KATHOLIEKE UNIVERSITEIT LEUVEN

Faculteit der Landbouwwetenschappen

Afdeling Bio-Industriele Wetenschappen

STUDY OF THE SHRIMP-CAROTENOIDS AND OF THEIR RADIATION SENSITIVITY IN VIVO AND IN VITRO

Leden van de jury :

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F. Dhiehl, Prof. I.S.T. Karlsruhe
H. Hendrickx, Prof. R.U.G.
P. Tobback, Docent K.U.L.
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Proefschrift voorgedragen tot het bekomen van de graad van Doctor in de Landbouwwetenschappen

door

Frans SNAUWAERT

juli 1974

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Aan Joke,

Koen en Katia.

Bij het tot stand komen van dit werk, hou ik eraan mijn oprechte dank te betuigen aan al diegenen met wie ik mocht samenwerken, in het bijzonder de leden van de Afdeling Bio-Industriële Wetenschappen.

Mijn bijzondere dank en erkentelijkheid gaat uit naar Prof. E. Maes en docent P. Tobback voor hun voortdurende hulp en hun stimulerende belangstelling en naar alle Professoren die tot mijn vorming hebben bijgedragen.

De samenwerking met meerdere studenten zal voor mij een aangename ervaring blijven : ir. Anthonissen, ir. Van Ginderachter, ir. Van de Casseye en ir. Van Gestel zullen op vele plaatsen in dit werk hun bijdrage terugvinden.

De hulp van Mevr. Knaepen-Milis, Mevr. Ramon-De Paepe, Juff. Raye en J. Terclavers, wordt in dankbaarheid erkend.

Verder dank ik Mevr. Massie-Verstraeten voor het typen van het manuscript.

Het beeindigen van dit werk was onmogelijk geweest zonder de steun van het "Nationaal Fonds voor Wetenschappelijk Onderzoek" dat mij achtereenvolgens een beurs als navorsingsstagiair en aspirant bezorgde, waarvoor mijn oprechte dank.

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"Quia mihi pulchrum in primus videtur non pati occidere".

Pliny the Younger, circa C.E. 98.

I N T R O D U C T I O N.

The increasing need for protein-rich food to improve the nutritional state of some population-groups in the world has repeatedly been stressed upon during the last decade.

The sea offers a unique source of proteins that could be able to meet the worlds' need.

Due to the high perishability of fish, a major problem however still exists: how to transport fresh fish from the point of origin to the areas of consumption.

This unfavourable situation has stimulated research on new methods for preserving protein-rich foodstuffs.

Gamma irradiation has suscitated much interest during the past ten years.

The recognition of the unique role of fish and seafoods in the world protein supply on one side and the potentialities of gamma irradiation as a food preservation method on the other has stimulated the elaboration of research and development programs in many countries all over the world.

These programs have been pursued with three main objectives :

a. To develop radiation-pasteurization procedures for seafoods, especially those which have defied other methods of preservation.

b. To use an appropriate combination of conventional preservation techniques with irradiation so as to minimise the limitations of either method, thereby improving product quality and extending shelf-life.

c. To demonstrate to the satisfaction of health authorities that no deleterious effects result from a feasible radiation processing.

The many symposia and meetings during the last two decades yet indicate that the field of radiation preservation of foods is a cosmopolitan one.

On this world basis the radiation processing of foods has progressed to the stage that approval has been given in certain countries for the marketing and limited consumption of individual types of food, such as : potatoes and onions irradiated for sprout inhibition ; wheat and wheat products, dried fruits and dried food concentrates irradiated to extend the market life.

Although extensive work has been done on fresh fish, shrimp, crab, lobster and other types of fishery products it was realized that more fundamental work was needed to resolve the many problems connected with te irradiation of these products.

This work aims to contribute to the fundamental knowledge of the food irradiation process.

S C O P E O F T H E W O R K.

1. Problems in preserving shrimps.

The brown shrimp (Crangon vulgaris Fabr.) is an important and typical crustacean in Western Europe. This crustacean is classified in the sub-class of the Malacostraca, order of the Eucarida, sub-order of the Decapoda.

Shrimp landings represent only 1% of the total catch of marine products in the E.E.C. (Tab.I) ; however, since the Crangon vulgaris Fabr. landings are mainly concentrated in the Common Market countries, the above figure represents in fact 90% of the shrimp catch on a world scale (Hovart et al. 1972).

During the last five years landings averaged 19,000 tons a year in the E.E.C. and were equivalent to a sum of 12 million dollar.

Unpreserved, boiled shrimps are highly perishable and often spoil within a few days after catch, even when stored at chilling temperatures.

The major factors that influence the keeping qualities of shrimps can be summarised as follows :

- A high content of extractable nitrogen compounds and a relatively large body surface causes the micro-organisms, especially the psychrophiles, to proliferate, causing putrifaction.

- After the boiling process on board, the shrimps are cooled with sea-water. Even when sea-water is not used reinfection from the air occurs.

Table I Landings of-and yield from marine products per country during the years 1967-1969 (a).

Country	Landings of marine products in million kg. (b)				Yield in 1000 $			
	1967	1968	1969	Average	1967	1968	1969	Average
Belgium	56.7	60.4	51.4	56.2	16,990	16,845	16,766	16,867
France	656.1	617.9	613.8	629.3	262,892	237,423	225,910	242,075
Italy	343.0	333.7	325.3	334.0	171,088	170,868	184,534	175,497
The Netherlands	252.5	2666.6	268.2	262.4	58,203	59,278	63,624	60,368
West Germany	465.1	460.0	436.1	453.7	84,689	83,561	91,246	86,499
Total	1,773.4	1,738.6	1,694.8	1,735.6	593,862	567,975	582,080	581,306
Country	Landings of crustacea in million kg.				Yield in 1000 $			
Belgium	1.8	1.6	2.0	1.8	1,240	1,360	1,457	1,352
France	19.4	19.1	24.6	21.0	24,233	23,107	24,315	23,885
Italy	13.4	14.3	14.6	14.1	14,555	16,077	18,016	16,216
The Netherlands	7.5	6.6	7.9	7.3	4,788	4,576	3,583	4,316
West Germany	7.4	8.6	10.6	8.9	2,348	4,125	4,868	3,780
Total	49.5	50.2	59.7	53.1	47,164	49,245	52,239	49,549
Country	Landings of shrimps in million kg.				Yield in 1000 $			
Belgium	1.3	1.0	1.4	1.2	869	922	1,039	943
France	2.0	2.3	2.3	2.2	2,511	2,686	2,485	2,560
The Netherlands	7.4	6.6	6.8	6.9	4,811	4,520	3,535	4,289
West Germany	4.7	7.8	8.8	7.1	2,139	3,924	4,727	3,597
Total	15.4	17.7	19.3	17.4	10,330	12,052	11,786	11,389

a) F.A.O. Yearbook of Fishery Statistics Vol 28, 1969.
F.A.O. excl. fresh water fish, fish not destinated for human consumption and shrimps destinated for drying.

- After landing arbitrary methods of handling and processing besides the prevalence of unhygienic practices, favour several microbial reinfections and increase the bacterial load of the shrimps to 10^6/g before being sold at the retail outlet (Early et al. 1970, Van Spreekens et al. 1970 and Vyncke et al. 1972).

- Ludorff et al. (1958) and Degkwitz et al. (1954) showed the importance of an active proteolytic enzyme system that is not fully destroyed during the boiling process.

The boiling time and the boiling temperature are in this case at least two important factors to control enzymatic deterioration.

- During periods of glut (hand)-pealing of shrimps causes serious problems due to a lack of adequate pealing machines with an adverse effect on the hygienic and organoleptic quality of shrimps destined for inland distribution.

- Hitherto, the shrimp has been preserved for longer periods by the use of antiseptics (usually benzoic acid or its salts, sorbic acid, parahydroxybenzoic acid esters (PHB) etc.) although this method has its limits as well.

Ice-storage does not extend the storage life beyond 7 days for there is rapid spoilage due to psychrophilic organisms.

Canning, freezing or freeze-drying change the quality to a greater or less extent.

Some of the problems encountered in conventional preservation of the shrimp are listed in Tab. II.

Table II Problems in preserving the brown shrimp.

Process	Defects (Storage properties)
Refrigeration (5-10° C)	Short shelf-life (3-4 days)
Ice storage (0-2° C)	Limited shelf-life (5-7 days)
Deep freezing (-20° C)	Oxydation and deshydratation alter textural-, and organoleptic quality (flavor, color)
Heat pasteurization (70-80° C)	- Extended shelf-life (20-30 days) - Perceptible alteration in organoleptic properties
Chemical preservation	- Limited effectiveness - Shift in microflora - Chemical deterioration.

2. Radurization of the brown shrimp.

In view of the shortcomings of the conventional methods in preserving this for the E.E.C. economically important crustacean, application of irradiation was expected to offer very promising advantages, especially to Belgium, the Netherlands and west-Germany.

Meantime it is commonly accepted that radappertisation of marine products is not a feasible commercial procedure, though it is of academic interest, since during this process undesirable sensory- and flavour changes are produced (Coleby et al. 1961, Kumta et al. 1966 and Shewan et al. 1970).

Interesting information can be drawn from the radurization of a variety of the commoner commercial crustacea, all of them having a high content of extractable nitrogen compounds, a high content of unsaturated fatty acids and containing typical carotenoid pigments (Tab. III).

Although radurization appears feasible, additional research is needed to solve specific problems and to obtain the information necessary for a better understanding of the mechanisms involved in each biological system.

Table III Summary table of storage life of blanched or boiled crustacea after irradiation.

Crustacean	Storage temp. ° C	Packaging	Dose level of irradiation in Mrad	Shelf-life (days)	References
Pandalus jordani	3	tin	0	42	Scholz et al (1962)
		vacuum	0.50-0.75	126	
peneaus stylifera and Metapeneaus species					
- fresh	0-2	polyethylene bags	0	7	Kumta et al (1970)
	0-2	"	0.15-0.25	21	
	10-12	"	0.15-0,25	14	
- blanched (100° C/4 min.)	10-12	"	0	21	
	10-12	"	0.15-0.25	56	
	0-2	"	0.15-0.25	84	
- boiled (100° C/ 100 min.)	28-34	"	0	3	
	28-34	"	0.15-0.25	56	
Homarus americanus	0	cellophane-polyethylene	0	14-21	Slavin et al (1966)
	0	"	0.075	28-35	
	1	"	0.15	30	
Nephrops norvegicus	0-1	polyethylene	0	28	Hanneson et al (1971)
Cancer magister	0.5	polyester-polyethylene (vacuum)	0	6-14	Miyauchi et al (1967 and 1968)
		"	0.1	14-35	
			0.2	21-56	
	5.5	"	0	2-9	
		"	0.1	14-21	
		"	0.2	21	

3. Research project.

The research of the Laboratory of Food Preservation is mainly focused on programs studying the effect of physical preservation methods on the organoleptic quality parameters of foodstuffs.

The colour of food is an important factor determining its quality and acceptability. Changes in colour are often considered to be a possible sign of spoilage, poor processing or as an indication of adulteration.

A fundamental study of the influence of gamma irradiation on the carotenoid pigments in the brown shrimp (*Crangon vulgaris Fabr.*) could be useful to determine the optimum irradiation dose for inactivating psychrophilic spoilage organisms and for maximum retention of the organoleptic attributes.

Since the pigments of the brown shrimp have as far as we know never been studied before, purification and identification of the carotenoids in boiled and unboiled shrimps were carried out.

The influences of gamma irradiation on carotenoids *in vivo* and *in vitro* were compared.

C H A P T E R I

GENERAL ASPECTS OF CAROTENOID CHEMISTRY.

1. Introduction.

In the first half of the 19th century the occurrence of carotenoid pigments in the vegetable- and animal kingdom was detected.
It has been estimated that the annual production of these pigments in nature amounts to about 100 million tons (Isler, 1971).

The carotenoids belong to the class of polyenes and represent the most unsaturated mass products of biosynthesis (Zechmeister, 1962).

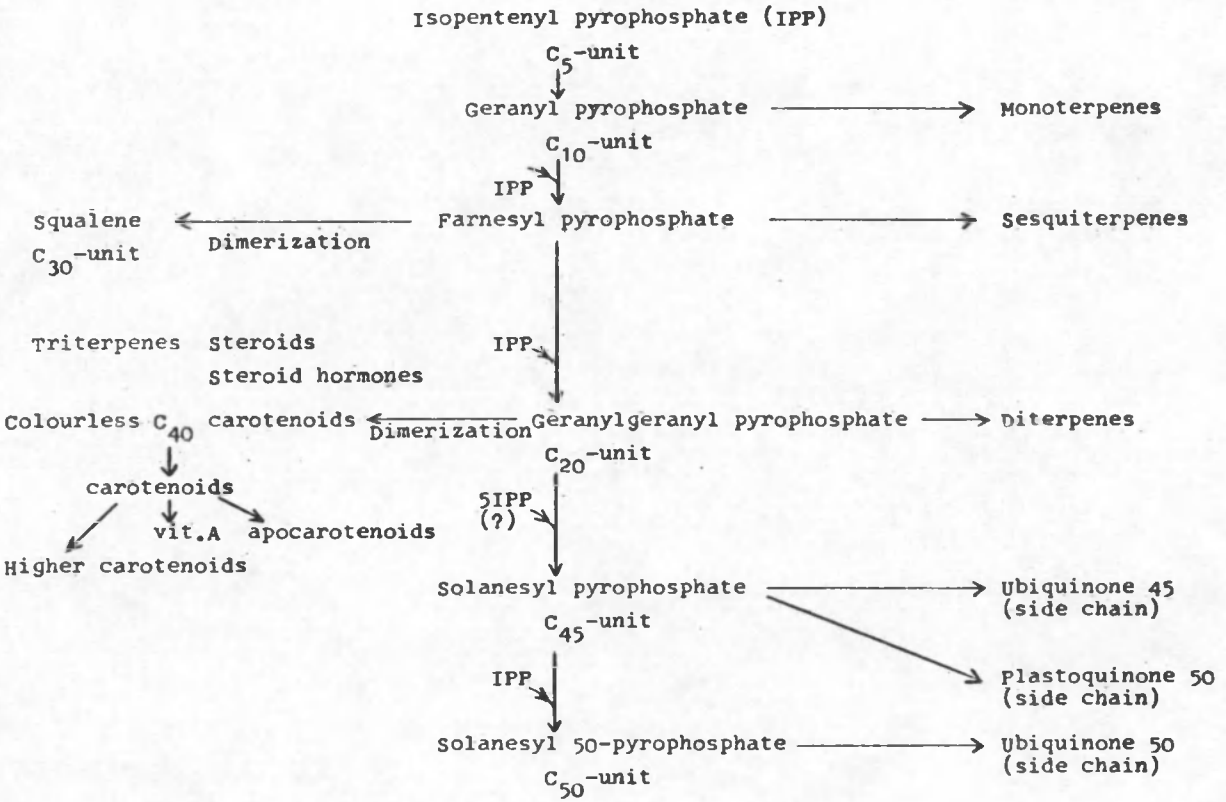
Scheme 1 places carotenoids in a wide biosynthetic perspective.

Most naturally occurring carotenoids are tetraterpenes, having 8 isoprenic building blocs, assembled in such a way that the linking of the isoprene units is reversed in the middle of the molecule (Goodwin, 1965).

Attention must be drawn to the fact that two C_{15} units combine similarly to give squalene, being a key compound in the synthesis of sterols. Although cyclization is not so extensive as in the triterpenes (e.g. sterols) carotenoids may be cyclized.

A detailed discussion of the biosynthesis and the *in vitro* synthesis of the carotenoids goes beyond the scope of the present survey and the reader is referred to pertinent reviews (Mayer et al. 1971 and Goodwin, 1971).

The ability to produce carotenoids seems to have been developed at an early stage in evolution. Some bacteria, the algae and higher plants preserve this capability, but animals, certainly those of the higher



Scheme 1. Generalized scheme for biosynthesis of terpenoids (Goodwin 1965 and 1971).

orders, seem to depend for their carotenoids on those present in their diet. However, subsequent transformation of carotenoids, obtained from the diet, sometimes leads to characteristic animal pigments that are not found in organisms capable of carotenogenesis de novo (Weedon, 1971).

2. Carotenoid nomenclature.

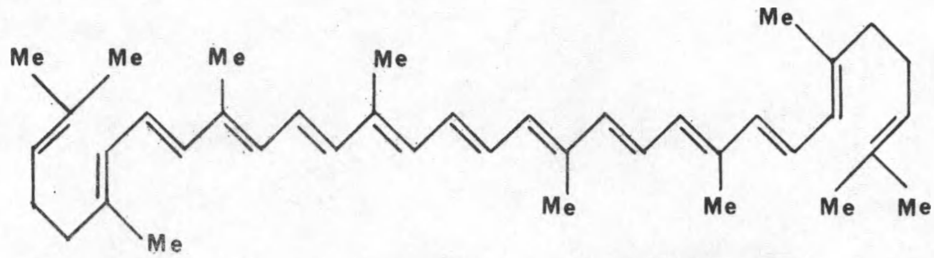
Carotenoids can be subdivided into two main groups :

A. Hydrocarbons, which are called "carotenes".

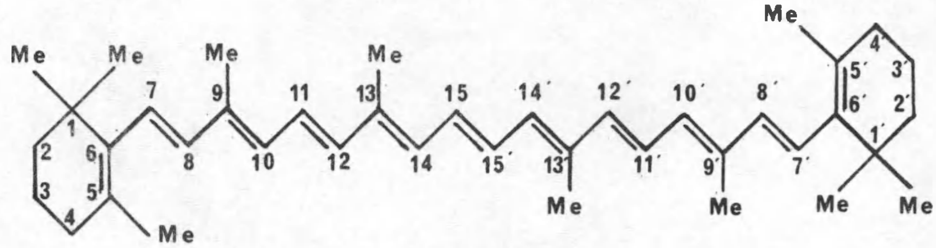
B. Oxygen-containing derivates, which are called "xanthophylls".

A. Carotenes.

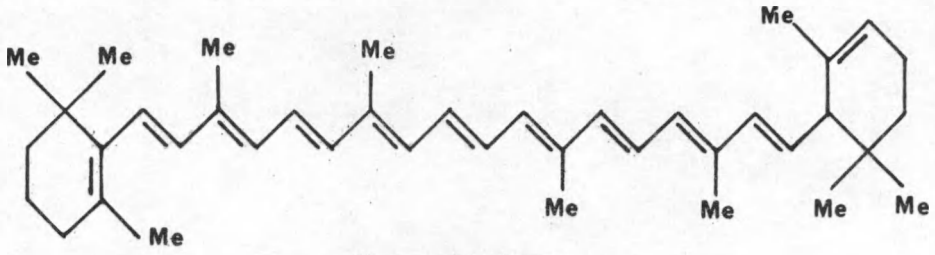
Many natural carotenoids can be formally derived from the "parent" carotenoids listed below.



Lycopene



β-carotene



α - carotene

The used numbering of the atoms on the carotenoid skeleton in this work is illustrated in the example of β-carotene.

In many ways the acyclic compound lycopene can be taken as the prototype of the carotenoids. Other carotenoids can then be considered as related to it by means of structural changes in one or both halves of the molecules.

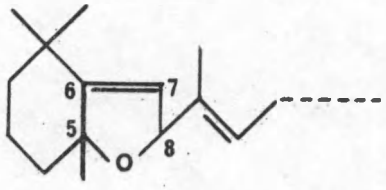
Thus β-carotene can be regarded as a bicyclic lycopene. Though it is often convenient to stress such structural relationships to identify different classes of carotenoids, it is not intended to suggest that in nature the corresponding transformations necessarily involve the specific precursor mentioned, nor that the transformations themselves are as simple as a superficial inspection of the gross structures might imply.

B. Xanthophylls.

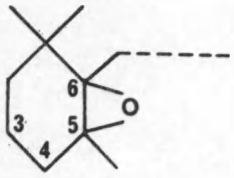
Though the numbers of basic structural modifications are comparatively few, their occurrence in different combinations accounts for the remarkable variety of natural carotenoids. The majority of the 300 known carotenoids are oxygenated and called xanthophylls. The oxygen in xanthophylls may occur as hydroxy-, carboxy-,

keto-, methoxy- or epoxy- groups.

Most substituents are in the position 1 to 6 and 1' to 6' although the 5-8 epoxydes (being the furanoid transformation products of the carotenoid 5-6 epoxydes) are the main exceptions of this generalisation.



furanoid ring



epoxide ring

In most C_{40}-carotenoids such as α-carotene, β-carotene, their hydroxylated derivates etc. the bond between C_6 and C_7 is a single bond and the compounds have the normal (cyclohexenyl) structure.

In less frequent instances a double bond occurs between C_6 and C_7 and then a retro (cyclohexylidene) structure is obtained.

The structure of carotenoids with somewhat special structures can be found in an excellent review by Isler (1971).

3. Cis-trans isomerism.

Carotenoids and related multiconjugated systems constitute a unique case among low-molecular weight substances because calculation of the numbers of geometrical isomers, theoretically possible for a given carotenoid, leads to some rather daunting figures (Zechmeister et al. 1943 and Kuhn et al. 1928).

For a conjugated system with n-non-cyclic double bonds, the number of stereoisomers N is given by the equations :

$N = 2^n$ for unsymmetrical systems.

$N = 2^{(n-1)/2} \cdot (2^{(n-1)/2} + 1)$ for symmetrical systems, n odd.

$N = 2^{n/2-1} \cdot (2^{n/2} + 1)$ for symmetrical systems, n even.

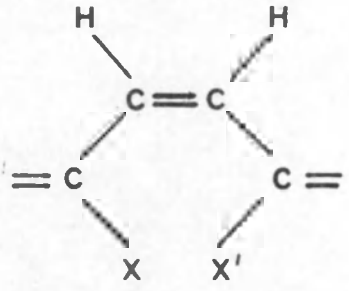
Fortunately a wide gap exists between the number of calculated- and observed spatial forms of carotenoids.

Pauling (1939) divided the double bonds in the acyclic polyene chain of carotenoids into two types : those for which the adoption of a cis-configuration involves very little steric hindrance (between two hydrogen atoms), and those for which a cis-configuration leads to a serious clash between a methyl- and a hydrogen atom.

The methyl-substituted double bonds of β-carotene for example fall into the unhindered category as do the 15-15' double bonds present in most carotenoids.

All other disubstituted double bonds are adjacent to a methyl side-chain and therefore fall into the second category.

A cis double bond located in a polyene chain will have the orientation :



X and X' represent either hydrogen atoms or methyl groups.

The Pauling classification is useful to indicate the double bonds about which stereomutation is most likely to occur.

Furthermore, in practice, relatively few isomers constitute the bulk of the mixtures produced by stereomutation, as would be expected on purely statistical grounds.

As it is indeed difficult or impossible to overcome the energy barrier in a direct process, trans-hindered cis, a number of these sterically hindered isomers have been obtained by total synthesis (Mayer et al. 1971).

Zechmeister and his collaborators (1962) have shown that most, if not all, carotenoids in vitro can be converted into mixtures of geometrical isomers by several methods.

The carotenoids, so far studied, conform to the general rule that in each stereochemical set the least soluble and the highest melting member is the all-trans compound.

Because of stereomutation on fusion, some cis-isomers exhibit the phenomenon of a double melting point.

In the presence of an asymmetric carbon atom, trans-cis rotations involve changes in optical activity.

Empirically it has been shown, in the absence of a pertinent theory, that the geometrical configuration of a carotenoid has a profound influence on its adsorption affinity ; this means that the separation of even complicated mixtures of isomers can be easily realized by chromatography.

Unfortunately theory is lagging behind practical experimentation in this field, probably because very little is known about the orientation of individual carotenoid isomers on active surface and about the strenght of the adsorption forces as a function of the molecular form (Zechmeister, 1962).

4. Visible and U.V. absorption spectrophotometry.

Since the absorption spectrum of a carotenoid (shape of the curve, position and intensity of the maxima) affords the best indication of the chromophore present, the carotenoids have received a good deal of attention from spectroscopists. Some of the correlations of absorption properties with structure will be briefly summarized since they have greatly helped in the identification of individual pigments.

The structural features in hydrocarbons, affecting the absorption, are the number of conjugated linkages, open chain-ring isomerism and cis-trans isomerism.

The effects of auxochromic substituents (hydroxyl-, carbonyl-, allenic-, acetylenic- or aromatic groups) have also been studied. Finally it must be kept in mind that the absorption spectra of all carotenoids are dependent on the solvent (Much of the data have been collated by Karrer and Jucker (1948), Davies (1965) and Zechmeister 1960 and 1962).

a) Of the C_{40} carotenoids, lycopene, possessing a chromophore that consists of an all-trans polyene system, exhibits a spectrum with well defined fine structure and with absorption maxima at the longest wavelengths. When the chromophore extends into one or two cyclic end

groups (γ-carotene and β-carotene) a partial loss of fine structure, a hypsochromic- as well as a hypochromic shift are observed. These shifts are due to the steric hindrance between the ring methyl groups and the acyclic polyene chain, resulting in a non planar structure that limits the overlap of the orbitals associated with the ring double bond and those of the polyene chain.

In Fig. I_1 the ultraviolet and visible light absorption spectra of γ-carotene, β-carotene and lycopene are drawn.

Conjugation of the polyene-chain with a keto-group in C_4 (echinenone) results in a hypochromic effect, while bathochromic shifts are observed with a complete loss of fine structure. The introduction of a second conjugated keto-group in the C_4' position causes a small bathochromic displacement of the broad absorption maximum.

Fig. I_2 shows the ultraviolet and visible light absorption spectra of β-carotene, echinenone and cantaxanthin.

Introduction of a hydroxyl group in C_3 and/or in C_3' results in very little change either in the position of the absorption maxima or in the spectral shape.

Epoxidation of a carotenoid removes an ethylenic linkage from the conjugated system and causes a corresponding hypsochromic shift.

b) Cis carotenoids exhibit light absorption of lower intensity than their all-trans isomer and as a general rule, the principal light absorption maxima are shifted to shorter wavelengths (ca. 2-5 nm for one - and ca. 10 nm for two sterically unhindered cis double bonds).

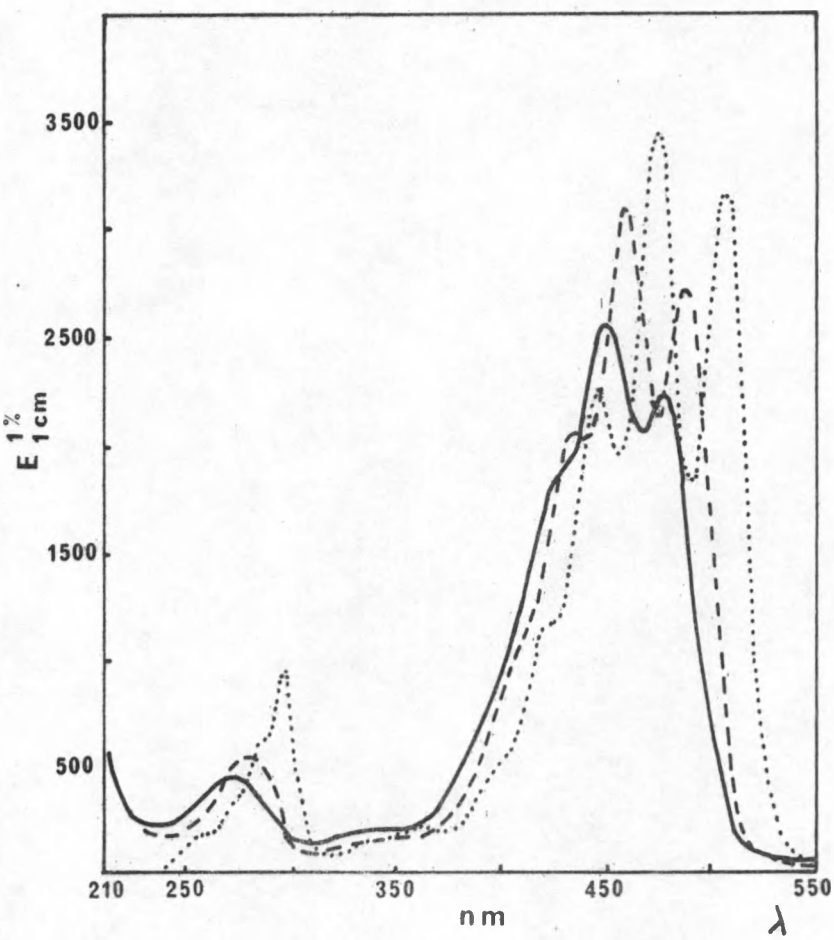


Fig. I_1 Ultra violet and visible light absorption spectra of lycopene (.....), γ-carotene (-----) and β-carotene (———) in petroleum ether.

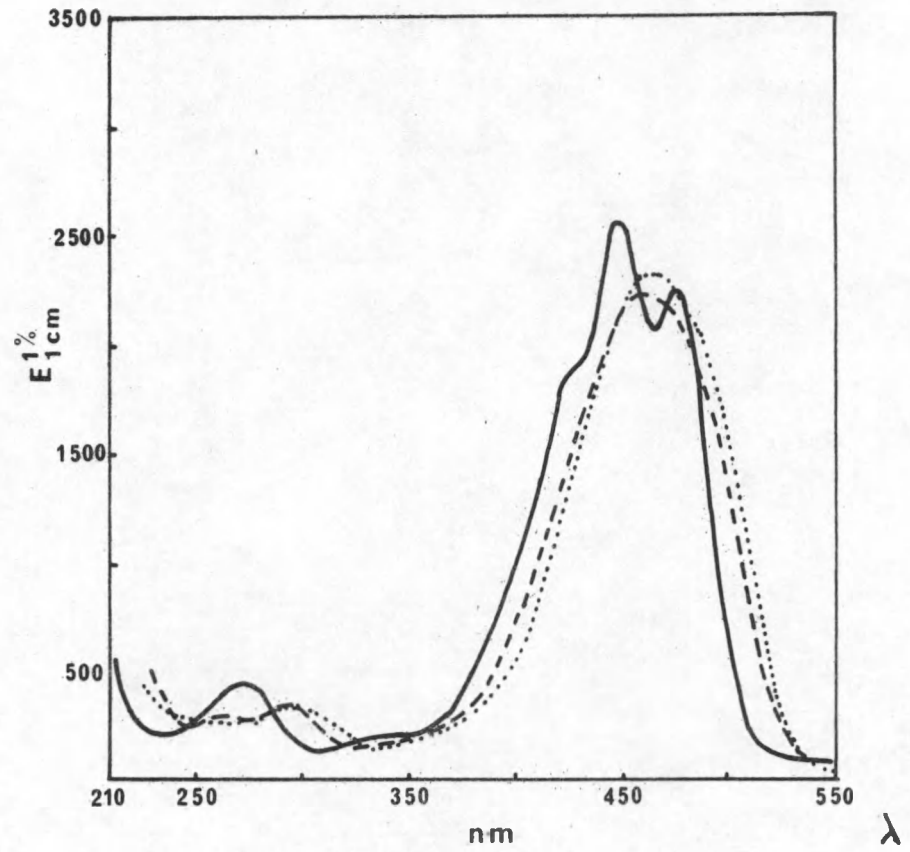


Fig. I_2 Ultra violet and visible light absorption spectra of β-carotene (———), echinenone (– – – –) and canthaxanthin (.....) (in petroleum ether).

One of the most noticeable features in the spectra of mono- (unhindered) cis carotenoids is the appearance of a subsidiary peak in the near ultraviolet region. The wavelength difference between this 'cis-peak' and the longest wavelength maximum of the all-trans compound is practically a constant (142 ± 2 nm for C_{40} carotenoids possessing 10 or 11 conjugated double bonds (Zechmeister et al. 1943).

This effect is demonstrated for β-carotene and its central 15-15' cis-isomer in Fig. I_3.

Theoretically it has been predicted that the intensity of the cis-peak will be roughly proportional to the square of the distance between the centre of the conjugated system and the mid-point of a straight line joining its two ends (i.e. zero in the case of the all-trans isomer).

According to this prediction the central-cis isomers, which have been prepared in a number of series by unambiguous total synthesis, have more intense cis-peaks than any of the other possible cis-compounds (Zechmeister, 1962).

Since the intensity of the cis-peak seems to depend primarily on the overall shape of the chromophore, it is hardly surprising that neither the poly-cis, nor the authentic di-cis isomers exhibit a significant absorption in this region. In both instances the molecules approximate the linear shape of the all-trans form.

Few authentic examples are known of carotenoids with hindered-cis bonds. It is obvious however, that sterically hindered isomers exhibit "degraded spectra" with little fine structure and with maxima that are of much lower wavelength and intensity than the all-trans isomer. Although the evidence is not conclusive, mono-hindered cis

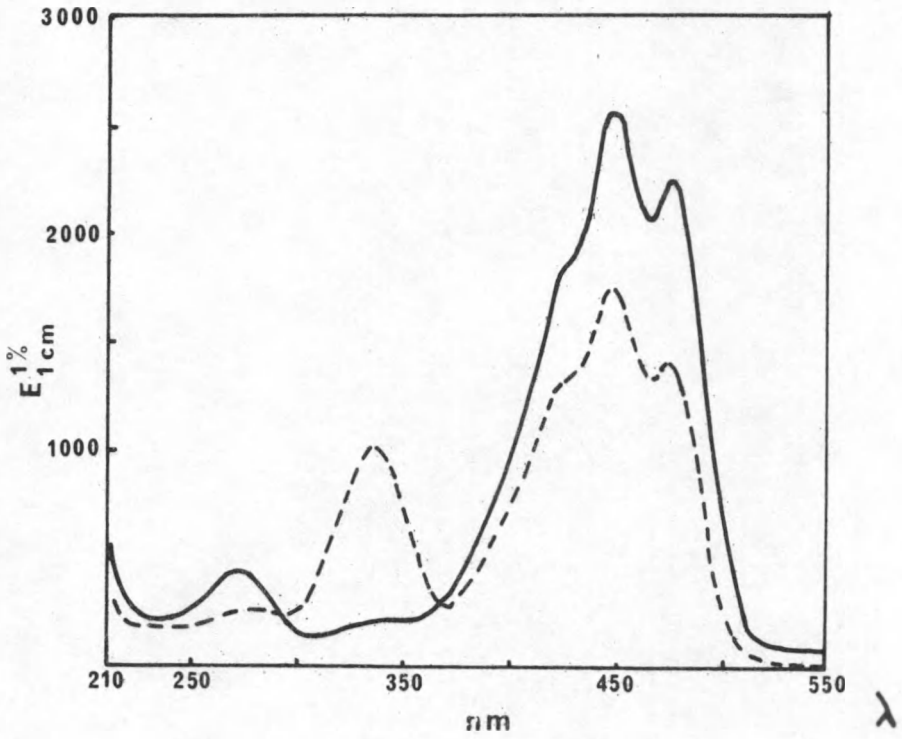


Fig. I_3 Ultra violet and visible light absorption spectra of β-carotene (———) and 15, 15' - cis - β-carotene (-----) (in hexane).

compounds also seem to exhibit cis peaks (Zechmeister, 1962).

c) When the absorption spectrum of a pure carotenoid is determined in different solvents slight differences both in the location and in the intensity of the absorption bands are usually observed. These solvent shifts should be correlated to the solvent-solute interactions in the ground state and in the excited state. Such interactions depend on the polar or apolar natures of both the solvent and the solute and also on any change in polarity of the solute on excitation. Hence the direction of the solvent shift may depend on either the dipole moment of the chromophore increases or decreases during transition (Mousseron-Canet et al. 1969, Stern and Timmons 1970).

In carotenoids the transitions $\pi \longrightarrow \pi^*$ usually undergo red shifts with increasing dielectric constant of the solvent.

Solvent effects on the location of absorption bands of carotenoids are tabulated by Davies (1965).

5. Carotenoproteins.

A. Nature of the compounds.

It has long been argued whether carotenoids participate in biological processes in the free state or as conjugates. On that basis the carotenoproteins, defined as those proteins in which carotenoids are present in stoichiometric proportions as prosthetic groups, acquire considerable importance.

Although more than 30 years have passed since the isolation of ovoverdin, the carotenoprotein of lobster egg, (Kuhn et al. 1938 a and 1938 b), remarkably little is known of the chemistry and biochemistry of carotenoproteins. Reviews of the earlier observations are made by Verne (1926, 1930) and by Lederer (1935).

It is now found that carotenoproteins occur widely among invertebrates, particularly in crustacea, but it may be assumed that their occurrence is ubiquitous in nature (Cheesman et al. 1967).

In their review the same authors define the different types of carotenoproteins.

a) In 'true carotenoproteins' there is a well-established stoichiometric relationship between carotenoid and protein. A relationship as such must involve the presence of specific sites for attachment of the carotenoid on the protein, resulting in a far-reaching change in the absorption spectrum of the former. The authors postulate that a true carotenoprotein is present in a crude aqueous extract when treatment with heat or organic solvents results in liberation of the carotenoid, the spectral characteristics of which are masked in such a complex. So far, only three carotenoproteins have been isolated in a state in which they satisfy the requirement of stoichiometry.

- Crustacyanin, the blue protein of lobster carapace (Wald et al. 1948, Jencks et al. 1964, Cheesman et al. 1966) seems, after removing the carotenoid, to be a simple protein with no components other than amino acid residues.

- Ovoverdin, the green storage protein of lobster eggs (Kuhn et al. 1938, Stern and Salmon, 1937, 1938) has been proved to be a lipoprotein with a large

prosthetic group, particulary rich in phospholipids (approximately 22% in weight) (Zagalsky, 1964 and Ceccaldi et al. 1966). Furthermore, the complex contains a carbohydrate component consisting of hexosamine and a non-amino-sugar residue, amounting to approximately 4.8% of the whole.

- Ovorubin is a red glycoprotein isolated from the gastropod Pomacea canaliculata (Cheesman, 1958 and Norden, 1962).

b) In the so-called lipoproteins with a lipid-associated carotenoid, the carotenoid is part of a large lipid prosthetic group. Hereby stoichiometric relationship between carotenoid and protein remains unproved. Nevertheless such substances sometimes appear to show specificity in their affinity for carotenoids.

In such complexes, the carotenoid may include a number of molecular species in different proportions and it might seem that the molecules were 'dissolved' more or less indiscriminately in the lipid component (Cheesman, 1967).

In the absence of more precise analysis several of the carotenoproteins of invertebrates listed by Cheesman (1967) are included in the category of relatively unspecific complexes.

B. General properties.

All the carotenoproteins yet examined are readily split into carotenoid and apoprotein by treatment of an aqueous solution with acetone or ethanol, which suggests that covalent bonds are not involved in the combination. Relatively few data are available with respect to the molecular dimensions of carotenoproteins. The chemical

and physico-chemical properties of crustacyanin only, have been intensively investigated by a number of workers (Jencks et al. 1964, Cheesman et al. 1966, Kuhn and Kühn 1967, Buchwald and Jencks 1968). The native form of the protein, α-crustacyanin, shows by a combination of sedimentation- and diffusion measurements a molecular weight of about 380,000 (Gammack et al. 1971).

With respect to the molecular dimensions of the other proteins pertinent references are compiled in the reviews of Cheesman et al. 1967 and Thommen 1971.

Carotenoids, isolated from carotenoproteins of invertebrates, include astaxanthin and its esters, canthaxanthin, β-carotene, xanthophylls, bile pigments and others, found as accompanying or associated pigments (Cheesman et al. 1967).

A characteristic of the spectra of the majority of carotenoproteins is a more or less pronounced bathochromic shift of the main absorption bands of the carotenoids ; rather exceptionally, a hypsochromic effect is noticed (Jencks and Buten 1964).

From literature it is also evident that a high variability of spectra can be obtained with closely related- or identical prosthetic groups and a series of different apoproteins (Cheesman et al. 1967).

The absorption spectra of a well-established carotenoprotein is shown in Fig. I_4.

Only different hypotheses about the nature of the carotenoid-protein linkages have been argued by several authors (Cheesman et al. 1967).

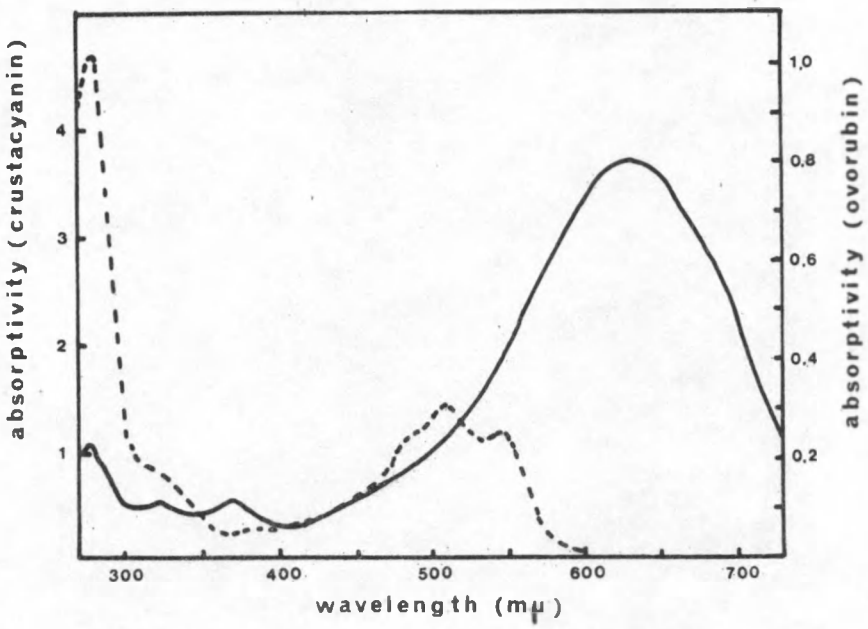


Fig. I_4 Absorption spectra of crustacyanin and ovorubin.
——— Crustacyanin (1mg/ml) in 0.2M-Na-K phosphate buffer, pH 7.
----- Ovorubin (1mg/ml) in water.
(after Cheesman, Zagalsky and Ceccaldi, 1966 and Cheesman, 1958, respectively).

The nature of the carotenoid prosthetic group as well as the amino acid composition and properties of the apoprotein moiety determine the conditions of protein-carotenoid interaction (Zagalsky et al. 1970 and 1972).

C. Possible functions of carotenoproteins.

1) The stabilizing influence of the carotenoid on the protein structure, particularly the control of the protein configuration, has recently been reviewed by Cheesman et al. 1967 and Zagalsky et al. 1972.

Such observations, together with the common experience that protein-associated carotenoids are much less susceptible to photo-oxydation than those in free form, constitude ample evidence for mutual stabilization of carotenoids and proteins.

2) Carotenoproteins are generally found in two body areas :

- the exoskeletons and epidermis
- the eggs and ovaries.

This occurrence and the characteristic change in colour often accompanying the combination of carotenoids and proteins reveal the probable role of carotenoproteins in protective coloration.

This protective coloration can conveniently be grouped into two categories. The first category involves the use of colour as a means of concealment, associated with predator-prey relationships.

In the second category carotenoproteins may protect several biological tissues or functions from the deleterious effects of radiation, although in many papers

(mentioned by Cheesman et al. 1967) no experimental evidence verifies the suggested effects.

No reports available, in which the occurrence and distribution of carotenoids in reproductive structures are discussed, give an adequate explanation for this phenomenon in terms of a fundamental participation of carotenoids in reproduction (Krinsky, 1971 and Cheesman et al. 1967).

3) Miscellaneous functions.

The possibility that carotenoids instead of vitamin A may be coupled to a photosensitive pigment complex in crustacea has been stressed by Wolfe and Cornwell (1965).

Isolation of a carotene-protein complex from spinach chloroplasts that catalyses the photoreduction of cytochrome indicates the enhanced electron donor and electron acceptor capacities of carotenoids by their binding to proteins (Kahn and Chang, 1965 and Krinsky, 1971).

A discussion of further participation of carotenoids in a variety of possible functions goes beyond the scope of this work and is amply discussed by Cheesman et al. (1967) and Krinsky (1971).

C H A P T E R II

STUDY OF THE CAROTENOIDS AND CAROTENOPROTEINS IN THE BROWN SHRIMP (CRANGON VULGARIS FABR.).

Introduction.

Although the carotenoids of many species of Crustacea have been studied in detail, no work is available on the Brown Shrimp (Crangon vulgaris Fabr.).

In the majority of the investigations on the pigmentation of the crustacea, astaxanthin was found to be very abundant and more or less characteristic of the class.(Isler, 1971, Czerpak and Czeczuga, 1969, Mc Beth, 1970, Tsukuda, 1963 and Czeczuga, 1973).

Furthermore the presence of astaxanthin in the form of protein complexes seems likewise to be characteristic.

The present chapter deals with the quantitative extraction of carotenoids from boiled unpeeled shrimps. Research on the identification and purification of the carotenoids in the boiled shrimps is reported.

The extraction of carotenoproteins from unboiled shrimps and the carotenoids bound to proteins in these complexes are discussed.

A. Carotenoids in the boiled shrimp.

1. Materials and methods.

1.a. Materials.

The common shrimp was caught in the North Sea a few miles off-shore by the "Ostend 100" fishing boat and boiled aboard in brine for an average time of 7 minutes.

Following the catch the shrimps were frozen in liquid nitrogen and stored. In this way the samples were transported to our laboratory for further analysis.

Samples of authentic carotenoids were used as standards for comparison with the shrimp pigments : β-carotene and canthaxanthin were kindly supplied by F. Hoffman-La Roche, Basel (Switzerland). Astaxanthin was extracted from Salmo Salar samples, kindly supplied by dr. ir. W. Vyncke (Ministry of Agriculture, Fisheries Research station, Ostend Belgium). Astacene was formed by saponification of astaxanthin. Isozeaxanthin was obtained after borohydride reduction of canthaxanthin.

1.b. Methods.

1.b.1. Extraction of carotenoids from boiled shrimps.

All extractions were carried out in dim light.

a. Extraction according to Bligh and Dyer (1959).

Several authors (Damman and Salwin, 1966, Lusk et al. 1964, Feys, 1973) have used this method for total lipid extraction.

In this study the method was modified as follows : 40 g aliquots, mixed with 80 ml of chloroform (Merck p.a.) and 100 ml of methanol (Merck p.a.) were homogenized for 4 minutes using a Sorvall omni-mixer. To the homogenate an additional 80 ml of chloroform was added and blended for 1 minute ; subsequently 80 ml of distilled water was added and blending was continued for another minute. The homogenate was filtered through a S&S n° 604 filter on a Büchner funnel under slight suction.

The filtrate was transferred into a separatory funnel and given a few minutes to stand for complete separation and clarification ; subsequently the chloroformic layer was removed. This fraction was dried over anhydrous sodium sulphate (Merck 6649), evaporated to dryness under reduced pressure and redissolved into 5 ml of P.E*.

b. Continuous extraction procedure.

An adaption of the method as described by Maes (1962) was used. Extraction was carried out using 300 ml of the following solvent systems :

1) P.E.
2) hexane - acetone - ethanol - toluene (10 : 7 : 6 : 7).
3) Hexane* - acetone (3 : 7).
4) P.E. - acetone (1 : 2).
5) Acetone.

A 40g sample was dried by homogenizing with 100g of anhydrous sodium sulphate in a Sorvall omni-mixer. The dried sample was furtherly ground in a mortar with 100g of seasand (Merck 7712) and transferred into the extraction column (length 30 cm ; diameter 3.5 cm). Of the solvents listed above, 50 ml were added to the column and allowed to stand for 30 minutes ; subsequently the remaining 250 ml

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* The abreviation P.E. is used to characterise the petroleum ether fraction with a boiling range of 40°-60° C ; by high-boiling P.E. the petroleum ether fraction with a boiling range of 100°-140° C is meant.

* The hexane used in all our experiments in this work was n-hexane.

were added. The pigments extracted in this way were transferred into P.E.

c. Extraction by homogenizing the tissues with acetone.

The material to be extracted was repeatedly homogenized in acetone until no further pigments could be extracted (Holter, 1969). The different acetone solutions were combined and concentrated under a stream of nitrogen or under reduced pressure. The pigments were transferred to P.E. as described above.

Fox et al. (1962, 1965, 1967) usually used ethanol instead of acetone as an extraction agent since the latter, while excluding phospholipids, tends to create troublesome emulsions, when diluted with water and shaken with P.E. Furthermore, in contrast to ethanol, acetone is more difficult to wash free of P.E.

1.b.2. Quantitative determination of carotenoids.

The total pigment content was determined spectrophotometrically using the equation as described by Davies (1965)

$$x = \frac{E.y}{E^{1\%}_{1cm} \cdot 100}$$

In this formula x expresses the weight (in g) of a carotenoid dissolved in y ml of a given solvent. Values of the $E^{1\%}_{1cm}$ are available for most carotenoids (Davies, 1965).

Since in the case of shrimps neither the structure of the different carotenoids present, nor the differences

in molar extinction coefficient were known, the pigment concentrations were arbitrarily expressed as μg β-carotene per gram fresh or dry weight.
A value of 2505 was taken for the $E^{1\%}_{1cm}$ of β-carotene in P.E. at λ_{max} of 451 nm.
Spectrophotometric measurements were carried out using a Hitachi-Perkin Elmer, Model 124,,double beam spectrophotometer.

1.b.3. Chromatographic analysis.

a. Column chromatography.

The initial separation of the carotenoids from the crude shrimp extract was made on silicagel (Machery, Nagel and Co.). The following procedure was developed, using glass columns, 2.5 cm in diameter and 20-30 cm in length (type VEL, 93D x 30 with a P_1 filter).
Prior to use, the silicagel was washed with methanol in order to elute all impurities. After drying the silicagel in an oven at 120° C overnight, the columns were filled with a slurry containing 24g silicagel in high-boiling P.E. A freshly prepared carotenoid extract in P.E. was transferred into the column and eluted with high-boiling P.E. containing increasing amounts of acetone. The eluted fractions were evaporated under vacuum to dryness and the residues redissolved in a few ml of hexane or P.E.

b. Thin-layer chromatography.

Thin-layer chromatography was also used to separate and identify the carotenoid pigments and the lipid fractions in a total pigment extract.

60g Silicagel G (Merck 7731) was slurried in 120 ml of distilled water and spread on 5 plates (glass,

20x20 cm) with a layer thichness of 0.5 mm. After drying the plates in an oven at 60°C for 4 hours, they were washed by developing in methanol p.a. in order to remove any trace of impurity from the adsorbing layer. The plates were then activated in an oven at 120°C for two hours and stored in a dessicator at room temperature.

An initial separation of the crude extract was achieved by TLC with the solvent mixture high-boiling P.E.-isopropanol (Merck p.a.) and distilled water in the proportions : 100/12/0.5.

In order to liberate the different carotenoid fractions from lipid components different solvent mixtures were used as mentioned in the discussion.

The presence of lipids was checked on the thin-layer plates by spraying with 2',7' - dichlorofluorescein.

c. Gas-liquid chromatography.

The fatty acid esters of the carotenoid fractions were converted into their respective methylester derivatives using the micromethod for interesterification with HCl or H_2SO_4 in anhydrous methanol as described by Stoffel et al. 1959 and Marinetti 1967.

When the esterification was completed the reaction mixture was cooled at room temperature ; two volumes of distilled water were added and the methylesters were extracted three times with 3 ml portions of P.E. The combined P.E. extracts were furtherly washed with water, dried over anhydrous sodium sulphate and transferred into a micro-sublimation apparatus. After evaporating the P.E. fraction, the methylesters residue was sublimated at 60°C and at a pressure of 0.2 - 0.25 mm Hg. At the end of the distillation period (30 minutes) the sublimate on the cold

finger was carefully washed down with P.E. in a vial. Aliquots of this solution were taken for further gas chromatographic analysis under the following experimental conditions :

Instrument : Varian Aerograph, Model 1400.
Detector : F.I.D. (Flame Ionization Detector).
Column : stainless steel, 6'x1/8".
Solid support : Chromosorb W 80/100 mesh.
Stationary phase : DEGS 10%.
Injection temperature : 200°C.
Detector temperature : 260°C.
Oven temperature : 160°C and 190°C.
Carrier gas : nitrogen.
Flow of nitrogen, hydrogen and air : respectively 25, 20 and 300 ml/min.

2. Results and discussion.

2.a. Qualitative and quantitative comparison of the different extraction methods.

Most of our experiments were carried out on unpeeled shrimps. However in order to check the distribution of the pigments in the entire organism, the shrimp meat and the peel were extracted separately. The absorption spectra of an extract of unpeeled shrimps, shrimp meat and shrimp peels were identical. Furthermore the absorption maxima were identical for all extraction procedures. The absorption spectrum of a shrimp extract in P.E. is given in Fig. II_1.

Using TLC or column chromatography identical pigments were found regardless of the extraction method used on entire shrimps, the shrimp meat and the shrimp peels.

In the course of the investigations it was noticed that the pigment content in the shrimp samples varied with the shrimping season. Fig. II_2. The highest pigment content was found in the late spring ; this level decreased afterwards until the month of November.

A comparative study of the different extraction methods on identical batches of shrimps showed that the continuous column-extraction method with acetone gave the most reproducible results (Tab. II_1). In this table it is shown that a continuous extraction on column with acetone gave the highest yield.

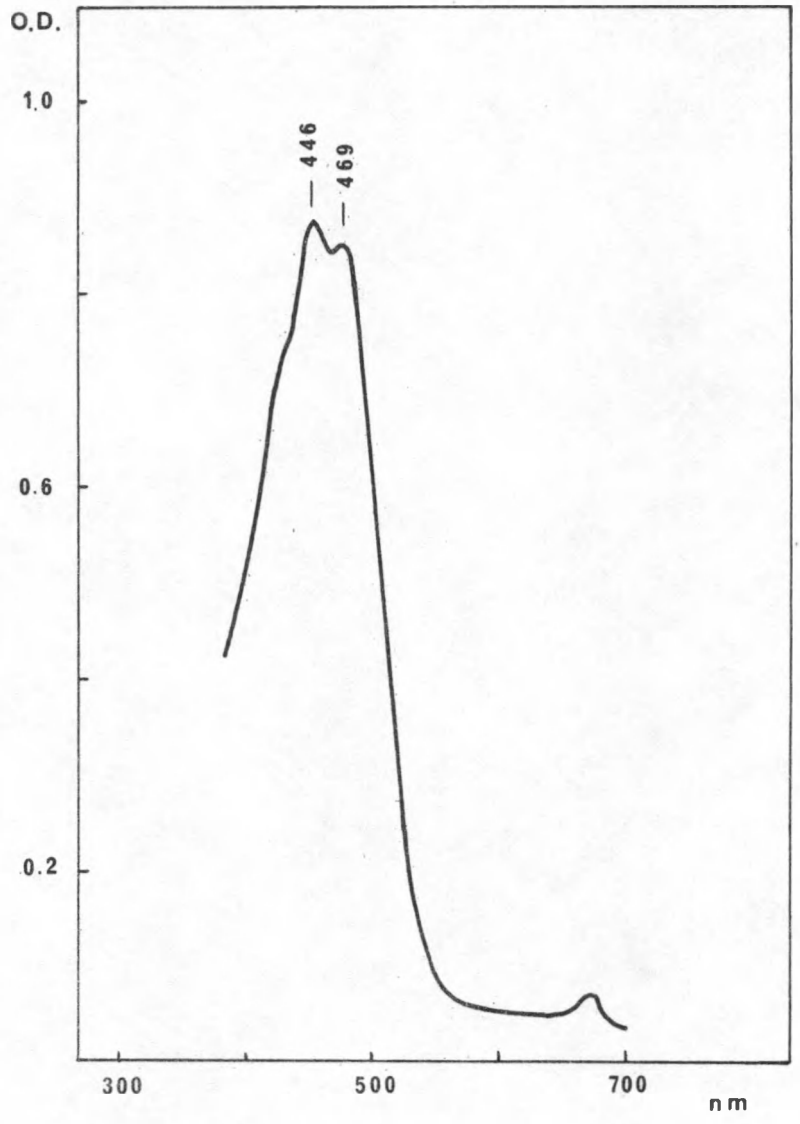


Fig. II_1 Total absorption spectrum of a shrimp extract (in petroleum ether).

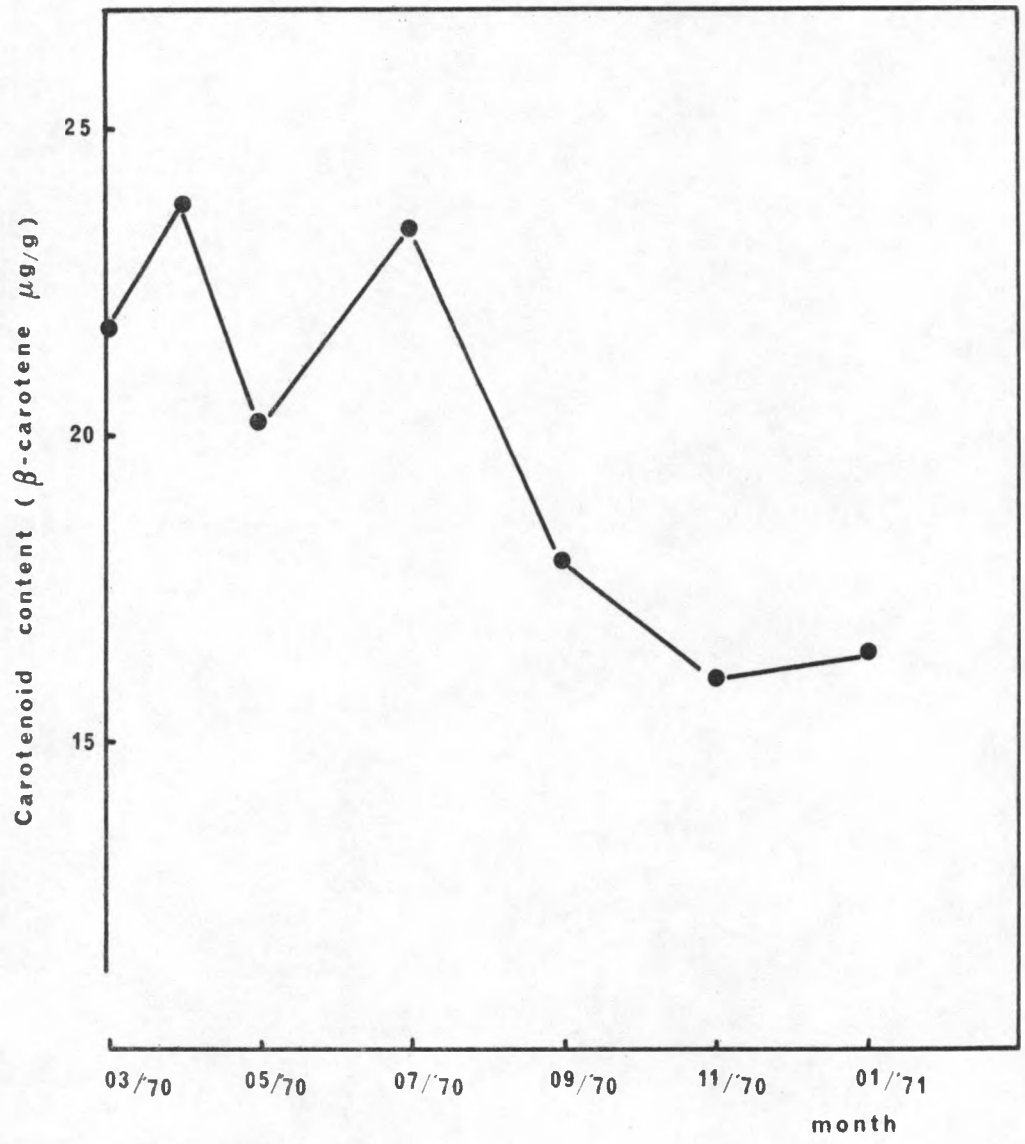


Fig. II_2 Seasonal variation of the total pigment content of shrimps.

Tabel II.1 Quantitative comparison of the experimented extraction procedures on entire boiled shrimps.

Extraction method	Average pigment content : μg β-carotene per g fresh weight	standard deviation	variation coefficient
2b1a	4.23	0.043	0.038
2b1b1	4.11	0.067	0.065
2b1b2	4.17	0.083	0.075
2b1b3	4.76	0.074	0.062
2b1b4	4.79	0.075	0.063
2b1b5	5.89	0.066	0.044
2b1c	4.76	0.076	0.041

Extraction was carried out as described in the experimental section.

The values in pigment contents are an average of 8 determinations. The moisture content in the shrimp was 71%.

2.b. Purification and identification of the carotenoids from boiled shrimps.

2.b.1. Column elution pattern and identity of the shrimp carotenoids.

A total pigment extract was separated by column chromatography with an elution solvent of increasing polarity. The different pigment fractions obtained had the following characteristics :

Fraction 1 : was a yellow band that eluted from a silicagel column with 3% acetone in high-boiling P.E. It gave a typical colour reaction with ninhydrin ; when spectrophotometrically analyzed it lacked any typical absorption spectrum. So far this fraction remains unidentified.

Fraction 2 : this yellow band eluted distinctly separated from the preceding and succeeding fractions with 3% acetone in high-boiling P.E.

- When partitioned between P.E. and 90% and 95% methanol respectively the fraction was entirely epiphasic.

- The pigment exhibited a typical carotenoid absorption spectrum in P.E. with maxima at λ 448 nm and λ 476 nm and a shoulder at λ 422 nm. This spectrum was not altered by saponification and its characteristics were identical to those reported for β-carotene (Wolfe et al. 1965, Gilchrist et al. 1967, Gilchrist, 1968 and Czeczuga, 1970).

- Co-chromatography of this fraction with authentic β-carotene (Merck 2236) on silicagel G layers, with the solvent mixture high-boiling

P.E., isopropanol, H_2O, (100:12:0.5) failed to separate the two components. Large quantities of neutral lipids accompanied the pigment and therefore co-chromatography was carried out on both the saponified and unsaponified fractions.

Fraction 3 : eluted as a yellow band with 5% acetone in high-boiling P.E., slightly ahead of fraction 4.

- On partition between P.E. and 95% methanol this fraction was entirely epiphasic.

- The fraction had an absorption spectrum in P.E. with maxima at λ 472 nm and λ 445 nm.

- After saponification of this fraction, chromatography on silicagel G (solvent system : 25% acetone in high-boiling P.E.) yielded five yellow bands with lower R_f values than the R_f value of the unsaponified fraction.

- The identification of these carotenoids was very difficult due to a lack of references.

a) A minor fraction was identified as iso-zeaxanthin. This could be concluded from co-chromatographic analysis on thin-layer with iso-zeaxanthin. Iso-zeaxanthin was obtained by borohydride reduction of canthaxanthin.
Moreover this fraction exhibited absorption spectra that were identical to those of iso-zeaxanthin as described by Davies (1965).

b) A major fraction was identified as zeaxanthin. The spectral patterns of these fractions were identical to those of zeaxanthin, as reported by Davies (1965).

c) Finally the absorption maxima of another fraction corresponded to those of lutein (Davies, 1965).

From this spectrophotometric results it was concluded that fraction 3 would be a mixture of zeaxanthin, iso-zeaxanthin and lutein esters.

Fraction 4 : eluted immediately behind fraction 3. This 4^{th} fraction exhibited a wide pink to red zone on the column. The pigments eluted slowly with 7% acetone in high-boiling P.E.

- TLC analysis on silicagel G in the solvent high-boiling P.E., isopropanol, H_2O (100:12:0.5) revealed the presence of three separated bands with decreasing R_f values :

a) a yellow pigment, being the zeaxanthin ester that was identified in fraction 3.

b) a pink epiphasic di-ester of astaxanthin.

c) a minor yellow unsaponifiable fraction.

- On TLC analysis the pink astaxanthin ester band was shown to be composed of three coloured zones, probably due to the nature of the fatty acid ester groups.

- The absorption spectrum of this pigment in P.E. had a single maximum at λ467 nm. On partition between P.E. and 90% methanol the pigment was epiphasic.

- After saponification an acidic hypophasic product was obtained which was not separable from astacene on TLC.

- Borohydride reduction of this fraction is discussed below.

- The absorption spectrum of the yellow unsaponifiable fraction in P.E. had maxima at λ444 nm and λ472 nm. The identification of this minor

fraction remained conjectural due to a lack of references.

Fraction 5 : after elution of fraction 4 and addition of a mixture of 25% acetone in high-boiling P.E. to the column a pink streaking pigment was eluted.

This component had a broad symmetrical absorption spectrum in P.E. with a maximum at λ468 nm. As will be discussed below, this component was probably a mono-ester derivative of astaxanthin.

After saponification this fraction yielded astacene.

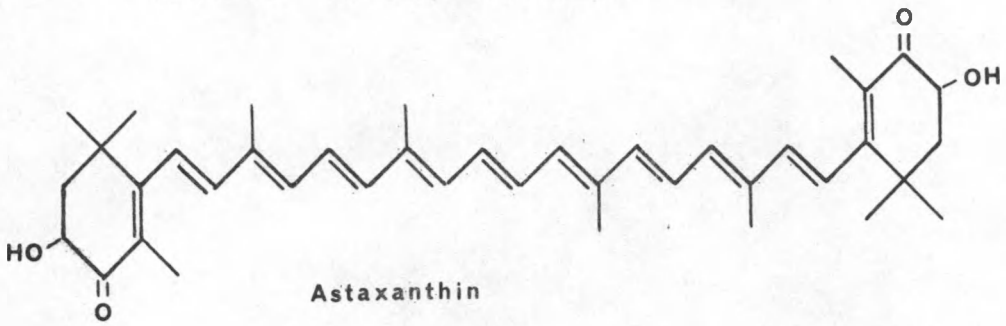
Fraction 6 : by adding a mixture of 50% acetone in high-boiling P.E. to the column a fully hypophasic pink fraction was eluted well behind fraction 5. Its symmetrical absorption spectrum in P.E. had a maximum at λ468 nm.

Saponification yielded a product unseparable from astacene on TLC analysis.

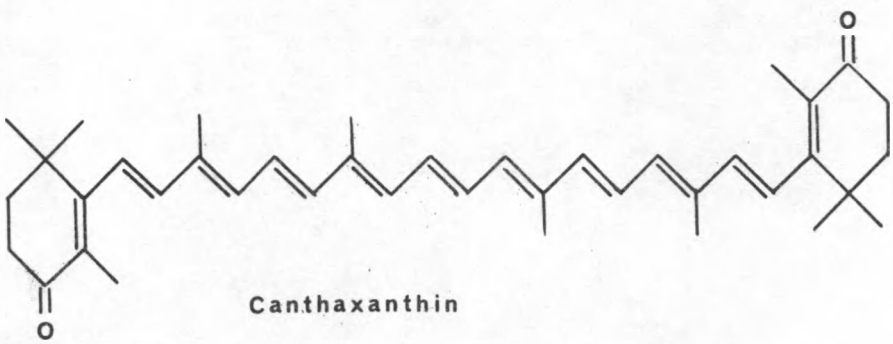
These characteristics were typical for unesterified astaxanthin.

The advantage of using silicagel instead of alumina as adsorbens for column chromatography is that there is no danger of hydrolysis of the astaxanthin ester derivatives or of oxydation of astaxanthin to astacene (Holter, 1969, Herring, 1968, Lee, 1967, Czeczuga et al. 1968). When using alumina columns we always found that a red pigment (astaxanthin or astacene) remained on the top of the column. This fraction could only be eluted by adding a few drops of glacial acetic acid to the elution solvent whether it was acetone or methanol.
On p. 46b. the structure of some carotenoids is given.

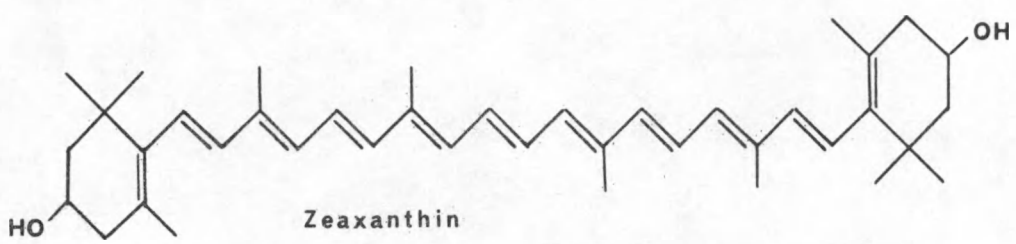
Structure of some carotenoids.



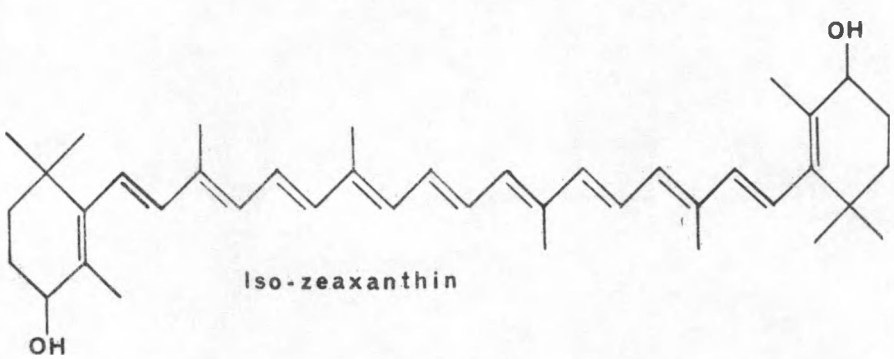
Astaxanthin



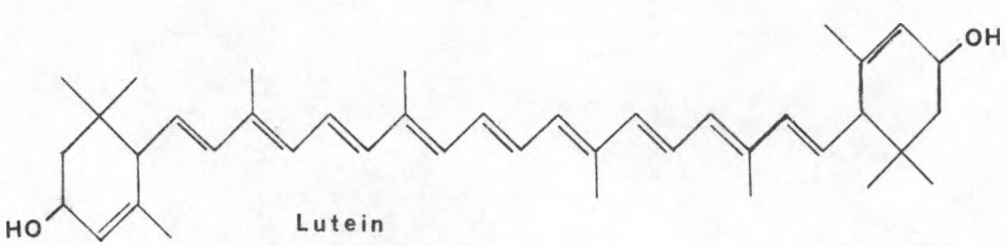
Canthaxanthin



Zeaxanthin



Iso-zeaxanthin



Lutein

2.b.2. Analysis of the astaxanthin fractions.

a) The astaxanthin-ester fractions were analyzed by TLC using the method as described by Lambertsen et al. (1971).

The fractions were applied on silicagel G plates and eluted with a solution of 25% isopropylacetate in benzene. Well-defined spots were obtained with R_f values of 0.96-1 for the di-esters, 0.80 for the mono-esters and 0.3 for free astaxanthin. Pure canthaxanthin ran between the mono- and di-esters of astaxanthin. These results were in full agreement with those of Lambertsen et al. (1971).

b) Reduction of the astaxanthin fractions.
Aliquots of each fraction were transferred to ethanol and reduced with a few grains of potassium borohydride at room temperature. The appearance of a pure yellow colour indicated that the keto groups were reduced to hydroxy groups. Visual observation was generally sufficient for determination of the end of the reduction reaction.

Reduced astaxanthin has the same chromophore as β-carotene. The absorption curves of original and reduced astaxanthin in ethanol is shown in Fig. II_3.

c) Thin-layer chromatography of the reduced astaxanthin fractions.
Borohydride reduction and subsequent TLC analysis on silicagel G with 25% acetone in P.E. was an elegant method for purification of the astaxanthin ester fractions from contaminating lipid material.

The dihydroxy di-fatty acid ester gave a R_f value of 0.75-0.80, the trihydroxy mono-fatty acid ester had a R_f value of 0.30-0.40 and for tetrahydroxy β-carotene a R_f value of 0 was found.

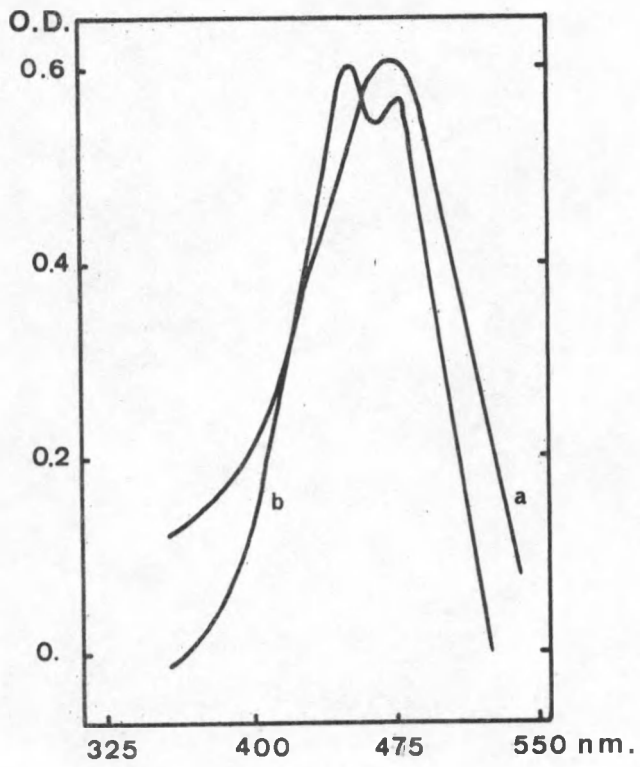


Fig. II_3 Absorption spectra of astaxanthin (a) and reduced astaxanthin (b) in ethanol.

The presence of lipids on the thin layer was checked by spraying it with 2',7'-dichlorofluorescein.

Tab. II_2 compiles the data of the column chromatographic analysis and the identity of the carotenoids present in the boiled shrimps.

2.c. Identification of the fatty acids esterified with the carotenoids.

2.c.1. Identification of the contaminating lipid material upon TLC analysis of the shrimp pigments.

Upon TLC analysis of a total pigment extract on silicagel G plates in the solvent mixture high-boiling P.E., isopropanol, H_2O (100:12:0.5) a clear separation of the carotenoid fractions was obtained.

The R_f values of the carotenoid fractions in this experiment are given in Tab. II_3.

Identification of the fatty acids bound to carotenoid pigments, necessitated the purification of the individual pigments from contaminating lipidic material.

The latter were identified by co-chromatography with standards of triglycerides, diglycerides, monoglycerides, fatty acids and fatty acid esters in the solvent mixture high-boiling P.E., isopropanol, H_2O (100:12:0.5).

The R_f values of the used standard lipids in this solvent are also compiled in Tab. II_3. Variation in the chain length of the different reference lipids did not give any change in R_f value on the thin layer plate.

Tabel II.2 Column elution pattern and identity of the shrimp carotenoids.

Fraction number	elution mixture	colour in P.E.	Identity
1.	3% acetone in high-boiling P.E.	yellow	unknown
2.	3% acetone in high-boiling P.E.	yellow	β-carotene
3.	5% acetone in high-boiling P.E.	yellow-orange	mixture of xanthophyllesters (zeaxanthin esters, isozeaxanthin, lutein esters)
4.	7% acetone in high-boiling P.E.	pink	a) zeaxanthin ester b) astaxanthin ester c) unsaponifiable xanthophyll
5.	25% acetone in high-boiling P.E.	pink	astaxanthin mono-ester
6.	50% acetone in high-boiling P.E.	pink	astaxanthin

Table II.3 Comparison of the R_f values of the carotenoid pigments and the reference lipid components in the solvent high-boiling P.E.-isopropanol-H_2O (100 : 12 : 0.5).

Carotenoids	R_f value	lipids	R_f value
astaxanthin	0.07	monopalmitin	0.02
astaxanthin mono-ester	0.26	dipalmitin	0.20
unsaponifiable xanthophyll	0.30	palmitic acid	0.26
astaxanthin di-ester	0.66	palmitic acid methyl-ester	0.70
mixture of xanthophyll-esters	0.77	tripalmitin	0.77
β-carotene	0.90		

Using the solvent mixture P.E., diethyl ether, acetic acid (80:20:1) a R_f value of 0.85 was found for the fatty acid methylesters on the silicagel plate.

As this value was much greater than the R_f values of the different carotenoid fractions and the triglycerides in this solvent, we concluded that no fatty acid methylesters are present in the shrimp extract.

From these experiments it could be stated that the astaxanthin-diester fraction and the xanthophyllester fraction are mainly contaminated with triglycerides.

The astaxanthin mono-ester fraction mainly contains free fatty acids and diglycerides.

2.c.2. Purification of the carotenoid ester pigments.

a) The mixture of xanthophyllesters when chromatographed on a silicagel G plate with P.E.-ethyl acetate (92:8) as solvent system gave a R_f value of 0.50.
For the triglycerides a R_f value of 0.75 was found. As described, interesterification of the xanthophyllesters yielded the fatty acid methyl esters that were analyzed by GLC.

b) Purification of the astaxanthin-di-ester fraction was achieved on the thin-layer plate by elution with high-boiling P.E.-acetone (97:3). A very sharp separation was obtained although the differences in R_f value was small (R_f value for the triglycerides : 0.35 and 0.30 for the astaxanthin-di-ester).
The astaxanthin di-ester was furtherly checked for purity by borohydride reduction and subsequent thin layer chromatography in the solvent mixture P.E.-acetone (100:25).

c) Separation of the astaxanthin mono-ester from free fatty acids and diglycerides was carried out on silicagel G with P.E.-ethylacetate (84:16) as solvent system. The fatty acids and diglycerides showed a R_f value of 0.40, while the astaxanthin mono-ester gave a value of 0.20.

2.c.3. Gas chromatographic analysis of the fatty acid methylesters.

1. Fatty acids present in a total lipid extract.

The fatty acids present in a total lipid extract from shrimps (obtained by extraction according to the Bligh and Dyer method) were studied in collaboration with Feys (1973). Identification of the fatty acid methylesters was carried out in the following steps :

i. Comparison of the relative retention time of known reference compounds. The retention times were calculated relatively to C_{19}:0 chosen as internal standard methylester (nonadecanoic methylester), since this compound was never found in the lipid extract.

ii. Calculation of the log retention time of the standard components as a function of the number of C-atoms. Under isothermic conditions a lineair relation exists between these two variables (Tranchant, 1968).

By calculation of the log retention time of the fatty acid methylesters in the shrimp extract the number of C-atoms of the different components was determined.

iii. In order to decrease the complexity of the mixture, separation of the methylated lipid extract was carried out by silver ion (argentation) thin-layer chromatography. The plates were prepared according to Lindsay (1964) and

Kuksis (1971). The solvent system was a mixture of chloroform-methanol in variable proportions (0 to 5% methanol). Components were located under U.V. light after spraying with a solution of 2',7'-dichlorofluorescein in ethanol. In Fig. II_4 and Fig. II_5 a survey is given of the identified fatty acids in a total lipid extract ; the column temperatures were respectively 160°C and 190°C.
About 30 fatty acids were identified. Quantitatively the $C_{16}:0$, $C_{18}:1\omega_9$, $C_{20}:5\omega_5$ and $C_{22}:6\omega_3$ were the most important fatty acids found in shrimps.
In the Alaska Pink Shrimp these four fatty acids were also the most important fractions (Krzeczkowski, 1970).

2. Fatty acids esterified with the carotenoids.

In Figs. II_6 and II_7 a typical chromatogram of the fatty acid methylester derivatives of the astaxanthin di-ester fraction was shown. All compounds identified in the total lipid extract were also present as esters of astaxanthin. An increase in the ratio lauric acid : palmitic acid and myristic acid : palmitic acid for the di-esters of astaxanthin in comparison to the total extract was found.
A relatively large amount of oleic acid was also typical for the astaxanthin di-ester fraction.
As to the higher fatty acids eikosapentanoic acid ($C_{20}:5\omega_5$) remained the most important.
Identical results were obtained for the mono-ester fraction of astaxanthin (van de Casseye 1974).
The xanthophyllester fractions revealed a still greater increase in the ratio lauric acid : palmitic acid and myristic acid : palmitic acid.
Oleic acid and eikosapentanoic acid were important fatty acids as well.
The fatty acids $C_{18}:2\omega_6$, $C_{19}:2\omega?$, $C_{17}:0$, $C_{17}:1\omega?$ and $C_{20}:0$ were only present in very minor proportions in the

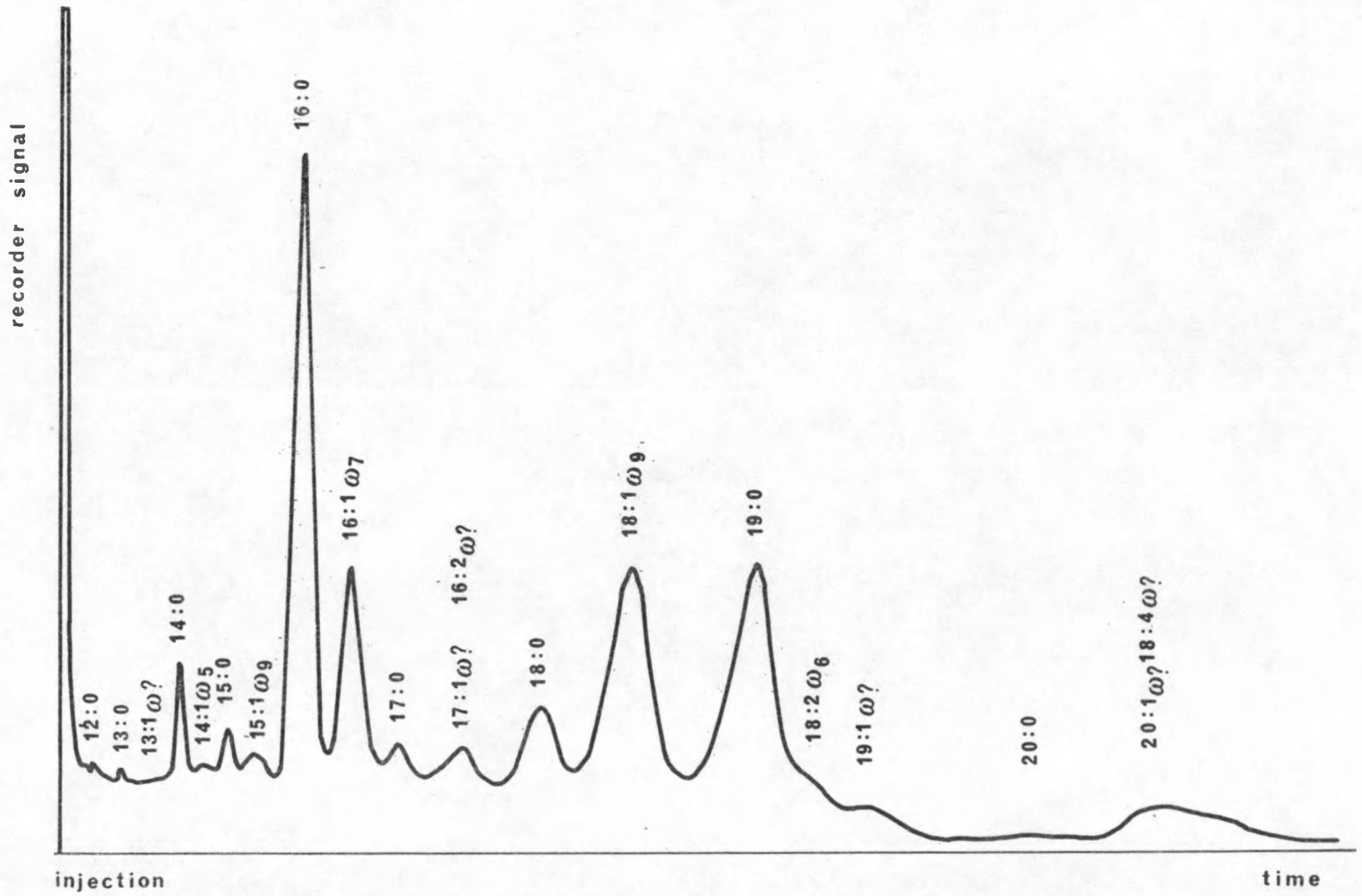


Fig. II_4 Chromatogram of the fatty acids in a total lipid extract (column temperature 160°C).

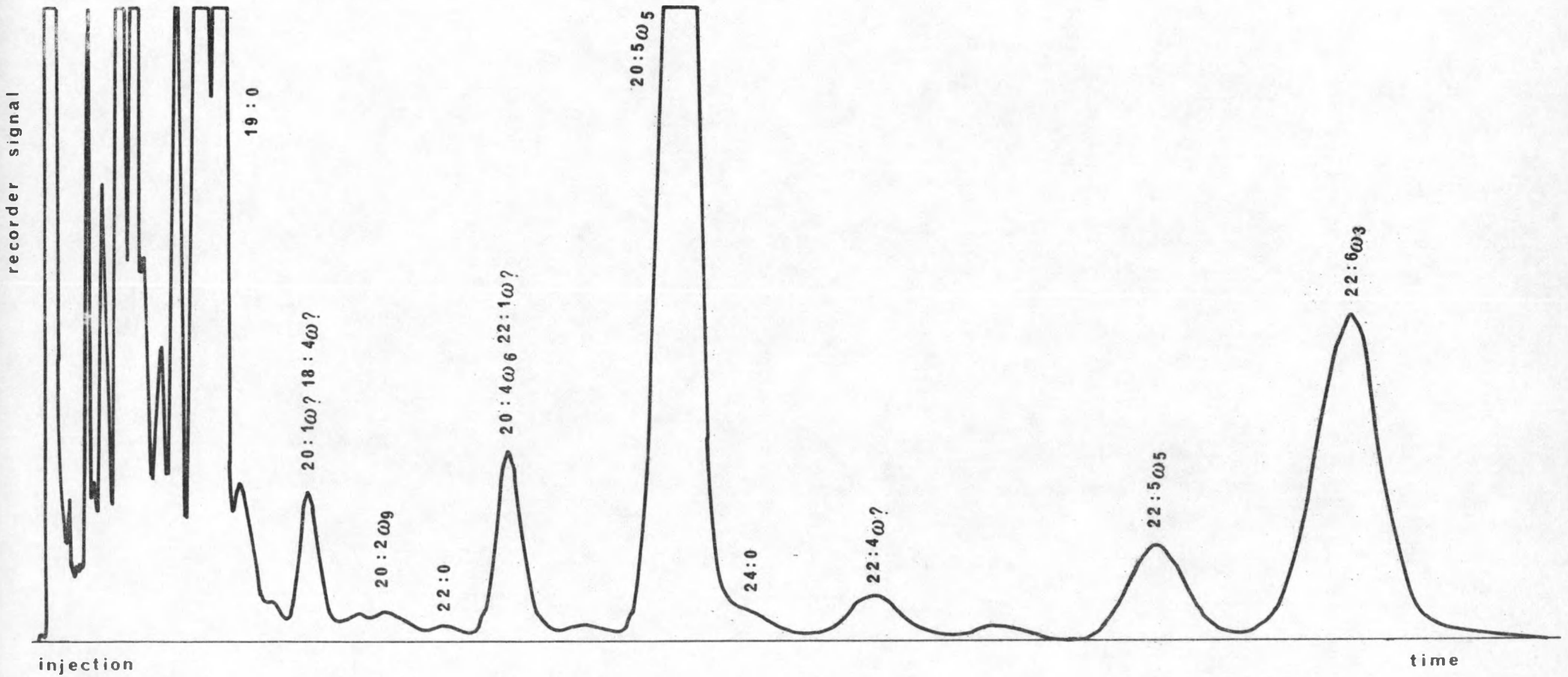


Fig. II_5 Chromatogram of the fatty acids in a total lipid extract (column temperature 190°C).

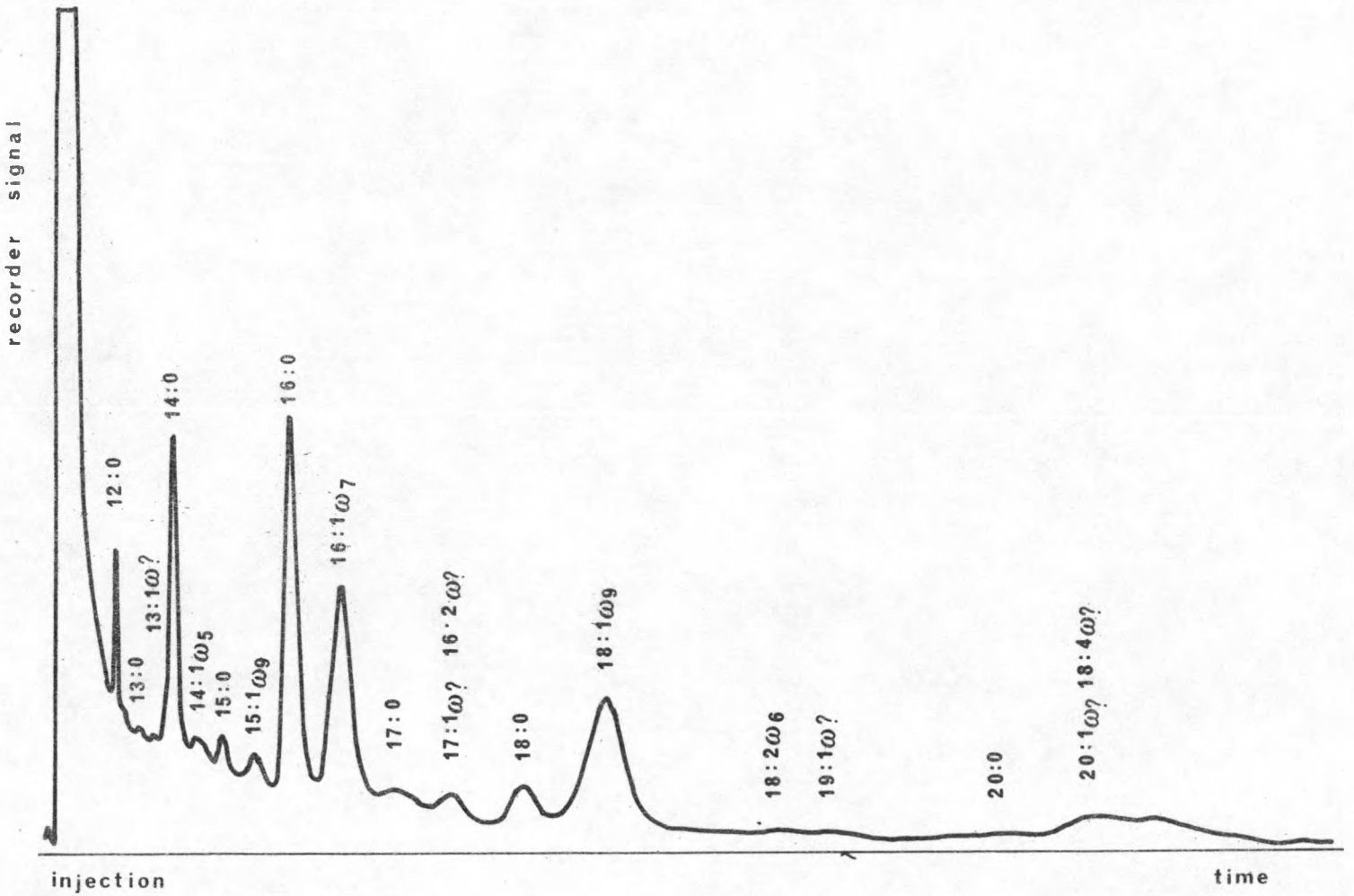


Fig. II_6 Chromatogram of the fatty acids in the astaxanthin di-ester (column temperature 160°C).

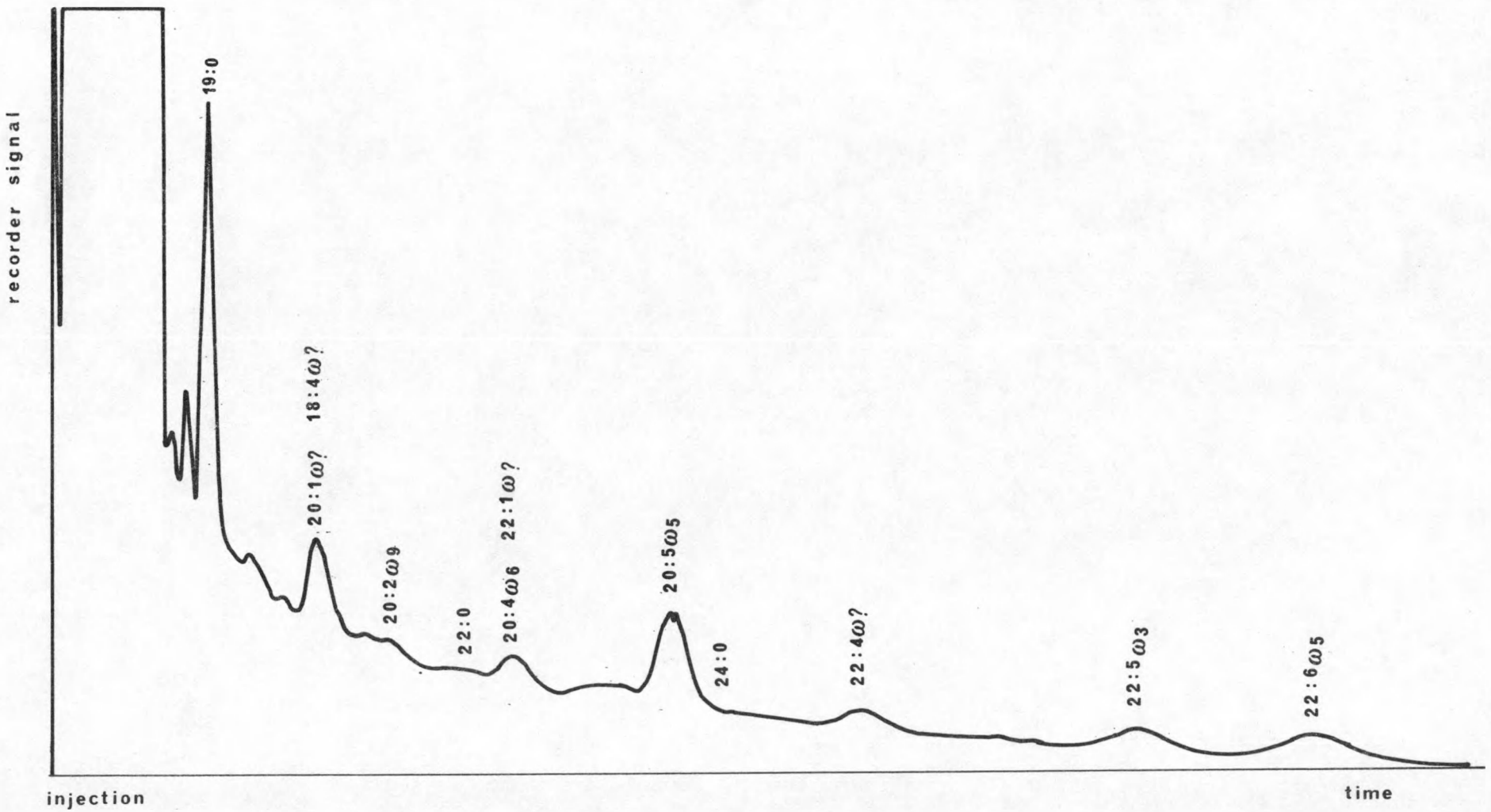


Fig. II_7 Chromatogram of the fatty acids in the astaxanthin di-ester (column temperature 190°C).

carotenoidesters and could only be detected at very high sensitivity operating conditions.

In conclusion : saturated as well as unsaturated fatty acids were found as carotenoid esters.

B. Carotenoids in the unboiled shrimp.

Despite the recent interest in the discovery, elucidation and biochemistry of animal carotenoids and animal carotenoproteins it is rather surprising that no attention has been paid so far to the carotenoproteins in the Crangon vulgaris Fabr.

There are reasons to believe that some types of combination of carotenoids with proteins exist, because of the failure of the carotenoids to be extracted from the blue-grey unboiled shrimp in P.E.

This chapter reports on a preliminary study on the carotenoproteins in the entire unboiled shrimp.

1. Materials and methods.

1.a. Materials.

Immediately after landing, the unboiled shrimps were frozen at -40°C and transported to the laboratory for analysis.

1.b. Methods.

1.b.1. Extraction of the carotenoproteins from unboiled shrimps.

In literature several procedures are described for extraction of carotenoproteins from different pigmented parts of some Decapod crustacean (Zagalsky et al. 1970).

In our experiments modified extraction procedures were adopted.

i. Carotenoproteins were extracted with different phosphate buffers (KH_2PO_4 and Na_2HPO_4) at pH 8, respectively 0.01, 0.1 and 0.2 in molarity.

For this purpose 500 g unboiled shrimps and 500 g seasand (Merck 7712) were homogenized with 1 liter of buffer solution for 4 minutes using a Sorvall omni-mixer.

Care was taken that the temperature of this mixture during homogenizing was kept around 5°C.

Afterwards the extraction mixture was given to stand in the refrigerator at 4°C during one night.

ii. Extraction was also identically performed by only changing the extraction solvents ; citrate buffers of pH 5 and pH 5.5, 0.01, 0.05 and 0.1 in molarity were used (Zagalsky et al. 1970).
Extractions with phosphate and citrate buffers in the presence of 10% dioxane (Zagalsky et al. 1970) were also carried out.

1.b.2. Purification of the carotenoproteins.

In order to remove the non-soluble residu, the crude extracts were centrifuged at 8,500 rpm (11,700 g) in a Sorvall SS-3, automatic superspeed centrifuge (Sorvall GSA rotor, d = 14.6 cm) at a temperature of 2°C.

Progressive saturation of this protein solution with $(NH_4)_2SO_4$ up to 5%, 10%, 40%, 60% precipitated the different protein fractions. The precipitates were suspended in 0.05 M phosphate buffer pH 7.

The carotenoproteins were subsequently applied on anionic and cationic Dowex ion exchangers (Serva Feinbiochemica Heidelberg 41351 and Fluka 44500). Adsorption and desorption of the carotenoproteins were tried in a pH range from 5 to 9.

Purification experiments of the carotenoproteins were also performed using calciumphosphate gel chromatography.

Finally dextran-gel chromatography on Sephadex G-75 and G-200 was carried out.

1.b.3. Carotenoid composition of the carotenoproteins.

The carotenoids present as prosthetic groups in the carotenoprotein fractions were identified by thin layer chromatography of the extracted carotenoids.

Carotenoids were liberated from the carotenoproteins in P.E. by addition of a suitable volume of acetone and methanol to the carotenoprotein solutions.

Identification of the carotenoids was performed as described in the experimental section of chapter II.A.

1.b.4. Molecular weigth determinations of the carotenoproteins.

Estimates of the molecular weights of the carotenoproteins were obtained from gel filtration studies.

Columns of Sephadex G-200 (2.4 x 21.3 cm) were equilibrated with 0.05 M phosphate buffer, pH 7, and calibrated with a series of reference proteins of known

molecular weight : urease (483,000), lactate-dehydrogenase (150,000) and serum albumin (68,000).

Molecular size estimations were derived by plotting the log of the molecular weight against the elution volume (Andrews, 1965).

1.b.5. Lipid analysis of the carotenoprotein fractions.

Upon liberation of the carotenoids from the carotenoproteins by "acetonolyse" lipid fractions simultaneously passed into the P.E. fraction.

Thin-layer analysis of those lipid components was carried out as described in the experimental section of chapter II.A.

2. Results and discussion.

2.a. Comparison of the different extraction methods.

Comparison of the different extraction procedures revealed that the method with citrate buffer (pH 5) 0.05 in molarity was the most reproducible and quantitative method.

Addition of 10% dioxane to the extraction solvent remarkably enhanced the efficiency of the extraction procedure.

However about 3 hours after extraction, liberation of the carotenoids was observed due to a destructive effect of dioxane on the carotenoproteins.

2.b. Fractionation of the carotenoproteins.

After centrifugation of a total carotenoprotein extract a minor flocculent was found in the upper phase of the supernatant (fraction I), indicating the presence of lipoproteins.

Saturation of the supernatant with 5% $(NH_4)_2SO_4$ and subsequent centrifugation yielded a grey-purple precipitate (fraction III) and a white floating flocculent on the surface (fraction II).

Subsequent addition of $(NH_4)_2SO_4$ to the supernatant gave a large precipitate (fraction IV) between 10% and 40% saturation.

The four fractionally precipitated fractions had different densities, and contained carotenoproteins as was indicated by carotenoid analysis.

At higher $(NH_4)_2SO_4$ saturations no further carotenoproteins were found neither in the precipitate nor in the supernatant.

2.c. Purification of the different carotenoprotein fractions.

The carotenoproteins in the four fractions described above were insoluble in all the phosphate and citrate buffers mentioned in the experimental section. Even in a medium of high ionic strength, obtained by addition of 10% $(NH_4)_2SO_4$ or NaCl, the carotenoproteins remained insoluble, since after centrifugation (8,500 rpm - 20 minutes) no carotenoproteins were found in the supernatant.

By addition of 1 g sodiumdodecylsulphate (SDS) per 100 ml citrate buffer pH 5.5 only fraction I and II dissolved.

Out of this effect we concluded once more that fraction I and II contained lipoproteins since SDS is known to interact preferentially with the lipoidic fraction of carotenoproteins.

Only at higher SDS concentrations dissociation of carotenoproteins in sub-units occurred as was estimated by gel chromatographic analysis.

The carotenoproteins of fractions III and IV could be solubilized with Triton X-100 (in a concentration of 0.1 g/100 ml).

However dissociation of the carotenoproteins in sub-units of varying molecular weight was not to be avoided under these experimental conditions.

Fraction I and II, solubilized by addition of SDS, were applied to anionic and cationic Dowex. However the carotenoproteins failed to elute with several phosphate buffers with a pH value between 5 and 9 and with different molarities.

This should be explained by the high polarity of the solubilized carotenoproteins.

Probably for the same reason adsorption chromatography on calcium phosphate gel remained unsuccessful.

By dextran-gel chromatography on Sephadex G-200 all proteins with different molecular weight than the carotenoproteins were separated. The fractions I and II partially purified in this way were indicated as

fraction I_P and II_P.

Fraction III, the blue-grey precipitate after centrifugation of an extract saturated with 5% ammonium sulphate, and fraction IV, the precipitate obtained after centrifugation of the supernatant saturated up to 40% ammonium sulphate, remained insoluble in phosphate buffer, as outlined above.

The only way to purify those fractions was by separating all compounds soluble in phosphate buffer, from the precipitated carotenoproteins (fraction III_P and fraction IV_P).

2.d. Characteristics of the carotenoproteins in unboiled shrimps.

2.d.1. Molecular weight determination.

The molecular weight estimates of the carotenoproteins derived from gel filtration are those of globular proteins, used to standardize the Sephadex G-200 columns, having identical elution volumes.

The actual molecular weights may be considerably lower, especially if the carotenoproteins are asymmetric (Andrews, 1965).

Therefore the accuracy of our molecular weight estimates should be compared with other methods for molecular weight determination.

On Fig. II_8 standardizing of elution volume versus molecular weight of known references is shown.

It was found by gel filtration on Sephadex G-200 that the carotenoproteins of fraction I_P and fraction II_P have the same molecular weight.

On Fig. II_9 the O.D. as a function of the elution volume is drawn for fraction I_P.

The molecular weight of fraction IV was estimated as follows :
a total carotenoprotein extract, saturated with 10% ammonium sulphate, yielded after centrifugation a supernatant (fraction IV_S) containing in solution the carotenoproteins of fraction IV as well as other proteins.

Gel chromatographic analysis of this fraction gave an estimate of the molecular weight of the carotenoproteins precipitating at 40% ammonium sulphate saturation (Fig. II_{10}).

Denaturation of carotenoproteins by Triton X-100 clearly resulted in fractions of lower molecular weight.

Fig. II_{11} shows the effect of Triton X-100 on the molecular weight of the carotenoproteins of fraction IV.

Tab. II_4 summarises the molecular weight values obtained by gel filtration of the described carotenoprotein fractions.

Carotenoproteins, built up of n sub-units, dissociate in a medium of low ionic strength, resulting in a molecular weight of 1/n molecular weight of the carotenoproteins.

Since after dialysis of the shrimp carotenoproteins identical molecular weight values were obtained by gel filtration it was concluded that carotenoproteins in shrimps did not exist of sub-units.

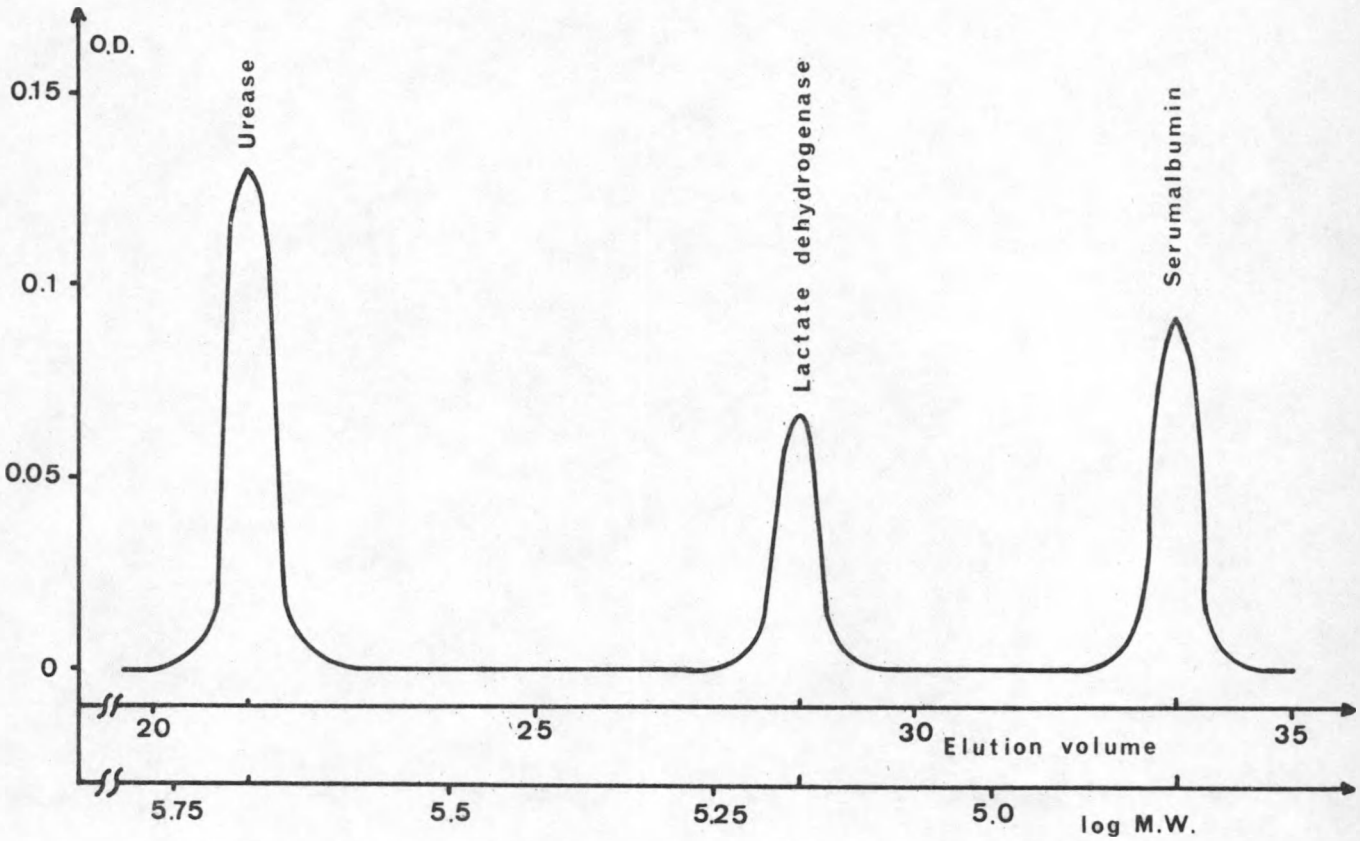


Fig. II_8 Standardizing elution volume versus log. molecular weight.

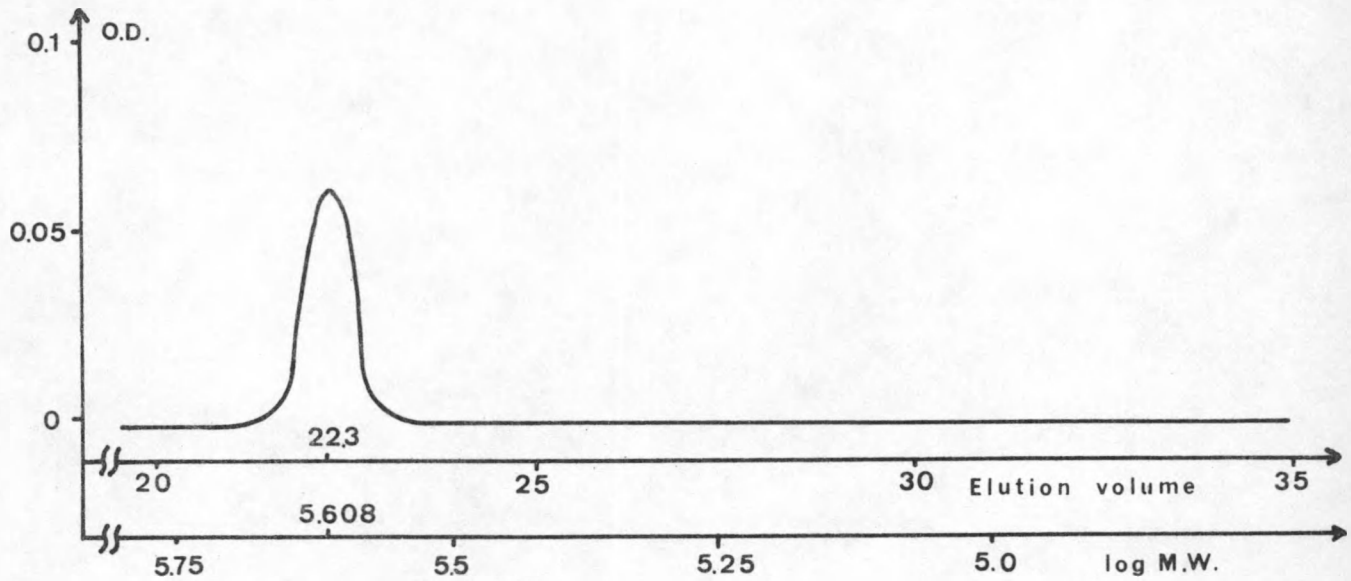


Fig. II_9 Estimation of the molecular weight of fraction I_p on G-200 sephadex.

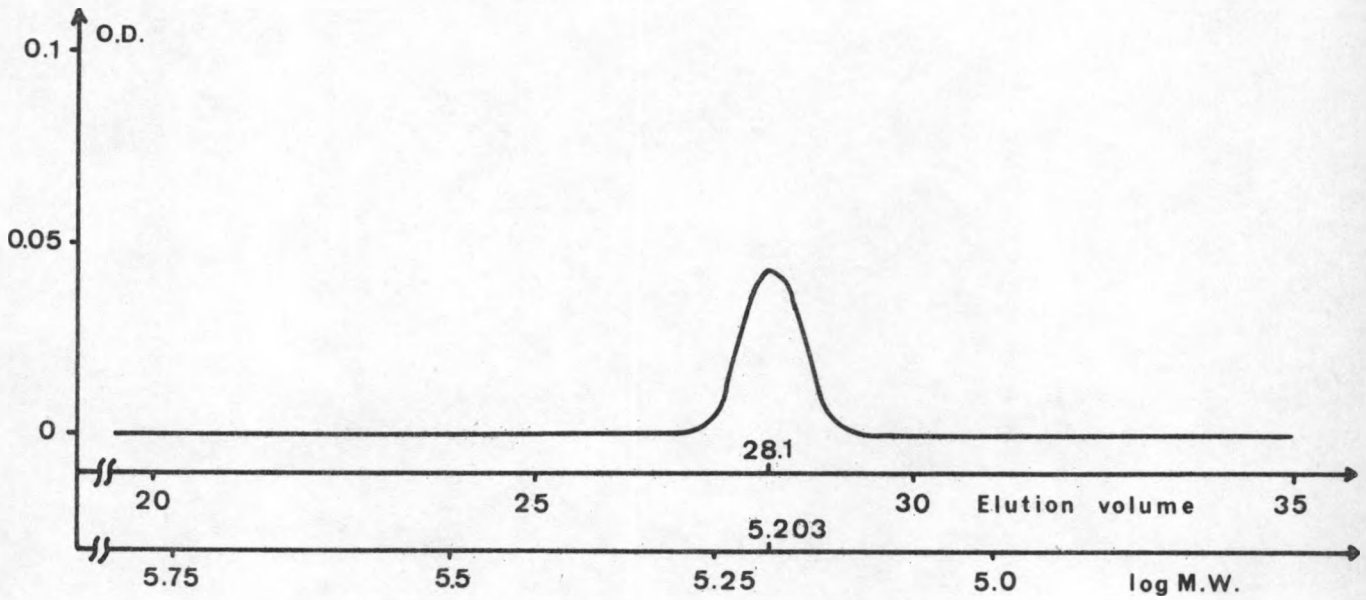


Fig. II_{10} Estimation of the molecular weight of fraction IV_S on G-200 sephadex.

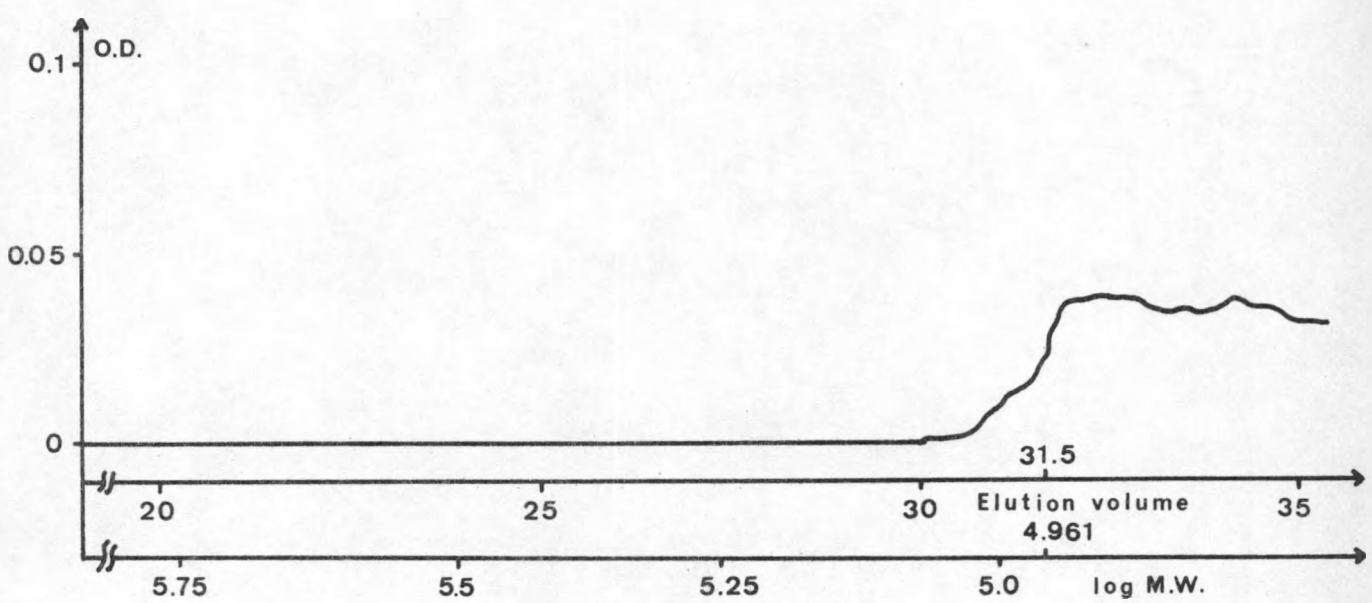


Fig. II_{11} Effect of denaturation by Triton X-100 on the molecular weight of the carotenoproteins of fraction IV.

Table II.4 Elution volumes and molecular weights of the carotenoprotein fractions determined upon gel-chromatographic analysis.

Fraction	Elution volume	Log molecular weight	Molecular weight
I_P	22.3	5.608	405,500
II_P	22.3	5.608	405,500
IV_S	28.1	5.203	159,600
IV_T	31.5	4.961	91,411

2.d.2. Carotenoid composition of the carotenoproteins.

By weight, the fractions I_P and II_P contained more carotenoids than the fractions III_P and IV_P. Probably this should be ascribed to a greater contamination with proteins of the fractions III_P and IV_P.

Thin layer chromatographic analysis revealed that the carotenoproteins in fractions I_P and II_P contained all carotenoids, identified in the boiled shrimp, with the exception of astaxanthin while fractions III_P and IV_P contained all carotenoids.

For the fractions III_P and IV_P the ratio astaxanthin and its ester derivatives / other carotenoids was greater than for the fractions I_P and II_P.

From this preliminary experiments we only could state that all carotenoids are bound to proteins. The problem whether these carotenoids occurred in true carotenoproteins or in lipoproteins with lipid associated carotenoids still exists.

2.d.3. Lipid analysis of the carotenoprotein fractions.

Only a qualitative analysis of the lipids in the carotenoprotein fractions was performed by TLC.

Hereby it was found that fraction I_P and fraction II_P contained all lipid fractions, as identified in the fat extract of boiled shrimps, with the exception of monoglycerides.

Fraction III contained, besides all other lipid fractions, an excess of mono-glycerides.

Finally in fraction IV, all known lipid fractions were identified.

From Fig. II_{12} it can be summarised that all carotenoproteins in unboiled shrimps contain lipoproteins.

From this preliminary study it was concluded that : the carotenoproteins in unboiled shrimps contained astaxanthin as well as the other carotenoids, present in shrimp, as prosthetic group.

More experimental work should be carried out in order to divide the shrimp carotenoproteins in true carotenoproteins and lipoproteins with lipid associated carotenoids.

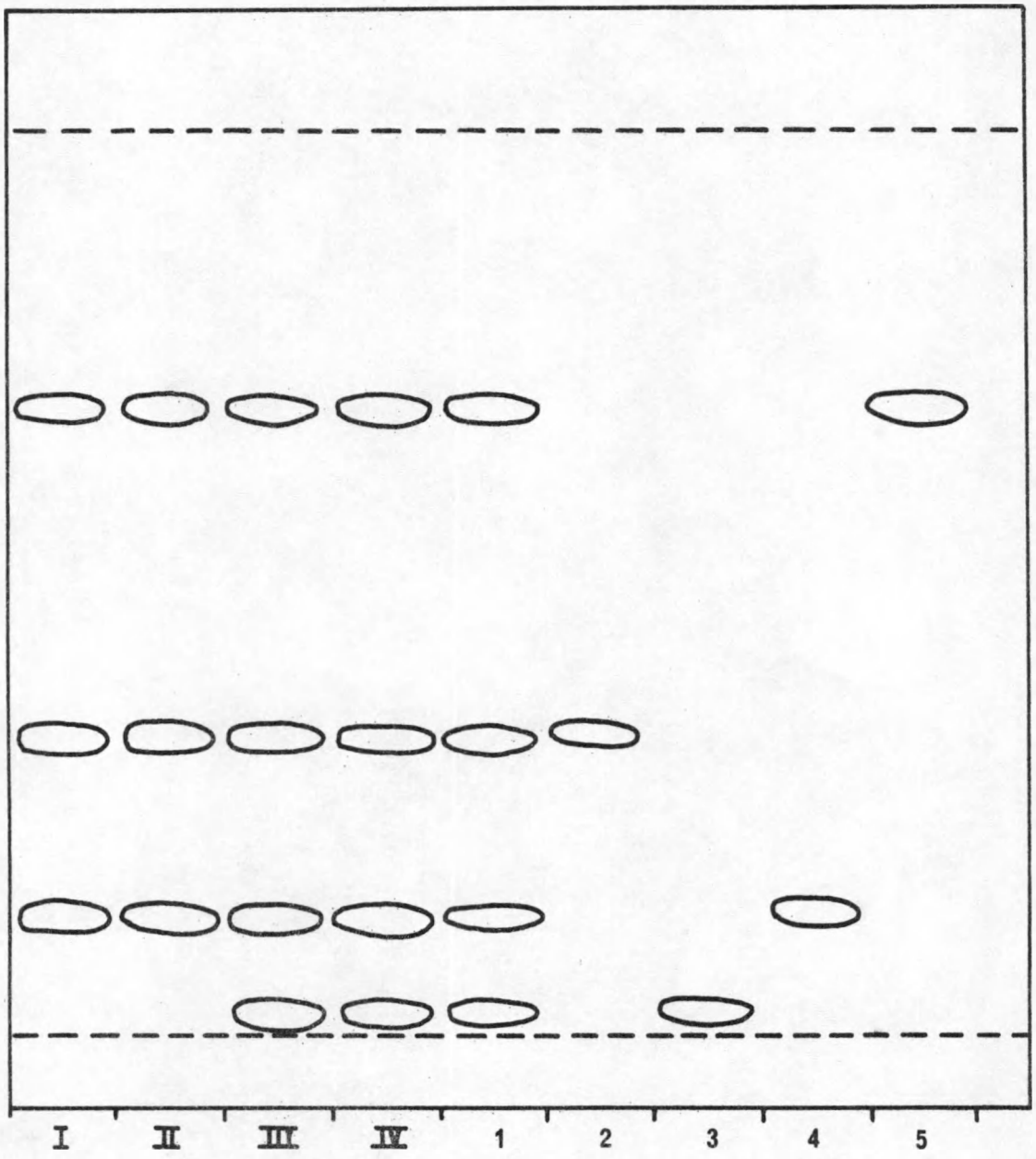


Fig. II_{12} Thin layer analysis of the lipids present in the carotenoprotein fractions of unboiled shrimps.

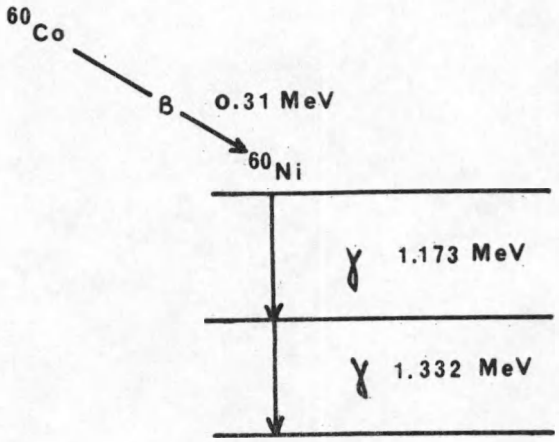
I	fraction I_p	1	Total fat extract of boiled shrimps
II	fraction II_p	2	Free fatty acid
III	fraction III_p	3	Mono-glyceride
IV	fraction IV_p	4	Di-glyceride
		5	Tri-glyceride.

C H A P T E R III

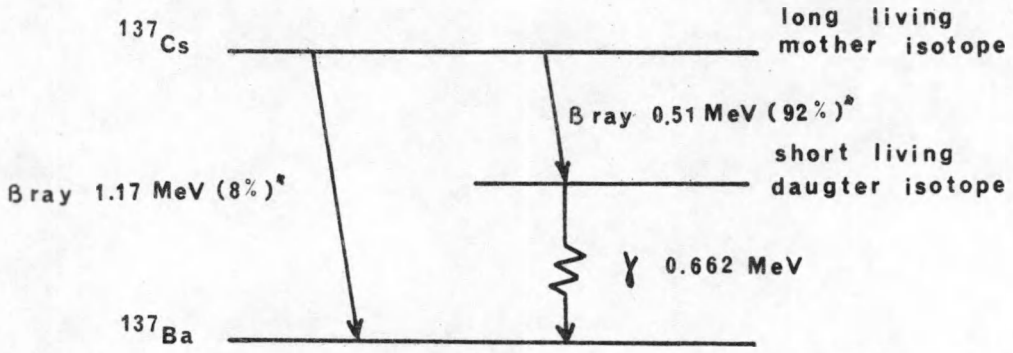
INTERACTION OF GAMMA RAYS AND MATTER.

1. Introduction.

Gamma rays are electromagnetic radiation of nuclear origin with short wavelengths in the region of 3×10^{-9} cm to 3×10^{-11} cm. In terms of energies this wavelength range represents an approximate value of 40 KeV to 4 MeV. The γ-rays emitted by radio-active isotopes are either mono-energetic or have a small number of discrete energies. For example, the most widely used isotope at present, the radioactive ^{60}Co, decays by emission of a β-particle to an excited ^{60}Ni nucleus that immediately achieves stability by emitting an equal number of γ-photons of energy 1.332 MeV and 1.173 MeV (Denaro and Jayson 1971). These processes may be represented by the following decay scheme :



The radiactive decay of ^{137}Cs is shown below.



Gamma energy is partially transferred to orbital electrons.

As a result of energy absorption "excitation" occurs when an electron has been moved into an orbit more distant from the atomic nucleus. Ejection of an electron from the atom following the absorption of photon energy, is termed 'ionization". The parent molecule-ion can be regarded as a positively charged free radical and it represents an important intermediate species. From this point of view it is convenient to group ionization with the more familiar heterolysis and homolysis (Williams, 1963).

$RX \longrightarrow RX^{+\circ} + e^{-}$ Ionization.

$RX \longrightarrow R^{+} + X^{-}$ Heterolysis.

$RX \longrightarrow R^{\circ} + X^{\circ}$ Homolysis.

The ejected electron will then dissipate the energy that has been transferred to it by further collisions with other atoms in the material.

Absorption of gamma rays by matter obeys a fundamental law :

$$I = I_{o}e^{-\mu x}$$

where I, I_o are the intensities of the transmitted and incident radiations respectively, x is the thichness of the absorber and μ is the total linear absorption coefficient. If the thickness of the absorber is expressed in cm, μ will have the units cm^{-1}.
The linear absorption coefficient depends on the density of the absorber and it is convenient to define the total mass absorption coefficient by

$$\mu mass = \mu linear \, / \varrho$$

where ϱ is the density of the material.

The total absorption coefficient ($cm^2.g^{-1}$) is the same for the different physical states, i.e. gaseous, liquid and solid state of the absorber.

Also useful are the atomic absorption coefficient μ_a (cm^2/atom) and the electronic absorption coefficient μ_e(cm^2/electron) that are respectively the absorption coefficients per atom and per electron. They are related to the linear and mass absorption coefficient in the following manner :

$$\mu_a = \frac{\mu A}{\varrho N_o} \qquad cm^2/atom \quad (1)$$

$$\mu_e = \frac{\mu A}{\varrho N_o Z} \qquad cm^2/electron \quad (2)$$

where ϱ is the density, A the atomic weight and Z the atomic number of the absorbing material, and N_o is Avogadro's number.

The mass, atomic and electronic absorption coefficients involve the dimensions of an area and are frequently called cross-sections, as they refer to the probability of absorption.

Photons will penetrate deeply into matter because they only lose their energy through direct interaction with orbital electrons ; even atoms of the most dense matter are largely composed of empty space and thus there is a high probability of a given photon passing through without interaction since it has no particular affinity (such as electrical charge) for any of the atomic parts. The type and number of interactions are dependent on the energy of the photon and the density of the matter and on the atomic number of its components. In this way the total absorption coefficient is the sum of separate coefficients representing the main processes of energy absorption.

These processes are the photo-electric effect, the Compton effect and pair production.

2. The photo-electric effect.

In the photo-electric effect, the entire energy of the γ-ray photon is transferred to an orbital electron with subsequent ejection of the electron, usually from the K-shell.

The ejected electron proceeds in the medium with a maximum energy E_e that is equal to that of the original quantum E_o less the binding energy of the electron in the atom.

$$E_e = E_o - E_b$$

Binding energies range from 100 eV for low atomic weight materials to 100 keV for high atomic weight materials.

The inner orbital vacancy is filled by an outer electron of the atom with consequent characteristic X-ray emission or, more rarely, by the ejection of one of the other electrons in the atom. In absorbers of low atomic number the binding energy of the K-shell is small and thus the secundary X-rays and electrons will only have low energies and will be absorbed locally.

As in the case for all physical interactions the photo-electric effect is also governed by the law of conservation of momentum. It is found that the scattering angle is distributed over a large number of values. This involves that the nucleus itself takes part in the process, carries away a part of the initial momentum and thus undergoes a recoil effect.

This means that the photo-electric effect can only occur with electrons which lie close to the nucleus (K and L electrons). At higher photon energy the electron will have a large probability of being ejected in a straigth forward direction.

The photo-electric effect is most probable for low photon energies 0.1 MeV, and for elements of a high atomic number (Hughes, 1973).

3. Compton effect.

In the Compton effect, photons of higher energies give up only part of their energy to an electron, which may be free or loosely bound in an atom. Hereby the electron is accelerated or ejected from the atom. The photon itself will be deflected (hence "scattered") by the interaction and now degraded in energy by the amount lost to the electron, will undergo additional Compton interactions with other atoms until its energy is lost or until it is absorbed in a photo-electric interaction. In this way energy and momentum of the incident photon are shared between the scattered photon and the recoil electron.

Compton interactions predominate for photon energies between 1 and 5 MeV in high-atomic number materials and over a much wider range of energies in low-atomic number materials.

In water, for example, Compton interactions predominate from about 30 kev to 20 MeV (Spinks and Wood 1964).

The electron-positron pair production and the photo-nuclear reactions are so unimportant, except at

very high photon energies (Spinks and Wood 1964, O-Donnell and Sangster 1970) that they do not need to be considered here.

The cross-sections of the three processes, the photo-electric effect, the Compton effect and pair production for water are shown as a function of photon energy in Fig. III_1. For a more extensive survey of the interaction of gamma rays with matter the references cited above should be consulted.

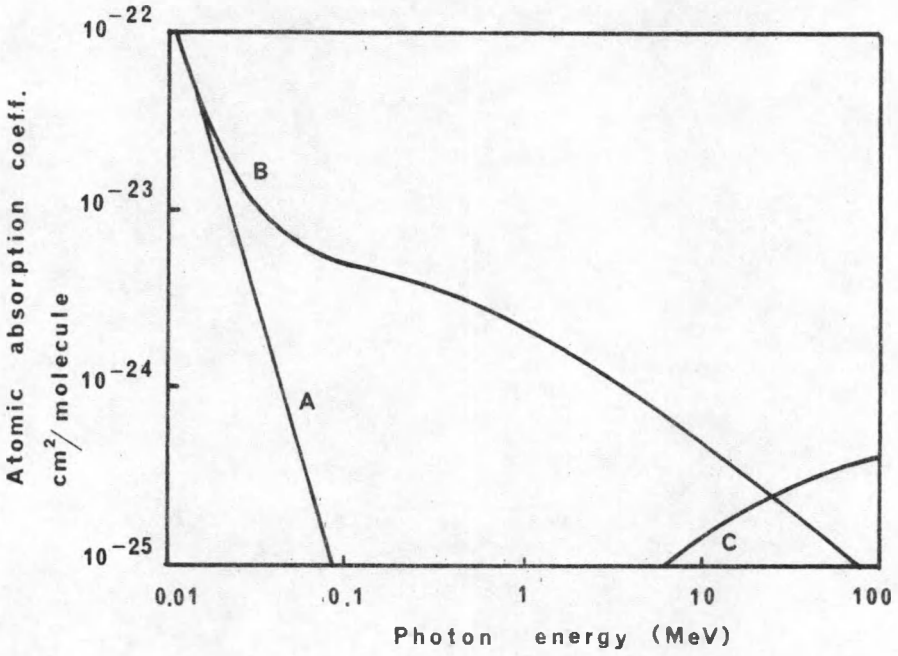


Fig. III_1 Cross sections of the photo-electric effect (A), the Compton effect (B) and the pair production (C) for water (Denaro and Jayson 1972).

4. Interaction of electrons with matter.

High-energy electrons are produced by Compton and photo-electric processes in material irradiated by gamma-rays. Those electrons interact with matter in several ways, mainly by inelastic and elastic collisions and by the emission of electromagnetic radiation.

The relative importance of these processes varies strongly with the energy of the incident electrons and, to a smaller extent, with the nature of the absorbing material.

i. High energy electrons, are decelerated in the field of nuclei and atomic electrons, and, according to classical physics, give X-rays called Bremstrahlung, which may be later absorbed by the material. A small amount of energy is also lost as Cerenkov radiation- the spectacular blue glow produced as electrons slow down to the speed of light in material of refractive index greater than one (O'Donnel and Sangster 1970).

ii. Electrons can also lose energy in matter through Coulomb interactions with electrons in the stopping material, producing excitation and ionization. Thus the primary electrons are slowed down and cascades of secundary, tertiary etc. electrons are produced. Towards the end of the tracks, where the electrons are moving more slowly and make more frequent collisions, the ionization density is the highest.

iii. For low electron energies and for high atomic number material, electrons may easily be deflected by the electrostatic field of an atomic nucleus (elastic scattering). The range of high-energy electrons in a material is in direct proportion to their energy and in inverse proportion to the density of the material.

For 1 MeV electrons the maximum penetration in water is 5-6 mm.

5. Behaviour of ions, excited states and free radicals.

The primary effect of gamma irradiation on matter is the production of ions, excited states and electrons (which may initially have excess kinetic energy).

Their formation is related to the absorption of energy by the material and their numbers are directly proportional to the absorbed dose. The situation is complicated by the inhomogeneous deposition of energy that results in the formation of these species in groups or spurs.

These processes may be represented (1) :

$$A \longrightarrow A^*$$

$$A \longrightarrow A^+ + e^-$$

$$A \longrightarrow (A^+)^* + e^-$$

These species subsequently react in a number of ways, summarised below.

---

(1) The symbol $\longrightarrow$ is used to denote : yields under the influence of gamma irradiation.
* is used for an excited state of a molecule.
$^\circ$ designates a free radical.

5.1. The electrons.

The ejected electrons are rapidly thermalized, and, in a polar medium, the molecules of the liquid become oriented around them, forming so-called "solvated electrons" (hydrated electrons in the case of aqueous solutions).

- If the electron is within the field of a positive ion, it will diffuse to the ion and neutralize it, forming an excited state that may be either the singlet or the triplet state and that may contain sufficient energy for spontaneous re-ionization.

$$A^+ + e^- \longrightarrow A^* \quad (3.1)$$

- Electrons can become attached to neutral molecules to form negative ions.

$$A + e^- \longrightarrow (A^-)^* \quad (3.2)$$

This process is most likely to occur when molecule A has a pronounced electron affinity such as oxygen, the halogens, the halogen containing organic compounds, aromatic compounds etc.

Electron attachment of solvated electrons to aromatic hydrocarbons has been demonstrated in pulse radiolysis studies.

$$e^- + C_{12}H_{10} \longrightarrow C_{12}H_{10}^- \quad : \text{the biphenylide ion}$$

is formed in the pulse radiolysis of a solution of biphenyl in alcohol.

The anions that are formed by electron attachment to hydrocarbons can either accept a proton from the alcohol

or react with other solute molecules by electron transfer. (Holroyd, 1968).

If the electron affinity of a molecule, due to the presence of an electronegative atom or group, is greater than the bond strength between that group and the rest of the molecule, the capture of an electron may immediately result in dissociation. This process may be respresented :

$$AB + e^- \longrightarrow A^- + B^\circ \quad (3.3)$$

$$e^- + CH_3Cl \longrightarrow CH_3^\circ + Cl^- \text{ (in the solvent cyclohexane).}$$

5.2. Reactions of ions.

Direct spectroscopic evidence for the presence of positive ions in organic liquids is scant but there is chemical evidence for their reactions. Positive ions can undergo fragmentation, charge transfer, ion-molecule-, and neutralization reactions. An evaluation of the role of ions as reactive intermediates hinges upon the life-time of a positive molecular ion before neutralization. If ion reactions are of significance for liquids, they must in general occur within about 10^{-12} sec (Williams, 1963).

5.2.a. Fragmentation of excited molecular cations occurs in the gas phase during electron bombardment (Meisels, 1968).

Such fragmentation is expected to be less important in the liquid phase relative to other reactions of the cations since the excited ions can be deactivated by collisions.

5.2.b. Ion-Molecule Reactions.

The role of ion-molecule reactions could more readily be established if it were known what ions were present during radiolysis. Although some fragmentation does occur in liquids, a large percentage of excited molecular ions are apparently stabilized by collision to groundstate ions. Thus it is important to consider the possible reactions that molecular ions might undergo before neutralization.

- Proton transfer reactions. Molecular ions are Brönsted acids in that they can give a proton to molecules with intrinsic basicity. This proton transfer reaction is expected to occur readily in liquids in which the molecules have a permanent dipole moment.

In the radiolysis of ethanol for example, proton transfer reaction followed by neutralization reaction has been proposed (Holroyd, 1968).

$$C_2H_5OH^{\delta} + C_2H_5OH \longrightarrow C_2H_5OH_2^+ + C_2H_5O^{\circ} \quad (3.4)$$

$$e^-_{sol} + C_2H_5OH_2^+ \longrightarrow H + C_2H_5OH \quad (3.5)$$

- Hydrogen-atom transfer reactions for odd-electron ions of the type

$$X^+ + R\text{-}H \longrightarrow X\text{-}H^+ + R^{\circ} \quad (3.6)$$

widely occur in the gasphase as discussed by Williams (1963).

$$\text{e.g. } C_3H_6^{+\circ} + C_3H_6 \longrightarrow C_3H_7^+ + C_3H_5^{\circ}$$

The reactions of even-electron positive ions are, of course, well known in conventional chemistry, where carbonium-ions

are postulated as intermediates in many organic reactions (Ingold, 1953). In the gasphase, the main type of reaction observed is hydride-ion transfer from a hydrogen carbon resulting in a more stable even electron ion. Exothermicity implies a lower bond heterolysis energy for the formation of the product ion, relative to the initial ion, from their respective hydrocarbons.

The reaction of the fragment ion $C_3H_5^+$ with neo-pentane could be an illustration to this.

$$C_3H_5^+ + \text{neo-}\ C_5H_{12} \longrightarrow C_3H_6 + C_5H_{11}^+$$

A more extensive discussion of the reactions of ions is given by Williams (1963), Holroyd (1968) and Spinks and Woods (1964).

5.3. Reactions of excited molecules.

The characteristics of excited atoms and molecules are known from spectroscopic and photochemical studies in which the excited species are produced by the absorption of visible or U.V. light (Mousseron - Canet and Mani 1969). The same excited species are formed by interaction of gamma rays with matter, though irradiation may also produce more highly excited states, with more intrinsic energy and states (e.g. triplet) which are not formed directly by the absorption of light. Reactions observed in photochemistry, therefore, are not necessarily the only ones stemming from excited molecules which occur upon radiolysis (Denaro and Jayson, 1970).

The similarities and dissimilarities of visible and ultraviolet light absorption compared with the photoelectric absorption of gamma radiation are discussed in the surveys of Spinks and Woods (1964) and Williams (1963).

The representation of the behaviour of excited states of polyatomic molecules can be quite complicated, but an understanding of the processes involved can be gained by considering the properties of the excited states of simple diatomic molecules.
The conversion of an excited state to the ground state can be achieved by

i. fluorescence (radiative conversion to the ground state).

ii. internal conversion (non-radiative conversion to a lower state of the same multiplicity).

iii. intersystem crossing (non-radiative conversion to a state of different multiplicity) and phosphorescence.

iiii. non-radiative energy transfer to a neighbouring molecule described by Mousseron-Canet and Mani (1969) and Denaro and Jayson (1970).

Excited molecules can also return to the ground state in a unimolecular reaction (dissociation or isomerisation of the excited molecule) and in a bimolecular reaction (chemical reaction involving a second molecule) briefly discussed below.

5.3.a. Unimolecular reaction.

The most usual unimolecular reactions undergone by excited states result in the dissociation of the molecule into two fragments, which may be free radicals or stable molecules.

If an excited state has sufficient energy, the molecule may break at a covalent bond to give two radical

fragments.

$A^* \longrightarrow R^\circ + S^\circ$ (3.7) e.g. $CH_3\text{-}CO\text{-}CH_3 \xrightarrow{h\nu} CH_3CO^\circ + {}^\circ CH_3$

If the excited state is an ion :

$(A^+)^* \longrightarrow R^{\circ +} + S^\circ$ (3.8)

The situations that lead to dissociation are illustrated by the potential energy curves in Fig. III_2.

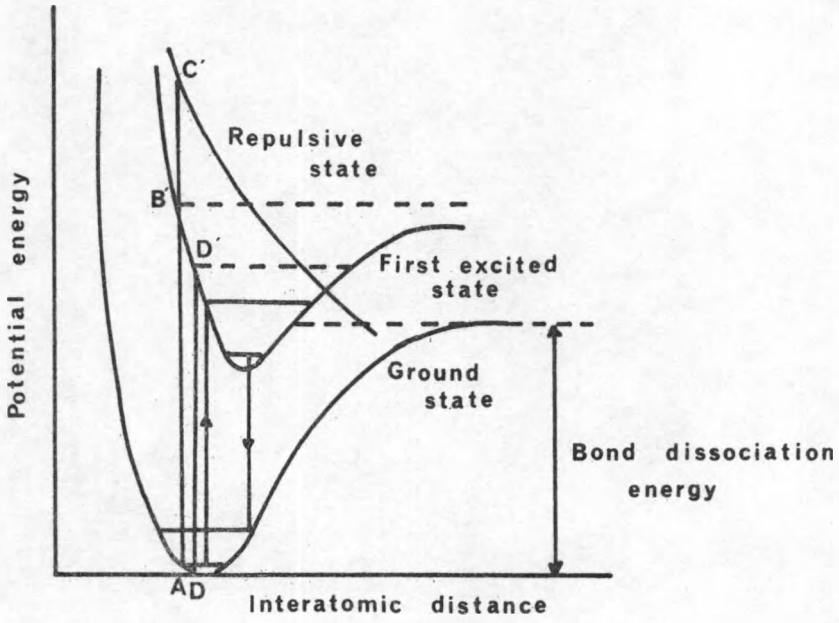


Fig. III_2 Potential energy curve for a diatomic molecule (Spinks and Woods 1964).

Excitation to a high vibrational level (transition AB'), excitation to a repulsive state (transition AC') and predissociation (transition D-D' crossing into the repulsive curve C) are three various routes accomplishing homolytic fission.

The radicals themselves may be excited or may have an appreciable amount of kinetic energy.

Radicals formed in liquids with only very small kinetic energies will be "caged" by the liquid molecules and will recombine dissipating the dissociation energy as heat (called the Franck-Rabinowith effect or cage effect) and no net chemical change is observed (Denaro and Jayson 1970).

When the radicals formed have a fair amount of kinetic energy, which allows them to escape from the cage, chemical changes are more likely to be produced.

The dissociation process involves a highly excited state, such as results from charge neutralisation as in reaction (3.1).

In addition to the dissociation into free radicals, an excited state may dissociate into stable molecules, although this event is less frequent (Spinks and Woods 1964).

$$A^* \longrightarrow B + C \quad (3.9)$$

In the radiolysis of cyclohexane the decomposition reactions :

$$C_6H_{12}^* \longrightarrow H_2 + C_6H_{10} \quad (86\%)$$

$$C_6H_{12}^* \longrightarrow H^\circ + C_6H_{11} \quad (14\%)$$

probably account for a significant part of the cyclohexene and hydrogen formed.

5.3.b. Bimolecular reactions.

The four main classes of bimolecular reactions described by Denaro and Jayson (1970) are :

- electron transfer reactions
- abstraction reactions
- addition reactions
- Stern-Volmer reactions.

6. Free Radical reactions.

Chapter III 5.3. made it clear that the homolytic decomposition of an excited molecule may lead to the production of free radicals.
Free radicals then are very rarely primary products of the radiolysis but arise from subsequent decomposition of the ions and excited states initially produced.
Typical reactions are :

$$C_6H_{14}^{*} \longrightarrow C_6H_{13}^{\circ} + H^{\circ}$$

$$C_2H_6^{+} \longrightarrow C_2H_5^{\circ} + H^{+}$$

$$e^{-} + CH_3Cl \longrightarrow CH_3^{\circ} + Cl^{-}$$

Properties and reactions of free radicals with particular reference to radiation chemistry are discussed below.

In Tab. III_1 a number of reactive radicals are arranged approximatively* in order of decreasing reactivity

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* The radicals (R) were grouped by comparing the bond dissociation energies in the compounds R-H and R-halogen and assuming that the largest dissociation energies signified the most reactive radicals.

Table III.1 Relative stability of reactive free radicals.
(The radicals are arranged from top to bottom in order of increasing stability and decreasing reactivity. The relative positions are approximate and, for the radicals in parentheses, questionable) (From Spinks and Woods 1964).

R=alkyl radical	Atoms and inorganic radicals	Alkyl radicals	Halogen derivatives	Unsaturated and oxygenated radicals	Aromatic compounds
-	H.,.OH	-	-	CH_2=CH.	Ph. o,m,p, $CH_3C_6H_4$.
R.,RCO_2.	F.	.CH_3,.C_2H_5	-	(CH_3CO_2.)	-
RCO.	-	n-C_3H_7.	.CF_3	CH_3CO.	-
		n-C_4H_9.	$ClCH_2CH_2$		
RO.	Cl.	i-C_3H_7.	$ClCH_2$.	(CH_3O.)	-
-	-	t-C_4H_9.	Cl_2CH.	-	-
-	-	-	.CCl_3,Br_2CH	-	PhCO.
-	Br.	-	.CBr_3	CH_2=CH-CH_2	$PhCH_2$.
RS.,RO_2.	I.,(HO_2.)	-	-	(CH_3S.)(CH_3O_2.)	-

respectively increasing stability.

- Radical stability increases in the series primary ($-CH_2^{\circ}$) < secondary ($=CH^{\circ}$) < tertiary ($\equiv C^{\circ}$).

- Stability will also be affected by the nature of the substituents. Fluorine has only a small effect when it replaces hydrogen but the effectiveness of the halogens increases in the series F<Cl<Br<I ; stability also increases as the number of halogen substituents is increased.

- Unsaturated substituents increase stability when they involve carbon atoms adjacent to that carrying the odd electron, but not when this carbon atom itself is part of the unsaturated groups. For instance $CH_2 = CH - CH_2^{\circ}$ is much more stable than $CH_2 = CH^{\circ}$ or (Ph°)*. In all cases the effectiveness of the stabilizing substituent decreases as it moves farther from the carbon with the odd electron.

- In general, radicals are less selective at high temperatures ; on the contrary even very reactive radicals may be quite selective at very low temperatures.

Other factors affecting radical reactions with a substrate besides temperature include the reactivities of the attacking and displaced radicals, the dissociation energies of the bonds broken and-formed and polar effects.

Polar fractions, which means effects due to the differences in electron density in the reactants and media,

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* Ph : means in this text : phenyl.

such as the type of solvent, pH, inductive and hyperconjugation effects, may influence radical reactions, though to a smaller extent than heterolytic reactions, which involve transfer of electron pairs in bond breaking and bond formation.

- Radicals can be classified as electron-acceptors or electron donors.

Halogen radicals for instance, tend to gain electrons and preferentially attack points of high electron density in the substrate. Methyl radicals on the other hand are relatively nucleophilic and rather tend to attack electron deficient centers in the substrate.

Radicals react either unimolecularly (rearrangement and dissociation) or bimolecularly with other radicals or with stable molecules.

6.1. Unimolecular reactions.

6.1.a. Radical rearrangement.

Reactive radicals can sometimes attain more stable structures by molecular rearrangement. Such reactions may be represented as

$$AB^{\circ} \longrightarrow BA^{\circ}$$

in which the migration of a group or atom within the radical causes a change in position of the unpaired electron.

For example $Ph_3C - CH_2^{\circ} \longrightarrow Ph_2C^{\circ} - CH_2Ph$

6.1.b. Dissociation.

Organic radicals can characteristically dissociate into a smaller radical and a stable molecule if such a process is energetically favourable.

$$AB^{\circ} \longrightarrow A^{\circ} + B$$

The stable molecule B, is usually an unsaturated compound.

$$Br_2CH - {}^{\circ}CHBr \longrightarrow \overset{\circ}{Br} + BrCH = CHBr$$

6.2. Bimolecular reactions.

6.2.a. Radical attack on substrates.

Addition : A common reaction of radicals is an addition to an unsaturated compound

$$A^{\circ} + >C=C< \longrightarrow >\overset{A}{\underset{|}{C}}-C^{\circ}<$$

Many examples of addition reactions are known and include radical - induced halogenation and the radical - initiated polymerization of unsaturated compounds e.g.

$$-CH_2 - {}^{\circ}CHR + CH_2 = CHR \longrightarrow CH_2-CHR - CH_2 - {}^{\circ}CHR \text{ etc.}$$

Abstraction : Radicals can abstract atoms from saturated organic compounds to form a stable molecule and another radical (having a greater stability)

$$A^{\circ} + BC \longrightarrow AB + C^{\circ}$$

The abstracted atom B is usually a halogen or hydrogen atom.

Example $^{\circ}OH + CH_3OH \longrightarrow H_2O + {}^{\circ}CH_2OH$

$H^{\circ} + CH_3I \longrightarrow HI + {}^{\circ}CH3$

Thus most radicals attack tertiary - in preference to secondary - or primary hydrogen atoms, though other factors (e.g. polar and energy considerations) will also influence the reaction.

6.2.b. Radical destroying processes.

- Combination : Two free radicals can combine their unpaired electrons together to form a bond.

$$R^{\circ} + S^{\circ} \longrightarrow RS$$

Reactions of this type are energetically favoured since they have little or no activation energy and energy is equally liberated to the dissociation energy of the bond formed.
Combination of methyl radicals to form ethane for example occurs in the gas phase at almost every collision between radicals.

$$2{}^{\circ}CH_3 \longrightarrow C_2H_6$$

If the resultant molecule is large the energy is delocalized and subsequently lost. Small molecules, having fewer possibilities of distributing the energy will normally dissociate again unless they can quickly lose energy. In a gaseous system, for instance, combination will only give a stable molecule if it occurs on a surface (e.g. a wall of the reaction vessel).

- Disproportionation - An alternative to the combination of two radicals to give a single stable molecule

is the transfer of an atom (generally hydrogen) from one radical to the other, giving two stable molecules, one of which is unsaturated :

$$2R^{\circ}H \longrightarrow RH_2 + R$$

$$2^{\circ}C_2H_5 \longrightarrow C_2H_6 + C_2H_4$$

- Reactions of radicals with oxygen to form peroxy radicals are most important in radiation chemistry.

- Reactions grouped under the heading "radical attack on substrates" are characterized by the appearance of a radical among the products. In some situations the newly formed radical may also react with the substrate and, with favourable conditions, the sequence of reactions between that radical and substrate may be repeated several times before the radical is destroyed. In other words, a chain reaction takes place.

A chain reaction, considered from kinetic point of view is divided into initiation, propagation and termination steps.

Typical examples of chain reactions are the radical induced polymerization.
A typical propagation step for styrene looks as follows :

$$-CH_2 - \overset{\overset{Ph}{|}}{\underset{\circ}{CH}} + CH_2 = \overset{\overset{Ph}{|}}{CH} \longrightarrow - CH_2 - \overset{\overset{Ph}{|}}{CH} - CH_2 - \overset{\overset{Ph}{|}}{\underset{\circ}{CH}} \quad \ldots$$

Chain reactions, involving successive abstraction reactions, are shown in the bromination of toluene.

$$\overset{\circ}{Br} + PhCH_3 \longrightarrow HBr + PhCH_2^{\circ}$$

$$PhCH_2^{\circ} + Br_2 \longrightarrow PhCH_2Br + \overset{\circ}{Br}$$

The repeated sequence of reactions may be more complex than the examples shown above.

C H A P T E R IV

INFLUENCE OF GAMMA IRRADIATION ON THE PROVITAMIN A, β- CAROTENE AND RELATED CAROTENOIDS IN MODEL SYSTEMS.

Introduction.

A number of different carotenoids occur in foods in a variety of forms and each of them exhibit significantly wide variations in their sensitivities toward radiation (Knapp et al. 1961, Lukton et al. 1956 and Franceschini et al. 1959).

Recognition of the different degradation products is important since some of the carotenoids have a special function as precursors of vitamin A.
In order to define suitable conditions for minimizing the loss of colour and nutritive value upon food irradiation it is necessary to understand the factors that govern the radiation sensitivity of the major carotenoid fractions.
However, because of the complexity of food products it is impossible to detect the mechanisms of the different degradation reactions in the natural product.
This made the development of suitable model systems necessary in order to understand the different factors that influenced the radiation sensitivity of carotenoids.
This chapter deals with the destruction mechanisms of β-carotene upon irradiation in crystalline form and in solution in different solvents.

A. Materials.

The carotenoids used in our experiments were commercially available β-carotene (Merck 2236) and cantha-xanthin (kindly supplied by Hoffman La Roche and Co, Basle, Switzerland). The solvents used in all our experiments were p.a grade products. Bovine albumin (Sigma A-4503), oleic acid (Merck 471) and stearic acid (Merck 673) were also used in some experiments.

Irradiation was carried out in boro-silicate-glass-stoppered (B-10) vials (13 mm o.d., 36 mm high) with a total content of 2.5 ml. Identical vials were used for the dosimetric determinations.

The irradiation unit.

All experiments were carried out in a Gammator M34-3 irradiation unit, "HELGA", manufactured by the Radiation Machinery Corporation, New Jersey, U.S.A. with the following specifications :

The exposure chamber is a cylinder, 3 inches in diameter and 4 inches high. The irradiation intensity at the centerline of the exposure chamber was 102.5 krad/hr. on August 6, 1970.

The irradiation chamber is housed in a movable rotor and can be brought into irradiation position by turning the rotor over an angle of 180°. Uniformity of the irradiation dose is achieved by rotation of the sample at 4.5 rpm on a turntable situated at the bottom of the irradiation chamber. Control of the amount of radiation exposure to the sample is achieved by means of the exposure time and the dose-rate.

The exposure time is controlled by an electric interval timer that operates a micro-switch ; when the pre-set exposure-time is elapsed a brake is released and the rotor returns automatically to the load position.

The dose rate can be varied by locking the rotor (and the irradiation chamber) at different distances from the source. These different positions can be chosen by a dose factor dial. In this way the dose rates can be varied between zero and 100%.

The unit has a ^{137}Cs-chloride source with a total activity of 1200 Ci ± 5%.
The ^{134}Cs content is less than 1%. A plan of the unit is shown in Fig. IV_1.

Dose- distribution in the gammator M-34-3 unit.

Dosimetry of the irradiation chamber was carried out in air by Neels (1971). The Fricke dosimeter, as described by the ASTM method, was used as a primary standard.
A phosphate glass dosimeter was chosen as a secondary standard.
The dosimeters are 24 mm long, 12 mm wide and 5 mm thick and have the following composition :

P_2O_5 70.5%, K_2O 12%, Al_2O_3 9%, MgO 4%, B_2O_3 3%,

various compounds 1.5%. The glass dosimeters were calibrated against the Fricke dosimeter as described by Madsen et al. (1968).
The first set of dosimetric experiments was carried out along the central axis of the unit up to about 2/3 of the height. The second series of experiments was carried out along the periphery of an imaginary cylinder axed on the central axis of the exposure chamber, with a radius of 27 mm. Therefore six-boro-silicate-glass stoppered (B-10) vials (13 mm o.d) with a total content of 2.5 ml were fixed in a stainless steel cage that fitted in the irradiation chamber. The centre of the vials was placed on a circle with a radius of 27 mm (measured from the centre of the bottom plate). The height of the vials above the bottom plate could also be varied.
Since the bottom plate of the exposure chamber rotates at a constant speed (4.5 rpm) during irradiation all points of the cylinder area situated at a given height above the

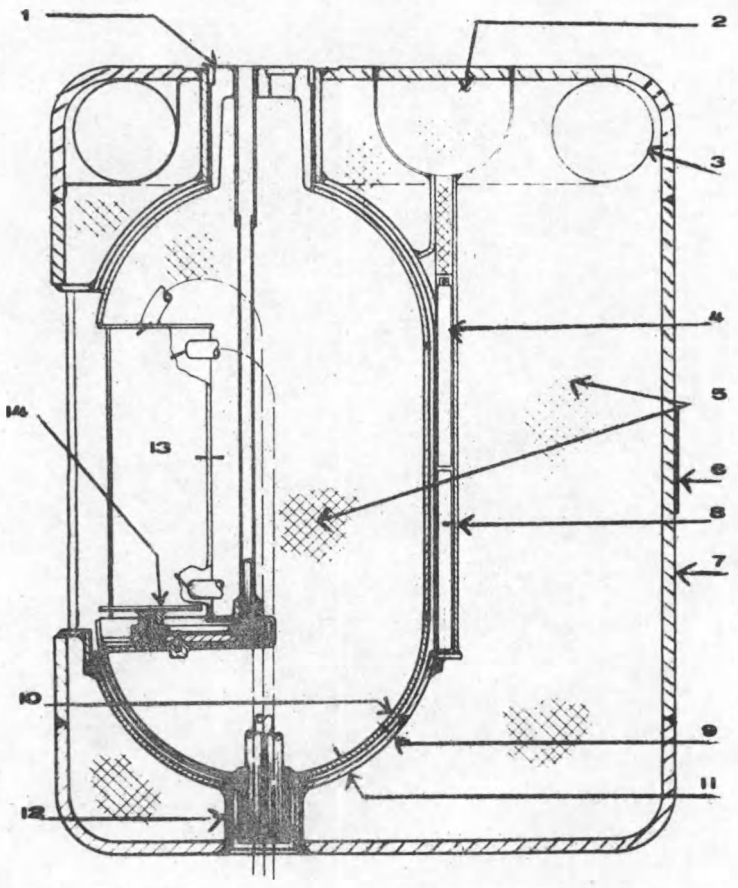


Fig. IV_1 Plan of the irradiation unit.

1. Rotor suspension
2. Access to ^{137}Cs sources
3. Collapsible aluminium torus for lead expansion
4. Radioactive sources (doubly encapsulated)
5. Rotor and shield (lead)
6. Source- identification data
7. Steel shielding
8. Source container wall
9. Rotor housing
10. Rotor wall
11. Space
12. Rotor suspension
13. Irradiation chamber
14. Turntable

bottom plate will receive the same average dose.
In Fig. IV_2 the dose-distribution in the irradiation chamber as determined by Neels (1971) is shown.
At regular time intervals the dose distribution was checked.

B. Methods.

Very little information is available on the radiation chemistry of carotenoids either in situ or in fat solubilizing solvents.

Therefore a study of the radiation degradation products of β-carotene in modelsystems was made.
In a first series of experiments β-carotene was irradiated in the crystalline form, or freely dissolved in the apolar solvents hexane, cyclohexane and benzene. These three solvents are comparable as far as chemical structure and composition are concerned. Literature extensively decribes the radiolysis of these solvents (Spinks and Woods 1964 and Ausloos 1968).

The good solubility of β-carotene in these solvents also explains our choice.

Irradiation of β-carotene in a more polar medium was the first step to simulate the food product.
However, irradiating β-carotene in methanol, ethanol and higher alcohols was impossible because β-carotene is only sparingly soluble in those solvents. We were able to carry out irradiation experiments in the mixtures benzene and methanol only. To solutions of β-carotene in cyclohexane fatty acids were added prior to irradiation.

Finally the presence of water soluble carotenoproteins in the food products was simulated by modelsystems

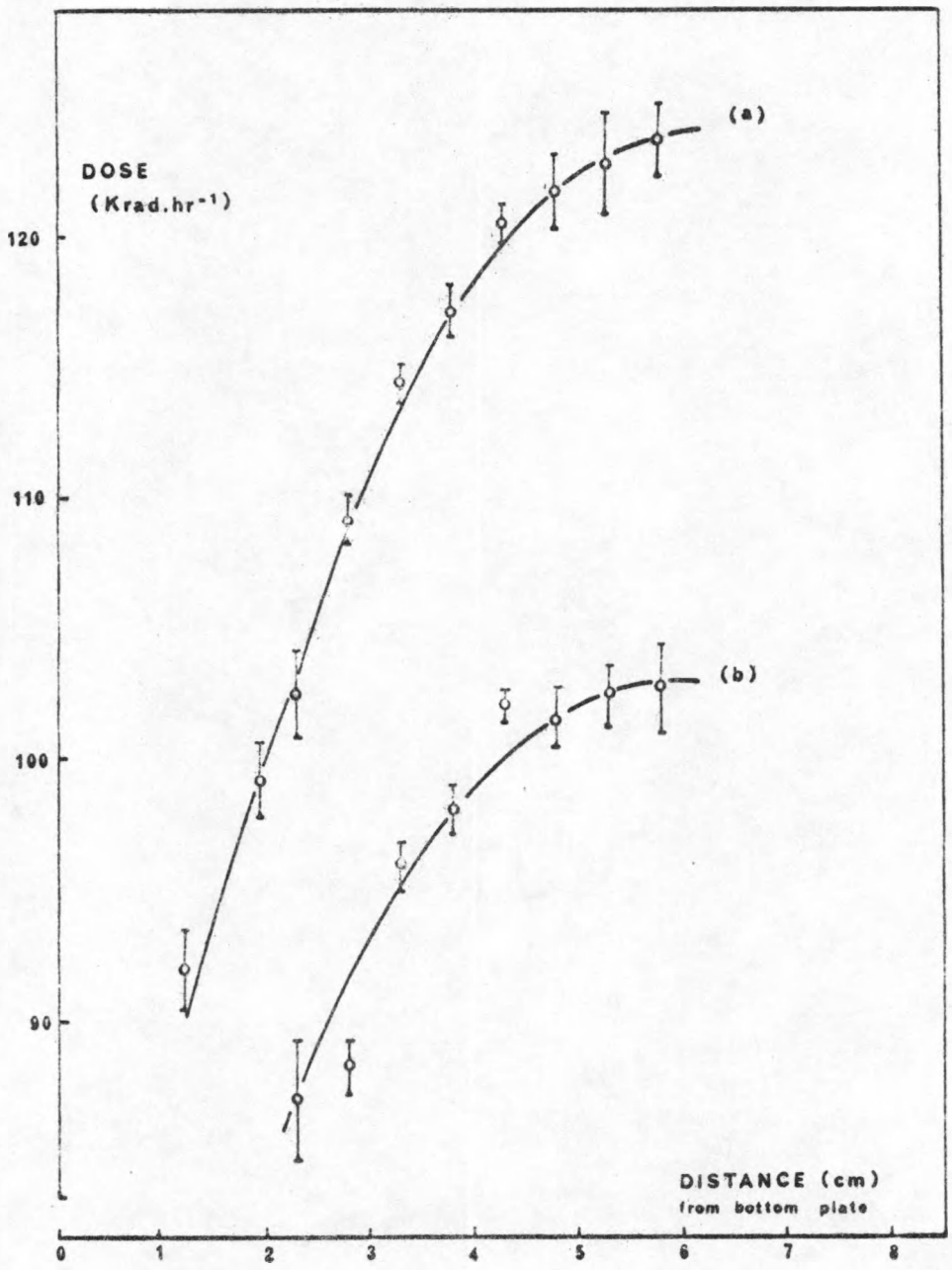


Fig. IV_2 Dose rate profile of the irradiation chamber
a) along the periphery of an imaginary cylinder axed on the central axis of the exposure chamber, with a radius of 27 mm.
b) along the central axis.

in which β-carotene and canthaxanthin were adsorbed to albumin.

B.1. Irradiation of β-carotene in organic solvents.

The β-carotene solutions were irradiated in anaerobic conditions. Before dissolving the carotenoid the various solvents were degassed at room temperature by passing through a current of pure nitrogen. The air-free solutions with concentrations of 5, 25, 50, 75 and 100 μM were irradiated in boro-silicate glass vials with a total content of 2.5 ml.
Spectrophotometric analysis of the irradiated solutions was performed using a Hitachi-Perkin Elmer Model 124, double beam spectrophotometer.

Crystalline β-carotene was irradiated in boro-silicate glass vials under a nitrogen atmosphere. Immediately after the irradiation the samples were dissolved in the oxygen-free solvents and analysed spectrophotometrically and by TLC.

B.2. Irradiation of water soluble carotenoprotein complexes.

To study the radiation sensitivity of β-carotene and canthaxanthin in aqueous systems the modified method of Ramakrishnan (1970) was adopted. The carotenoids (35 mg) dissolved in 70 ml P.E. were added to 2 g albumin (albumin v, Sigma A4503). This mixture was stirred at a temperature of 2°C during 2 hours. Afterwards the P.E. was evaporated under vacuum. This dried coloured powder was subsequently dissolved in distilled water and this solution was filtered on a S & S blauband 5893 filter. The filtered solution was suitably diluted to 100 ml and stored

in the refrigerator.
Boro-silicate-glass vials containing the carotenoprotein solution were exposed to doses up to 1500 krad.
From 10 ml of the irradiated solution the carotenoid content was determined as follows : in a separatory funnel 10 ml acetone, 10 ml methanol and 5 g $(NH_4)_2SO_4$ (Merck 1217) were added to 10 ml carotenoprotein solution to precipitate the proteins.
The liberated carotenoids were dissolved in P.E. A more quantitative extraction in P.E. was achieved by addition of distilled water, redissolving the protein precipitate. This procedure was repeated three times. The recovery of carotenoids before and after irradiation was compared spectrophotometrically.

B.3. Addition of other fat soluble compounds to a solution of β-carotene in cyclohexane.

In order to study the possible modifying effects of some free fatty acids on the radiation stability of β-carotene in solution oleic acid (1 ml, 2 ml, 5 ml and 10 ml respectively per 100 ml solution) was added to a solution of β-carotene of different concentrations in cyclohexane. The effect of identical quantities of stearic acid (calculated in mM) was compared.

B.4. Thin-layer chromatographic analysis.

For the TLC analysis the irradiated solutions were evaporated under reduced pressure. The precipitate was subsequently dissolved in a few millilitres of P.E. and spotted on a silicagel G plate. As elution solvent a mixture of P.E. (100-140)-acetone (100:12) was used. After the separation, the different fractions were scraped off the plates and extracted from the adsorbent with acetone.

The adsorbent was removed by filtration and the fractions evaporated to dryness and redissolved in a suitable solvent for spectrophotometric analysis. The method of Hager and Meyer-Bertenrath (1972) was used to separate α-carotene and β-carotene. Six plates (layer thickness 0.5 mm) were prepared with 29.5 g $CaCO_3$ (Merck 2066), 6 g MgO (Merck 5868), 5 g $Ca(OH)_2$ (Merck 2047) and 120 ml distilled water. A P.E. (100-140)-benzene-acetone (40:10:1) mixture was used as elution solvent.

B.5. Determination of the G value of β-carotene irradiated in the solvents hexane, cyclohexane and benzene.

In the present study the G values are expressed as the number of β-carotene molecules degraded for an energy absorption of 100 eV and determined by the variation in the optical density at the main absorption maxima of the carotenoid spectrum.

The molar extinction coefficients of β-carotene in the various solvents were determined at room temperature. The best-fit line through the experimental figures (for solutions up to 7μM) was calculated according to the least squares methods (Snedecor et al. 1968).

Accepting that a variation in optical density at the two absorption maxima can be interpreted as caused by a modification of the β-carotene molecule after irradiation, the G value was calculated as follows :

$$C = \frac{E_o - E_1}{\varepsilon} \qquad (1) \text{ (in mole/litre)}$$

where E_o and E_1 are the absorption at λ max before and after irradiation ; ε is the molar extinction coefficient of β-carotene ; C is the concentration (mole/litre) of the degraded β-carotene.

In the equation (1) the exact value of E_1 should be written as :

$$E_1 = \varepsilon_1 C_1 \, l + \varepsilon_2 C_2 \, l + \varepsilon_3 C_3 \, l \ \ldots \ \varepsilon_n C_n \, l$$

$\varepsilon = \varepsilon_1$ = the molar extinction coefficient of β- carotene.

$\varepsilon_2, \ \varepsilon_3 \ \ldots \ \varepsilon_n$ are the molar extinction coefficients of the different degradation products formed upon irradiation of β-carotene.

In the equation (1) we have supposed that $\varepsilon = \varepsilon_1 = \varepsilon_2 \ \ldots \ \varepsilon_n$, in other words : after irradiation we have calculated our results as β-carotene

$$C' = \frac{E_o - E_1}{\varepsilon} \times \frac{N}{10^3 d} \quad \text{(in molecules per gram)}$$

where N is Avogadro's number and d is the density of the solution.

$$G = \frac{E_o - E_1}{\varepsilon} \times \frac{N}{10^3 d} \times D^{-1} \times 10^2 \text{ eV molecules per 100 eV}$$

D is the dose expressed in eV/g.

The greater the difference between the molar extinction coefficient of β-carotene and the molar extinction coefficients of the degradation products, formed upon irradiation, the more our approximative calculation method can be justified.

C. Results and discussions.

C.1. Irradiation of crystalline carotenoids.

Crystalline β-carotene and canthaxanthin were irradiated under anaerobic conditions with doses of 50, 100, 200, 500 and 1500 krad. In none of these cases any breakdown of the carotenoid molecule could be detected neither by spectrophotometric analysis nor by TLC analysis. According to Willard (1968), in samples of the vapor, the liquid and the solid state of a compound, exposed for equal time to the same flux of γ-radiation, the energy deposited per gram is essentially the same for each. Differences caused by the small shifts in ionization potential and energy levels resulting from stronger intermolecular forces in the condensed phases are negligible. The energy of gamma ray photons is converted into kinetic energy of electrons by the Compton and photo-electric processes, and this energy is dissipated by ionization and excitation of molecules within 10^{-15} sec or less.
In contrast to these primary processes, the subsequent chemical events evidently depend on the phase.
In this way the stability of carotenoids upon irradiation in crystalline form, can be explained by the restricted mobility of the active species in this solid phase.

C.2. Irradiation of β-carotene in solution (in benzene, cyclohexane and hexane).

At a chemical level a solute can be affected by radiation in two ways : either directly, in which case the initial process of energy deposition occurs within the solute molecule, or, indirectly by the reactive moieties formed by the deposition of energy in the solvent (Alexander et al. 1967). The alternatives of direct and indirect

action are of course not mutually exclusive ; both probably contribute importantly, except in the cases of very dilute solutions and of pure substances.

Most of our experimental work in this chapter relates to the measurement of the overall chemical change upon irradiation of β-carotene in model solutions. No techniques were available for the direct identification of transient intermediates.

C.2.a. Dilution test upon irradiation of β-carotene in benzene, cyclohexane and hexane.

A first series of experiments with diluted solutions of β-carotene in the three solvents benzene, cyclohexane and hexane was carried out in function of the following parameters : the nature of the solvent, the concentration of β-carotene and the dose.

Air-free solutions with concentrations of 5, 25, 50, 75 and 100 μM were irradiated with increasing doses up to 200 krad. Immediately after the irradiation the percent degradation and/or the G value for each experiment was calculated using the extinction coefficients of β-carotene as listed in Tab. IV_1.

The variation of the G values for the different β-carotene concentrations in the three solvents as a function of the dose are compiled in Tabs. IV_2, IV_3 and IV_4. Each G value is calculated as the mean value of a series of eight experiments.

Figs. IV_3, IV_4 and IV_5 show the variation of the G value as a function of the concentration for the doses of 14, 52 and 105 krad.

Table IV.1 Calculated molar extinction coefficients of β-carotene at the wavelengths of maximum absorption.

Solvent	$\lambda_{Max.}$ (nm)	a_o	a_1	correlation coefficient
n-Hexane	449	7.64×10^{-3}	1.2876×10^{5}	0.9999
	476	5.6428×10^{-3}	1.123×10^{5}	0.9999
Cyclohexane	454	-4.9285×10^{-3}	1.2442×10^{5}	0.9994
	482	-6.5714×10^{-3}	1.0768×10^{5}	0.9995
Benzene	463.5	-8.9285×10^{-3}	1.1826×10^{5}	0.9989
	491.5	-9.214×10^{-3}	1.0214×10^{5}	0.9993

The values listed in this table are calculated from the standard plots of β-carotene in the given solvents, according to the equation $y = a_o + a_1 x$. The coefficient a_1 expresses the molar extinction coefficient.

Table IV.2 Mean G-value for various β-carotene concentrations in benzene as a function of dose. Concentration in μM β-carotene.

Dose (krad)	5 μM		25 μM		50 μM		75 μM		100 μM	
	$G_{\lambda 463.5}$	$G_{\lambda 491.5}$	$G_{\lambda 463.5}$	$G_{\lambda 491.5}$	$G_{\lambda 463.5}$	$G_{\lambda 491.5}$	$G_{\lambda 463.5}$	$G_{\lambda 491.5}$	$G_{\lambda 463.5}$	$G_{\lambda 491.5}$
3.36	0.132	0.141	0.325	0.336	0.550	0.560	-			
6.72	0.122	0.122	0.321	0.336	-	-	-			
10.09	0.117	0.112	0.310	0.313	0.650	0.620	0.553	0.543		0.571
13.45	0.116	0.119	0.311	0.313	0.605	0.582	0.465	0.562	0.573	0.563
16.81	0.115	0.122	0.282	0.311	0.568	0.550	0.463	-	0.562	0.541
25.22	0.108	0.110	0.271	0.266	0.347	0.390	0.459	0.452	0.458	0.442
33.62	0.098	0.102	0.295	0.311	0.369	0.359	0.453	-	0.432	0.438
42.03	0.092	0.095	0.272	0.253	0.334	0.341	0.416	0.411	0.425	-
50.43	0.083	0.086	0.264	0.269	0.350	0.349	0.393	0.396	0.423	0.438
105.53	-	-	0.199	0.204	0.310	0.314	0.346	0.362	0.420	0.430
158.29	-	-		0.156	0.286	0.297	0.339	0.345	0.384	0.398
205	-	-			0.235	0.243	0.285	0.306	0.375	0.381

Table IV.3 Mean G-value for different β-carotene concentrations in hexane as a function of dose. Concentration in μM β-carotene.

Dose (krad)	5 μM		50 μM		75 μM		100 μM	
	$G_{\lambda 449}$	$G_{\lambda 476}$	$G_{\lambda 449}$	$G_{\lambda 476}$	$G_{\lambda 449}$	$G_{\lambda 476}$	$G_{\lambda 449}$	$G_{\lambda 476}$
3.36	0.142	0.151	0.862		1.015	0.997	-	-
6.72	0.114	0.125	0.727			-		-
10.05	0.107	0.112	0.721		0.860	0.842	1.283	1.754
13.45	0.107	0.106	0.608	0.537	0.828	0.849	1.502	1.593
16.81	0.111	0.115	0.616	0.558	0.824	0.830	1.312	1.380
25.22	0.102	0.104	0.631	0.660	0.802	0.800	1.249	1.274
33.62	0.096	0.099	0.533	0.529	0.795		1.242	1.267
42.03	0.087	0.089	0.538	0.527	0.730	0.714	1.195	1.081
40.43	0.084	0.086	0.502	0.550	0.760	0.721	1.137	1.0735
105.53	0.061		0.400	0.446	0.619	0.630	0.908	0.839
158			0.305		0.541	0.565	0.730	0.736
205			0.230		0.455	0.456	0.628	0.624

Table IV.4 Mean G-value for different β-carotene concentrations in cyclohexane as a function of dose.

Concentration in μM β-carotene.

Dose (krad)	5 μM		50 μM		75 μM		100 μM	
	$G_{\lambda 454}$	$G_{\lambda 482}$	$G_{\lambda 454}$	$G_{\lambda 482}$	$G_{\lambda 454}$	$G_{\lambda 482}$	$G_{\lambda 454}$	$G_{\lambda 482}$
3.36	0.189	0.190	1.131	1.051	1.482	-		
6.72	0.177	0.170	1.111	1.103	1.485	1.274	1.985	1.887
10.09	0.147	0.144	1.050	1.043	1.559	1.375	1.815	
13.45	0.149	0.146	0.931	0.924	1.522	1.420	1.836	1.778
16.81	0.135	0.135	0.904	0.915	1.309	1.343	1.717	
25.22	0.118	0.121	0.848	0.865	1.211	1.255	1.596	1.682
33.62	0.106	0.104	0.804	0.810	1.183	1.151	1.479	
42.03	0.095	0.091	0.741	0.748	1.137	1.098	1.423	1.425
50.43	0.088	0.087	0.701	0.707	1.024	1.082	1.379	
105.53					0.760	0.750	1.020	1.205
158					0.558	0.542	0.809	0.960
205.					0.438	0.432	0.620	0.727

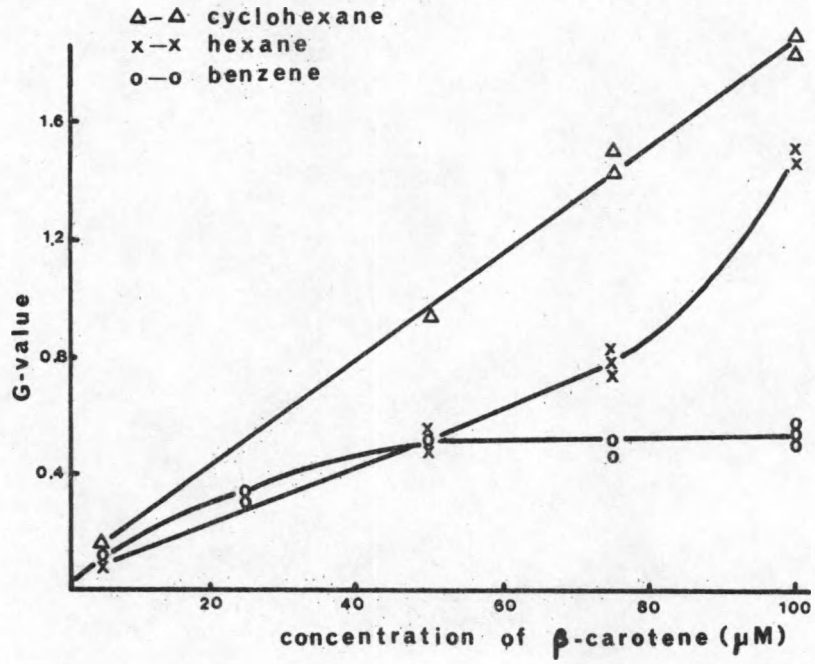


Fig. IV_3 Influence of the concentration of β-carotene on the G-values (calculated for each solvent at the highest absorption maximum) for a dose of 14 krad.

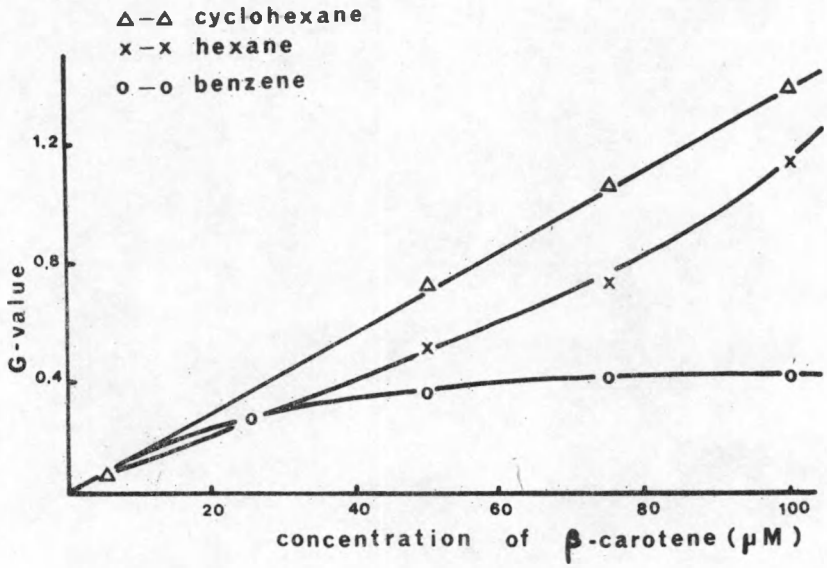


Fig. IV_4 Influence of the concentration of β-carotene on the G-values (calculated for each solvent at the highest absorption maximum) for a dose of 52 krad.

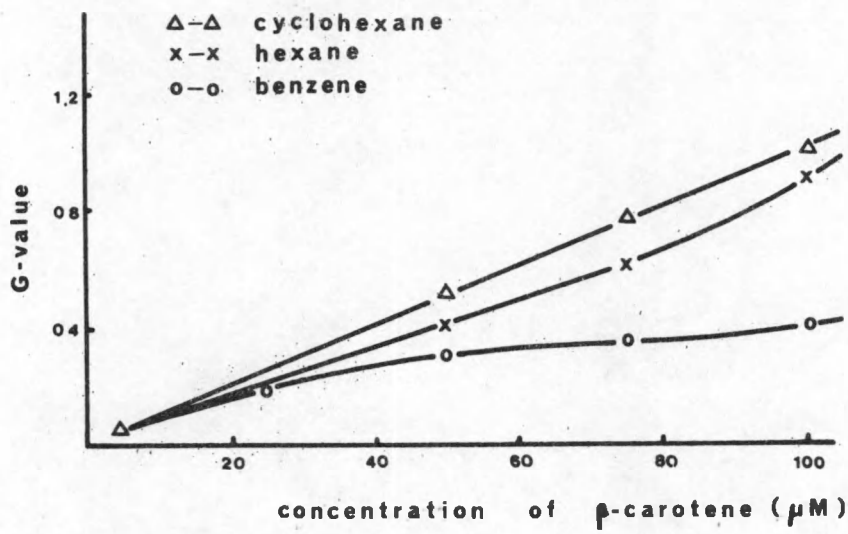


Fig. IV_5 Influence of the concentration of β-carotene on the G-values (calculated for each solvent at the highest absorption maximum) for a dose of 105 krad.

From the data in Tabs. IV_2, IV_3, IV_4 and the Figs. IV_3, IV_4, IV_5 we can state that :

(1) The G values calculated from the variation in optical density at both absorption maxima compare very well.

(2) For each of the carotenoid concentrations studied the G values decrease with the dose.

(3) For a given dose the G values increase to a marked extent with the concentration.

(4) The nature of the solvent has a pronounced effect on the G value.

(5) For β-carotene in benzene and for a given dose the G value tends to remain constant for concentrations above $50_{\mu}M$. If the degradation of β-carotene is the result of an indirect effect only, for each concentration and for a given dose the same G value should be found. For low concentrations ($5_{\mu}M$) the degradation might mainly be caused by the indirect effect.

Calculating the percent degradation and the absolute quantities of the degradation of β-carotene (Tab. IV_5) the contribution of the direct degradation mechanism with increasing solute concentration is evidenced.

We can conclude from all those data that β-carotene is most stable in benzene and most radiosensitive in cyclohexane. The relative radiation stability of β-carotene in benzene (except for the $5_{\mu}M$ concentrations) should be ascribed to the aromatic character of the benzene molecule.

Hereby the aromatic compound could share its relative stability towards radiation with the dissolved β-carotene molecule.

Table IV.5 Percent degradation of β-carotene as a function of the concentration.

Dose (krad)	Concentration of β-carotene in μM	(a) Degradation of β-carotene		(b) Degradation of β-carotene		(c) Degradation of β-carotene	
		in %	in mg/l	in %	in mg/l	in %	in mg/l
50	5	78.40	2.11	75.20	2.02	55.07	1.48
	50	36.45	9.78	63.25	16.98	37.80	10.14
	75	25.00	10.07	56.40	22.71	35.56	14.32
	100	20.00	10.74	54.07	29.04	37.18	19.09
100	5						
	50	48.06	13.89	91.02	24.44	59.92	16.09
	75	44.99	18.12	82.62	33.27	58.55	23.58
	100	42.50	22.82	83.08	44.61	58.82	31.58
150	5			-	-		
	50	78.91	21.19	-	-	67.88	18.22
	75	69.24	27.88	91.52	36.86	73.84	29.74
	100	56.68	30.44	96.32	51.72	74.59	40.05
	5			-	-		
	50	78.43	21.09	-	-	86.00	23.09
	75	75.96	30.59	96.68	38.33	84.25	33.93
	100	72.59	38.98	96.32	51.72	85.50	45.91

(a) irradiation in benzene, (b) irradiation in cyclohexane, (c) irradiation in hexane.

Physical protection by the benzene molecule should involve the transfer of energy from an energy-rich species of β-carotene that might be either an ion or an excited molecule. Moreover, comparison of the yiels of radiolysis products from benzene, cyclohexane and hexane shows that benzene gives appreciably smaller product yields, than cyclohexane and hexane, particularly when it is irradiated as a liquid (Spinks and Woods 1964). Hereby it should be emphasized that by this stability of the aromatic compound, probably less reactive intermediates (especially H° radicals) are available for radical attack of the dissolved β-carotene molecule.

Those data are in contrast with the G values found for the irradiation of the 5 μM solutions of β-carotene in benzene and hexane. While for low concentrations the contribution of the indirect action is expected to be important, a greater G value is found for the experiments in benzene than in hexane.

This might be explained by the fact that the life time of a thermal hydrogen in benzene is greater than in hexane and cyclohexane (Holroyd, 1968).

Phenyl radicals were never observed in the radiolysis studies of benzene. This is not surprising since phenyl radicals add rapidly to benzene.

In conclusion the reactions H° + solvent radical, competing with the reaction H° + β-carotene, will be more important for the experiments in cyclohexane and hexane.

Finally the decrease in G value as a function of the dose for a given concentration can be explained by the attribution of several processes.

1) Termination of radical reactions.

2) Decrease in the β-carotene concentration during irradiation and consequent decrease in the degradation rate.

3) To calculate the G values the optical density at λmax was measured. As described earlier in the text it was assumed that the absorption of an irradiated β-carotene solution was the result of the presence of β-carotene molecules only.

C.2.b. TLC analysis of the irradiated β-carotene solutions.

Comparing the total absorption spectrum of γ-irradiated β-carotene as a function of the nature of the solvents, the dose and the β-carotene concentration we could summarise that most considerable distortions in the spectral characteristics were obtained for the experiments in cyclohexane.
Figs. IV_6, IV_7 and IV_8 compare the spectra of 100 μM solutions of β-carotene in benzene, cyclohexane and hexane after the irradiation with doses of 100, 50 and 100 krad, respectively.

It appears from these data that the spectrum of irradiated solutions in benzene is hardly deformed, whereas for the experiments in cyclohexane important changes in the ultraviolet region are already observed at a dose of 50 krad. At doses above 200 krad the typical carotenoid spectrum entirely disappears.

The degradation products of the irradiated solutions, giving the spectral pattern represented in Figs. IV_6, IV_7 and IV_8 were separated by TLC as described in the experimental section of this chapter. As will be discussed below, probably similar degradation products are formed upon irradiation of β-carotene in the three solvents chosen.

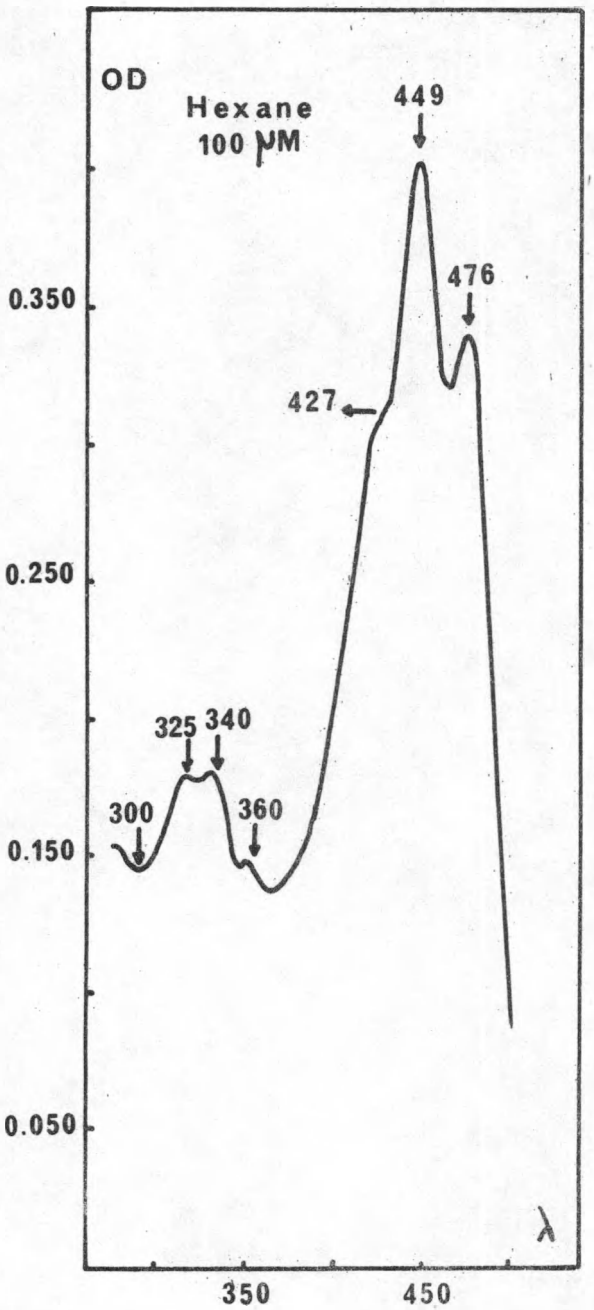


Fig. IV_6 Absorption spectrum of a 100 μM solution of β-carotene in hexane after irradiation with a dose of 100 krad.

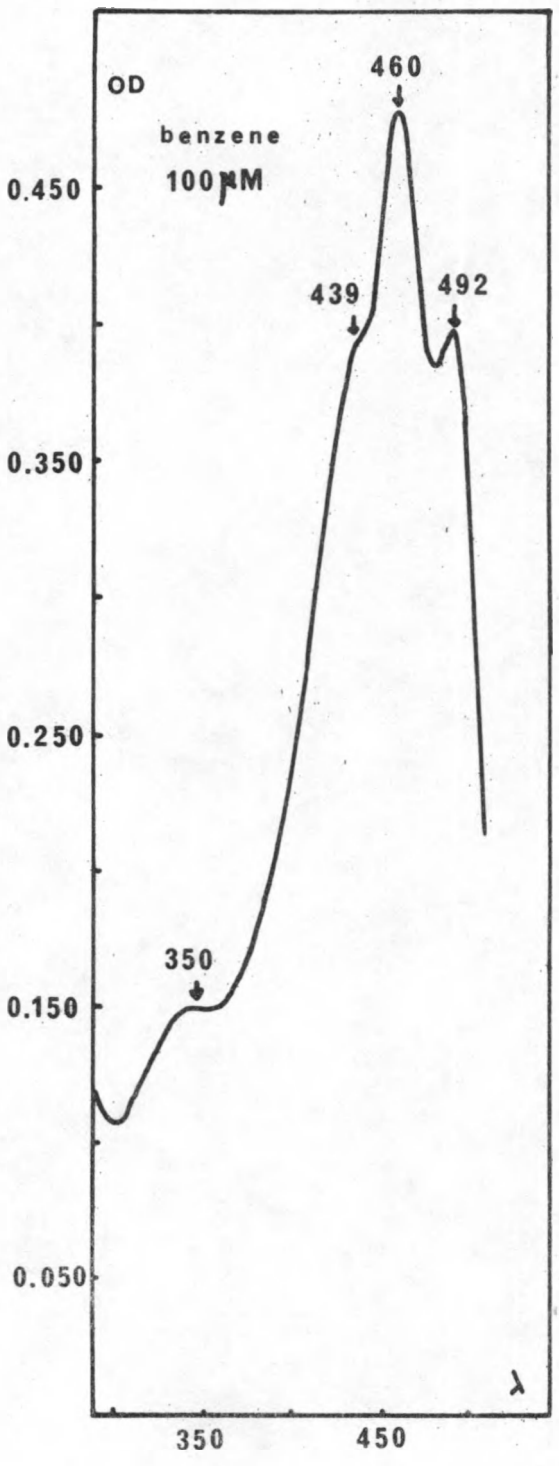


Fig. IV_7 Absorption spectrum of a 100 μM solution of β-carotene after irradiation with a dose of 100 krad.

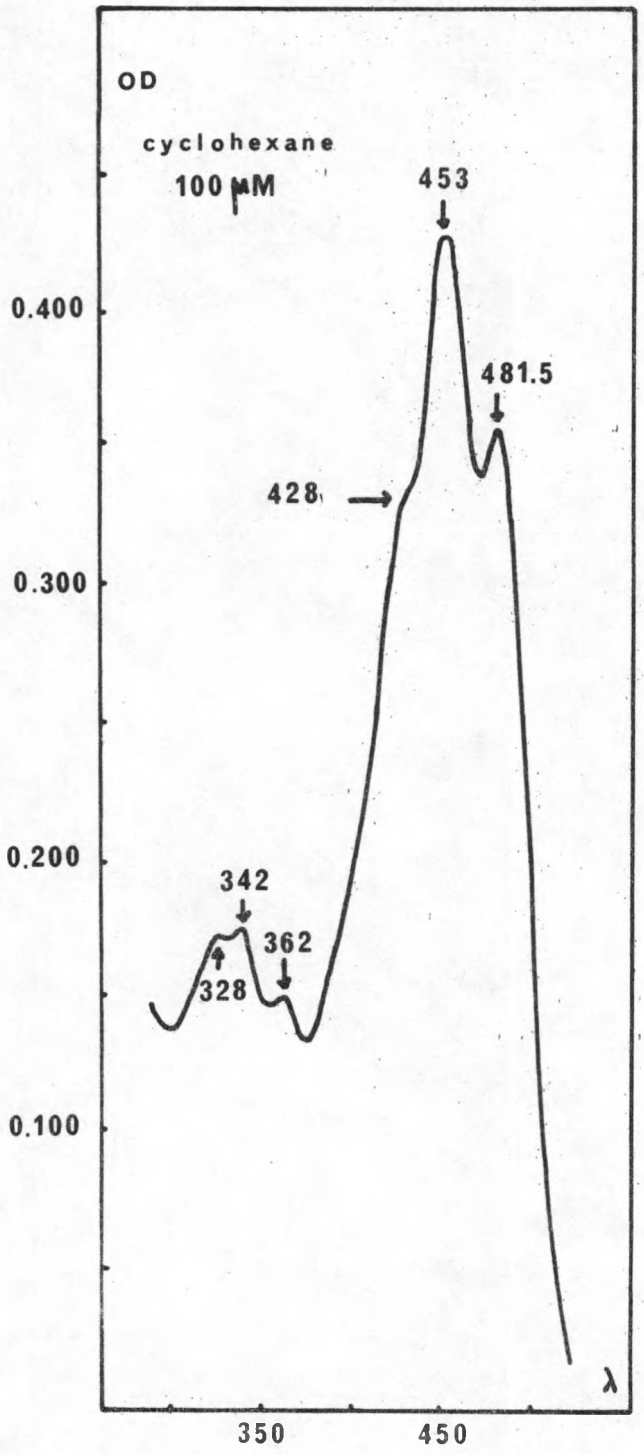


Fig. IV_8 Absorption spectrum of a 100 μM solution of β-carotene after irradiation with a dose of 50 krad.

On the chromatographic plate eight yellow bands appeared with decreasing R_f values. Tab. IV_6 compiles the characteristics of the degradation products, after the irradiation of β-carotene in the three solvents, separated by TLC.

C.2.c. Discussion of some mechanisms involved in the degradation of β-carotene upon irradiation in benzene, cyclohexane and hexane.

The distribution of certain structural indices in β-carotene, which can be used for the description of the electronic distribution in conjugated molecules, are reproduced in Fig. IV_9 (Pullman and Pullman, 1963). For the sake of simplicity only one half of the molecule has been drawn, the other half being exactly symmetrical.

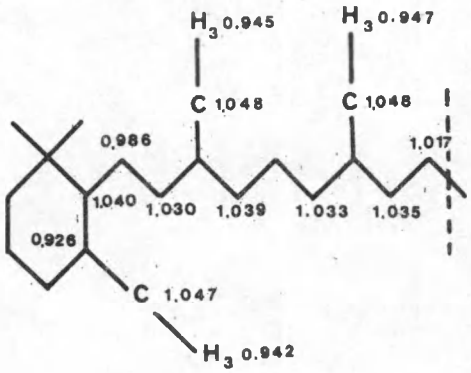
The energy of the highest filled molecular orbital (0.079 β-units) and the lowest empty molecular orbital (-0.183 β-units) indicate that β-carotene should be both an excellent electron donor and an excellent electron acceptor. Pullman and Pullman (1963) have also calculated the localization energies of the carbon atoms in β-carotene for the different types of attack, nucleophilic, electrophilic or radical. The values of these energies are listed in Tab. IV_7. This table definitely confirms that the main reactive center of the molecule (aside from the cyclohexene rings) toward any type of attack is position 7, as was already indicated by the high value of its free valence.

C.2.c.1. Cis-trans isomerization.

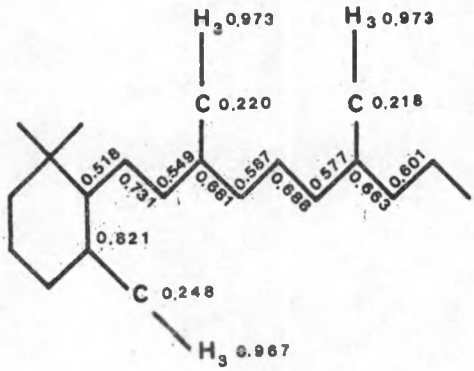
Cis-forms of carotenoids absorb light of lower wavelength and lower intensities than the corresponding all-trans isomers (Davies, 1965). The λshifts for different

Table IV.6 The characteristics of the degradation products, separated by TLC, after irradiation of β-carotene in benzene, cyclohexane and hexane.

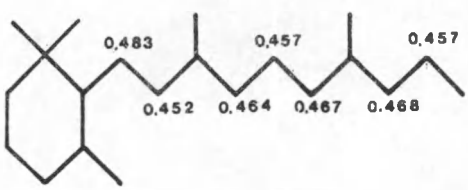
		shifts of the main absorption maximum of the various degradation products towards shorter wavelengths in nm compared with the main absorption maximum of unirradiated β-carotene.		
Band	R_f-value	Experiments in benzene	Experiments in cyclohexane	Experiments in hexane
1	0.98	2.40-1.60	1.80-1.40	1.50-1.
2	0.84	30.20-28.50	27.70-26.50	26.70-26.21
3	0.72	61.50-55.70	58.70-52	55.20-51.30
4	0.50	65-60	-	-
5	0.42	90	84	81
6	0.30	35-28	-	-
7	0.25	6.00-5.80	4.30-4.00	3.70-4.50
8	0.00	90	84	81



Electrical charges



Bond orders



Free valences

Fig. IV$_9$ Electronic indices in β-carotene (Pullman and Pullman 1963).

Table IV.7 Carbon localization energies in β-carotene (in β-units).

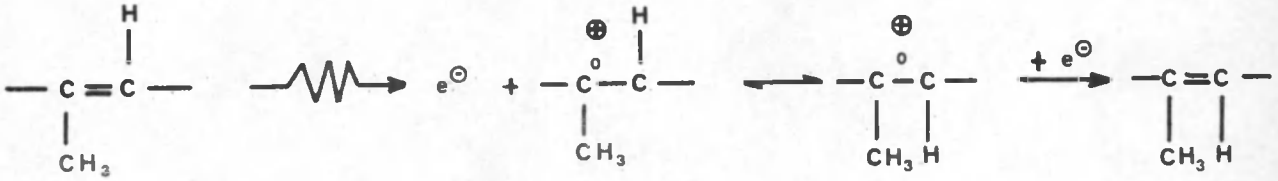
Localization energies			
Carbon	Nucleophilic	Radical	Electrophilic
15	2.12	2.06	2.00
14	2.13	2.06	1.99
12	2.15	2.09	2.03
11	2.07	2.03	1.98
10	2.21	2.13	2.06
8	2.29	2.23	2.17
7	1.95	1.92	1.88

cis-isomers are described by Zechmeister (1962) and Schwieter et al. (1968). Comparing the absorption spectra of the first band on the thin-layer (R_f 0.98) of β-carotene irradiated in the three solvents we can state that :

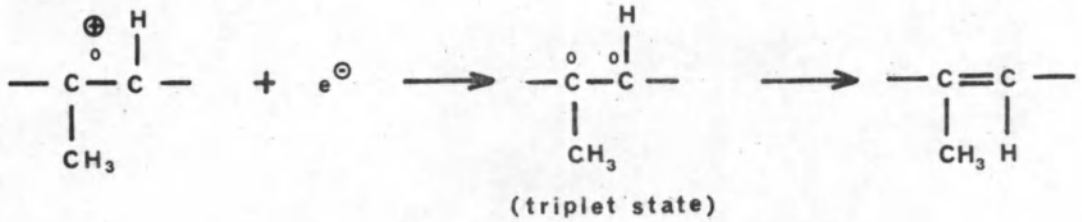
- the absorption maxima are shifted by 1 to 2.4 nm to shorter wavelengths.
- a subsidiary cis-peak appears in the ultraviolet region (around 340 nm).

This may probably be due to cis-isomers. Therefore it was concluded that this fraction on the thin-layer plate was a mixture of β-carotene and one or more cis-isomers.
This isomerization can be explained in two ways :

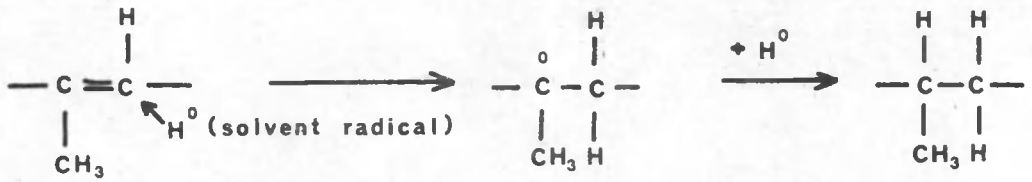
a) By direct interaction of the γ-rays with the β-carotene molecule an electron from a molecular orbital can be ejected and the ionized species in which the electron hole is localized at any particular double bond site might appear to be a likely candidate for the role of an intermediate leading to cis-trans isomerization.

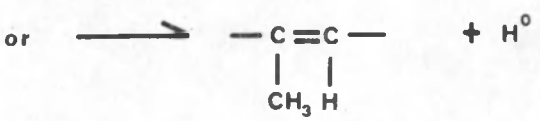


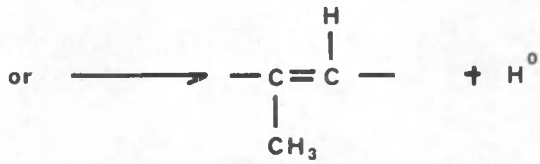
It could be argued that the three electron carbon-carbon bond involved here retains considerable rigidity. However charge neutralization of such cationic radicals leads with high probability to a triplet excited state invoked in the cis-trans isomerization.



b) Indirect radiation induced cis-trans isomerization. Even at low concentration ($5_{\mu}M$) a cis-peak appeared in the total absorption spectrum of the irradiated solutions. TLC analysis of these irradiated low concentration solutions revealed the presence of cis-isomers in band 1. Therefore it was proposed that cis-trans isomerization probably occurs as the result of the indirect effect as well.







C.2.c.2. Shortening of the conjugated systems and formation of α-carotene.

The position of the long wavelength absorption bands (usually three) of the carotenoid spectrum is a function of

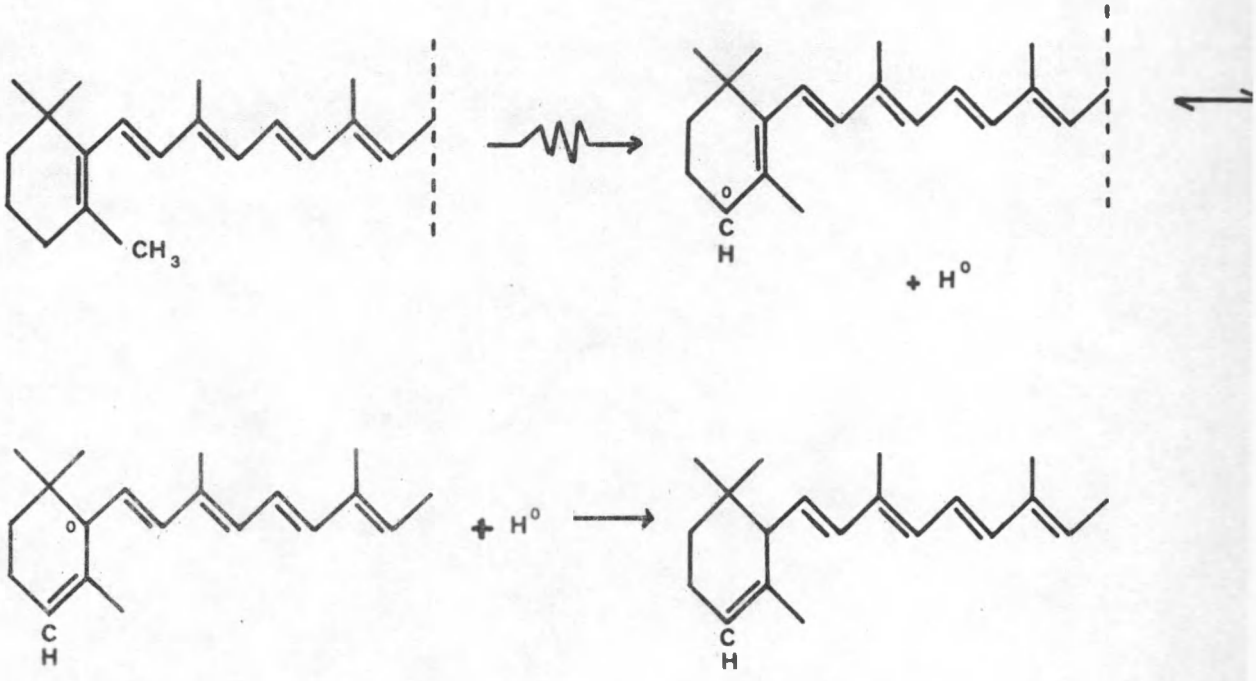
the number of conjugated double bonds in the molecule. In compounds containing two β-ionone residues, the absorption maximum at the shortest wavelength is reduced to a shoulder, whereas for an α-carotenoid spectrum this peak is more or less pronounced.

By replacing one aliphatic double bond by a single bond the spectrum is shifted by 7-35 nm to the shorter wavelengths. By replacing an ethylenic bond in the 6-membered ring this shift ranges from 5 to 9 nm.(Schwieter et al. 1968).

From the shifts in absorption maxima of the degradation products of β-carotene compiled in Tab. IV_6 and the shape of the spectrum it was supposed that the conjugated system in the β-carotene molecule is shortened upon irradiation.

In our opinion these results can be explained by either an initially direct or an indirect reaction mechanism.

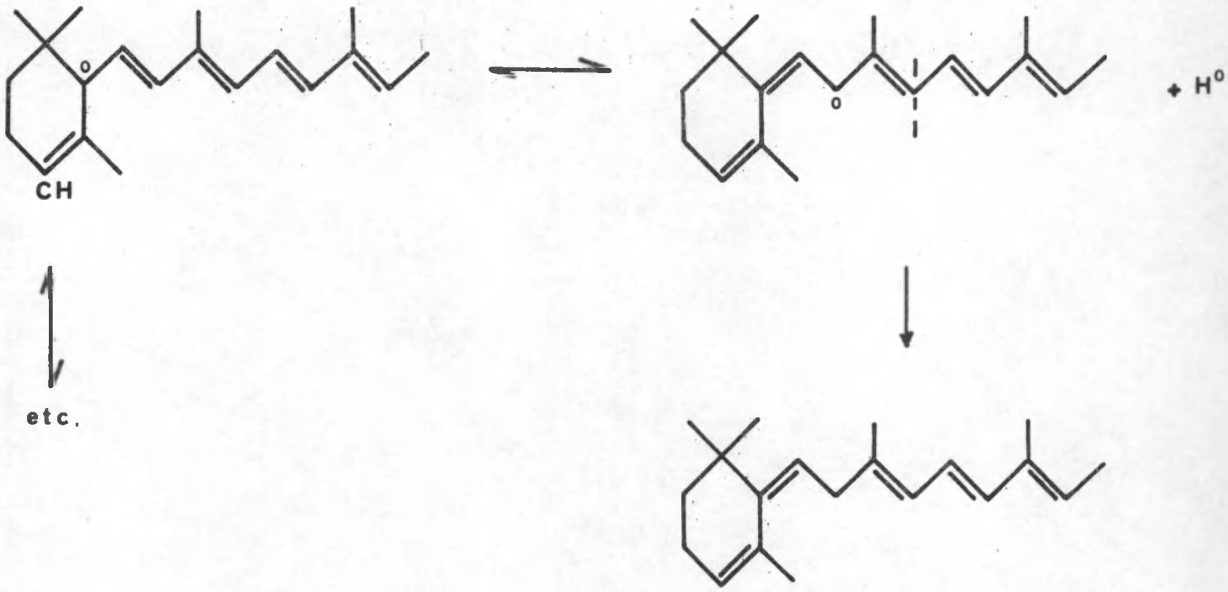
Direct action on the β-carotene molecule can initially result in the homolytic scission of a C-H bond, apparently adjacent to a double bond.



α-carotene

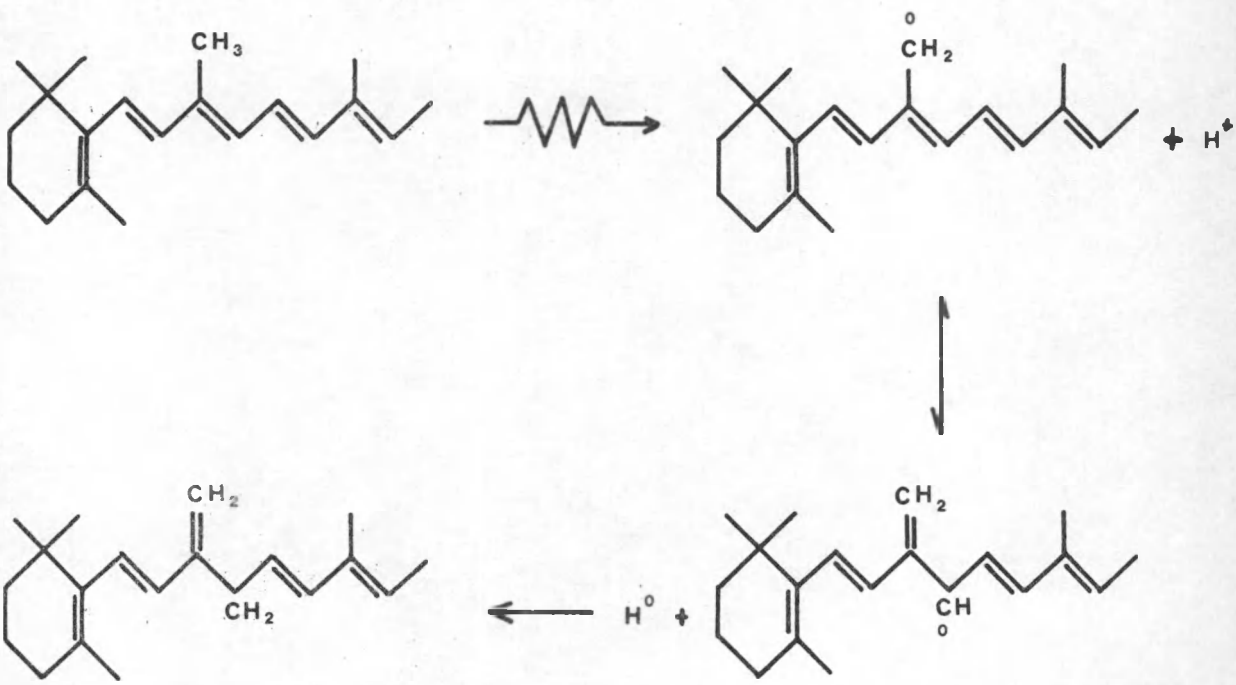
Upon the TLC analysis of the reaction products after the irradiation in the three solvents, band 7 (R_f 0.25) is probably α-carotene.

Using the method of Hager and Meyer-Bertenrath (1972) -carotene ran above β-carotene. The spectral pattern of this fraction indicated that α-carotene was formed upon irradiation.



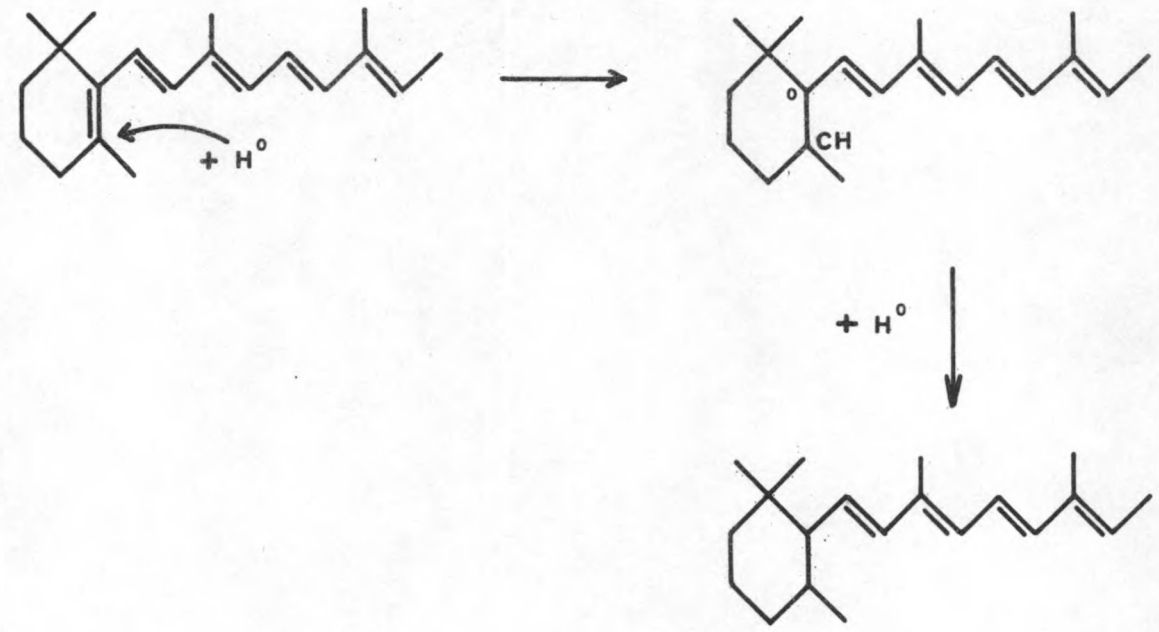
α-carotene with two double bonds out of conjugation

Homolytic scission of a C-H bond can also occur in the methyl groups adjacent to a double bond.

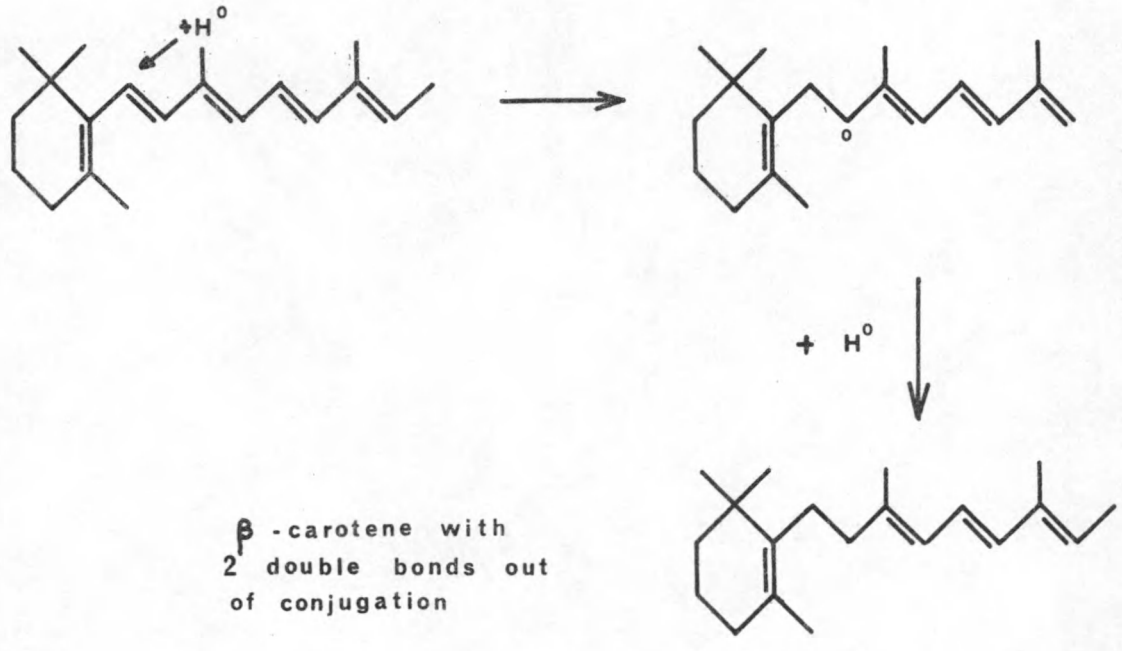


This type of reaction can be written for each of the methyl-groups adjacent to a double bond. In this way two or more double bonds are moved out of conjugation.

Hydrogen radical addition can also occur on a double bond.

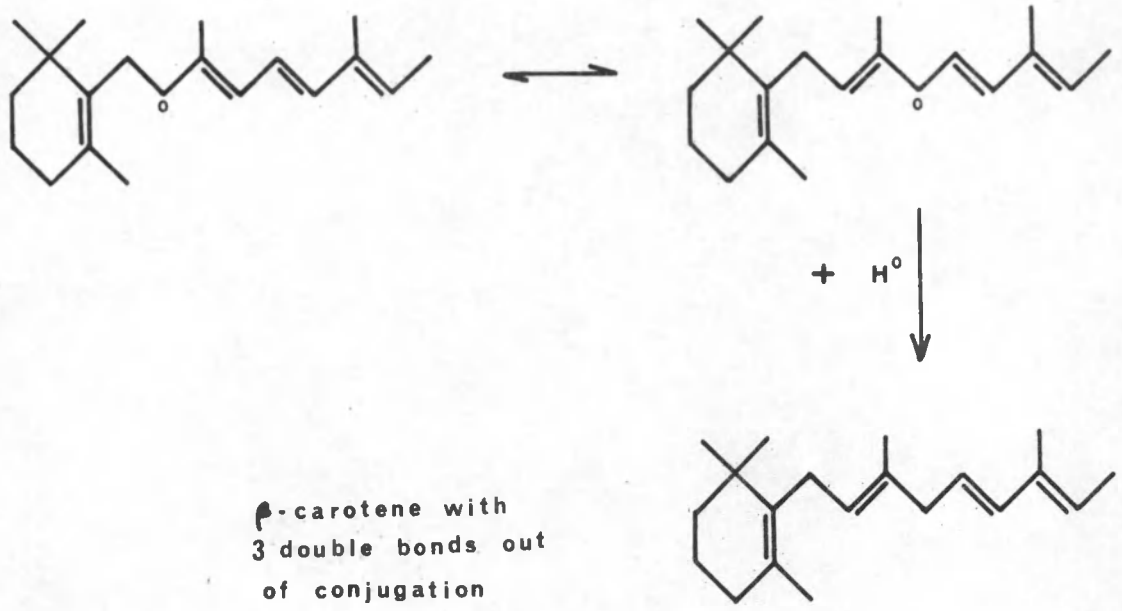


or



β -carotene with 2 double bonds out of conjugation

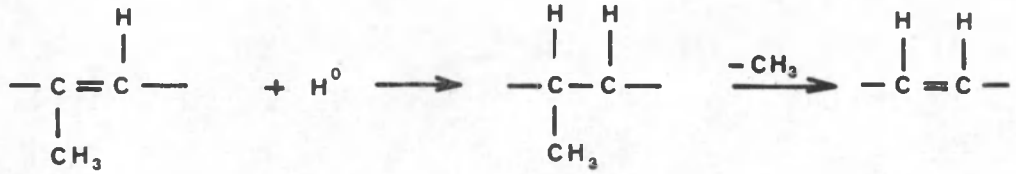
or



β-carotene with 3 double bonds out of conjugation

etc.

Further by indirect action a methylgroup can also be split off.



All reactions, represented above, between the β-carotene molecule and β-carotene radical and the solvent radical H° are expected to predominate any reactions with other solvent radicals for the following reasons :

- In the solvent H° radicals will be formed in preference since the splitting of a C-C bond is more endothermic than the splitting of a C-H bond.

- The mobility of hydrogen radicals in the medium is much greater than that of alkylradicals.

- Upon addition of H° on a C=C bond in the β-carotene molecule less steric hindrance will occur than by addition of alkylradicals.

Considering the spectral patterns of the degradation products separated by TLC analysis and some possible reaction mechanisms outlined above we can summarize:

- fraction 1, is a mixture of unaltered β-carotene and one or more cis-isomers.
- fraction 7, shows a typical spectrum of α-carotene.
- fraction 2, the λ shift of about 28-30 nm can be ascribed to a conjugated system with 9 double bonds. (The ring double bond, and the ethylenic bond between C_7 and C_8 are moved out of conjugation.)
- fraction 3 and fraction 4, the λ shift being twice that of fraction 2, indicates that terminal attack at both sides of the molecules has occurred. Their absorption maxima should correspond to a polyene chain of 7 double bonds.
- fraction 5 and fraction 8 no longer show the typical carotenoid spectrum, probably due to a large hydrogenation of the conjugated system.
- fraction 6, corresponds to a compound with a polyene chain containing less than 7 double bounds.

C.3. Irradiation of β-carotene in benzene/methanol mixtures.

C.3.a. Dilution test.

Upon irradiation of β-carotene in benzene/methanol mixtures the behaviour of β-carotene in a more polar medium

was studied.

The percent degradation of β-carotene was studied as a function of both the β-carotene and the methanol concentration. β-carotene dissolved in benzene, benzene/methanol (75:25) and benzene/methanol (50:50) was irradiated in anaerobic conditions as described in the experimental section. From the variation in optical density at the two absorption maxima the percent degradation was calculated. Assuming once more that the degradation products do not influence the extinction of β-carotene, at both absorption maxima, which might represent an oversimplification, the relative contribution of direct and indirect interaction can be demonstrated from the calculated percent degradation values. In Tabs. IV_8, IV_9 and IV_{10} the degradation of β-carotene as a function of the β-carotene concentration is compared in the three solvent systems.
In these tables it is shown that for a concentration of 5 μM β-carotene the degradation decreases with increasing methanol concentration.

The percent degradation was found to be the highest upon irradiation of low concentrations of β-carotene (5 μM) in benzene. These results compare very well with the conclusions drawn in Chapter IV, C.2.a. where it was stated that the indirect effect in benzene was more pronounced than in cyclohexane and hexane.
Despite the higher radical and molecular yields for methanol, cyclohexane and hexane, the radiosensitivity of β-carotene is the highest in benzene, at low β-carotene concentrations. The only possible explanation is that radical reactions between solvents fragments and ion-molecule reactions predominate for the solvents benzene/methanol, cyclohexane and hexane.
Radicals formed in benzene should preferentially interact with the dissolved β-carotene.

Table IV.8 Percent degradation of β-carotene in benzene as a function of the irradiation dose and the concentration.

Dose (krad)	5 μM		25 μM		50 μM		75 μM		100 μM	
	λ_{463}	λ_{491}	λ_{463}	λ_{491}	λ_{463}	λ_{491}	λ_{463}	λ_{491}	λ_{463}	λ_{491}
3.32	6.14	7.94	2.16	2.32	1.18	1.74	1.69	1.93	1.64	1.52
6.64	14.40	15.30	3.59	3.48	3.86	4.06	3.01	3.23	3.25	3.37
9.96	20.78	20.88	7.04	7.36	5.70	6.96	5.36	3.81	4.36	4.38
13.28	26.80	27.68	10.56	10.44	9.88	9.48	6.49	6.97	7.12	7.45
16.6	35.34	36.98	12.44	11.56	13.06	13.94	7.41	8.12	9.22	9.34
24.9	48.08	48.34	18.80	18.60	16.18	18.58	12.07	13.16	14.88	14.14
33.2	58.62	57.12	29.36	29.04	19.94	20.28	15.64	16.77	17.58	18.04
41.5	66.66	67.02	36.56	36.40	25.95	25.96	19.64	20.13	19.94	19.46
49.8	78.40	77.44	43.88	43.36	36.43	36.12	25.04	24.34	20.00	21.72
99.6			74.82	72.05	48.06	48.16	44.99	45.12	42.50	43.14
149.4					71.11	72.12	69.07	69.24	56.68	57.34

Table IV.9 Percent degradation of β-carotene in benzene/methanol 75/25 as a function of the dose and the concentration.

Dose (krad)	5 μM		25 μM		50 μM		75 μM		100 μM	
	λ_{461}	λ_{489}	λ_{461}	λ_{489}	λ_{461}	λ_{489}	λ_{461}	λ_{481}	λ_{461}	λ_{481}
3.32	5.40	5.84	1.04	1.94	2.96	3.11	3.25	3.24	1.91	1.65
6.64	10.80	18.48	6.26	7.00	5.92	4.83	5.15	5.22	4.86	5.13
9.96	17.77	18.28	12.19	14.33	9.93	9.72	9.91	9.87	7.97	6.35
13.28	22.81	23.92	16.37	17.5	12.76	13.6	12.54	11.28	8.94	9.75
16.6	28.29	27.40	18.81	19.34	17.94	17.5	17.32	18.41	13.09	14.00
29.4	44.29	42.20	29.96	30.34	27.35	25.48	26.59	25.79	21.68	21.42
33.2	50.56	50.92	42.50	43.18	33.79	33.46	33.12	34.07	33.45	32.37
41.5	62.02	62.24	51.96	51.72	46.86	46.48	39.59	39.43	39.28	38.93
49.8	69.04	68.74	62.67	62.66	52.12	53.96	49.72	49.61	47.28	46.99
99.6			87.80	87.32	79.54	80.00	75.07	75.04	73.10	73.25
149.4					91.40	92.4	94.65	93.21	85.86	86.76

Table IV.10 Percent degradation of β-carotene in benzene/methanol 50/50 as a function of the dose and the concentration.

Dose (krad)	5 μM		25 μM		50 μM		75 μM		100 μM	
	λ_{458}	λ_{485}	λ_{458}	λ_{485}	λ_{458}	λ_{485}	λ_{458}	λ_{485}	λ_{458}	λ_{485}
3.32	4.30	4.34	2.26	2.36	1.39	1.18	1.49	1.45		
6.64	9.38	8.90	5.90	6.32	3.94	4.16	3.77	3.56	3.44	3.36
9.96	15.28	13.84	7.08	7.52	6.68	6.92	5.37	5.27	6.52	6.32
13.38	18.36	19.18	9.96	11.48	9.44	9.28	8.24	8.69	8.42	8.30
16.6	22.14	22.34	14.4	15.00	11.34	11.26	10.55	10.67	10.12	9.68
24.9	32.44	33.40	20.24	22.12	21.46	21.34	15.27	15.55	17.16	17.00
33.2	41.88	42.88	27.64	28.84	30.38	26.68	22.67	23.32	26.96	27.28
41.5	51.16	52.18	37.40	38.32	39.96	40.12	27.22	26.98	29.36	29.84
49.8	59.68	58.36	46.64	45.28	42.10	40.18	35.15	36.01	34.00	34.18
99.6					84.38	82.20	82.92	81.97	78.80	79.84
149.4					97.04	96.4	96.57	95.23	97.16	97.86

From 25 μM β-carotene concentrations the lowest degradation of β-carotene is found in benzene. For higher β-carotene concentrations the degradation is mainly affected initially by the direct effect. As stated, the aromatic character of the benzene molecule protects the β-carotene molecule against the direct interactions of the gamma rays.
It was found peculiar that for the experiments in benzene/methanol (75:25) the percent degradation was higher than for those in benzene/methanol (50:50).
The radiosensitivity of β-carotene is about the same in the two mixtures benzene/methanol for the experiments with 75 μM and 100 μM β-carotene concentrations.
As a conclusion Figs. IV_{10} and IV_{11} compare the influence of the solvent upon the radiation degradation of β-carotene as a function of the concentration.

For these doses the radiation sensitivity of β-carotene in cyclohexane, hexane and benzene/methanol mixtures seems to be about the same.

The presence of methanol in benzene enhanced the radiosensitivity of β-carotene.

C.3.b. Comparison of the total carotenoid spectrum and TLC analysis of the irradiated β-carotene solutions.

In an analogue way as in Chapter IV, C.2.b. the deformation of the total absorption spectrum upon irradiation was studied as a function of the nature of the solvent, the β-carotene concentration and the irradiation dose.

For a 5 μM β-carotene concentration the deformations of the absorption spectra for the experiments in benzene and in benzene/methanol mixtures compare very well.

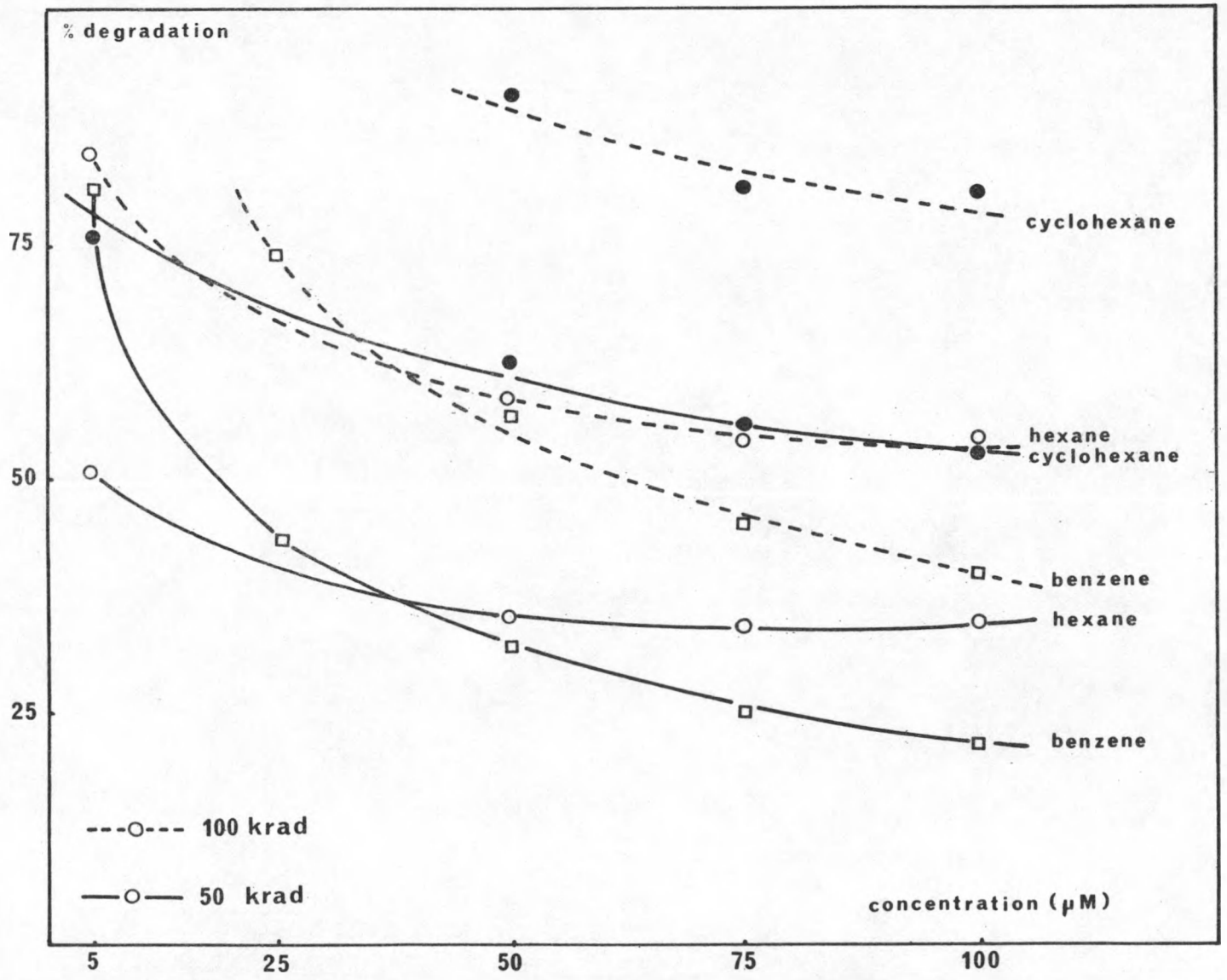


Fig. IV_{10} Influence of the solvent upon the radiation degradation of β-carotene as a function of the concentration.

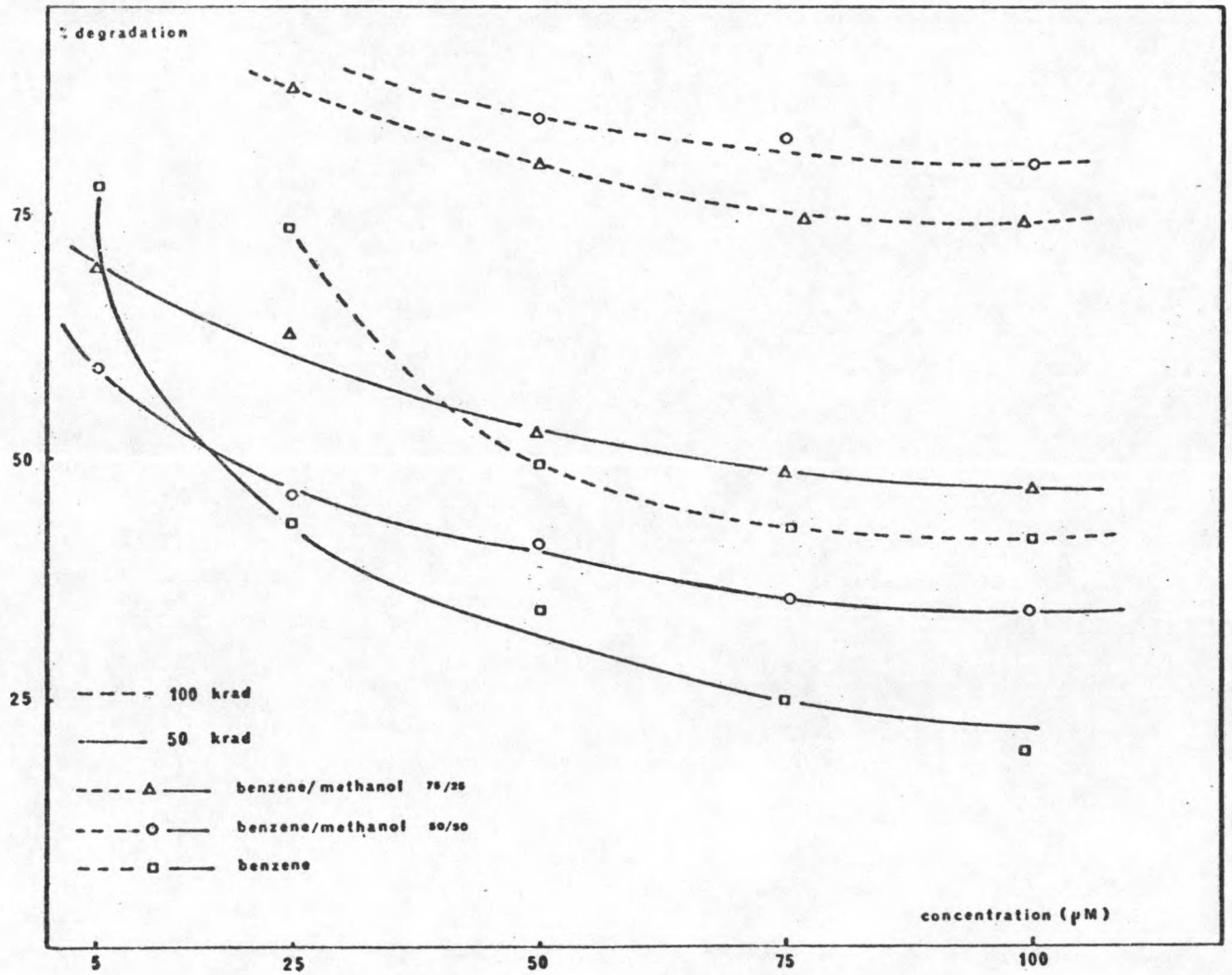


Fig. IV$_{11}$ Influence of the solvent upon the radiation degradation of β-carotene as a function of the concentration.

For the higher β-carotene concentrations (50 μM, 75 μM, 100 μM) the deformations of the spectra and the hypsochromic shifts of the absorption maxima were more pronounced for the experiments in benzene/methanol especially for doses up to 100 krad.

The carotenoid spectra disappear entirely for the irradiation experiments in benzene/methanol at a dose of 150 krad.

For the experiments in benzene, on the contrary, only minor shifts were noticed.

These results confirm that from a concentration of 25 μM on, β-carotene is most radiation stable in benzene.

TLC analysis of an irradiated solution of 100 μM β-carotene with a dose of 100 krad in the solvent mixtures benzene/methanol also revealed the presence of eight coloured fractions.

The spectral pattern of these fractions also indicated the formation of cis-trans isomers, a progressive shortening of the conjugated system and the presence of α-isomers.

Using the procedure of Jamamoto (1965) no positive indication of the presence of epoxide groups were found.

The possibility of the presence of aldehyde- and keto groups was tested according to the method of Bancher et al. (1972) without positive result.

As a conclusion it can be stated that : probably analogue mechanisms are involved leading to the formation

of degradation products when irradiating β-carotene in benzene and benzene/methanol mixtures.

C.4. Irradiation of β-carotene in the presence of fat soluble compounds.

Fats and fatty acids form an important constituent of foods and can be one of the variable factors in meat- and fish products. The influence of the incorporation of oleic- and stearic acid on the radiation sensitivity of β-carotene in cyclohexane was studied in some experiments. Cyclohexane was chosen as solvent, because the previous experiments have proved an enhanced radiation sensitivity of β-carotene in cyclohexane.

C.4.a. The extent of degradation of β-carotene in the presence of mono-unsaturated fatty acids is exemplified in the system oleic acid in cyclohexane.

For a concentration of 5 μM, respectively 50 μM of β-carotene in cyclohexane it was observed that the percentage loss of β-carotene was not altered in the presence of 1% and 2% oleic acid when exposed to a radiation dose up to 93 krad.

On the contrary concentrations of 5% and 10% oleic acid remarkably enhanced the percent degradation of β-carotene. In Figs. IV_{12} and IV_{13} the influence of the oleic acid concentration is shown.
For an initial pigment concentration of 50 μM β-carotene underwent less destruction in a solution containing 5% oleic acid than for an initial pigment concentration of 5 μM (Tab. IV_{11}).

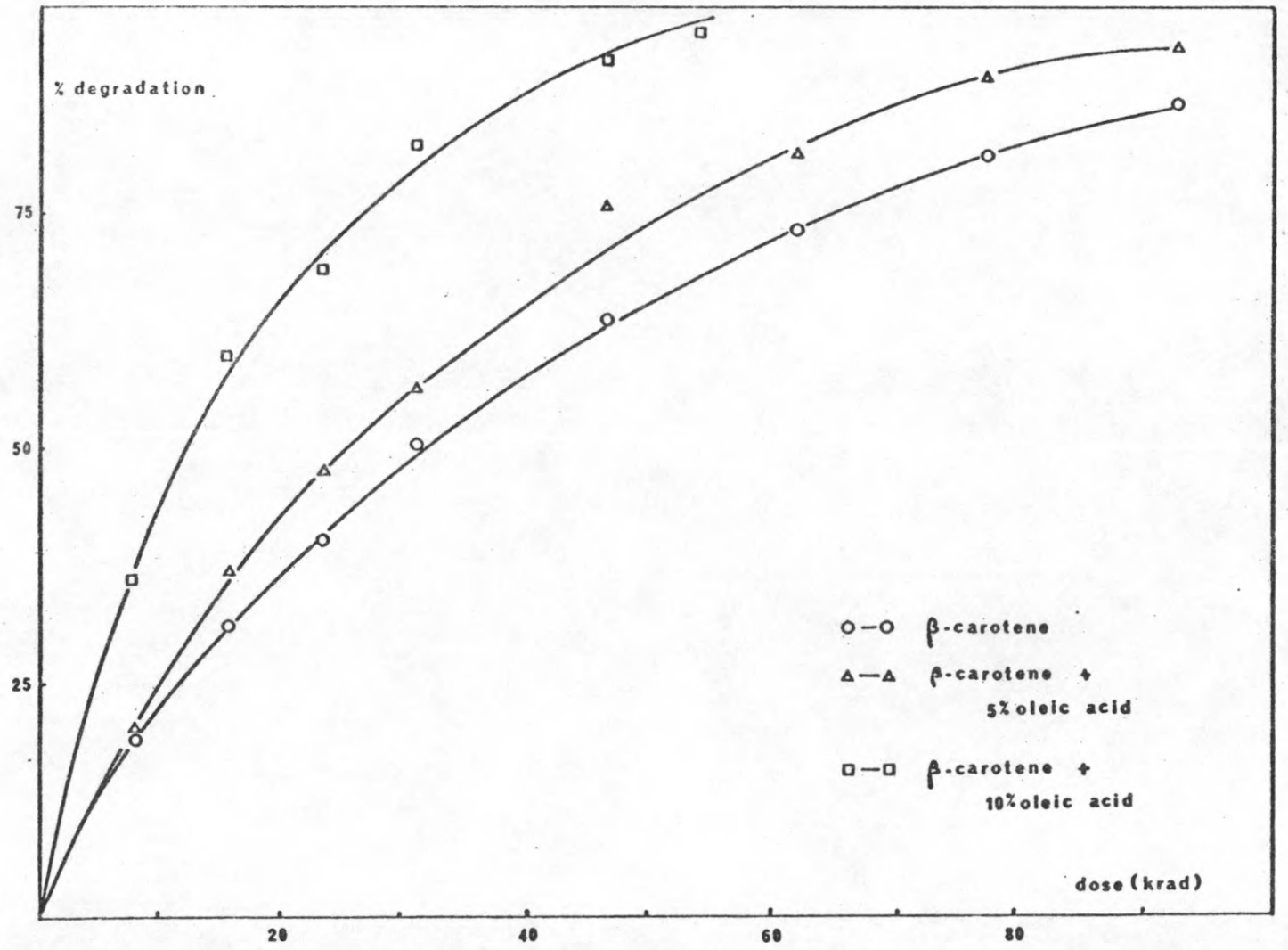


Fig. IV 12 Influence of the oleic acid concentration on the radiation degradation of a 5μM solution of β-carotene in cyclohexane.

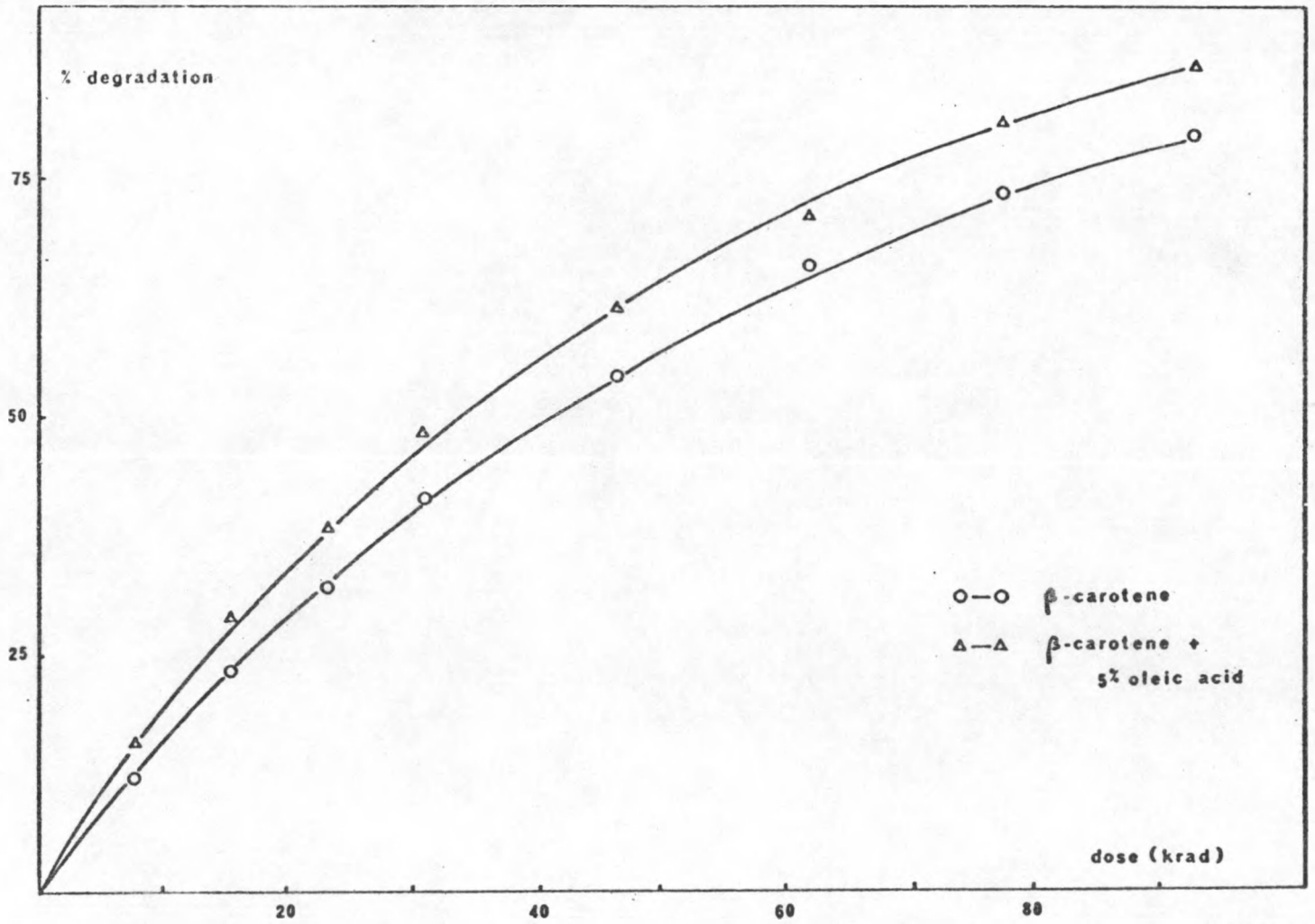


Fig. IV 13 Influence of the oleic acid concentration on the radiation degradation of a 50μM solution of β-carotene in cyclohexane.

Table IV.11 Influence of the initial pigment concentration on the percent degradation upon irradiation of β-carotene in solution in cyclohexane containing 5% oleic acid (157.52 mM oleic acid).

Dose krad	5 μM β-carotene	50 μM β-carotene
7.75	19.97	15.76
15.5	37.56	29.11
23.25	47.41	38.39
31	56.74	48.39
46.5	76.20	61.28
62	81.19	71.18
77.5	89.12	81.27
93	92.15	85.76

Oleic acid, being a H° acceptor as well as a H° donor upon irradiation as will be amply discussed by Beke (1974), should be responsible here for the enhanced radiation sensitivity of β-carotene.

By introducing a H° donor in the medium, radical reactions between β-carotene and H° will be responsible for the enhanced radiation degradation of β-carotene by mechanisms previously outlined in the text.

C.4.b. Addition of stearic acid to a solution of β-carotene in cyclohexane upon irradiation.

Exposure of oleic acid to γ-rays under anaerobic conditions mainly results in polymerization, cis-trans isomerization, decarboxylation and hydrogenation (Howton and Wu 1967) while stearic acid under identical conditions mainly yields decarboxylation and polymerisation.

In both cases hydrogen is an important constituent of the mixture of gaseous products.

The possibility of oleic acid to take up H° (to undergo hydrogenation) is considered to be one of the reasons why β-carotene was less degraded in the presence of oleic acid than in the presence of stearic acid.

Experiments with stearic acid were only carried out at one concentration of 1.7922 g/l (63.01 mM) because higher stearic acid quantities remained insoluble in cyclohexane.

Tabs. IV_{12} and IV_{13} compare the radiation sensitivity of β-carotene in cyclohexane in the presence of a saturated and an unsaturated fatty acid of equal chain length.

Table IV.12 Percent degradation of β-carotene (5 μM) in cyclohexane in the presence of oleic acid and stearic acid, as a function of the dose.

Dose krad	5 μM β-carotene	5 μM β-carotene + 63.01 mM stearic acid.	5 μM β-carotene + 157.52 mM oleic acid.
7.75	18.65	23.41	19.97
15.5	31.14	36.67	37.56
23.25	40.5	47.87	47.41
31	50.05	55.79	56.74
46.5	63.84	67.89	76.20
62	73.32	76.84	81.19
77.5	80.09	84.36	89.12
93	86.06	89.79	92.15

Table IV.13 Percent degradation of β-carotene (50 μM) in cyclohexane, in the presence of oleic acid and stearic acid, as a function of the dose.

Dose krad	β-carotene	β-carotene 63.01 mM stearic acid.	β-carotene 157.52 mM oleic acid.
7.75	12.44	17.55	15.76
15.5	23.69	30.81	29.11
23.25	32.10	41.10	38.39
31	41.32	48.78	48.74
46.5	54.11	62.27	61.29
62	65.79	74.73	71.18
77.5	73.52	81.80	81.27
93	79.91	87.03	85.76

Radical H° addition on a double bond in oleic acid probably will compete with the radical reaction : H° + β-carotene and in this way a lower percent degradation of β-carotene was found in the system oleic acid in cyclohexane upon irradiation.

C.4.c. Irradiation of β-carotene in cyclohexane paritally saturated with hydrogen.

By deducing probable mechanisms of the radiolysis of β-carotene in organic solvents it was believed that reactions between H° and β-carotene or β-carotene radicals importantly contributed to the radiation degradation of β-carotene.

To study the possibility of this mechanism experiments were carried out with a solution of β-carotene in cyclohexane, partially saturated with hydrogen.

Before the irradiation a constant stream of hydrogen (60 bubles/min) was passed through the β-carotene solution in the irradiation vial for 10 minutes.

Tab. IV_{14} shows the effect of the presence of H_2 on the percent degradation of β-carotene upon irradiation in cyclohexane.

Hereby it is demonstrated that the presence of hydrogen increased the degradation of β-carotene in cyclohexane upon irradiation.

Table IV.14 Effect of the presence of H_2 on the percent degradation of β-carotene (5 μM) in cyclohexane.

Dose krad	β-carotene	β-carotene + H_2 gas
7.75	18.65	19.43
15.5	31.14	33.18
23.25	40.15	46.54
31	50.05	57.35
46.5	63.84	70.33
62	73.32	81.73
77.5	80.09	83.08
93	86.06	94.32

C.4.d. Comparison of the total absorption spectra and TLC analysis of irradiated solutions of β-carotene in the presence of fatty acids.

As far as the total absorption spectra are concerned we can state that the addition of fatty acids did not influence the spectral patterns of irradiated β-carotene solutions.

TLC analysis did show that the same degradation products were formed upon the irradiation of β-carotene in cyclohexane whether fatty acids are present or not.

However the separation of the different fractions was very difficult because of the presence of the fatty acids, masking the different fractions.

C.5. Irradiation of β-carotene and canthaxanthin adsorbed on albumin.

The presence of watersoluble carotenoproteins in food products was simulated by modelsystems in which β-carotene and canthaxanthin was adsorbed to albumin.

It appeared that in the aqueous solutions of β-carotene-albumin and canthaxanthin-albumin, the pigments form a complex with the protein. This was evidenced by the fact that addition of methanol, acetone and $(NH_4)_2SO_4$ was necessary to achieve complete re-extraction in petroleum ether. Analysis of the spectral characteristics of the pigments revealed that those were not altered after the adsorption on albumin. On the other hand the spectral patterns of the adsorbed pigments were obviously different from those in the common organic solvents (Figs. IV_{14} and IV_{15}). It was found that canthaxanthin adsorbed more readily than β-carotene. After the irradiation of the

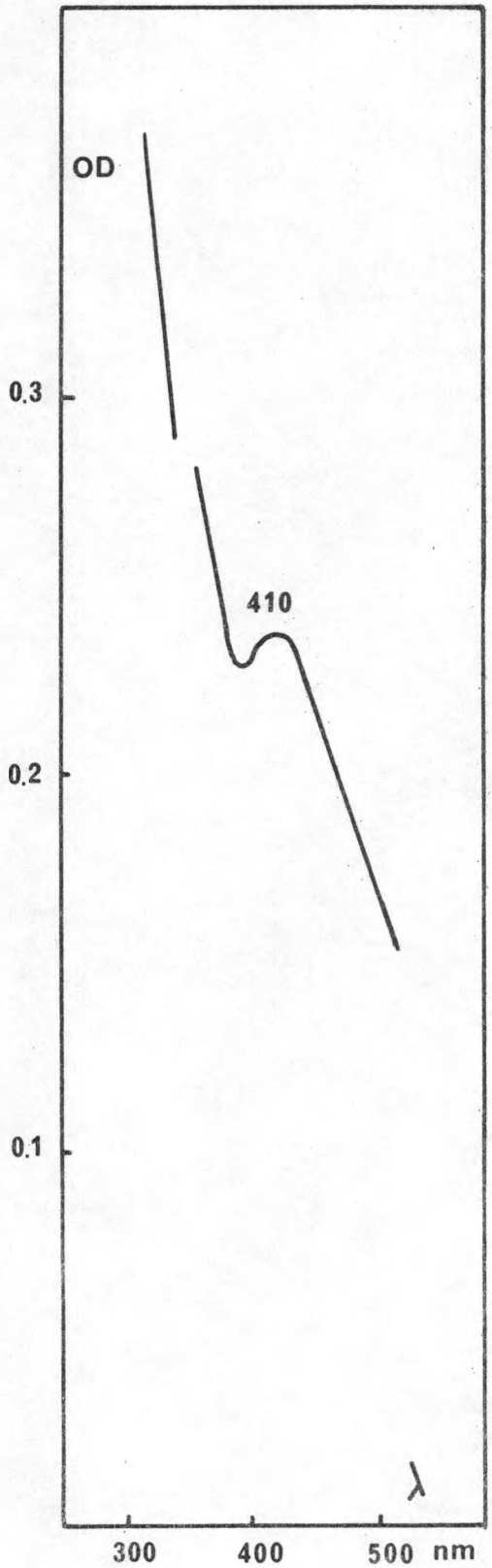


Fig. IV_{14} Absorption spectrum of β-carotene adsorbed to albumin.

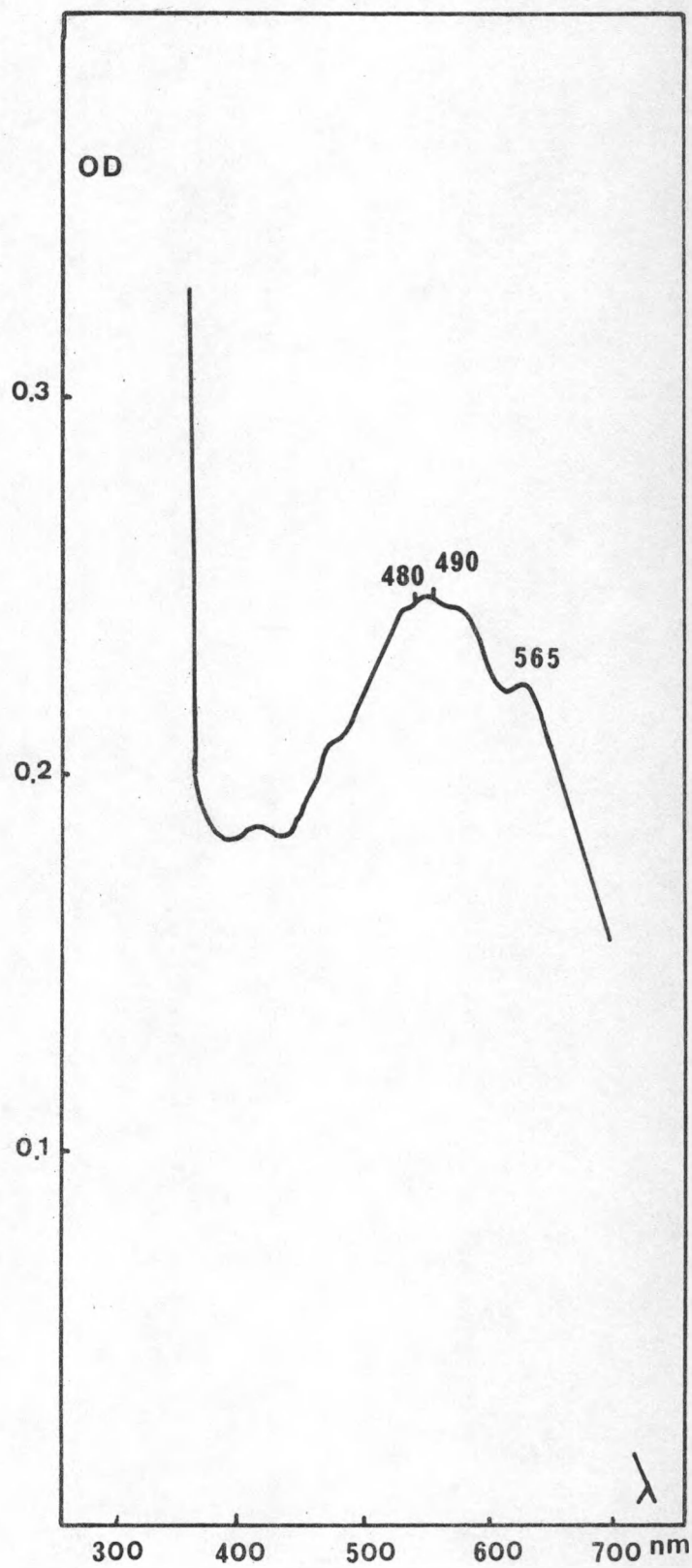


Fig. IV_{15} Absorption spectrum of canthaxanthin adsorbed to albumin.

water soluble carotenoprotein complexes, the carotenoid content was recorded.

C.5.a. Radiation lability of β-carotene and canthaxanthin.

In Tab. IV_{15} the percent degradation of β-carotene as a function of the dose for two different concentrations is listed.

After the irradiation with a dose of 500 krad the production of H_2S was noticed.
Increasing viscosity of the carotenoprotein solutions was observed as a function of the dose.
After the irradiation with a dose of 1500 krad a solid gel was obtained.
The extent of degradation upon irradiation of canthaxanthin adsorbed to albumin is shown in Tab. IV_{16}. In this protein complex canthaxanthin seemed to be more stable than β-carotene. Here too H_2S formation was characteristic after the irradiation with a dose of 500 krad. An increase in viscosity was also observed although to a lesser extent than with β-carotene.

The spectral recordings of the pigments after the extraction from the irradiated aqueous solutions indicated that β-carotene and canthaxanthin had not undergone any qualitative change after the irradiation in contrast with the considerable distortions in the spectra after the irradiation in apolar solvents.
No degradation products were detected upon the thin layer chromatography of the pigments extracted from the protein complexes after the irradiation.

Table IV.15 Degradation of β-carotene adsorbed on albumin as a function of the dose for an initial concentration of 8,7 μM (a) and 4,8 μM (b).

Dose krad	% degradation (a)	(b)
100	2.12	3.23
200	2.45	8.21
300	4.36	9.38
400	8.36	22.29
500	21.44	22.46
600	26.50	27.75
700	30.62	30.31
800	38.43	45.18
900	45.93	52.48
1000	51.59	55.57

Table IV.16 Degradation of a 9,2 μM solution of canthaxanthin adsorbed to albumin as a function of the dose.

Dose krad	% degradation
20	1.42
400	1.45
600	4.64
800	5.37
1000	7.39
1500	9.45

C.5.b. Discussion.

From an analysis of the aqueous solutions of carotenoproteins it is evident that β-carotene and cantha-xanthin in these systems are quite resistent toward radiation damage in contrast to the behaviour of these pigments in a polar solvent.

In the carotenoid-protein complex it is reasonable to expect a protection of the pigments from radiation damage by thiol containing amino acids (sulphur - containing amino acids being the principal points of damage upon irradiation of proteins).

As to us the reactions accounting for the increased carotenoid stability upon irradiation of carotenoproteins can be summarized as follows :

$$RSH \longrightarrow RS^{\circ} + H^{\circ}$$

$$2RS^{\circ} \longrightarrow RSSR$$

Sulphydryl containing substances having a labile hydrogen atom can also give a hydrogen atom to radiation induced radicals converting them into a stable molecule, i.e. (abstraction of hydrogen occurs both by direct and by indirect action).

$$RH \longrightarrow R^{\circ} + H^{\circ}$$

$$R^{\circ} + XSH \longrightarrow RH + XS^{\circ}$$

$$XS^{\circ} + XS^{\circ} \longrightarrow XSSX$$

The polymerization reactions are responsible for the increased viscosity, noticed in the experiments.

Formation of H_2S results from the following reactions :

$$RSH \xrightarrow{\sim} R^\circ + SH^\circ$$

$$SH^\circ + H^\circ \longrightarrow H_2S$$

The nature of the carotenoprotein bond seems to qualify for the radiation-protective agent. β-carotene and canthaxanthin appear to form a complex with albumin as is evident from the need for protein denaturating agents to get complete recovery.

The spectra of the aqueous carotenoprotein solutions indicate a hypsochromic shift in the spectrum of the β-carotene-albumin complex and a bathochromic shift for the canthaxanthin-albumin complex.

Specific adsorption of the carotenoids to the proteins can account for the specific structure of the spectrum of the carotenoproteins.

Considering the electronic indices of β-carotene the electronic deficient C_7 can easily interact with the nucleophylic groups of the proteins. The hypsochromic shift and the loss of fine structure in the spectrum of the β-carotene-albumin complex might typify a complex with a considerable distortion in the conjugated system.

Due to the presence of two ketonic functions in canthaxanthin more places for interaction with albumin are present than in the case of β-carotene.

The bathochromic shift of the spectrum of the canthaxanthin-protein complex can be explained by the overlapping of some orbitals of the amino acids and of canthaxanthin resulting in a smaller excitation energy.

Besides formation of a complex, the orientation of β-carotene and canthaxanthin (which are essentially lipid soluble molecules) may account for an increased stability.

It is possible that in the β-carotene-albumin complexes and canthaxanthin-albumin complexes the sensitive loci of the carotenoid molecule may be kept away from the proximity of free radicals produced in the aqueous phase.

A better molecular protection mechanism in the canthaxanthin-albumin system might explain the higher radiation stability of this complex in comparison to the β-carotene-albumin complex.

The most reactive centers for radical attack being protected in the carotenoprotein complex also explains the absence of degradation products.

C H A P T E R V

CAROTENOID STABILITY DURING RADURIZATION OF THE BROWN SHRIMP - (*CRANGON VULGARIS FABR.*).

In the introduction the importance of extending the shelf-life of the brown shrimp by gamma irradiation has been discussed.

Vyncke and Declerck (1972) evaluated the usefulness of several commonly used objective quality indices in determining the quality of irradiated shrimps.

Their tests and organoleptic judgment showed the shelf-life of brown shrimps to be extended by at least 12 days by gamma irradiation. The optimal dose appeared to be 100 krad. With doses of 300 krad and higher, unacceptable off-flavours were noticed. After irradiation the shrimps were kept at 0°C.

Especially TVN (total volatile bases) and ammonia appeared to be fairly reliable indices of spoilage for irradiated shrimps.

Parallelling these investigations the influence of gamma irradiation on the carotenoid stability in boiled shrimps was studied.

This chapter deals with the stability of carotenoids in gamma irradiated (Co^{60}) boiled and unboiled unpeeled shrimps. The carotenoid stability was also studied upon irradiation of salmon species.

Finally a crude extract of shrimp carotenoproteins in phosphate buffer was irradiated.

A. Materials.

The boiled shrimp samples in this study were identical to those described in chapter II. These shrimps were kept at 0°C during transport. Irradiation was carried

out about 40 hours after catch.

Samples of Salmo salar and Orchorynchus nerka were kindly supplied by Dr. W. Vyncke of the Fisheries Research Station at Ostend.

B. Methods.

B.1. Irradiation procedure.

The shrimps were packed in paper bags of 1 kg each which were then wrapped in polyvinylchloride stretch film to prevent dessication. Radurization was carried out in a research irradiator consisting of a watertight container lowered between a Co^{60} source stored in the hydraulic channel of the BR_2 reactor at the I.R.E. (Mol).

Five kg of shrimps were irradiated with doses varying from 50 krad to 300 krad and the measured over-dose ratio was 1.3

The importance of accurately determining this ratio and of keeping it at a minimum level was stressed by Leone (1970) and Schiettecatte (1969). The source activity was 100,000 Ci ; the dose-rate 498 krad/hr.

During irradiation, the temperature of the shrimps increased by a few degrees only.

B.2. Analysis of the pigments in the irradiated shrimp.

The continuous column-extraction method with acetone, as described in the experimental section of chapter II, has been used whenever quantitative analysis

was needed to be done.

The total pigment content of the irradiated and unirradiated samples was determined spectrophotometrically and expressed arbitrarily as μg β-carotene per gram dry weight as is also outlined in chapter II.

Qualitative pigment analysis of the irradiated and non-irradiated samples was carried out by TLC as described in chapter II.

C. Results.

C.1. Effects of irradiation on the pigments of boiled shrimps.

In Tab. V_1 the effect of gamma irradiation on the pigment content of different shrimp samples was compared. Post-operative effects were measured after a storage of 20 days at 4°C.

A perusal of the data in this table shows that immediately after the irradiation there is no difference in the pigment content between the irradiated and the non-irradiated samples. At sight no noticeable changes in colour were observed. These results show that the carotenoids in shrimps are remarkably stable toward gamma rays.

Contrary to our expectations an increase of extractable pigments was found in the non-irradiated samples after a storage of 20 days at 4°C. This could be ascribed to changes in internal structure of the shrimp that would facilitate the extraction. Losses of relatively great magnitude were found in the irradiated samples after

Table V.1 Direct- and post-operative effect of gamma-irradiation on the carotenoid content of shrimp (Crangon vulgaris Fabr.).

Exp. N°	Date of analysis	Dose in krad 0		50		100		200		300	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1.	14.4.1970	23,8	100	-	-	23,6	98,9	23,2	97,8	22,9	96,1
	after 20 days of storage	24,6	100	-	-	21,9	89	21,4	87	20,4	82
2.	20.5.1970	21,4	100	-	-	21,7	100	21,2	99,5	21,1	98,4
	after 20 days of storage	21,3	100	-	-	18,5	86,6	16,8	78,8	15,8	74
3.	11.6.1970	23,3	100	-	-	23,2	99,7	23,2	99,6	23,0	99
	after 20 days of storage	26,0	100	-	-	20,9	80,5	21,0	80,8	19,3	74,5
4.	22.9.1970	18,5	100	18,6	100,2	19,2	103,1	18,4	99,2	-	-
	after 20 days of storage	19,4	100	16,6	85,7	15,6	75,8	13,3	68,6	-	-
5.	13.11.1970	16,2	100	15,4	95,2	15,5	95,6	15,6	96,1	-	-
	after 20 days of storage	16,7	100	12,7	76	11,2	66,9	10,9	65,1	-	-
6.	19.1.1971	16,7	100	15,5	92,6	16,4	98,2	16,0	96,1	-	-
	after 20 days	17,0	100	14,2	83,0	13,5	79,7	11,9	69,1	-	-

The figures given in this table are expressed as µg β-carotene per g dry weight or as percent of the pigment content in the non-irradiated samples.
Storage time : 20 days at a temperature of 4° C.

a 20 days' storage at 4°C. These post-operative losses were mainly pronounced in autumn - (the season during which the shrimp contains less carotenoids).

However despite these changes in the pigment content no noticeable visual discoloration was observed. This judgment on colour was performed by a panel of four members scoring the colour of irradiated shrimp as excellent.

The pigment extracts of all samples were analyzed by TLC. So far and contrary to the results with prawns (Kumta et al. 1966) and salmon species (chapter V.c.3.) none of the different pigments in shrimps were destroyed preferentially. On TLC the same fractions were obtained for the irradiated samples compared with the untreated ones.

No degradation products were found upon TLC. After a 20 days' storage the absorption spectrum of the total extract as well as the spectra of the different carotenoids after the chromatographic separation remained unchanged.

Those results clearly show that changes in colour will certainly not be the limiting factor when radurizing shrimps.

C.2. Irradiation of unboiled shrimps.

In an analogous way unboiled shrimps were irradiated with doses of 100 krad up to 350 krad. Quantitative determination of the carotenoids after the irradiation showed there was no loss of pigment content. At sight no discolorations were observed. TLC analysis revealed no degradation products.

Storage experiments after the irradiation were not carried out because pigment degradation and - alterations were performed enzymatically (i.e. hydrolysis of the ester pigments).

In this way post-operative effects of radurization could not be measured.

C.3. Effect of irradiation on the shrimp carotenoproteins in 0.1 M phosphate buffer.

The crude extract of the carotenoproteins of shrimps in 0.1. M phosphate buffer (pH 7) was irradiated in an identical manner as the β-carotene-albumin and the canthaxanthin-albumin complexes.

From the results in Tab. V_2 it is apparent that the carotenoproteins, irradiated as described above, are very stable toward gamma rays. In this way the experiments with model systems and carotenoproteins isolated from shrimps compare very well.

C.4. Effect of irradiation on Salmon carotenoids.

In the case of Salmon species and the Penaeidae shrimps, carotenoid degradation may limit acceptability of the product after the irradiation. Kumta et al. (1970) stated that in prawns the naturally occurring astaxanthin pigment is readily destroyed at radiation doses above 250 krad.

By way of comparison ice-cooled Salmo salar and Orchorynchus nerka samples were also irradiated at a dose of 300 krad.

Table V.2 Degradation of the carotenoids upon gamma irradiation of the watersoluble carotenoprotein complexes.

Dose in krad	% degradation
200	1.45
400	4.73
600	7.55
800	10.32
1000	16.21
1500	26.63

The concentration of the carotenoprotein solution expressed as β-carotene was 4 μM.

The results are a mean value of 4 experiments.

Those irradiated samples exhibited a pronounced discoloration. Spectrophotometric analysis did not reveal the typical symmetrical absorption curve of astaxanthin any more.

Based on the changes in optical densities almost 90% of the pigment content was lost after the irradiation.

It is shown that as to the brown shrimp only, radurization will have no influence on the colour as quality parameter. Otherwise it is peculiar that astaxanthin present in the three groups of sea foods is destroyed in a different way.

D. Discussion.

Since the shrimp and salmon are complex systems and since upon irradiation a variety of factors operate simultaneously, it is very difficult to define per se the influence exerted by any single factor.

The variation in the pattern of degradation of carotenoids could be due to the differences in nature of the carotenoids, - in their concentration, - in their compositional and physico-chemical variations within biological systems etc., and to the influence either sensitizing or protective, exerted by other food components in the medium.

Considering the results obtained from the irradiation of shrimps and salmon it was thought that model systems were useful in elucidating some factors affecting the radiation sensitivity of carotenoid pigments.

From our modelsystems with β-carotene-albumin complexes and canthaxanthin-protein complexes it was

deduced that the effective number of vulnerable sites in the carotenoid molecule accessible to free radical attack may differ greatly resulting in a varied response toward radiation injury.

In the case of shrimps analogue protective mechanisms by proteins might be postulated to explain the radiation stability of carotenoids upon irradiation.

The difference in radiation sensitivity of shrimp- and salmon pigments should be explained as follows :

Astaxanthin and its ester derivatives in salmon species are nor bound to proteins. Moreover in contrast to shrimps (containing only 2% fat) salmon species contain up to 18% fat.

Probably the orientation of the astaxanthin molecules should favour the direct action of the γ-rays and the reactions between astaxanthin and the free radicals produced in the medium.

The absence of degradation products upon storage of irradiated boiled shrimps should be due to a variety of uncontrolable mechanisms.

GENERAL CONCLUSION.

In the introduction it was discussed that there exists a need for extending the market life of various fishery products to increase the nutritional status of the population in various countries.

World wide reports have constantly indicated that fishery products can be treated with low doses of radiation to extend their market life.

presently, in the field of food irradiation there is little standardization as to the measurement and expression of either the radiation dose or the product quality.

In this study some more fundamental work was carried out on the colour, as quality factor, of the shrimp.

In chapter II.A. an extensive analysis of the carotenoids in boiled shrimps was carried out. It was shown that a series of carotenoids are responsible for the colour of the brown shrimp : astaxanthin, astaxanthin-monoester, a minor unsaponifiable xanthophyll, astaxanthin-diester, a mixture of zeaxanthin-, isozeaxanthin- and lutein esters, and β-carotene.

Hereby the fatty acids, esterified with the carotenoids, were also qualitatively determinated.

- In chapter II.B. some of the molecular properties of the different carotenoproteins in the unboiled shrimp were studied.

An estimation of the molecular weight of the carotenoproteins was performed.

From the carotenoid analysis of the different shrimp carotenoprotein fractions it was concluded that all carotenoids are bound to proteins in the unboiled shrimp. The problem whether the shrimp carotenoproteins are true carotenoproteins or lipoproteins with lipid associated carotenoids still exists.

- Because of the complexity of food products it was impossible to detail the mechanisms involved in the degradation of carotenoids in the shrimp medium.

A progressive development of irradiating β-carotene in model systems was performed in order to understand the different factors that determine the radiation degradation mechanisms.

Irradiation experiments of β-carotene in organic solvents revealed that the carotenoid concentration, the nature of the solvent and the radiation dose are three important factors determining the radiation sensitivity of β-carotene.

From the degradation mechanisms, amply discussed in the text, we can summarize that :

by direct and/or indirect interaction of the gamma rays a progressive shortening of the conjugated polyene chain is found, as well as the formation of α-isomers and cis-trans isomers.

By addition of radical donors, as outlined for oleic- and stearic acid, the radiation sensitivity of β-carotene is enhanced.

To underline the eventuality of the reaction mechanisms between H° radicals and β-carotene molecules or β-carotene radicals, β-carotene was irradiated in cyclohexane partially saturated with hydrogen.

- The presence of watersoluble carotenoproteins in food products was simulated by modelsystems in which β-carotene and canthaxanthin was adsorbed to albumin.

A protection of the pigments from radiation damage by thiol containing amino acids was discussed.

The importance was stressed that in carotenoproteins the sensitive loci of the carotenoid molecule are kept away from the proximity of free radicals produced in the surrounding medium upon irradiation.

- Finally as a matter of application the radiation sensitivity of carotenoids in the boiled shrimps and in salmon species was studied.

It was found peculiar that astaxanthin, present in shrimp and salmon, was destroyed in a different way.

In elucidating the factors that affect the different radiation sensitivity of respectively shrimp- and salmon carotenoids, the results obtained from irradiation of carotenoids in modelsystems were very useful.

The difference in radiation sensitivity of shrimp and salmon pigments should be explained as follows :

Astaxanthin and its esters in salmon species are not bound to proteins. In this lipid-rich medium (18% lipids) the orientation of the astaxanthin molecules should favour the direct action of the γ-rays and the reactions

between astaxanthin and the free radicals produced in the medium.

In unboiled as well as in boiled shrimps the vulnerable sites of the carotenoids accessible to free radical attack are more or less protected by the associated or surrounding proteins.

It was clearly shown that changes in colour will certainly not be the limiting factor when radurizing shrimps.

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