentric cleavage of lycopene called acyclo-retinoic acid. This study investigated the effect of lycopene and its oxidative cleavage product on gap junction communication in human fetal skin fibroblasts. Lycopene stimulated gap junction communication, while a 10 fold concentration of the acyclolycopene was needed to produce a similar effect. It was concluded that in the case of lycopene, its breakdown to the retinoic acid analogue is of minor importance. Interestingly lycopene itself did not promote gene expression in this study. This would imply that lycopene acts in an independent way to retinoic acid. Could the carotenoid act at the membrane level to increase the level of communication between the cells?

Phase I and phase II enzymes

The chemopreventative properties of carotenoids, particularly β -carotene was thought to be linked with the synthesis of detoxification enzymes particularly cytochrome P450. Studies by

Astorg (1997) indicated that β -carotene itself did not affect the expression of rat cytochrome P450 or phase II enzymes, whereas canthaxanthin and an associated product of β -carotene namely β -apo-8'-carotenal both induced the CYP1A gene in mice. Further work (Gradelet, 1996) revealed that expression of this gene was mediated by activity following the binding of a ligand to a specific intracellular receptor designated AH. Although canthaxanthin and the carotenal were involved in the gene expression they did not bind to the receptor. In a separate study (Astorg, 1997) lycopene was demonstrated to protect against the initiation of preneoplastic foci by diethylnitrosamine treated rats. Lycopene did not appear to act through its antioxidant properties, but rather through its modulating effect on the liver enzyme activating, cytochrome P-450 2E1. It is unclear whether the active component was lycopene or one of its associated products.

The genotoxicity of nitrogen oxide has been shown to be inhibited by carotenoids in short term tests. Supplementation with β -carotene resulted in a significant decrease in inducible nitric oxide synthase in patients with nonatrophic gastritis (Mannick, 1996).

Research on the metabolic activities of both lycopene and β -carotene, suggest that the all trans parent compounds do not exhibit any metabolic activity. However associated products such as β -apo-8'-carotenal and 2,6-cyclolycopene-1,5-diols may prove to be metabolically active in the promotion of GAP junctions and enhancement of protective enzymes such as cytochrome P450.

Carotenoid interactions with free radicals

Lycopene as an antioxidant

In plants carotenoids play a role in helping to quench and prevent the formation of ROS, especially singlet oxygen that is formed during photosynthesis. The importance of this interaction in healthy animals is uncertain. However, singlet oxygen can be formed during the process of lipid peroxidation, and it has also been suggested by Tatsuzawa (1999) that singlet oxygen is also produced during the activation of neutrophils. This is thought to be due to the interaction of hypochlorous acid and hydrogen peroxide. The hypochlorous acid is produced following the activity of myeloperoxidase, which acts upon hydrogen peroxide. This ROS is produced following the activation of neutrophil NADPH oxidase.

Singlet oxygen quenching by carotenoids occurs via physical or chemical mechanisms. The efficiency of the physical quenching greatly exceeds that of the chemical quenching. The former process relies on the transfer of excitation energy from singlet oxygen to the

carotenoid resulting in ground state oxygen and excited triple state carotenoid. The energy is dissipated through rotational and vibrational interactions between the excited carotenoid and the surrounding solvent to yield ground state carotenoid and thermal energy. Isomerisation of the carotenoid may occur during this process. The quenching activity will largely depend upon the number of conjugated double bonds and is influenced to a lesser extent by acyclic or cyclic end-groups. Lycopene has 11 conjugated and 2 non-conjugated double bonds, and is one of the most efficient singlet oxygen quenchers.

Chemical quenching is a minor process but can lead to the decomposition of lycopene to yield 2-methyl-hepten-6-one and apo-6'-lycopenal (Ukai, 1994). The decomposition products may offer some biological activity, but has this any physiological relevance? Bearing in mind the minor importance of singlet oxygen in humans, and the low incidence of the chemical quenching process.

As previously reported lycopene appears to be an excellent quencher of singlet oxygen (Di Mascio, 1989; Conn, 1991) in vitro ($17 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) compared to β -carotene ($13 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). Exposure to sunlight can deplete carotenoid levels in the plasma and skin, and this scavenging mechanism may be important in the eye.

Carotene-oxygen radical interactions have been studied with lycopene and β -carotene. The second order rate constants for the electron transfer from a carotenoid radical anion to oxygen have been determined (Conn, 1992).



For lycopene the rate constant was $2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and for β -carotene it was $25 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. This means that for β -carotene the equilibrium lies to the right and more in favour of the formation of superoxide. For lycopene the reaction is less efficient and electron transfer is observed in both directions. The electron transfer rate constant for lycopene is $1/10^{\text{th}}$ that of β -carotene, and will be important in evaluating their antioxidant properties.

There is some tentative evidence to suggest that lycopene and other carotenoids can also interact with peroxynitrite. Panasenko et al. (2000) demonstrated that lycopene was an effective scavenger of peroxynitrite in a model system employing LDLs.

Lycopene is largely transported around the body bound to LDL particles. The oxidative modification of LDL is thought to be central to several theories accounting for the initiation of atherosclerotic events. Lipid peroxidation is an essential mechanism in LDL oxidation, and it is thought that lycopene and β -carotene play a role in the prevention of lipid peroxidation.

Lipid peroxidation of LDL is thought to be initiated by the presence of the hydroxyl radical or peroxynitrite. The end result is that H is abstracted from a polyunsaturated lipid to form a radical e.g. arachidonate radical. Following a structural reorganisation of the lipid radical and the addition of oxygen, a lipid peroxy is formed.

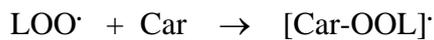


Peroxy radicals can react with carotenoids by one of two mechanisms

a) formation of a carotenoid cation radical



b) Addition reaction



The radical formed following the addition reaction can react with either other peroxy radicals or with oxygen.

The carotenoid cation radical is then thought to be reduced by α -tocopherols in the LDL particle.



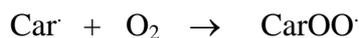
This reaction would be beneficial in two respects in that it terminates an initiation event, and secondly it would optimise the antioxidant capacity of vitamin E. Vitamin E is quite polar and resides towards the periphery of the LDL particle. In contrast the structure of the carotenoid will influence its location within the LDL particle. Lycopene and β -carotene are located within the hydrophobic core of the LDL, and so would be expected to interact with non-polar radicals. While zeaxanthin which contains two distal polar groups is located near the periphery so that it may have greater access to radicals or reactive species that initiate lipid peroxidation (Rice-Evans, 1997).

Isolated human LDL can be oxidised when they are challenged with copper ions. The lipoperoxidation of lipids is usually monitored by the TBARS assay or following the formation of conjugated dienes. Esterbauer (1992) assessed the depletion of carotenoids during this process. In this *in vitro* system there is no regeneration of tocopherol by water soluble antioxidants and as expected the lipid soluble antioxidant was the first to be depleted. The initial carotenoid to be depleted was lycopene, while β -carotene was relatively stable. If the carotenoids are located in a position that makes their interaction with tocopherol unlikely. Then this raises the possibility of a synergistic activity between the carotenoids and the more polar xanthophylls present, prior to the interaction with tocopherol. Indeed Stahl (1998) indicated that an antioxidant synergism between lycopene and lutein exists when multilamellar liposomes were oxidised using AMVN. Again the discussion focused on the orientation of the xanthophyll, with the polar groups being anchored at polar sites in the membrane whilst lycopene resided in the inner part of membranes and retained a substantial degree of mobility.

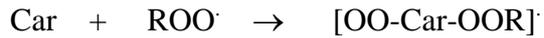
Many groups have investigated the number of antioxidant components present in a LDL particle. It has been estimated that there are 3-15 molecules of α -Tocopherol per LDL particle, whilst β -carotene and lycopene are present at 0.3 and 0.16 molecules respectively. These figures refer to a healthy subject not consuming vitamin E or carotenoid supplements (Esterbauer, 1992). Although lycopene may play a role in protecting LDL against peroxidation, the figures would suggest that carotenoids are not essential. Dietary intervention studies have also yielded conflicting results as reported in chapter 4.

Prooxidant effects

The potential prooxidant effects of the carotenoids depends upon the formation of carotenoid peroxy radical. This specie is easily formed as carbon centred radical and usually very stable due to extensive delocalisation of electrons within the polyene structure. This radical will interact with oxygen to form the peroxy radical.



Alternatively the carotenoid may interact with a lipid peroxy radical to form a carotenoid radical adduct. This may then go on to react with molecular oxygen to yield reactive peroxy radical.



The generation of these carotenoid peroxy radicals could propagate lipid peroxidation within membranes. This prooxidant effect is dependent upon several factors such as oxygen partial pressure, concentration of the carotenoid, synergism with other antioxidants.

Partial Pressure of Oxygen - *In vitro* studies indicate that at low oxygen tensions, carotenoids behave as a chain breaking antioxidants whilst at higher tensions they exhibit prooxidant behaviour. This was demonstrated by Hill when β -carotene was reacted with a trichloroperoxy radical. At low partial pressures of oxygen only electron transfer occurred to produce a carotenoid cation radical. When the same reaction was repeated in air and oxygen saturated solutions a radical cation and a second species [CCl₃OO-car] were detected.

Following the outcome of several recent intervention trials, β -carotene (as a supplement) has attracted some adverse publicity in its role as a cancer chemopreventive agent. The Alpha Tocopherol, Beta-Carotene cancer prevention study (ATBC) indicated that male Finnish smokers who received β -carotene had an 18% increase in the incidence of lung cancer. This outcome was confirmed during the US Carotene and Retinol Efficiency Trial (CARET) study, in which a group of smokers receiving the combination of β -carotene and retinol had a 28% increase in the incidence of lung cancer, compared to the control group. In addition, the large U.S. Physicians Health Study indicated that there was no overall effect of supplemental β -carotene on lung or other cancers.

This raises a dilemma in that a substantial body of evidence indicates that dietary carotenoids are important in reducing the incidence of certain cancers, whilst supplements of an individual carotenoid demonstrated apparent prooxidant activity increasing cellular damage.

There is an uneven distribution of oxygen partial pressures in the human body, e.g. 100 mmHg at the alveoli in the lung, approximately 40 mmHg in venous blood and as low as 5-15 mmHg in the tissues. However, *in vitro* studies regarding the antioxidant nature of the carotenoids are normally performed under atmospheric partial pressures of oxygen, which would promote the prooxidant nature of the carotenoids (see also chapter 4). Clearly this is not representative of the *in vivo* situation. In the light of the CARET study this is of particular importance, as the increased partial pressure of oxygen at the lung surface would have potentiated the pro-oxidant effect of the carotenoid in smokers.

Carotenoid Concentration and Interaction with Other Antioxidants - Many dietary intervention studies that have employed high concentrations of carotenoids, failed to report any effects. This has also been reported for other antioxidants such as vitamin E. Killion and colleagues (1996) reported that patients suffering atherosclerosis exhibited altered levels of vitamin E. Their data also demonstrates a strong association between tissue vitamin E levels and tissue lipid peroxidation. Similar results were obtained when rats were fed with large amounts of β -carotene, increased concentrations of peroxides were found in the rat plasma and liver.

Any effective synergism between the carotenoids, xanthophylls, tocopherols and other co-oxidants would depend upon a balance between all of these components. An increase in carotenoid concentration could disturb this balance and promote lipid peroxidation. The induction of lipid peroxidation would generate carotenoid radical adducts. The prevention of any prooxidant activity would therefore depend upon the ability of tocopherols to reduce the radicals, and the regeneration of the tocopherols by water soluble antioxidants. Therefore an increase in the carotenoid concentration or a decrease in associated antioxidants would enhance the probability of prooxidant activity.

Another untoward effect of an increased concentration of carotenoids is the destabilisation of plasma membranes. van De Ven (1984) and colleagues showed that when β -carotene was incorporated into phosphatidylcholine membranes, the orientation of the carotenoid was dependent upon the composition of the lipid bilayers. In the case of dioleoyl lecithin bilayers the carotenoids had a parallel orientation and a perpendicular orientation was observed in a soybean lecithin lamellar membranes. The addition of β -carotene to phosphatidylcholine membranes decreased the rigidity of the membrane by increasing the motional freedom of the polar lipid headgroups. β -Carotene is distributed homogeneously with a membrane with no preferred orientation, thus its effect is to decrease the rigidity of the membrane. Whereas, xanthophylls would behave as 'gate posts' spanning the entire membrane bilayer. This rigidifying effect would decrease the penetration of oxygen in the lipid bilayer. This in turn would then limit the extent of lipid peroxidation.

Recently lutein and zeaxanthin were incorporated by Sujak (1999) into phosphatidylcholine liposomes. The liposomes were then challenged with either UV or AAPH (2,2'-azobis(2-methylpropionamide)dihydrochloride). The conclusions of this study indicated that there was a decreased penetration barrier for the oxidant AAPH, and the xanthophylls acted by free radical scavenging mechanisms to protect the liposomes against oxidative damage.

In a recent study (Lowe, 1999) adenocarcinoma HT29 cells were supplemented with either β -carotene or lycopene. The cells were then challenged with superoxide and hydrogen peroxide generated by xanthine/xanthine oxidase. Cellular damage was assessed using ethidium bromide to monitor insult to the plasma membrane, whilst the comet assay was used to assess DNA single strand breaks. Low concentrations ($1\mu\text{M}$) of carotenoid afforded protection to the cells. This may reflect the efficiency of the carotenoid to scavenge radicals in the depth of the membrane and prevent the onset of lipid peroxidation. When greater concentrations were employed ($> 4\mu\text{M}$) the membrane would be expected to become more fluidic and allow greater oxidant damage to occur. Following free radical challenge the cells were not necrotic nor did they release significant amounts of LDH, but ethidium bromide uptake was increased and this indicated that the cells had indeed become permeabilised particularly when higher doses of carotenoids were used. This would allow the uptake of small molecules in a non-regulated manner, therefore exacerbating further ROS damage to the cell.

Another protective mechanism by which either lycopene and β -carotene may reduce the incidence of atherosclerosis is by the reduction of circulating plasma cholesterol. Work by Fuhrman (1997) suggests this may be achieved by the carotenoids regulating HMGCoA reductase expression. This inhibition is believed to occur by a post transcriptional mechanism (Moreno, 1995).

Synergy of carotenoids with xanthophylls and other antioxidants.

A healthy diet of fruit and vegetables results in approximately 19 different carotenes and xanthophylls being present in human blood and tissues. These and other carotenoids in the diet have quite different structures and therefore different biological properties. The structure and properties of carotenes and xanthophylls determines their location and orientation in the plasma membrane, so that carotenes are embedded deep in the hydrophobic core whilst the more polar xanthophylls span the membrane (Subczynski, 1992; Gabrielska, 1996). This not only will affect the properties of the membrane itself but also the possible interaction of these molecules with other antioxidants and indeed different ROS. A combination of carotenes and xanthophylls in the plasma membrane may provide optimal protection against free radicals, for example the carotene lycopene and the xanthophyll lutein act in a synergistic manner to protect liposomes against oxidative damage (Stahl, 1998).

There is an increasing interest in flavonoids as dietary antioxidants, but little is known of their *in vivo* mechanisms or their metabolites. Work by Pietta (1998) suggests that flavonoids may

act as antioxidants, with regard to hydroxyl radicals. The resulting flavonoid radical may then be regenerated by its interaction with either ascorbate or urate. This activity may also be extended to the regeneration of tocopheryl radicals. The overall activity of the flavonoids in this study was to preserve carotenoids and vitamin E levels. This would be beneficial in the case of LDL oxidation, and the onset of atherosclerosis.

Other components

Folate - Folate acts as methyl donor and also prevents hypomethylation which is an early step in the colon carcinogenesis (Mouzas, 1998). Folate deficiency has been associated with a variety of human malignancies including colon cancer. In a recent study, 24 chronic ulcerative colitis patients were administered folate or a placebo. After three months of therapy the patients receiving folate demonstrated a reduction in proliferative changes whereas no change was observed in the placebo group. This finding suggests that folate supplementation may contribute to the regulation of rectal cell proliferation.

Olive oil is an important feature of the mediteranean diet. It has been recently shown that two phenolic components of olive oil have the ability to scavenge high concentrations of HOCl (Visioli 1998). Along with flavonoids the presence of polyphenols may act as antioxidants and thus supplement those that are derived from the tomato.

Chapter 4. Human Intervention Studies with Tomato Components

EFFECT OF TOMATO AND/OR LYCOPENE ON OXIDATIVE STRESS

Most of the literature on tomato refers to tomato and/or lycopene effects on different biomarkers linked to oxidative stress, and involves *in vitro* and animal studies; human studies are far from exhaustive. In general we have more information on lycopene than on the tomato itself.

Intervention trials are generally carried out on small groups of subjects and mainly scheduled to investigate bioavailability of lycopene from different tomato sources. In contrast there are very few human studies trying to support or confirm the effect of lycopene and tomato consumption on health.

The main biomarkers investigated are related to lipid peroxidation and DNA damage, but limited information is available on total antioxidant capacity of plasma, protein oxidation, and immune function. The methodologies to study such parameters generally involve *ex vivo* oxidative treatments. Actually, in some cases, *ex vivo* supplementation is preferred to *in vivo* supplementation with the disadvantage that results can only be referred to as the potential effect of a “non-physiological” enrichment and can not be related to the role of the tomato.

Lipid peroxidation

The results are still controversial, and there are no unequivocal conclusions on the possible protective role of tomato/lycopene against lipid oxidation. Several limitations in the methods for the quantification of lipid damage have to be considered (e.g. TBARS as previously reported).

It has been reported that subjects consuming a lycopene free diet had higher lipid peroxidation than those on their habitual diet (Rao and Argawal, 1998a), and that after an oxidative stress deriving from both the ingestion of a meal or a glucose solution and from cigarette smoking, serum lycopene concentration decreased.

Enrichment of LDL with lycopene was demonstrated to reduce their susceptibility to metal ion-dependent (CuSO_4) or -independent (2,2-azobis, 2,4-dimethyl-valeronitril) oxidation; this was selective of LDLs with high vitamin E content; this was selective to LDLs with high vitamin E content. A synergistic effect was confirmed as the combination of carotenoids together with vitamin E increased protection against LDL oxidation (Fuhrman et al, 1997). In contrast no effect was seen by Romanchick et al. (1997) as lycopene was destroyed very

quickly before the formation of lipid peroxidation products.

Sutherland et al. (1999) found no effect of tomato juice supplementation (2 x 200 mL/day) for 4 weeks on TBARS, FLOP (fluorescence index of lipid oxidation product) and the resistance of LDL to oxidation in patients with kidney graft. This, despite a doubling of plasma lycopene concentration after the treatment.

A significant decrease in lipid oxidation evaluated by TBARS (Rao and Agarwal, 1998b) and LDL oxidation evaluated by TBARS and conjugated dienes (Agarwal and Rao, 1998) was seen after the supplementation for 1 week with tomato juice (about 50 mg/day lycopene), spaghetti sauce (about 39 mg/day lycopene) or tomato oleoresin (about 75 mg/day lycopene). This decrease was not shown by Steinberg and Chait (1998) who evaluated lipid peroxidation by breath pentane excretion after tomato juice supplementation. It should be noted that some of these effects may be due to vitamin E which is present as an additive in the spaghetti sauces and oleoresin products.

DNA DAMAGE

Several studies support an antioxidant role for lycopene and/or tomato consumption on DNA protection from oxidative damage. Even if still insufficient to extrapolate conclusive remarks, a few intervention studies have been conducted with the aim to evaluate both the effect of lycopene on endogenous and exogenous DNA damage. Thus the potential to protect against "basal" and/or "induced" oxidative stress is considered.

The supplementation daily for 2 weeks with tomato juice (330 ml, 40 mg lycopene), but also carrots and spinach, was shown to decrease the levels of endogenous strand breaks in lymphocyte DNA evaluated with the comet assay, while after the *ex vivo* oxidative treatment with H₂O₂ only the carrot intervention was effective (Pool-Zobel et al., 1997). Subsequently the same authors suggested that the reduced genetic DNA damage in lymphocytes could be attributed to the enhancement of cytosolic GSTP1, and DNA repair proteins by tomato and carrot juices (Pool-Zobel et al., 1998).

Lower endogenous lymphocyte DNA oxidation evaluated by 8-OHdG was also reported by Rao and Agarwal (1998b) after the daily consumption for 1 week of two types of spaghetti sauces, a tomato juice and a lycopene rich oleoresin from tomato.

More recently it has been reported that also the consumption of a single serving of tomatoes was sufficient to decrease the level of the mutagenic oxidized purine base 8-OHG. If these results were confirmed there would be support for the ability of tomato in modulating oxidative damage (Rehman et al., 1999).

Further evidence on tomato/lycopene action have been reported by Riso et al. (1999) who demonstrated a significant reduction in DNA damage (evaluated by the comet assay) of lymphocytes challenged *ex vivo* with H₂O₂ after the consumption of both 60 g tomato puree (about 16.5 mg lycopene) for 3 weeks, and 25 g tomato puree (about 7 mg lycopene) for 2 weeks (Porrini and Riso, 2000). They also found an inverse relation between DNA damage and plasma and lymphocyte lycopene concentration (Porrini and Riso, 2000), supporting the hypothesis of a consistent contribution of lycopene on cell protection from the oxidative damage but not excluding the contribution of other tomato antioxidants. This inverse relationship was also demonstrated by the cell culture group in this Network.

Protein oxidation

More research is needed to investigate the possible effect of lycopene and tomato products on the prevention of protein oxidation as few data are available. Rao and Agarwal (1998b) found a trend towards lower protein oxidation estimated by measuring the loss of reduced thiol groups, after 1 week consumption of different tomato based products, as previously reported, however this decrease was not significant.

Immunomodulation

The first site of extracellular ROS attack on the cell is the poly unsaturated fatty acid-rich cell membrane which is very susceptible to oxidative stress. Lipid peroxidation alters membrane fluidity (Baker and Meydani, 1994) disrupts membrane integrity and modulates cell function, signal transduction and phagocyte response, to compromise all aspects of the immune system. Watzl et al. (1999) recently demonstrated that 2 weeks of tomato juice consumption significantly enhanced IL-2 and IL-4 secretion but not lymphocyte proliferation compared with values obtained after a low carotenoid diet. However the period of depletion itself suppressed cytokine secretion and cell proliferation, supporting an effect of restoring the original condition more than a specific or unique effect of tomato consumption.

Plasma antioxidant capacity and other protective effect

The total peroxy radical trapping potential of plasma (TRAP), as an index of antioxidant capacity, should provide indication of the modification in reactivity due to increased plasma antioxidants following an intervention with tomato products. However different authors suggest that TRAP can not be appropriate for studying the *in vivo* effect of the consumption of lipophilic antioxidant rich foods on plasma antioxidant capacity.

No literature reports any change in TRAP levels in response to daily consumption of tomato products providing different quantities of lycopene (Steinberg and Chait, 1998; Böhm and Bitsch, 1999; Pellegrini et al., 2000). However, trials by Rock et al. (1992), using subjects on self-selected tomato sauce-rich diets assessed uptake, bioavailability and half-life of blood carotenoids and yielded some useful, indirect evidence that lycopene may function as a plasma antioxidant. Lycopene repletion following a diet rich in pasta sauce stabilized at approximately 1 μM . After 7 days on a tomato/carotenoid free diet the lycopene levels had declined to around 100nM. Over the same period the levels of β -carotene and lutein had fallen from 200nM to 150nM. These interesting observations indicate that lycopene may possibly have a role as a sacrificial antioxidant by virtue of its increased sensitivity to oxygen and ROS/RNS as compared to the other carotenoids, thus sparing them to mediate their known antioxidant and other protective functions. Other results exist suggesting a role of lycopene from the diet in saving other antioxidant compounds and preventing tissue damage *in vivo*. Ribaya-Mercado et al. (1995) hypothesized that lycopene may protect other carotenoids and antioxidant substances as, in a human study, up to 46 % reduction in skin lycopene was observed after a single exposure to a dose of solar simulated light. This seems to suggest a role of this carotenoid in mitigating oxidative damage in tissues. Furthermore the exposure of human plasma to the gas phase of cigarette smoke produced the disappearance of most of the lipophilic compounds and firstly lycopene (Handelman et al., 1996). At this regard Clinton et al. (1996) and Rao et al. (1999) hypothesized a role of lycopene in prostate tissue, however more research is needed to clarify this action.

POTENTIAL CONTRIBUTION OF OTHER COMPOUNDS PRESENT IN TOMATO

Present research on the effect of tomato products against oxidative stress is focusing on the possible interaction among all the antioxidant compounds present in this food. It is argued that antioxidants such as β -carotene, vitamin C, vitamin E might work in synergy with lycopene and this would better explain the healthy effect of tomato consumption. These compounds are present in little and variable amount depending on the cultivar and/or the processing of tomato, however it is not excluded that tomato products may contribute significantly to their intake and, consequently, to the antioxidant protection.

Vitamin C, E and β -carotene have been all suggested as protective compounds in relation to lipid peroxidation and DNA damage. Many *in vitro* and *in vivo* studies have been conducted

as demonstrated by the consistent literature. As regards human intervention studies, generally supplements at high concentrations are used rather than dietary doses or rich foods. Thus definitive conclusions can not be drawn without considering the limits of interpretation of such works. However the most relevant literature on the evidence on vitamin E, vitamin C, and β -carotene against the major biomarkers of oxidative stress (lipid peroxidation and DNA damage) are reported.

Lipid peroxidation

Vitamin E - The most consistent data with respect to micronutrient antioxidants and atherosclerosis appear to relate to α -tocopherol, which is the quantitatively most important antioxidant present in LDLs, followed by retinyl stearate, γ -tocopherol, β -carotene, and lycopene (Esterbauer et al., 1990).

There is good evidence that α -tocopherol has significant protective effect against LDL oxidation. Apart from *in vitro* studies, several *in vivo* studies demonstrated that vitamin E supplementation increased the resistance of LDLs to oxidative modification, nevertheless the doses used were generally very high, also higher than those usually contained in α -tocopherol supplements. Furthermore, in many studies small numbers of subjects were supplemented and most had no randomized control groups.

Supplementation with 1200-1600 mg/day vitamin E led to a \approx 50% decrease in susceptibility of LDL to oxidation (Reaven et al., 1993; Princen et al., 1992; Reaven and Witztum, 1993; Fuller et al., 1998; Reaven et al., 1996; Dieber-Rotheneder et al., 1991).

A significant effect was reported also using lower amounts (Princen et al., 1992; Dieber-Rotheneder et al., 1991; Jialal and Grundy, 1992; Astley et al., 1999; Simons et al., 1996; Suzukava et al., 1995), however always greatly exceeding the RDA. 400 IU/day α -tocopherol for 8 weeks (Marangon et al., 1999) prolonged LDL lag time of lipid peroxide formation and conjugated dienes after copper-catalyzed LDL oxidation, decreased urinary F_2 -isoprostanes. The supplementation of 50 smokers with 280 mg α -tocopherol acetate daily for 10 weeks determined a reduction of plasma concentrations of lipid peroxides, thiobarbituric acid reactive substances and conjugated dienes (Brown et al., 1994). There was also a significant reduction in erythrocyte lipid peroxidation. In a similar study (Porkkala-Sarataho et al., 1998) 200 mg α -tocopheryl acetate/day elevated oxidation resistance of VLDL+LDL, prolonging the lag time by 34% when assessed with a copper-induced method and by 109% when assessed with a hemin + hydrogen peroxide-induced method. The same dose (200 mg/day)

had no effect in the study by Abbey et al. (1993). Supplements as low as 100 mg α -tocopheryl acetate/day were able to increase the resistance of LDL to oxidation when administered together with 15 g fish oil to 48 postmenopausal women both using and not using hormone-replacement therapy (Wander et al., 1996).

Few attempts have been done to ascertain the minimum dose of α -tocopherol that would decrease the susceptibility of LDL to oxidation. Dieber-Rotheneder et al. (1991) were the first, giving daily dosages of 150, 225, 800, or 1200 IU α -tocopherol for 21 days to 8 subjects (plus 4 placebo). They found that the oxidation resistance of LDL was higher during vitamin E supplementation, but they used only 2 subjects in each group and could not undertake statistical analyses. Jialal et al. (1995) made a dose-response study and evaluated the effect of the supplementation of healthy volunteers with 60, 200, 400, 800 and 1200 IU/day for 8 wk on copper-catalysed LDL oxidation, measuring the formation of conjugated dienes and lipid peroxides by TBARS. All doses resulted in significant increases in plasma and LDL concentrations of α -tocopherol, however the susceptibility of LDL to oxidation decreased only at doses \geq 400 IU/day. Princen et al. (1995) evaluated the effect of 25, 50, 100, 200, 400 and 800 IU/day α -tocopherol acetate in 20 healthy volunteers during six 2-week periods, and found that the resistance of LDL to oxidation was elevated dose-dependently (+28% after the last period) and differed significantly from the baseline resistance time even after ingestion of only 25 IU/day. However the progression of lipid peroxidation in LDL was reduced only after intake of 400 or 800 IU/day. The authors suggested that only at high dosages α -tocopherol becomes incorporated into the interior of the LDL particle in sufficiently amounts to retard the autocatalytic chain reaction of the propagation phase.

In many of the papers reported, a correlation was found between oxidative resistance and LDL- α -tocopherol concentration, however the correlation becomes weaker in unsupplemented subjects (Frei and Gaziano, 1993; Dieber-Rotheneder, 1991).

From these data it seems that the interest in the role of α -tocopherol in protecting LDL from oxidation is well documented. However, the examination of all data reported in literature, and in particular of data regarding the profile of lipid hydroperoxide (LOOH) accumulation in LDL oxidation, reveals that α -tocopherol can also be prooxidative. For example, it has been shown that supplementation of LDL with α -tocopherol increases the initial rate of lipid peroxidation; the rate of lipid peroxidation is higher in the presence of α -tocopherol than immediately after its depletion; the maximal rate of lipid peroxidation in the presence of the vitamin is independent from the rate of initiation of lipid peroxidation (Bowry and Stocker,

1993; Neuzil et al., 1997; Ingold et al., 1993; Witting et al., 1995; Witting et al., 1997). Consequently the characteristic of lipid peroxidation cannot be explained by the conventional antioxidant action of α -tocopherol (chain-breaking antioxidant). These discrepancies may be explained by the tocopherol-mediated peroxidation (TMP) model (Bowry and Stocker, 1993) which has been recently deeply explored and discussed (Upston et al., 1999). TMP is a general model for radical-induced lipoprotein oxidation and its prevention by coantioxidants; this could explain why vitamin E together with coantioxidants may be superior in protecting lipoproteins from oxidation (Upston et al., 1999). It should also be noted that α -tocopherol has additional metabolic roles, such as the regulation of smooth muscle cell proliferation (Azzi et al., 1999) which may complicate any assessment of its antioxidant function. The non-antioxidant effects of Vitamin E take place at the level of cell signalling and gene expression. Data suggest the existence of a ligand/receptor type of mechanism, where oxidant stress causes a loss of antioxidant molecules such as vitamin E, which at 10-50 μ M specifically increases protein phosphatase 2A1 activity. This activation is followed by PKC α dephosphorylation and by a decrease in PKC activity. This vitamin E downregulation of PKC activity modulates the expression of collagenase MMP-I and α -Tropomyosin. This same mechanism has also been demonstrated to prevent cell adhesion, to inhibit platelet aggregation, to prevent smooth muscle cell proliferation and to inhibit the PKC dependent oxygen burst (Azzi et al., 1999).

β -carotene - The "oxidative hypothesis of atherosclerosis" raises the possibility that also β -carotene may prevent or delay the progression of atherosclerosis. To support this hypothesis several observational, prospective cohort studies showing a risk reduction associated with the ingestion of foods rich in β -carotene have been advocated. However, the effects of β -carotene on LDL oxidation are not so evident. Supplementation of humans with β -carotene does not result in increased resistance of plasma-derived LDL to *ex vivo* oxidation (Reaven et al., 1993; Princen et al., 1992; Gaziano et al., 1995; Reaven et al., 1994). As in all these studies LDL was incubated under ambient pO₂ (approximately 150 mm hg), while it is known that β -carotene acts as an efficient antioxidant only at low physiological pO₂, Reaven et al. (1994) addressed this question by incubating LDL from β -carotene supplemented subjects with Cu²⁺ at the lower oxygen tensions typically present in the artery wall (35 mm hg and 15 mm hg); no increased resistance to oxidation of β -carotene-enriched LDL compared to control LDL was observed under all incubation conditions. Similar results were obtained *in vitro* by Hatta

and Frei (1995). All together these results do not suggest significant protection. Consequently, the protective effects of β -carotene against cardiovascular disease are most likely not mediated by increased protection of LDL against oxidation in the arterial wall. Alternative mechanisms, such as the reduced capacity of arterial wall cells to modify LDL, preservation of endothelial vasodilator function, and increased serum HDL levels, have been suggested (Hatta and Frei, 1995).

Vitamin C - Ascorbate is not carried within the LDL particle because of its high hydrophilicity, consequently there are other possible ways in which it may reduce LDL oxidative susceptibility (Frei et al., 1996): 1) by reducing preformed lipid hydroperoxides and preventing propagation of lipid peroxidation in LDL; 2) by scavenging of radicals and oxidants in the aqueous phase; 3) by regenerating the endogenous antioxidants (α -tocopherol, β -carotene) from oxidation; 4) by modification of histidine residues and other copper-binding sites on apo B (by dehydroascorbate or another oxidation product). Ascorbate has been shown to protect LDL from oxidation *in vitro* (Jialal and Grundy, 1991; Frei B, 1991; Frei et al., 1996), however results obtained by *in vivo* studies are conflicting and difficult to compare due to differences in the experimental protocol. Fuller et al. (1996) and Wen et al. (1997) performed a similar study supplementing smokers with 1000 mg ascorbic acid for 4 weeks and studying the resistance of LDL to copper induced oxidation compared to a control group. Fuller et al. (1996) found that the ascorbate-supplemented group had a significant reduction in LDL oxidative susceptibility as measured by thiobarbituric acid-reactive substances (TBARS) and the formation of conjugated dienes; Wen et al. (1997) found no effect on the lag phase of conjugated diene production or TBARS formation to copper induced oxidation, while plasma lipid peroxides measured as malondialdehyde were reduced significantly in the vitamin C supplemented group. This could be explained considering that a relatively large amount of free radicals could be scavenged by high plasma ascorbate concentrations, confirming that vitamin C is more effective in the aqueous phase. However, in the Fuller et al., study subjects followed a low ascorbate diet for 2 weeks before supplementation and this could have modified the baseline values of LDL oxidizability.

Short-term supplementation with vitamin C (1 - 1.5 g/day) for 2 weeks showed a protective effect on *in vivo* oxidation of LDL after smoking as measured by the content of TBARS in isolated LDL (Harats et al., 1990). Furthermore an increase in the lag period of LDL oxidation was evidenced (Harats et al., 1998) after a dietary enrichment with citrus fruit

(corresponding to 500 mg ascorbic acid) for 2 months. Sanchez-Quesada et al. (1998) demonstrated that oral supplementation with 1 g ascorbic acid protects LDL from the increased susceptibility to oxidation that occurs after intense aerobic exercise. This inhibition had been attributed to the antioxidant role of ascorbic acid in the seeding of LDL with hydroperoxide, and to the regeneration of oxidized α -tocopherol contained in LDL after its exercise-induced consumption.

Combined supplementation - Whether the capacity of each single antioxidant to reduce susceptibility of LDL to oxidation is additive when antioxidants are given in combined supplementation, has not been determined in depth. Jialal and Grundy (1993) studied the effect of combined supplementation with α -tocopherol (800 IU/day) plus ascorbate (1.0 g/day) and β -carotene (30 mg/day) on copper-catalysed LDL oxidation in a randomised, placebo-controlled study over a 3-month period, and found that the combined supplementation resulted in a twofold prolongation of the lag phase and a 40% decrease in the oxidation rate. The combined antioxidant group was also compared with a group that received 800 IU of α -tocopherol only, but no significant differences between the two groups with respect to LDL oxidation kinetics were observed. In a similar study with a daily supplement of 18 mg β -carotene, 900 mg vitamin C and 200 mg vitamin E over a 6-month period, Abbey et al. (1993) found a lengthened lag time before the onset of oxidation after antioxidant supplementation (28% after 3 months and 35% after 6 months), but no difference in the rate of oxidation. In addition there was a significant correlation between prolongation of the lag phase and LDL α -tocopherol content, but no correlation with β -carotene content. Mackness et al. (1993) supplemented 16 volunteers with selenium-ACE tablets (200 mg selenium, 18 mg β -carotene, 180 mg vitamin C and 74 mg vitamin E daily) for 20 days and provided further evidence that these substances can protect isolated LDL against Cu^{2+} catalysed oxidation. However, as in their experiments antioxidant supplements produced significant decreases in the rate of conjugated diene formation but had no effect on the subsequent generation of lipid peroxides, they suggested to be cautious in increasing antioxidant vitamin intake in order to make LDL in the artery wall more resistant to oxidation. It seems therefore that natural antioxidants such as vitamin E, are rapidly exhausted and may offer little long-term protection. In contrast, Nyssönen et al. (1994) supplemented 40 smokers with 400 mg ascorbic acid, 100 μg organic selenium, 200 mg α -tocopherol, 30 mg β -carotene and found a 27% increase in lag time to oxidation of VLDL+LDL: the increase in the oxidation lag time

resulted correlated with VLDL+LDL α -tocopherol ($r=0.72$) and β -carotene ($r=0.80$) concentration. The authors concluded that the supplementation with antioxidant vitamins and selenium increases the oxidation resistance of atherogenic lipoproteins in human plasma, and suggested that when α -tocopherol and β -carotene concentrations in plasma and lipoproteins are elevated to higher levels than usually achievable by diets, the plasma and lipoprotein status of the major dietary antioxidants almost entirely determines the oxidation resistance of VLDL and LDL.

Parfitt et al. (1994) found that plasma lipid peroxidation (conjugated dienes and lipid peroxides) was significantly lower in a group of healthy young persons from Naples with respect to a group from Bristol and this was related to a higher consumption of fresh tomatoes, olive oil and vitamin E.

Recently Steinberg and Chait (1998) investigated the effect on smokers of daily consumption of a tomato-based juice supplemented with vitamin C (600 mg), vitamin E (400 mg) and β -carotene (30 mg) on various indexes of lipid peroxidation (breath pentane excretion and susceptibility of LDL to copper-mediated oxidation). Compared with the results obtained with the placebo juice, the vitamin-supplemented juice resulted in an increased protection against lipid peroxidation. Furthermore, the increased concentration of plasma vitamin C, β -carotene and lycopene correlated significantly with the conjugated diene lag phase. These results suggest the importance of the concomitant presence of these antioxidants supporting the hypothesis of a mutual interaction.

DNA Damage

The antioxidant activity of compounds such as vitamin C, E and β -carotene in relation to DNA damage has been studied more than that of lycopene. Beyond the evaluation of the oxidized base 8-OHdG and the single strand breaks by the comet assay, DNA damage has been also investigated by means of bleomycin-induced chromosomal damage assay, nucleoid sedimentation, post-labelling, micronucleus assay and sister chromatid exchange.

Vitamin E - The association of dietary or plasma vitamin E concentrations and mutagen sensitivity in healthy populations has not been demonstrated.

High intake of vitamin E has been associated with reduced risk for cancer (Bostik et al., 1993; Salonen et al., 1985), however it is not known whether it can exert its effect directly by inhibiting the initiating and/or later stage of cancer or by enhancing the immune response

(Meydani and Tengerdy, 1993; Harapanhalli et al., 1994). For this reason the effect of vitamin E supplementation above normal dietary intake was tested with respect to genetic damage rate in human lymphocytes (Fenech et al., 1997). Despite the increase in plasma vitamin E concentration after the intake of 50 and 300 mg for 8 weeks, there was no apparent relation between plasma vitamin E and endogenous or exogenous (exposure to hydrogen peroxide) chromosome damage (micronuclei frequency) in healthy individuals. Also Goodman et al. (1998) found the same results evaluating the bleomycin-induced chromosomal damage after the supplementation with 400 IU α -tocopherol twice daily for 6 weeks. Higher doses (2 x 400 IU vitamin E for 6 weeks) did not reduce DNA damage evaluated in Peripheral Blood Leukocytes treated with H₂O₂ (Brennan, 1996). Hartmann et al. (1995) demonstrated that high doses of vitamin E (1200 mg /daily for 14 days) can prevent exercise-induced DNA damage while high doses of a multivitamin mixture increased the baseline level of DNA damage probably because of the large amount of trace metals also present.

It has been reported that dietary modification can affect DNA oxidative damage in healthy humans. For example the oxidation of cellular membranes (e.g. increased PUFA intake) has been hypothesized as an important aspect of the mechanism for DNA damage, thus compounds such as vitamin E that is a lipid peroxidation chain-breaking antioxidant in cell membranes may provide protection.

Recently a crossover design consisting in a solid diet (5 days) followed by 10 days of consumption of two different nutritionally complete liquid formula with low PUFA/high vitamin E (ratio: 1.1) or high PUFA/low vitamin E (ratio: 3.5) was used to verify whether this intervention could be related with a decrease in 8OHdG/dG. The results obtained were similar for both the formulas however the diet modification involved a 22% decrease in the ratio supporting the hypothesis that modest dietary changes can improve protection from DNA damage (Chen et al., 1999).

Jenkinson et al. (1999) also reported that a 15% increase in dietary PUFA determined higher endogenous and exogenous DNA damage that was reduced by adding 80 mg α -tocopherol/day in the diet, supporting the hypothesis that vitamin E may protect from DNA damage through the inhibition of membrane lipid peroxidation.

β -carotene - Several human intervention studies have been performed to investigate the potential protective effect of this carotenoid against some type of cancer, however the results

were not univocal and promising (ATBC, 1994; Omenn et al., 1996; Hennekens et al., 1996). Also the specific role of β -carotene in preventing DNA damage which is the base of cancer development has been investigated, but mainly using supplements more than foods. Pool-Zobel et al. (1997) found an effect of carrot juice intervention (330 ml, 22.3 mg β -carotene and 15.7 mg α -carotene for 2 weeks) on endogenous and exogenous levels of strand breaks in lymphocyte DNA and oxidative base damage.

The supplementation with 180 mg/week of β -carotene for 9 weeks was found to reduce the frequency of micronucleated exfoliated cells from the buccal mucosa of tobacco and areca-nut chewers (Stich et al., 1984). The same effect was found for vitamin A but not cantaxanthin suggesting that the inhibitory effect on the formation of micronuclei was due to a mechanism not involving the scavenging of free radicals. Van Poppel et al. (1992) giving 20 mg/day β -carotene to heavy smokers found a reduction of micronuclei in sputum, and hypothesized a role of β -carotene in preventing DNA damage in early stage of lung carcinogenesis. It has been also reported (Umegaki et al., 1994) that the supplementation with 30 mg β -carotene for 13 days was able to protect human lymphocytes from x-ray induced genetic damage (induction of micronuclei formation).

On the contrary the supplementation with 15 mg β -carotene twice daily for 6 weeks did not affect mutagen sensitivity (Goodman et al., 1998) despite the 6 times increase in the carotenoid plasma concentration. Other authors found no effect of β -carotene on oxidative DNA damage in humans evaluated as 8-OHdG (van Poppel et al., 1995). Differently Lee et al. (1998) reported a decrease of 8-OHdG in smokers after 9 mg β -carotene intake daily for 4 weeks even though the carotenoid was less effective with respect to vitamin E and C.

Vitamin C - Data in literature report that oxidative stress and smoking increase 8OHdG and that reduced intake of ascorbate (5 mg/day) in males is correlated to higher sperm DNA oxidation products that, on the contrary, are lowered after resupplementation with 60 mg/day vitamin C (Fraga et al., 1996). As also reported by a recent review by Carr and Frei (1999a), several studies showed an effect of vitamin C supplementation in the protection from DNA oxidative damage (Pohl and Reidly, 1989; Green et al., 1994; Fraga et al., 1991; Lee et al., 1998; Panayotidis 1997; Brennan, 1996) however at doses that exceed those in tomato products (60-1000 mg vitamin C). Other authors did not find any effect of vitamin C supplementation both on *in vivo* or *ex vivo* DNA damage (Prieme et al., 1997; Anderson et al., 1997) while in some cases there was a decrease or an increase depending on the marker

of DNA oxidation considered (Podmore et al., 1998; Cooke et al., 1998; Rehman et al., 1998). It has been also hypothesized that vitamin C can significantly influence mononuclear cell DNA, serum, and urinary 8OHdG levels *in vivo* and that, probably through its redox properties, may stimulates the repair of 8OHdG in DNA/nucleotide pool. In fact the authors found an increase of 8OHdG in urine several weeks after the end of vitamin C supplementation (500 mg/day), while the levels of the oxidized base decreased in DNA during supplementation and returned to basal level at washout. Thus, the authors suggested that vitamin C may have some form of residual effect, an apparent pro-oxidant effect. Another explanation was that vitamin C may not act as an antioxidant *per se* but may promote the removal of 8-oxodG from the DNA and/or nucleotide pool upregulating the repair enzymes (Cooke et al., 1998; Rehman et al., 1998).

Other authors reported that vitamin C supplementation may have prooxidant effect (Podmore et al., 1998); the interaction of vitamin C with metal ions produce damage but whether this mechanism occurs *in vivo* has not been definitively demonstrated (Carr and Frei, 1999b). Experiments using purified DNA or isolated nuclei (Drouin et al., 1996; Hu et al., 1997) confirm the pro-oxidant action of vitamin C in the presence of metal ions *in vitro*. In absence of metal ions, on the contrary, vitamin C seems to inhibit DNA oxidative damage *in vitro* (Fisher-Nielsen et al., 1992; Noorozi et al., 1998; Pflaum et al., 1998) but also some exception are reported (Singh, 1997; Anderson et al., 1994).

Recently the ingestion of 2 g vitamin C did not cause any adverse effect on chromosome damage, apoptosis and necrosis in lymphocytes of people with normal Fe levels and absence of excessive oxidative stress (Crott and Fenech, 1999).

By using the comet assay to quantify DNA damage, Anderson et al. (1994) by supplementing primary lymphocytes *ex vivo* showed that vitamin C had a protective effect at low doses (40 µmol/L) but an exacerbating effect at high doses (200 µmol/L).

Thus data on vitamin C and DNA damage after *in vivo* supplementation are still conflicting and not exhaustive to definitely demonstrate the role of vitamin C in antioxidant protection.

Combined supplementation - Just few data are available on the effect of combined supplementation against DNA damage in humans. The intake of an antioxidant mixture (vitamin A, C, E, β-carotene, folic acid, rutin) for 4 months by two groups of people (young and aged donors) decreased lymphocyte micronuclei induced *in vitro* by gamma-radiation (Gaziev et al., 1996).

Also Duthie et al. (1996) found a decrease in endogenous lymphocyte DNA damage

(oxidised bases) and an increased protection from *ex vivo* oxidative damage giving subjects (smokers and nonsmokers) vitamin C, E and β -carotene for up to 40 weeks.

Nutritional status - Several authors tried to demonstrate whether the levels of endogenous antioxidants are related to DNA damage with conflicting results (Lenton et al., 1999; Collins et al., 1998; Duthie et al., 1996).

In a cross-sectional analysis of heavy smokers, polycyclic aromatic hydrocarbons (PHA)-DNA adducts were inversely associated with plasma levels of β -carotene and α -tocopherol (Mooney et al., 1997). Furthermore the two micronutrients were significantly correlated, and when β -carotene was low, α -tocopherol had a significant protective effect on adducts but not when β -carotene was high. The interaction observed suggest that several micronutrients may act in concert to protect from oxidative damage.

A correlation was also found between plasma ascorbic acid and β -carotene and mutagen sensitivity assessed by bleomycin induced chromosomal breaks (Kucuk, 1995).

It has been reported that vitamin C and β -carotene are lower in smokers than in non smokers while no significant difference was found for α -tocopherol levels (Lee et al., 1998). These results were interpreted suggesting that vitamin C and β -carotene are more vulnerable to smoking-induced ROS.

Results from a recent study (Bianchini et al., 2000) show that women from Spain had higher levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine with respect to women from Sweden. This is in contrast with the hypothesis generally accepted that a Mediterranean diet rich in α -tocopherol and carotenoids protects cells against oxidative DNA damage. The authors also suggested that these results could be attributed to the consumption of foods other than fruit and vegetables, including fats. Further studies are necessary to evaluate whether mutagen sensitivity may be affected by plasma levels of certain nutrients and be modified by dietary intervention.

Immunomodulation

Vitamin C, has been shown, in animal and human studies to be rapidly depleted in several cancers, infectious diseases, and surgical trauma when oxidative stress was involved. It appears to be involved at all stages in the immune response. One important site is that of delayed hypersensitivity which recovers on Vitamin C repletion (Jacobet al, 1991).

Vitamin E, though very effective as a chain-breaking antioxidant in cell membranes, becomes a prooxidant in the absence of adequate concentrations of cytosolic antioxidants such as ascorbate and glutathione (Stocker, 1999). Vitamin E deficiency is associated with lowered B-cell antibody production and T-cell proliferation in response to mitogenic stimulation and an increase in rate of infection. Substantial protection is best demonstrated in supplementation studies with Vitamin E depleted subjects and in the elderly (Meydani and Heharka, 1996). The protective mechanism is thought to involve inhibition of 5-lipoxygenase thus decreasing leukotriene B4 levels, the agonist for IL-1B (Deveraj and Jialal, 1997). Inhibition of NF-kB activity *per se*, or by a feedback mechanism via IL-1B may be another immunoprotective function of Vitamin E in ROS initiated inflammatory processes (Jackson et al, 1998). β -carotene has been assessed in a large number of *in vitro*, *in vivo* studies and clinical trials, and may be protective against several cancers, CHD, stroke, ageing, sunburn and macular degeneration (Mayne 1996). Results are conflicting, but on balance, the major immunoprotectant mechanism appears to be via increases in T-helper lymphocyte (CD4+) numbers (Allard et al, 1998) and in increased natural killer cell activity. Again as with Vitamin E the most significant results are observed with elderly subjects.

Conclusion

Cumulative evidence from this review appears to support the requirement for a balanced supply of dietary-derived antioxidant and protective molecules. The natural stoichiometry of these components in tomatoes and other plant foods, allows an orderly detoxication and removal of deleterious products from the body without the build up of potentially toxic intermediates. Studies with megadoses of purified/synthetic antioxidants have not revealed the 'magic bullet' and serve to reinforce the argument for dietary modulation.

Overall Recommendations

Since the inception of this Network in 1997 there have been major changes in the perception of how we should investigate the role of phytochemicals with putative antioxidant properties in healthy diets. Salutary lessons have been learnt from major intervention studies and emphasis has moved from the study of high doses of purified compounds, to the more physiologically relevant investigation of delivery of these compounds as crucial components in the food matrix.

The function of WG4 has been to research the notion that oxidative stress is involved in many diseases and may result in tissue injury, and that dietary modification may ameliorate these conditions.

A study of the chemical properties of biological oxidants and their respective antioxidants leads us to predict the mechanisms by which they will interact in biological systems. Despite the innate complexities of even the most simple cellular systems, studies with animal models and cell culture systems have largely supported the basic mechanistic proposals for the mode of action of RS and free radicals in oxidative stress, and the protection conferred against this damage by exogenous and endogenous antioxidant systems. What has been less successful was the extrapolation of animal and cell culture data to design large scale human intervention studies such as the ATBC and CARET trials.

False inferences have been made based on major components in certain complex diets, leading to simplistic designs for the intervention trials. More recent mechanistic studies have revealed beneficial synergistic relationships which will have important bearing on the design of future intervention trials.

The overall message from this Network is to support the need for additional research on dietary intervention using whole foods to better define what is a basic healthy diet, and to

council against the search for that elusive ‘magic bullet’ lest we shoot ourselves in the foot (again)!

Proposals for future research

Health-related studies

Experimental design.

1. The use of food-derived, synergistic mixtures of AO/protective components, delivered to the appropriate animal or cell systems at physiologically relevant concentrations.
2. Establishment of base-line AO levels to avoid using AO-replete test systems.
3. More use of primate and pig model systems to simulate human studies.
4. Nothing less than a revolution is required in the field of cell culture where we currently use transformed cell lines to study the role of AO in cancer prevention.

It is a major priority in this field of research to switch to stem cell lines or SV40-immortalised cell lines if we are to study the effects of AO and oxidants on ‘normal’ rather than mutated cell lines.

5. If we are to determine the true AO or prooxidant properties of dietary components in cell culture systems, then the appropriate O₂ tension must be employed ie. 10-20ppm for body tissues vs 160mm atmospheric.
6. To move away from the simple chemical degradation products as biomarkers of protein and DNA damage to analyse macromolecular damage and gene-products induced by oxidant stress or AO protection.
7. To assess the role of population genetics in response to oxidative stress and dietary intervention. Recent advances in molecular biology will allow the rapid and simultaneous analysis of thousands of expressed genes using oligonucleotide arrays.

The advantage of this approach is two-fold. It will give an unequivocal response of healthy subjects to oxidative stress/AO protection in terms of gene expression, but will also reveal subjects with gene defects that place them at increased risk from oxidative stress eg. those with haemochromatosis, glutathione deficiency, methylene tetrahydrofolate reductase mutations etc for whom appropriate dietary strategies can be designed and prescribed.

Plant Production and Quality Control

The message from WG4 which is supported by much recent AO research, is that a tomato (product) with a balanced array of protective AO components is preferable to an engineered

strain which is simply enriched in lycopene. If all the useful components could be increased whilst maintaining their stoichiometry in the tomato this would be a worthwhile but extremely difficult target to attain. Perhaps the best efforts of the genetic engineers should be directed at producing disease and drought resistant strains without compromising the quality of the nutritional components.

Medical advances over the past century have led to significant increases in life expectancy in the 'developed' world. This does not, however, infer a corresponding improvement in the quality of life. The ravages of infectious diseases have been replaced by the chronic ailments of old age, such as arthritis, Alzheimer's disease, CVD, diabetes and macular degeneration etc., all of which have oxidative stress in their aetiologies, and are largely a result of inappropriate diets and lifestyles. Moreover, as the ratio of old:young increases in the population, there is an obvious need to maintain productivity. This can surely be done by improving the health and contribution of the older population.

It is incumbent upon us as scientists to provide insights into how the length and quality of life can be enhanced by optimising diet and lifestyle.

The tomato and its products can make a significant contribution by providing numerous healthy nutrients as part of a balanced diet.

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