SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of β-apo-8'-carotenal (E 160e) as a food additive[1]

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Additives and Nutrient Sources added to Food provides a scientific opinion re-evaluating the safety of β-apo-8'-carotenal (E 160e) as a food additive in the EU. β-Apo-8'-carotenal was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1974 and the EU Scientific Committee for Food (SCF) in 1975 and 2000. Both committees established an Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day, which was withdrawn by the SCF in 2000. The Panel concluded that the available in vitro and in vivo genotoxicity studies do not give reason for concern with respect to genotoxicity. Upon a public call for data two subchronic toxicity studies in rats performed according to OECD guidelines and under GLP became available for evaluation. Based on the 13-week study the Panel established that based on increased incidence of eosinophilic droplets in the kidneys the LOAEL was 10 mg β-apo-8'-carotenal active ingredient/kg bw/day. Upon a public call for data two additional studies on reproductive and developmental toxicity became available revealing a NOAEL of 500 mg/kg bw/day, the highest dose level tested. Overall, the Panel concluded that the present database on β-apo-8'-carotenal provides a basis to revise the ADI. The Panel concluded that based on the LOAEL of 10 mg/kg bw/day from the 13 week study in rats and an uncertainty factor of 200, an ADI for β-apo-8'-carotenal of 0.05 mg/kg bw/day can be established. Exposure estimates at Tier 3 indicate that the newly set ADI is reached for adults on average and exceeded by adults at the 95th percentile and by children on average and at the 95th/97.5th percentile.

KEY WORDS

β-apo-8'-carotenal, E 160e, CAS Registry Number 1107-26-2, food colour.

1 On request from the European Commission, Question No EFSA-Q-2011-00352, adopted on 07 December 2011.

2 Panel members: F. Aguilar, R. Crebelli, B. Dusemund, P. Galtier, J. Gilbert, D.M. Gott, U. Gundert-Remy, J. König, C. Lambré, J-C. Leblanc, A. Mortensen, P. Mosesso, D. Parent-Massin, I.M.C.M. Rietjens, I. Stankovic, P. Tobback, D. H. Waalkens-Berendsen, R.A. Woutersen, M. C. Wright.

3 Acknowledgement: The Panel wishes to thank the members of the ANS Working Group B on Food Additives and Nutrient Sources: M. Bakker, D. Boskou, B. Dusemund, D. Gott, T. Hallas-Møller, J. König, D. Marzin, D. Parent Massin, I.M.C.M. Rietjens, G.J.A. Speijers, P. Tobback, T. Verguieva, R.A. Woutersen for the preparatory work on this scientific opinion.

Suggested citation: EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Scientific Opinion on the re-evaluation of β-apo-8'-carotenal (E 160e) as a food additive. EFSA Journal 2012;10(3):2499. [46 pp.] doi:10.2903/j.efsa.2012.2499. Available online: www.efsa.europa.eu/efsajournal.htm

SUMMARY

Following a request from the European Commission to the European Food Safety Authority, the Panel on Food Additives and Nutrient Sources added to Food was asked to deliver a scientific opinion re-evaluating the safety of β-apo-8'-carotenal (E 160e) when used as food colour.

β-Apo-8'-carotenal (E 160e) is allowed as food additive in the EU and was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1974 and the EU Scientific Committee for Food (SCF) in 1975 and 2000. The SCF and JECFA both established an Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day, which was withdrawn by the SCF in 2000.

JECFA defined the ADI for the sum of the carotenoids β-apo-8'-carotenal, β-carotene, β-carotenoic acid methyl ester and β-carotenoic acid ethyl ester. However, in 2000 the SCF withdrew the entire group ADI based on a recent evaluation of β-carotene. The SCF decided that there was insufficient scientific basis, either from human or experimental studies, on which to set a new ADI for β-carotene and related carotenoids, but was nonetheless of the opinion that currently permitted food additive uses of β-carotene and related carotenoids are temporarily acceptable from a health point of view at the estimated levels of intake.

At present only β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester are specifically defined in Commission Directive 2008/128/EC and JECFA. According to specifications, β-apo-8'-carotenal has to comprise at least 96% of the final product in commercial β-apo-8'-carotenal products. The sum of all subsidiary colouring matters is less than 3% and these subsidiary colouring matters are related carotenoids, mainly β-carotene. The Panel noted that the specifications should be updated to more clearly define the purity of the material.

The Panel concluded that the available data indicate that after oral administration absorption of β-apo-8'-carotenal and/or its metabolites is at least 15%. The absorbed β-apo-8'-carotenal is metabolically converted to β-apo-8'-carotenoic acid as well as to its ethyl and methyl esters in rats.

The Panel noted that the SCF has questioned the suitability of rodents as a test species for evaluating the bioavailability and effects of β-carotene in human, since rodents were considered to convert β-carotene to vitamin A much more efficiently than humans. Most laboratory animals were reported to degrade β-carotene in their intestines and absorb almost no β-carotene intact, due to high dioxygenase activity converting β-carotene to retinal, which, according to SCF, is in contrast to humans where β-carotene is mainly (20-75%) absorbed intact. Therefore rodent studies were considered to lack relevance for human risk assessment, and the Panel considered that such limitations may also apply to β-apo-8'-carotenal evaluated in the present opinion. Therefore, the Panel carefully evaluated the ADME characteristics for β-apo-8'-carotenal in both rodent and human focusing on possible differences.

An overview of the major results from an ADME study with β-apo-8'-carotenal in rats compared to those from a human study was made. According to the metabolite pattern in plasma the uptake and metabolism of β-apo-8'-carotenal seems qualitatively similar in rats and humans. Comparison of the plasma kinetic data from the rat with the data from the human study indicates also quantitative similarities between the two species.

Based on the metabolite pattern in blood plasma and based on the plasma kinetics the Panel concluded that for β-apo-8'-carotenal rats are a suitable model for humans concerning uptake and systemic exposure to β-apo-8'-carotenal and metabolites.

A direct comparison of the formation of vitamin A from β-apo-8'-carotenal in rats and humans is more difficult, since the experimental setup of the two studies was not identical. Results obtained reveal that in rats as well as in humans vitamin A is formed from β-apo-8'-carotenal. From the existing data a quantitative comparison can not be made.

No acute oral toxicity of β-apo-8'-carotenal was observed at relatively high doses.

Studies on genotoxicity provided upon a public call for data revealed generally negative results in *Salmonella typhimurium/Escherichia coli* reverse mutation assays, an in vitro genotoxicity assay using Chinese hamster ovary (CHO) cells and an in vivo rat bone marrow micronucleus test.

A few more recent studies have addressed the genotoxicity of β-carotene cleavage products. In a study with primary rat hepatocytes, statistically significant increases of micronuclei and chromosomal aberrations were observed when cells were treated with a mixture of β-carotene cleavage products or apo-8'-carotenal at concentrations from 0.01 to 10 μM. In the same experimental conditions, a dose related increase of sister chromatid exchanges, which attained statistical significance at top dose, was also observed. β-Carotene, tested in the same dose range, induced neither significant cytotoxic nor genotoxic effects. Concerning the significance of these experimental findings, the Panel noted that there is limited experience with cytogenetic assays in primary rat hepatocytes, which show a very high spontaneous incidence of both micronuclei and chromosomal aberrations. Moreover the increases in the frequency of micronuclei and chromosomal aberrations observed in presence of β-carotene cleavage products, and micronuclei in the presence of apo-8'-carotenal, were not dose-related over a 10 000-fold concentration range. The statistically significant increases in the frequency of micronuclei are only 20 and 11% (at 0.1 and 1 μM, respectively) over control incidence, which is within the range of experimental variation for the end-points studied, and thus has limited or no biological significance. The frequencies of chromosomal aberrations in the presence of apo-8'-carotenal show an apparent treatment-related increase, but data are unreliable since they are based on only 20 metaphases/culture. The increase in sister chromatid exchanges observed in presence of both β-carotene cleavage products and apo-8'-carotenal is more credible, but the biological significance of this indicative assay, in relation to genotoxicity is indirect.

In another study the genotoxic effects of the β-carotene breakdown product β-apo-8-carotenal in Human Retinal Pigment Epithelial Cells (ARPE-19) was investigated using the Comet assay. The authors concluded that their results suggest that β-apo-8-carotenal, when applied at partially toxic doses, is genotoxic inducing DNA single strand breaks, prevented by high levels of GSH. The authors however also stated that the mechanism of genotoxicity of β-apo-8-carotenal, i.e. whether direct via DNA damage and apoptosis, or indirect through plasma membrane damage and necrosis, could not be disclosed. Thus no firm conclusion on the genotoxic potential of the β-apo-8-carotenal can be drawn from this study.

The Panel concluded that the genotoxicity studies of the β-carotene breakdown products in primary rat hepatocytes and in ARPE-19 cells in the studies quoted above, provide very limited evidence of genotoxicity.

The induction of DNA strand breaks by β-apo-8'-carotenal, but not by β-carotene, was reported in another in vitro Comet assay on A549 cells.

Overall the Panel concluded that the available in vitro genotoxicity studies and the in vivo micronucleus study with β-apo-8'-carotenal do not give reason for concern with respect to genotoxicity.

Upon a public call for data two subchronic toxicity studies in rats performed according to OECD guidelines and under GLP became available for evaluation, including a 4 weeks toxicity study in rats and a 13 weeks study in rats both using β-apo-8'-carotenal 10% WS/N. Five groups of 10 male and 10 female Sprague-Dawley rats were dosed continuously by diet for 13 consecutive weeks at levels of 0, 0 (placebo), 10, 30 and 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. There were no signs of neurotoxicity, body weight or food consumption effects that could be attributed to administration of β-apo-8'-carotenal 10% WS/N. Statistically significant increases in white blood cells and some of the associated parameters were seen in males receiving 30 and 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. Minor increases were seen in AST and ALT levels in females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. These differences were not evident after the recovery phase. Histopathologically, administration of β-apo-8'-carotenal 10% WS/N in the diet was associated with eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above, multinucleate hepatocytes in the liver of females at 30 mg β-apo-8'-carotenal

active ingredient/kg bw/day and above, and with increased numbers of inflammatory cell foci in the liver of females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day.

The finding of eosinophilic droplets at 10 and 30 mg β-apo-8'-carotenal active ingredient/kg bw/day was minimal in degree in all affected animals and, in the absence of other changes in the kidney, was considered by the authors not to be an adverse effect.

Overall, the authors concluded in first instance that the No Observed Adverse Effect Level (NOAEL) was considered to be 10 mg β-apo-8'-carotenal active ingredient/kg bw/day.

An amendment to this first description of the study was also submitted. In this report it was stated that it was realized that the originally defined NOAEL in the 13-week rat study for β-apo-8'-carotenal (Edwards et al., 2007) may be open to question. Specifically the histopathological change of multinucleated hepatocytes (MNHs) was reported as an adverse change whereas there are literature references to this change being adaptive and not adverse (Williams and Iatropoulos, 2002). Accordingly, a limited peer review was organized. Based on the peer review, expert opinion and also taking into account published information on the long half life of multinucleated hepatocytes (MNHs), the study pathologist and the peer review pathologist came to the mutually agreed-upon conclusion that the change of MNHs as observed in this study is not adverse. Accordingly the overall interpretation of the NOAEL in this study has been reviewed.

The amendment indicates that histopathologically, administration of β-apo-8'-carotenal 10% WS/N in the diet was associated with eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above, for which there was no NOAEL. The finding of eosinophilic droplets at 10 and 30 mg β-apo-8'-carotenal active ingredient/kg bw/day was minimal in degree in all affected animals and, in the absence of other changes in the kidney, was considered in the amendment not to be an adverse effect.

Administration of β-apo-8'-carotenal 10% WS/N was also associated with increased numbers of inflammatory cell foci in the liver of females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day, for which the NOAEL was 30 mg β-apo-8'-carotenal active ingredient/kg bw/day. Increased incidence of multinucleate hepatocytes in the liver was seen in females at 30 mg β-apo-8'-carotenal active ingredient/kg bw/day and above, for which the NOAEL was 10 mg β-apo-8'-carotenal active ingredient/kg bw/day.

The amendment report concluded that overall, for males the NOAEL was 100 mg β-apo-8'-carotenal active ingredient/kg bw/day and for females it was 30 mg β-apo-8'-carotenal active ingredient/kg bw/day (based on increased hepatic inflammatory cell foci).

The Panel closely evaluated the data on the eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above. The Panel concluded that the data were not suitable for a BMD analysis, but that a LOAEL of 10 mg/kg bw/day could be identified for these histopathological changes in the kidneys.

Two studies on reproductive and developmental toxicity have been conducted well before the introduction of (OECD) GLP guidelines, both in rats (Anonymous, 1966). Although on the basis of these two studies, there appears to be no direct reason for concern, it should be considered that the available studies were conducted well before the introduction of (OECD) GLP guidelines, and that in addition, the studies have been described in very little detail in an evaluation conducted over three decades ago.

Upon a public call for data two additional studies on reproductive and developmental toxicity became available for evaluation; a range-finding study and a subsequent study investigating the effects of β-apo-8'-carotenal 10% WS/N on embryonic and fetal development of the rat when administered by the oral route (dietary admixture). These studies were performed according to OECD guidelines and under GLP. The NOAEL for maternal toxicity was considered by the authors to be the dietary concentration of 2064 mg/kg diet (20 mg β-apo-8'-carotenal/kg bw/day). The Panel noted that the reduction in mean body weight gain was observed during the first 9 days of treatment (days 6 to 15 of gestation) in the 100 and 500 mg/kg bw/day groups compared with the vehicle control group and that this effect

attained statistical significance only between days 6 and 11 of gestation. As a consequence, net mean body weight gain during the treatment period (days 6 to 20 of gestation) was slightly but not statistically significantly lower in these groups. The Panel therefore considered the slight reduction in mean body weight and food intake not as adverse and concluded that the NOAEL of this study was 500 mg/kg bw/day.

Long term carcinogenicity studies on β-apo-8'-carotenal were made available to the Panel upon a public call for data and were performed well before the introduction of (OECD) GLP guidelines. A summary of these chronic toxicity studies with β-apo-8'-carotenal was also provided. β-Apo-8'-carotenal was administered at 0.1% in the diet to the first generation of Wistar rats and their offspring (second generation) for a period of 2 years and to a third generation for a period of 1 year. The average intake of β-apo-8'-carotenal was about 40 mg/kg bw/day. The spontaneous tumours were distributed evenly over both groups (11/147 treated rats and 10/122 untreated rats) and consequently the authors concluded that their occurrence was not connected with the application of β-apo-8'-carotenal. Based on these results the Panel considered that there were no adverse effects in this chronic toxicity study at the single dose level tested amounting to about 40 mg/kg bw/day.

Overall, the Panel concluded that the present database on β-apo-8'-carotenal provides a basis to revise the ADI of 5 mg/kg bw/day.

The Panel concluded that based on the LOAEL of 10 mg/kg bw/day from the 13 week study in rats showing an increased incidence of eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and an uncertainty factor of 200, an ADI for β-apo-8'-carotenal of 0.05 mg/kg bw/day can be established. The Panel considered an uncertainty factor of 200 to derive the ADI from the LOAEL sufficient, given the fact that the increase in the eosinophilic droplets in the kidneys at the LOAEL was minimal.

The Panel also considered whether this carotenoid could be included in a group ADI with β-carotene.

However, since the re-evaluation of β-carotene concluded that an ADI for β-carotene could not be established the Panel concluded that a group ADI including β-apo-8'-carotenal and β-carotene can not be established.

Exposure estimates based on Tier 2 using maximum permitted levels would result in exposures to β-apo-8´-carotenal of 0.9 mg/kg bw/day for adults on average and of 3.3 mg/kg bw/day at the 95th percentile. For children, Tier 2 estimates would result in exposures to the colour in the range of 0.5-3.4 mg/kg bw/day on average and in the range of 1.2-7.2 mg/kg bw/day at the 95th/97.5th percentile.

It was indicated by food industry however that the colour is relatively rarely used due to its comparability to β-carotene. Based on this information and the maximum reported use levels provided, Tier 3 estimates lead to exposure to β-apo-8´-carotenal of adults of 0.05 mg/kg bw/day on average and of 0.19 mg/kg bw/day at the 97.5th percentile with non-alcoholic flavoured drinks being the main contributor (92%). Exposure estimates at Tier 3 for children were calculated across European countries in the range of 0.02-0.22 mg/kg bw/day on average and in the range of 0.09-0.71 at the 95th/97.5th percentile. Main contributors for children´s exposure to β-apo-8´-carotenal were non-alcoholic flavoured drinks (50-91%) and fine bakery wares (11-50%).

The Panel concluded that exposure estimates at Tier 3 using the maximum reported use levels for the few food categories where food industry reported the use of β-apo-8´-carotenal are at the level of the ADI of 0.05 mg/kg bw for adults on average and exceed this ADI for adults at the 95th percentile and for children at both average and 95th/97.5th percentiles for all European countries.

The Panel noted that the specifications should be updated to more clearly define the purity of the material.

The Panel noted that specifications should be extended to include maximum residue limits for residual solvents.

The Panel noted that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$ 10 mg/kg.

TABLE OF CONTENTS

Abstract 1
Summary 2
Background as provided by the European Commission....7
Terms of reference as provided by the European Commission....7
Assessment....8
1. Introduction 8
2. Technical data....8
2.1. Chemistry....8
2.2. Specifications....8
2.3. Manufacturing process....9
2.4. Methods of analysis in food....10
2.5. Reaction and fate in food....10
2.6. Case of need and proposed uses....10
2.7. Information on existing authorisations and evaluations 11
2.8. Exposure 12
2.8.1. Crude estimates (Budget Method) for β-apo-8'-carotenal....12
2.8.2. Refined estimates for β-apo-8'-carotenal 13
3. Biological and toxicological data 14
3.1. Absorption, distribution, metabolism and excretion....15
3.2. Toxicological data....18
3.2.1.Acute oral toxicity 18
3.2.2.Short-term and subchronic toxicity 18
3.2.3.Genotoxicity 23
3.2.4.Chronic toxicity and carcinogenicity....27
3.2.5.Reproductive and developmental toxicity 29
3.2.6.Allergenicity, hypersensitivity and intolerance 31
4. Discussion....31
Conclusions 36
Documentation provided to EFSA 37
References 40
Annex A 43
Glossary / Abbreviations....45

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 1333/2008[4] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union. In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under Regulation (EU) No 257/2010[5]. This Regulation also foresees that food additives are re-evaluated whenever necessary in light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[6] of 2001. The report "Food additives in Europe 2000[7]" submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with the highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of the adoption of Regulation (EU) 257/2010 the 2003 Terms of Reference are replaced by those below.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedure and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

[4] OJ L 354, 31.12.2008, p. 16.
[5] OJ L 80, 26.03.2010, p19
[6] COM(2001) 542 final.
[7] Food Additives in Europe 2000, Status of safety assessments of food additives presently permitted in the EU, Nordic Council of Ministers. TemaNord 2002:560.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of β-apo-8'-carotenal (E 160e) when used as a food colour.

β-Apo-8'-carotenal (E 160e) is authorised as a food additive in the EU and has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1974 and the EU Scientific Committee for Food (SCF) in 1975 and 2000.

The Panel on Food Additives and Nutrient Sources added to Food (ANS) was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following a public call for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

2. Technical data

2.1. Chemistry

β-Apo-8'-carotenal (E 160e) is a carotenoid food colour with the chemical name (2E, 4E, 6E, 8E, 10E, 12E, 14E, 16E)-2,4,6,8,10,12,14,16-heptadecaoctaenal, 2,6,11,15-tetramethyl-17-(2,6,6-trimethyl-1-cyclohexen-1-yl)-. The CAS Registry Number is 1107-26-2 the EINECS number is 214-171-6, and its colour index number is 40820. The molecular formula is C30H40O, its molecular weight is 416.65 g/mol and its structural formula is presented in Figure 1.

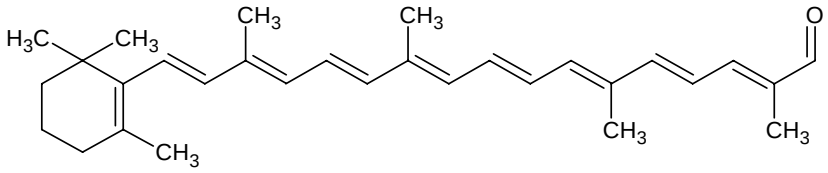


Figure 1: Structural formula of β-apo-8'-carotenal

β-Apo-8'-carotenal consists of dark violet crystals with metallic lustre or is a crystalline powder. The Panel noted that the compound is essentially insoluble in water (US EPA, Syracuse Research Corporation), and that the compound is slightly soluble in ethanol and soluble in lipids. Synonyms include: CI Food Orange 6, C Orange 16.

2.2. Specifications

β-Apo-8'-carotenal is specifically defined in Commission Directive 2008/128/EC[8] and by JECFA (JECFA, 2011). In the JECFA and in the EU specifications, it is indicated that the specifications apply to predominantly the all-trans (E) isomer of β-apo-8'-carotenal together with minor amounts of other carotenoids. Diluted and stabilized forms are prepared from ß-apo-8'-carotenal meeting these specifications, and include solutions or suspensions of β-apo-8'-carotenal in edible fats or oils, emulsions and water dispersible powders; these preparations may have different cis/trans isomer ratios (JECFA, 2011; Directive 2008/128/EC).

[8] Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. OJ L 6, 10.1.2009, p. 20-63

The β-apo-8'-carotenal content in the additive should be not less than 96% of the total colouring matters.

Table 1 presents the specifications for β-apo-8'-carotenal according to Commission Directive 2008/128/EC and JECFA (JECFA, 2011).

Table 1: Specifications for β-apo-8'-carotenal according to Commission Directive 2008/128/EC and JECFA (JECFA, 2011)

	Commission Directive 2008/128/EC	**JECFA (2011)**
Assay	Not less than 96% of total colouring matters $E_{1\ cm}^{1\%}$ 2 640 at ca 460-462 nm in cyclohexane	Not less than 96% of total colouring matters
Identification	Maximum in cyclohexane at 460-462 nm	Determine the absorbance of the sample solution (See Method of Assay) at 461 nm and 488 nm. The ratio A_{488}/A_{461} is between 0.80 and 0.84
Purity		
Subsidiary colouring matters	≤ 3.0%f total colouring matters may be carotenoids other than β-apo-8'-carotenal	≤ 3% of total colouring matters may be carotenoids other than β-apo-8'-carotenal
Sulphated ash	≤ 0.1%	≤ 0.1%
Arsenic	≤ 3 mg/kg	-
Lead	≤ 10 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb)	≤ 40 mg/kg	-

According to the specifications and the purity criteria, β-apo-8'-carotenal should comprise at least 96% of total colouring matters in commercial products. As, however, the amount of total colouring matters is not quantified, the amount of β-apo-8'-carotenal as well as the major part of the commercial product, remain undefined in the present specifications. Upon request EFSA was informed that the pure, crystalline β-apo-8'-carotenal final product complies with this criterion of "not less than 96%". According to EU Directive 2008/128/EC this is determined by absorption (E1cm, 1% 2640 at ca 460 – 462 nm) measured in cyclohexane. The sum of all subsidiary colouring matters is less than 3% and these subsidiary colouring matters are related carotenoids, mainly β-carotene. The Panel noted that the specifications should be updated to more clearly define the purity of the material.

The Panel noted that the JECFA specification for lead is ≤ 2 mg/kg whereas the EC specification is ≤ 10 mg/kg.

The Panel noted that specifications should be extended to include maximum residue limits for residual solvents.

2.3. Manufacturing process

β-Apo-8'-carotenal is produced by chemically synthesis. The detailed production process description for β-apo-8'-carotenal was made available to EFSA. The solvent employed in the production process are methanol, iso-propanol, acetone and water and do not give reason for concern provided that specifications are extended to include residue limits for these solvents. The pure, crystalline β-apo-8'-carotenal final product complies with the criterion of "not less than 96%".

2.4. Methods of analysis in food

Several methods for the determination of β-apo-8'-carotenal in foods are described in public literature, of which variations of High Performance Liquid Chromatography (HPLC) appear to be most generally employed (Oliver and Palou 2000). In addition, according to JECFA (JECFA, 2011) β-apo-8'-carotenal can be analyzed by means of spectrophotometry. The analytical methods described for the parent colour are not necessarily suitable for the determination of impurities in the stabilized forms.

EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued an opinion on the safety of use of colouring agents, including β-apo-8'-carotenal, in animal nutrition (EFSA, 2009). In this opinion it is indicated that the CRL (European Union Reference Laboratory for GM Food and Feed formerly known as Community Reference Laboratory) reports that no ISO and CEN methods could be found as the official analytical method for the determination of β-apo-8'-carotenal in feeding stuffs or other relevant matrices, and that HPLC is currently the method of choice for carotenoid analysis since it gives the most accurate, sensitive and reproducible quantitative analyses of carotenoid content and composition. β-Apo-8'carotenal was analyzed by using thin layer chromatography (TLC) and HPLC with UV detection from red pepper (Minguez-Mosquera et al., 1995). The opinion also indicated that β-apo-8'-carotenal was analysed from retail foods and beverages by using HPLC and photodiode array (Scotter et al., 2003). The limit of detection (LOD) and the limit of quantification (LOQ) of β-apo-8'-carotenal in their method were 0.01 and 0.1 mg/kg, respectively. The response was linear over the range of 0–50 mg/l. β-Apo-8'-carotenal was well separated from the *trans* and *cis* isomers of β-carotene.

2.5. Reaction and fate in food

No data were available on the reaction and fate in food of β-apo-8'-carotenal. However, in general the majority of colour additives are unstable in combination with oxidising and reducing agents in food. Since colour depends on the existence of a conjugated unsaturated system within the dye molecule, any substance which modifies this system (e.g. oxidising or reducing agents, sugars, acids, and salts) will affect the colour (Scotter and Castle, 2004).

2.6. Case of need and proposed uses

Authorised use levels have been defined in the EU legislation (Directive 94/36/EC)9.

Currently, β-apo-8'-carotenal is an authorised food colour in the EU with maximal permitted levels of 50 to 500 mg/kg food for various foodstuffs (individually or in combination with other food colours). β-Apo-8'-carotenal is also allowed in beverages at levels up to 200 mg/l (individually or in combination with other food colours). Table 2 summarizes those beverages and foodstuffs that are permitted to contain β-apo-8'-carotenal up to specified maximum permitted levels (MPLs) set by EC legislation (Council Directive 94/36/EC).

[9] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. OJ L 237, 10.09.1994, p.13-29.

Table 2: Maximum permitted levels (MPLs) of β-apo-8'-carotenal in beverages and foodstuffs according to Council Directive 94/36/EC and maximum reported use levels

Beverages	**Maximum Permitted Level** (mg/l)	**Maximum reported use levels for β-apo-8´-carotenal** (mg/l)
Non-alcoholic flavoured drinks	100	11.8
Spirituous beverages	200	0
Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails		0
Fruit wines, cider and perry		0
Foodstuffs	**Maximum Permitted Level** (mg/kg)	**Maximum reported use levels (mg/kg)**
Complete formulae for weight control intended to replace total daily food intake or an individual meal	50	0
Complete formulae and nutritional supplements for use under medical supervision		0
Soups		0
Flavoured processed cheese	100	0
Fish paste and crustaceans paste		0
Smoked fish		0
Savoury snack products and savoury coated nuts		0
Liquid food supplements/dietary integrators		0
Meat and fish analogues based on vegetable proteins		0
Edible ices	150	0
Desserts including flavoured milk products		0
Fine bakery wares	200	7.5
Candied fruit and vegetables, Mostarda di frutta		0
Preserves of red fruits		0
Extruded or expanded savoury snack products		0
Pre-cooked crustaceans	250	0
Confectionery	300	8.26
Mustard		0
Fish roe		0
Solid food supplements/dietary integrators		0
Decorations and coatings	500	0
Sauces, seasonings, pickles, relishes, chutney and piccalilli		0
Salmon substitutes		0
Surimi		0
Edible cheese rind and edible casings	*Quantum satis*	0

2.7. Information on existing authorisations and evaluations

Apo-8'-carotenal is authorised as a food additive in the EU under Directive 94/36/EC.

Based on the JECFA evaluation of 1975, a group Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day was estimated for the sum of the carotenoids β-carotene, β-apo-8'-carotenal, and β-apo-8'-carotenoic acid methyl and ethyl ester. Results from a four-generation rat study with β-carotene (Bagdon et al., 1960) were used for calculation of the group ADI. In this study, no adverse effects were seen at 50 mg β-carotene/kg bw/day, and an uncertainty factor of 10 instead of 100 was used because of the natural occurrence of carotenoids in the human diet and the low toxicity of carotenoids in animal studies.

In 1975, the SCF endorsed the ADI established by JECFA of 0-5 mg/kg bw/day as the sum of the carotenoids β-carotene, β-apo-8'-carotenal and β-apo-8'-carotenoic acid methyl and ethyl ester. The SCF in an accompanying comment (1975) mentions that only the ethyl ester is listed in the Community Directive and that this was the only compound considered. Consequently, the ADI was expressed as the sum of β-carotene, β-apo-8'-carotenal and the ethyl ester of β-apo-8'-carotenoic acid alone.

When however β-carotene was re-evaluated (SCF, 2000), the SCF decided to withdraw the group ADI of 5 mg/kg bw/day for β-apo-8´-carotenal, β-carotene and the ethyl ester of β-apo-8'-carotenoic acid. The ADI was withdrawn for two main reasons. First, the ADI was based on rodent studies, and since rodents were considered to convert β-carotene to vitamin A much more efficiently than humans, these studies were considered to lack relevance for human risk assessment. The second reason was the adverse findings in human smokers receiving β-carotene supplements at 20 mg/person/day or more, amounts that are much lower than the previously established ADI. The SCF considered the scientific basis to be insufficient to set a new ADI.

As, however, there were no indications that daily intakes of about 1-2 mg β-carotene and/or related carotenoids, as food additives, are harmful in the context of the overall dietary intake of these substances, and as, in addition, the scientific basis was considered to be insufficient to set a new ADI, the Committee decided that currently permitted food additive uses of β-carotene and related carotenoids would be temporarily acceptable (SCF, 2000).

An additional evaluation can be found in a report released by the Nordic Council of Ministers (TemaNord, 2002) who has taken into account the literature published until 2000.

Data on specifications and permitted levels have been defined in the EU legislation (in particular Directives 2008/128/EC and 94/36/EC) and by JECFA (JECFA, 2011).

EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued an opinion on the safety of use of colouring agents, including β-apo-8'-carotenal, in animal nutrition (EFSA, 2009). It was stated that data on the safety of β-apo-8'-carotenal for the target animals are not available, but that given the natural occurrence of the compound and considering the molecular structure of the carotenoid, the FEEDAP Panel does not see reasons for concern. It was also concluded that several in vitro studies, performed with β-apo-8'-carotenal in both prokaryotic and eukaryotic test systems, do not give rise to safety concerns with respect to the genotoxicity of the compound. The FEEDAP Panel also concluded that there are no safety concerns for the consumer from the consumption of eggs from hens fed β-apo-8'-carotenal supplemented diets and that although data to estimate human exposure from poultry tissues are not available, safety concerns are not likely.

2.8. Exposure

The Panel followed the principles of the stepwise approach, which were used in the report of the scientific cooperation (SCOOP) Task 4.2 (EC, 1997), to estimate intakes of food additives. For each successive Tier, this involved a further refinement of intake estimates. The approach progresses from the conservative estimates that form the first Tier of screening, to more realistic estimates that form the second and third Tiers. In the tiered approach, Tier 1 is based on theoretical food consumption data and maximum permitted levels (MPLs) for additives as permitted by relevant Community legislation. The second and third Tiers refer to assessment at the level of individual Member States, combining national data on food consumption with the maximum permitted levels for the food additive (Tier 2) and with its actual usage patterns (Tier 3).

2.8.1. Crude estimates (Budget Method) for β-apo-8'-carotenal

The dietary exposure to β-apo-8'-carotenal from the maximum permitted levels was estimated using the Budget method (Tier 1), which is based on the fact that there is a physiological upper limit to the amount of food and drink (for beverages 100 ml/kg bw and for solids 25 g/kg bw), and thus of food additives, that can be consumed each day. A further assumption is that only a certain proportion of the

diet is likely to contain food additives (25%). Full details on the budget method are described in the report of the SCOOP Task 4.2 (EC, 1997).

In the case of β-apo-8'-carotenal, the maximum permitted level in beverages was 200 mg/l (Directive 94/36/EC). The maximum permitted level in solid foods was 500 mg/kg.

The default proportion (25%) of beverages and solid food that could contain the additive was considered adequate. In fact, even though β-apo-8'-carotenal may be used in a variety of solid foods and beverages that could represent more than 25% of processed foods, it is unlikely that a person would systematically choose all processed foods with the same colour added even considering brand loyalty. This assumes that a typical adult weighing 60 kg consumes daily 1.5 liters of beverage and 375 grams of solid foods containing β-apo-8'-carotenal .

The overall theoretical maximum daily exposure to β-apo-8'-carotenal for adults would therefore be:

(200 x 0.1 x 0.25) + (500 x 0.025 x 0.25) = 5 + 3.12 = 8.1 mg/kg bw/day.

For children, the level of β-apo-8'8'-carotenal considered in beverages was 100 mg/l (after exclusion of alcoholic drinks) and in solid food was 500 mg/kg. As recommended by SCOOP task 4.2 (EC, 1997) for children, it is assumed that 100% of beverages contain the additive. This conclusion was derived from UK data on consumption of soft drinks by children aged under 5 years, where the 97.5th percentile of consumption was between 70 and 80 ml/kg bw/day. This assumes that a typical 3 year-old child weighing 15 kg consumes daily 1.5 litres of beverages and 94 g of solid foods containing β-apo-8'-carotenal.

The overall theoretical maximum daily exposure to β-apo-8'-carotenal in children would therefore be:

(100 x 0.1 x 1) + (500 x 0.025 x 0.25) = 10 + 3.12 = 13.1 mg/kg bw/day.

It was noted that β-apo-8'-carotenal may be used *quantum satis* in edible cheese rind and edible casings. As this is a very specific food category, which is unlikely to be consumed in high amounts on a daily basis, if at all, it was excluded from the Budget method calculation, since it is not expected to influence the outcome of this exposure calculation to any relevant extent.

2.8.2. Refined estimates for β-apo-8'-carotenal

Refined exposure estimates have been performed for Tier 2 using MPLs presented in Table 2, and for Tier 3 using the maximum reported use levels presented in Table 2, both combined with national consumption data for children and the adult population.

The Panel noted that its estimates could be considered as being conservative as it is assumed that all processed foods and beverages contain β-apo-8'-carotenal added at the maximum reported use levels.

For adults, the Panel calculated the exposure based on the UK consumption survey, as the UK population is considered to be one of the highest consumers of soft drinks in Europe, and also because detailed individual food consumption data (UK NDNS, 2000-2001) were available from the UNESDA report (Tennant et al., 2006) and the Natural Food Colours Association (NATCOL) report (Tennant 2007).

Exposure estimates for children (1-10 years old) have been performed by the Panel based on detailed individual food consumption data from eleven European countries (Belgium, France, the Netherlands, Spain, Italy, Finland, Greece, Cyprus, Czech Republic, Sweden and Germany) provided by the EXPOCHI ("Individual food consumption data and exposure assessment studies for children") consortium (Huybrechts et al., 2010). As the UK is not part of the EXPOCHI consortium, estimates for UK children (aged 1.5 - 4.5 years) were made by the Panel with the use of detailed individual food consumption data (UK NDNS, 1992-1993) available from the UNESDA report (Tennant et al., 2006) and the NATCOL report (Tennant, 2007). Table 3 summarises the anticipated exposure of children and adults to β-apo-8'-carotenal.

Tier 2

In the case of β-apo-8'-carotenal, when considering MPLs (Tier 2), estimates reported for the UK adult population give a mean dietary exposure to β-apo-8'-carotenal ranging from 0.9 to 3.3 mg/kg bw/day for high level (97.5th percentile) consumers. The main contributors to the total anticipated mean exposure to β-apo-8'-carotenal (>10%) were non-alcoholic flavoured drinks (47%).

The mean dietary exposure of European children (aged 1-10 years and weighing 16-29 kg) considered on the basis of the EXPOCHI data and UK children ranged from 0.5 to 3.4 mg/kg bw/day, and from 1.2 to 7.2 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to β-apo-8'-carotenal (>10% in all countries), were non-alcoholic flavoured drinks (14-55%), fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (13-46%), desserts, including flavoured milk products (12-50%) and sauces, seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney and piccalilli (11- 44%).

Tier 3

Further data suggest that reported use levels of β-apo-8'-carotenal in some food categories are lower than the MPLs. Therefore, it was decided that concentration data made available to the Panel by the food industry (Tennant 2008, updated in February 2011) would be used to refine the estimate of dietary exposure to β-apo-8'-carotenal (Tier 3).

When considering the maximum reported use levels from Table 2, estimates reported for the UK adult population give a dietary exposure to β-apo-8'-carotenal ranging from 0.05 mg/kg bw/day on average to 0.19 mg/kg bw/day for high level (97.5th percentile) consumers. The main contributors to the total anticipated mean exposure to β-apo-8'-carotenal (>10%) were non-alcoholic flavoured drinks (92%).

The mean dietary exposure of European children (aged 1-10 years and weighing 16-29 kg), considered on the basis of the EXPOCHI data and UK children, ranged from 0.02 to 0.22 mg/kg bw/day, and from 0.09 to 0.71 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to β-apo-8'-carotenal (>10% in all countries), were non-alcoholic flavoured drinks (50-91%), and fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (11-50%).

Table 3: Summary of anticipated exposure to β-apo-8'-carotenal. using the tiered approach (EC, 2001) in children and the adult population

	Adult UK population (>18 years old) mg/kg bw/day	**Children UK & EXPOCHI population** (1-10 years old, 15.8-29 kg body weight) mg/kg bw/day
Tier 1. Budget method	8.1	13.1
Tier 2. Maximum Permitted Level		
• Mean exposure	0.9	0.5 – 3.4
• Exposure 95th*or 97.5th percentile*	3.3	1.2 – 7.2
Tier 3. Maximum reported use levels		
• Mean exposure	0.05	0.02 – 0.22
• Exposure 95th*or 97.5th percentile*	0.19	0.09 – 0.71

* For the UK population, estimates are based on the UNESDA report which gives the 97.5th percentile (Tennant, 2006).

3. Biological and toxicological data

β-Apo-8'-carotenal (E 160e) has been previously evaluated by JECFA in 1974, the SCF in 1975 and 2000, and TemaNord in 2002. The present opinion briefly reports the major studies evaluated in these reports and describes additional newly reported literature data in some more detail.

The studies used for the JECFA and SCF evaluations were performed in the 1960s when Good Laboratory Practice (GLP) guidelines were not yet implemented. OECD GLP guidelines were not promulgated before 1981. It is unclear whether these reported studies comply with OECD and GLP guidelines.

In the new ADME and toxicity studies provided the β-apo-8'-carotenal tested was typically a standard commercial product form of beadlets, such as β-apo-8'-carotenal 10% WS/N with WS denoting water soluble. β-Apo-8'-carotenal 10% WS/N consists of free flowing particles (beadlets), containing β-apo-8'-carotenal dispersed in a cornstarch-coated matrix of gelatin, sucrose and corn oil.

In the ADME study radio-labeled material needed to be used, and thus no commercial formulation could be tested. However, the investigators of the ADME study in report no 2500426 used a preparation denoted as "simulated beadlets". The investigators of the study intended to express with the term "simulated beadlet" that they endeavoured as best as possible to formulate and test a material in the ADME study that was as similar as possible to the commercial product form, being the WS/N form of β-apo-8'-carotenal, a beadlet formulation.

3.1. Absorption, distribution, metabolism and excretion

JECFA in its evaluation in 1975 describes several studies on the toxicokinetic aspects of β-apo-8'-carotenal and the ethyl and methyl esters of β-apo-8'-carotenoic acid.

After dietary administration of β-apo-8'-carotenal to rats, some β-apo-8'-carotenal accumulates in the liver together with vitamin A (= retinol) and β-apo-8'-carotenoic acid (no further details provided) (Thommen, 1962; Brubacher et al., 1960).

When large doses (100 and 500 mg/kg bw /day) of a mixture containing β-apo-8'-carotenal and β-apo-8'-carotenoic acid methyl ester are given to rats, only a minor fraction is absorbed and most (76-104%) is excreted in the faeces. β-Apo-8'-carotenoic acid as well as its ethyl and methyl esters are normal metabolites of β-apo-8'-carotenal (Wiss and Thommen, 1963).

After dietary exposure of vitamin A-deficient rats to β-apo-8'-carotenal, only 4% is converted to vitamin A in the gut (compared to 10% after exposure to β-carotene). Carotenals are easily oxidized to carotenoic acids and less readily reduced to alcohols in vivo. This finding points to metabolic pathways other than β-oxidation (Wiss and Thommen, 1963; Glover, 1960).

In dogs, β-apo-8'-carotenal is poorly absorbed from the gastrointestinal tract and excreted in the urine together with β-apo-8'-carotenoic acid. No hypervitaminosis A was noted (Bagdon et al., 1960).

Dogs (2-4/group/sex) received 0, 100 or 1000 mg β-apo-8'-carotenal/animal/day for 14 weeks (no detail on route of administration). The level of β-apo-8'-carotenal in serum was elevated in the group receiving 1000 mg and there was an occasional trace in the group receiving 100 mg. The level of vitamin A in the kidney was increased three to five-fold compared to controls. Values of β-apo-8'-carotenal levels in other tissue were variable. Microscopic examination revealed pigmentation of the adipose tissue, kidney and adrenal cortex (Bagdon et al., 1962). The Panel noted that although the authors concluded that the content of vitamin A in the kidneys of β-apo-8'-carotenal treated dogs was 3 to 5-fold higher than in the kidneys of the control animals, close evaluation of the study results does not seem to support this conclusion. The control animal kidneys had a mean vitamin A concentration of 104 IU/g, with a range from 45 – 165 IU/g. The kidneys from the animals dosed with 100 mg β-apo-8'-carotenal/day had a mean concentration of 359 (204 – 580) IU/g and the dogs treated with 1000 mg β-apo-8'-carotenal/day a mean concentration of 227 IU/g with a range of 46 – 440 IU vitamin A/g. From this it appears that there was no dose dependency in the kidney vitamin A concentration, and the mean concentration from the high dose treatment appears to be even lower than that from the low dose treatment. The variations in the animals within a dose group are high and two from six animals from the high dose groups have kidney concentrations similar to the control animals.

β-Apo-8'-carotenal has been associated with hypercholerolemic activity (Wood, 1963).

The ester of β-apo-8'-carotenoic acid (not further specified as ethyl or methyl) was eliminated from the blood of human infants rapidly and proportionally to the blood concentration (Kübler, 1963).

TemaNord (2002) mentions an additional study which was allegedly derived from the JECFA evaluation. In this study in humans, a single oral dose of β-apo-8'-carotenal was extensively metabolised, mainly to the corresponding acid, alcohol, and palmitate ester (no specific reference given).

The following new study on absorption, distribution, metabolism and excretion has been provided upon a public call for data.

Five male rats received a single oral dose of [6,7-^{14}C]-β-apo-8'-carotenal by gavage formulated as "simulated beadlets" (for an explanation of this material see above at the introduction for section 3) at a dose of 1.3 mg/kg bw (Rümbeli et al., 2007). One animal was sacrificed 3 hours post dose and terminal blood was collected. From the other four animals multiple blood samples were taken at 1, 2, 4, 6, 8, 10 hours and 24 hours post dose. Urine and faeces were collected from 0-24 hours and 24-48 hours post dose. Those animals were sacrificed 48 hours post dose and the total radioactivity in excreta, plasma, liver, kidneys, fat, intestinal tract and remaining carcass were determined. After dosage of [^{14}C]-β-apo-8'-carotenal the total radioactivity in plasma reached a maximum concentration of 342 ng β-apo-8'-carotenal equivalents/g plasma after 10 hours and the radioactivity was eliminated with a half life of 21 hours.

The major part of the radioactivity applied was excreted within 0-48 hours via faeces (49%) and urine (15%) and 10% remained in the gastrointestinal tract. The concentration of radioactive residues was highest in liver (4.4% of dose), corresponding to 1.6 µg β-apo-8'-carotenal equivalents/g. The concentration in kidney, fat and blood was 1.1, 0.1 and 0.05 µg β-apo-8'-carotenal equivalents/g, respectively. Urine was analyzed by HPLC with radioactivity detection. At least 13 radioactive polar metabolite fractions were characterized each representing $\leq$ 2% of the dose applied. Faeces were extracted and the extracts were analyzed by HPLC with radioactivity and UV/VIS detection. The major radioactive residue eliminated via faeces was identified as β-apo-8'-carotenal, representing 18% of the dose applied. Additional metabolites identified in faeces were 8% β-apo-8'-carotenoic acid and 1% β-apo-8'-carotenol.

Liver was extracted which released 83% of the liver radioactivity. The extract was fractionated and the apolar fraction (67%) was analyzed by HPLC. The major radioactive residues were identified as retinol (16%), retinyl-palmitate (16%) and two other retinyl-fatty acid conjugates (17% and 10%, respectively). No β-apo-8'-carotenal, β-apo-8'-carotenol or β-apo-8'-carotenoic acid was found in liver.

Blood plasma from one animal sacrificed 3 hours post dose was extracted and the extract was analyzed by HPLC. Besides minor amounts of β-apo-8'-carotenal, significant amounts of β-apo-8'-carotenol and β-apo-8'-carotenoic acid were characterized.

The authors concluded that, based on the metabolite patterns in urine, plasma and liver the metabolic pathway of β-apo-8'-carotenal proceeds via oxidation to β-apo-8'-carotenoic acid or reduction to β-apo-8'-carotenol, cleavage at the 15-16 position followed by reduction to retinol, conjugation with fatty acids and excretion as polar metabolites via urine.

The Panel concluded that after oral administration, absorption of β-apo-8'-carotenal and/or its metabolites is at least 15%.

The Panel also noted that the SCF (2000) has questioned the suitability of rodents as a test species for evaluating the bioavailability and effects of β-carotene in human, since rodents were considered to convert β-carotene to vitamin A much more efficiently than humans. Most laboratory animals were reported to degrade β-carotene in their intestines and absorb almost no β-carotene intact, due to high dioxygenase activity converting β-carotene to retinal, which, according to SCF, is in contrast to humans where β-carotene is mainly (20-75%) absorbed intact. Therefore rodent studies were considered to lack relevance for human risk assessment, and the Panel considered that such limitations may also apply to β-apo-8'-carotenal evaluated in the present opinion. Therefore, the Panel carefully

evaluated the ADME characteristics for β-apo-8'-carotenal in both rodent and human focusing on possible differences.

Table 4 presents an overview of the major results from an ADME study with β-apo-8'-carotenal in rats (Rümbeli et al., 2007) compared to those from a human study (Zeng et al., 1992). The rats were dosed with a single dose of 3.1 µmol/kg bw ^{14}C- β-apo-8'-carotenal and the human subjects received a single dose of 1.6 µmol/kg bw. In both species β-apo-8'-carotenal was identified only as a minor component in blood plasma or serum but major metabolites in humans and rats were β-apo-8'-carotenol and fatty acid conjugates of β-apo-8'-carotenol. Additionally, significant amounts of β-apo-8'-carotenoic acid were found in rat plasma, which was only a minor metabolite in human plasma. According to the metabolite pattern in plasma the uptake and metabolism of β-apo-8'-carotenal seems qualitatively similar in rats and humans.

Table 4. Comparison of ADME data from β-apo-8'-carotenal in rat and human.

Species	**Rat**	**Human**
Study	Rümbeli R et al., DSM Nutritional Products Ltd Report, 2007, No. 2500426	Zeng S et al., Am. J. Clin. Nutr. 1992, 56, 433-439
Number of subjects	4	6
Dose	Single oral dose 3.1 µmol/kg bw ^{14}C-β-Apo-8'-carotenal	Single oral dose 1.6 µmol/kg bw β-Apo-8'-carotenal
Major plasma metabolites	β-Apo-8'-carotenol β-Apo-8'-carotenoic acid Fatty acid conjugates	β-Apo-8'-carotenol β-Apo-8'-carotenyl palmitate
Minor plasma metabolites	β-Apo-8'-carotenal	β-Apo-8'-carotenal β-Apo-8'-carotenoic acid
Tmax [h]	6 (total radioactivity) 10 (total radioactivity)	5.5 (β-Apo-8'-carotenol) 10.9 (β-Apo-8'-carotenyl palmitate)
Cmax [µmol/kg] or [µmol/l]	0.71 (total radioactivity) 0.82 (total radioactivity)	0.23 (β-Apo-8'-carotenol) 0.29 (β-Apo-8'-carotenyl palmitate)
AUC [µmol/h x kg] or [µmol/h x l]	19.7 (total radioactivity)	15.6 (β-Apo-8'-carotenol) 4.2 (β-Apo-8'-carotenyl palmitate)

Comparison of the plasma kinetic data from the rat with the data from the human study (see Table 4) indicates also quantitative similarities between the two species. In the human study a plasma maximum (Tmax) of 5.5 hours was found for β-apo-8'-carotenol and one of 10.9 hours for β-apo-8'-carotenyl palmitate. In the rat study the total radioactivity also showed two maxima, one at 6 hours and one at 10 hours post dose (mean plasma curve from all animals). The plasma maxima (Cmax) of the radioactivity in rat plasma corresponded to 0.71 and 0.82 µmol β-apo-8'-carotenal equivalents/l plasma and the human serum maxima corresponded to 0.23 and 0.29 µmol/l based on β-apo-8'-carotenol and β-apo-8'-carotenyl palmitate, respectively. Taking into account that the total radioactivity measurements in rats include additional minor metabolites besides the major metabolites β-apo-8'-carotenol and β-apo-8'-carotenyl palmitate as quantified in the human serum, it can be concluded, that the Cmax in rat plasma is roughly twice as high as the Cmax in human serum. This correlates with the dosing which was twice as high for the rats as for the humans.

A direct comparison of the formation of vitamin A from β-apo-8'-carotenal in rats and humans is more difficult, since the experimental setup of the two studies was not identical. In the human study only blood serum was analyzed for vitamin A and it was stated, that retinyl palmitate was slightly but significantly elevated in all subjects at 12-32 hours after dosing with β-apo-8'-carotenal, indicating that formation of vitamin A in humans can only be detected in blood at a relatively late time point

after dosing of β-apo-8'-carotenal. In the rat study blood plasma was analyzed at a very early time point post dose (2 hours after dosing β-apo-8'-carotenal) – and no retinol or metabolites of retinol were identified. It might be that analysis of the rat plasma at a later time point would also show the presence of vitamin A. Analysis of the radioactive rat liver metabolites showed that retinol and retinyl fatty acid conjugates were the major metabolites. This indicates that in rats as well as in humans vitamin A is formed from β-apo-8'-carotenal. From the existing data a quantitative comparison can not be made.

Also of importance is that literature suggests that β-apo-8'-carotenal can serve as a substrate for the cleavage enzyme β-carotene-15,15`-monooxygenase, but that in vitro studies show a lower enzyme activity though for the substrate β-apo-8'-carotenal as compared to β-carotene. Bachmann et al. (2002) in their feedback regulation study of the β-carotene-15,15`-monooxygenase used the liver storage assay to assess the amount of newly formed retinol in the liver of vitamin A depleted rats as well as retinol plasma levels. Even though not a physiological approach, these data show an increase of vitamin A in the liver of 1.5 – 3.5% of the administered dose after a single dose of β-apo-8'- carotenal. Serum retinol could be restored to approx. 80% compared to β-carotene.

3.2. Toxicological data

3.2.1. Acute oral toxicity

JECFA gives the results of acute toxicity tests with both β-apo-8'-carotenal in mice. In this study the LD50 was found to be >10,000 mg/kg bw (Anonymous, 1966).

The following additional studies on oral acute toxicity have been provided upon a public call for data.

A series of acute oral toxicity studies reported LD50 values of β-apo-8'-carotenal in rats of > 20,000 mg/kg bw (Bächthold, 1972; Bächthold, 1975; Bächthold, 1976), oral LD50 values > 10,000 mg β -apo-8'-carotenal/kg bw in rats (Bächthold, 1973; Bächthold, 1977; Bächthold, 1980; BASF 1972), and oral LD50 values > 10,000 mg β-apo-8'-carotenal/kg bw in mice (Bächthold, 1977).

In another study (Loget and Arcelin, 2006a) two groups, each of three female HanRoc:WIST rats were treated with β-apo-8'-carotenal 10% WS/N by oral gavage at a dose of 2000 mg/kg bw, corresponding to 232 mg β-apo-8'-carotenal/kg bw. The LD50 was higher than 2000 mg/kg bw, corresponding to 232 mg β-apo-8'-carotenal/kg bw.

A similar study with crystalline β-apo-8'-carotenal revealed an LD50 of higher than 2000 mg β-apo-8'-carotenal/kg bw (Loget and Arcelin, 2006b).

3.2.2. Short-term and subchronic toxicity

JECFA has evaluated two subchronic studies with β-apo-8'-carotenal.

Groups of male rats (16/group) were given β-apo-8'-carotenal by gavage at doses of 0, 100 or 500 mg/kg bw/day, 5 days/week, for 34 weeks. Testicular weight was significantly lower in the high-dose group compared to controls. Microscopic examination revealed granular pigment deposition in the liver and kidneys of treated animals. Fertility, as shown by monthly mating of four females, was not affected. No adverse effects were seen on body weight gain, general health, survival, liver and kidney function, or organ weights.

In an identical experiment with β-apo-8'-carotenoic acid methyl ester the results were similar to those described above. No adverse effects on mortality or weight gain were noted, but the male rats receiving 500 mg/kg bw/day showed reduced testicular weights compared with controls and granular pigment deposits in the liver and kidney. No deleterious effect on spermatogenesis was noted (Anonymous, 1962; Anonymous, 1966). According to TemaNord, these studies were performed with β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester instead of β-apo-8'-carotenoic acid methyl ester.

Groups of dogs (2-4/group/sex) received 0, 100 or 1000 mg β-apo-8'-carotenal/animal/day for 14 weeks (no detail on route of administration). Microscopic examination revealed pigmentation of the adipose tissue, kidney and adrenal cortex. No significant compound-related effects were noted regarding general health, post mortem pathological lesions, peripheral blood picture, liver function, serum enzymes, blood urea levels or organ weights (Bagdon et al., 1962).

The SCF (2000) refers to two short-term studies with β-apo-8'-carotenal.

In mice, supplementation with 300 mg β-apo-8'-carotenal/kg diet (supposedly equal to 37.5 mg/kg bw/day) for 15 days, had no effect on any phase I or phase II xenobiotic-metabolising enzymes in the liver (Astorg et al., 1994, 1997).

In a study in rats, animals were given 300 mg β-apo-8'-carotenal/kg diet (equivalent to 15 mg/kg bw/day) for 15 days. A significant induction on liver CYP-1A1 and -1A2 levels was observed (Gradelet et al., 1996).

The following new studies on subacute and subchronic toxicity have been provided upon a public call for data.

3.2.2.1. Rats

A 13 week rat toxicity study of β-apo-8'-carotenal and degraded β-apo-8'-carotenal was performed (Bagdon, 1964). The latter consisted of 75% β-apo-8'-carotenal and 25% degradation products. Four groups of 20 rats each (10 males and 10 females except for one group (pure β-apo-8'-carotenal) that contained 11 males and 9 females) were given diets containing 0 (control), 1% degraded β-apo-8'-carotenal, 0.5% degraded β-apo-8'-carotenal or 1% pure β-apo-8'-carotenal. The animals were weighed at weekly intervals and food consumption was measured. Complete blood counts were taken during the 2nd, 4th, 9th and 13th weeks. Measurement of liver function was ascertained after 6 and 12 weeks of treatment by determination of alanine aminotransferase (ALT) (SGP-T). Urinalysis was also performed. Upon sacrifice the animals were examined for gross pathological changes. Sections of several tissues (including brain, peripheral nerve, heart, lungs, liver, spleen, pancreas, stomach, small and large intestines, thyroids, adrenals, gonads, urinary bladder, kidneys, bone marrow, prostate, seminal vesicles or uterus) were examined microscopically. The study report stated that the results obtained indicated that rats tolerated 1% of β-apo-8'-carotenal administered in the diet and 1% degraded β-apo-8'-carotenal, 0.5% degraded β-apo-8'-carotenal (consisting of 75% β-apo-8'-carotenal and 25% degradation products) without toxic manifestations. The Panel established a No Observed Adverse Effect Level (NOAEL) of 1% β-apo-8'-carotenal in the diet equivalent to about 500 mg/kg bw/day.

In another study the subacute toxicity of β-apo-8'-carotenal and β-apo-8'-carotinic acid (C30)-methylester were investigated (Schärer et al. 1961). β-Apo-8'-carotenal and β-apo-8'-carotinic acid (C30)-methylester were administered by stomach tube to young male rats (24 animals per treatment group with the control group consisting of 12 animals) in daily doses of 1 g/kg bw 5 days a week for 4 weeks. These dosages were well tolerated. Weight development was not impaired. Liver function (bromsulphalein test) proved normal after 4 weeks. No effects on the testes were observed. Pigments deposits were observed in the kidneys of treated animals. No pigment deposits were observed in the liver although this organ was somewhat heavier in treated animals than in controls. Histopathological examination was performed on liver, kidney, spleen, testis, heart, lung, small intestine, large intestine and adrenals, and revealed no adverse effects.

In a more recent study four groups of 5 male and 5 female Sprague Dawley rats were dosed continuously by diet for at least 4 consecutive weeks with β-apo-8'-carotenal 10% WS/N, at levels of 0, 20, 100 and 500 mg β-apo-8'-carotenal active ingredient/kg bw/day (Loget and Morgan 2006). β-Apo-8'-carotenal 10% WS/N consists of free flowing particles (beadlets), containing β-apo-8'-carotenal dispersed in a cornstarch-coated matrix of gelatin, sucrose and corn oil. An additional group of 5 male and 5 female Sprague-Dawley rats received placebo in their diet over a similar treatment period. The animals were regularly monitored for any signs of ill health or reaction to treatment. Body

weights and food consumption were recorded once during pretrial, then twice weekly during treatment. Urine and blood samples were collected for laboratory investigations during week 4 of the study.

On completion of 28 days of treatment, all animals were killed and subjected to necropsy where pre-specified organs were weighed and/or placed in fixative. During necropsy, plasma and liver samples were obtained from each animal for analysis. Histopathological evaluation was performed on all animals receiving 0 or 500 mg β-apo-8'-carotenal active ingredient/kg bw/day.

Consistent with the nature of the test item, dietary administration with β-apo-8'-carotenal 10% WS/N for at least 4 consecutive weeks resulted in orange/red coloured faeces and skin in animals treated at 100 or 500 mg/kg bw/day. A slight reduction in body weight gain and food consumption was also recorded in animals treated at 500 mg/kg bw/day. There were no premature decedent animals in this study.

Increases in ALT and aspartate aminotransferase (AST) levels were noted in all treated groups along with increases in creatinine and total bilirubin levels in treated female animals. Increases in liver weight were also noted in all treated female groups. However, the authors indicated that toxicological significance of these changes was unclear.

The changes in AST were as follows: 87 $\pm$ 10 in male control rats, 87 $\pm$ 3 in male placebo control rats, 102 $\pm$ 4 ($p<0.05$) in male rats at a dose of 20 mg/kg bw/day, 96 $\pm$ 5 (not significant) in male rats at 100 mg/kg bw/day and 110 $\pm$ 14 ($p<0.001$) in male rats at 500 mg/kg bw/day, and 74 $\pm$ 3 in female control rats, 80 $\pm$ 8 in female placebo control rats, 77 $\pm$ 7 (not significant) in female rats at a dose of 20 mg/kg bw/day, 96 $\pm$ 27 ($p<0.05$) in female rats at 100 mg/kg bw/day and 92 $\pm$ 5 ($p<0.01$) in female rats at 500 mg/kg bw/day.

The changes in ALT were as follows: 77 $\pm$ 11 in male control rats, 77 $\pm$ 11 in male placebo control rats, 107 $\pm$ 14 ($p<0.01$) in male rats at a dose of 20 mg/kg bw/day, 125 $\pm$ 15 ($p<0.001$) in male rats at 100 mg/kg bw/day and 118 $\pm$ 12 ($p<0.001$) in male rats at 500 mg/kg bw/day, and 54 $\pm$ 2 in female control rats, 54 $\pm$ 16 in female placebo control rats, 62 $\pm$ 13 (not significant) in females rats at a dose of 20 mg/kg bw/day, 82 $\pm$ 21 ($p<0.01$) in female rats at 100 mg/kg bw/day and 77 $\pm$ 18 ($p<0.05$) in female rats at 500 mg/kg bw/day.

There were no other in-life findings that could be attributed to administration with β-apo-8'-carotenal 10% WS/N. Yellow or orange discolouration, abnormal colour or staining of the skin and body tissues was noted at necropsy in the majority of animals treated with β-apo-8'-carotenal 10% WS/N. One male and all female animals given the highest dose of β-apo-8'-carotenal 10% WS/N (500 mg/kg bw/day) exhibited minimal eosinophilic droplet formation in the tubular epithelial cells of the outer renal cortices and this was correlated with the increases in creatinine levels noted in the treated female groups. However in this study the incidence of eosinophilic droplets is unusual in that the females are more affected than the males. For this reason the Panel concluded that the effect should not be disregarded. The authors indicated that the severity of the finding is very minimal, and that therefore at this point, the finding is considered to be of equivocal toxicological significance. The Panel does not agree with this conclusion.

The authors concluded that administration of β-apo-8'-carotenal 10% WS/N in the diet at doses up to 500 mg/kg bw/day was associated with discolouration of the tissues and faeces of treated animals which is an expected effect of the test item. A slight reduction in body weight gain and food consumption was also recorded in animals treated at 500 mg/kg bw/day.

There was no histopathological correlation with necropsy findings. When considering histopathological changes, there were minimal eosinophilic droplets in the kidneys of animals treated at 500 mg /kg bw/day. This finding may be of equivocal toxicological significance, however, the authors indicated that under the conditions of the study, a NOAEL in both sexes was considered to be 100 mg/kg bw/day. The Panel noted that effects on AST and ALT activities were also observed in the new 13 week study described below but appeared to be reversible upon a 4 week recovery phase

included in this new study. Thus the Panel concluded that the NOAEL of this 28 week toxicity study was 100 mg/kg bw/day.

The new studies that became available following a public call for data also included a 13 week toxicity study on β-apo-8'-carotenal 10% WS/N in rats incorporating a neurotoxicity screen with administration by the diet with a 4 week recovery period (Edwards et al. 2007). The study design was in compliance with OECD guideline 408 and the study was performed in accordance with GLP. Five groups of 10 male and 10 female Sprague-Dawley rats were dosed continuously by diet for 13 consecutive weeks at levels of 0, 0 (placebo), 10, 30 and 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. In addition, a further 3 groups of 5 male and 5 female rats were assigned to the recovery study and dosed at 0, 0 (placebo), 100 mg β-apo-8'-carotenal active ingredient/kg bw/day for 13 weeks followed by a 4 week recovery period. The achieved levels of intake were very close to nominal. The rats were regularly monitored for any signs of ill health or reaction to treatment. Detailed functional observations were performed weekly, with additional functional investigations performed prior to dosing and week 12 of treatment. Body weights and food consumption were recorded at regular intervals until the end of the treatment period. Blood and urine samples were collected for laboratory investigations at the end of the treatment and recovery periods. Blood samples were also collected from all animals for analysis of the test item concentration in plasma. On completion of 13 weeks of treatment (main study) and a further 4 week treatment free period (recovery study), all rats were subjected to necropsy with pre-specified organs being weighed and/or placed in fixative. During necropsy, plasma and liver samples were obtained from each rat for analyses. Histopathological evaluation was performed on all animals receiving 0 and 100 mg β-apo-8'-carotenal active ingredient/kg bw/day; liver and kidneys were also examined from all animals receiving 10 and 30 mg β-apo-8'-carotenal active ingredient/kg bw/day.

Dietary administration with β-apo-8'-carotenal 10% WS/N for 13 weeks resulted in the expected orange/red colored faeces in all treated animals and orange colored skin in animals treated at 30 mg β-apo-8'-carotenal active ingredient/kg bw/day and above. In males at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day the discoloured skin lasted for 2 weeks after the completion of treatment. There were no signs of neurotoxicity, body weight or food consumption effects that could be attributed to administration of β-apo-8'-carotenal 10% WS/N.

Statistically significant increases in white blood cells and some of the associated parameters were seen in males receiving 30 and 100 mg β-apo-8'-carotenal active ingredients/kg bw/day. Minor increases were seen in AST and ALT levels in females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. These differences were not evident after the recovery phase.

Discolouration of body tissues was seen at necropsy at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day, which persisted to the end of the recovery period. There was an increase in liver weight in females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. This was not evident at the end of the recovery phase.

In groups receiving β-apo-8'-carotenal 10% WS/N, plasma and liver levels of β-apo-8'-carotenal showed a dose related increase. Also, mean plasma values at the different sampling points during the treatment period were similar at the respective dosages. Plasma and liver levels for three metabolites (β-apo-8'-carotenol, β-apo-8'-carotenoic acid and a polar metabolite) also increased with dose. Values for metabolites were generally up to 3 fold higher for females than for males. In the recovery study there was evidence at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day of complete plasma clearance for β-apo-8'-carotenal and the metabolite β-apo-8'-carotenol. For liver samples there was a significant clearance of β-apo-8'-carotenal and metabolites, although clearance was not totally complete over this 4 week time period.

Histopathologically, administration of β-apo-8'-carotenal 10% WS/N in the diet was associated with:

- eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above,

- multinucleate hepatocytes in the liver of females at 30 mg β-apo-8'-carotenal active ingredient/kg bw/day and above,

- increased numbers of inflammatory cell foci in the liver of females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day.

The finding of eosinophilic droplets at 10 and 30 mg β-apo-8'-carotenal active ingredient/kg bw/day was minimal in degree in all affected animals and, in the absence of other changes in the kidney, was considered by the authors not to be an adverse effect. The Panel does not agree with this conclusion.

Overall, the authors concluded in first instance that the NOAEL was considered to be 10 mg β-apo-8'-carotenal active ingredient/kg bw/day.

An amendment to this fist description of the study was also submitted (Perry and Shearer 2008). In this report it was stated that it was realized that the originally defined NOAEL in the 13-week rat study for β-apo-8'-carotenal (Edwards et al., 2007) may be open to question. Specifically the histopathological change of multinucleated hepatocytes (MNHs) was reported as an adverse change whereas there are literature references to this change being adaptive and not adverse (Williams and Iatropoulos, 2002). The Panel was also informed that a limited peer review was organized with a pathologist experienced with compounds from the same chemical category as β-apo-8'-carotenal. The liver slides from all females were reviewed by the pathologist. In addition, the input of an expert in the field of hepatotoxicity was sought.

The work carried out in these additional reviews was not part of the original study.

The Panel was informed that based on the peer review, expert opinion and also taking into account published information on the long half life of multinucleated hepatocytes (MNHs), the study pathologist and the peer review pathologist came to the mutually agreed-upon conclusion that the change of MNHs as observed in this study is not adverse. Accordingly the overall interpretation of the NOAEL in this study was reviewed. The Panel agreed that the change of MNHs as observed in this study is reversible, although, given the long half life of MNHs a study with a longer recovery period to demonstrate full reversibility would have been better in order to fully prove this assumption. The Panel however also noted that the finding of eosinophilic droplets in the kidney of all exposed groups point at the kidney as the target tissue.

The amendment indicates that histopathologically, administration of β-apo-8'-carotenal 10% WS/N in the diet was associated with eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above, for which there was no NOAEL. The finding of eosinophilic droplets at 10 and 30 mg β-apo-8'-carotenal active ingredient/kg bw/day was minimal in degree in all affected animals and, in the absence of other changes in the kidney, was considered by the study pathologist and the peer review pathologist not to be an adverse effect.

Administration of β-apo-8'-carotenal 10% WS/N was also associated with increased numbers of inflammatory cell foci in the liver of females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day, for which the NOAEL was 30 mg β-apo-8'-carotenal active ingredient/kg bw/day.

The amendment report concluded that overall, for males the NOAEL was 100 mg β-apo-8'-carotenal active ingredient/kg bw/day and for females it was 30 mg β-apo-8'-carotenal active ingredient/kg bw/day (based on increased hepatic inflammatory cell foci).

The Panel closely evaluated the data on the eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above. Table 5 presents the data which were not suitable for a BMD analysis. From these data the Panel concluded that 10 mg/kg bw/day is a lowest observed adverse effect level (LOAEL) for this effect.

Table 5: Dose-dependent incidence of eosinophilic droplets in the kidneys of rats exposed for 13 weeks to β-apo-8'-carotenal 10% WS/N, as reported by Edwards et al. (2007).

Gender	Dose level (mg/kg bw/day)	No of animals	No of animals with eosinophilic droplets in the kidney (minimal)	No of animals with eosinophilic droplets in the kidney (mild)	No of animals with eosinophilic droplets in the kidney (total incidence)
male	0	10	0	0	0
	0 (placebo)	10	0	0	0
	10	10	2	0	2
	30	10	1	0	1
	100	10	3	0	3
females	0	10	0	0	0
	0 (placebo)	10	0	0	0
	10	10	10	0	10
	30	10	10	0	10
	100	10	5	5	10

In rats given 100 mg β-apo-8'-carotenal active ingredient/kg bw/day followed by a recovery period, eosinophilic droplets were found in the kidney of 1/5 males and 4/5 females. The severity in males was minimal, and in females was minimal or mild.

3.2.2.2. Dogs

In addition the results of 14 week toxicity and metabolism studies of β-apo-8'-carotenal in dogs (strain and body weight not given) were provided (Bagdon 1961). The study report states that β-apo-8'-carotenal was well tolerated, that toxic manifestations were not detected in these animals despite prolonged treatment with large doses ranging to doses as high as 1000 mg per day. Three groups of dogs (6 animals/group) were given dose levels of 0 (control), 100 and 1000 mg per day β-apo-8'-carotenal given as a single administration orally in gelatin capsules each day for 14 weeks. The dogs were observed closely each day for signs of toxicity and body weights were recorded at weekly intervals. Blood counts, consisting of measurements of the haematocrit, haemoglobin, erythrocyte sedimentation rate, total and differential leukocytes were taken during the 0th, 6th and 13th experimental weeks. Also before treatment and after 6 and 13 weeks administration of β-apo-8'-carotenal a series of liver function tests was performed (including AST and ALT). At autopsy, the organs were weighed and examined for gross pathological changes. Sections of the tissues were examined microscopically. The results obtained did not show abnormalities. β-Apo-8'-carotenal was well tolerated. General health and growth was unimpaired. The occurrence of death in 1 control and 1 treated dog was unrelated to treatment. Haematopoietic tissues were unaffected. Also liver and kidney function tests did not deviate from normal. Gross and microscopic findings were not remarkable except for some deposition of pigment in the tissues of dogs given the highest dose. The Panel identified a NOAEL of 1000 mg/ dog/day which, assuming a body weight of 10-15 kg would amount to 67-100 mg/kg bw/day.

3.2.3. Genotoxicity

TemaNord mentions two in vitro genotoxicity studies with β-apo-8'-carotenal, an Ames test with *Salmonella typhimurium*, and a test with cultured hamster lymphocytes exposed for 48 hours (no further details). In both tests, β-apo-8'-carotenal was found to be non-genotoxic (BIBRA, 1996).

A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell lines was reported by Ishidate et al. (1988). On the basis of the evaluation of the results abstracted from the literature, they concluded that 'apocarotenal', assumed to be β-apo-8'-carotenal (250 and 1000 μg/ml), is not clastogenic in mammalian cell cultures.

In a study on the antimutagenic and anticarcinogenic effects of carotenoids, Azuine et al. (1992) also tested the mutagenicity of β-apo-8'-carotenal with and without metabolic activation in *Salmonella typhimurium* strains TA 98 and TA 100. β-Apo-8'-carotenal was not mutagenic in this test system.

Rauscher et al. (1998) examined β-apo-8'-carotenal at concentrations up to 100 µg β-apo-8'-carotenal per plate for mutagenicity in the histidine-deficient strains of *Salmonella typhimurium* TA98, TA 98NR and/or TA100. They found negative results.

Several new studies on genotoxicity have been published since the previous evaluations.

Three publications by Alija et al. (2004, 2005, 2006) are available, which describe experiments investigating the genotoxic potential of β-carotene and its oxidation products, among which β-apo-8'-carotenal.

Alija et al. (2004; 2005) investigated the genotoxic potential of β-apo-8'-carotenal, β-carotene, and a β-carotene cleavage product (CP) mixture in primary rat hepatocytes at concentrations of 0, 0.01, 0.1, 1, 5 or 10 µM. The endpoints tested were: the mitotic indices, percentage of necrotic and apoptotic cells, cells with micronuclei (MN), chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs). A statistically significant increase of micronuclei and chromosomal aberrations was observed when cells were treated with CP or β-apo-8'-carotenal at concentrations from 0.1 to 10 µM. In the same experimental conditions, a dose related increase of sister chromatid exchanges, which attained statistical significance at top dose, was also observed. In the same study, β-carotene (0.01 – 10 µM) induced neither significant cytotoxic nor genotoxic effects.

The Panel noted that the CP mixture is produced by degradation using NaClO. The similarity of the degradation products obtained by this method and those produced under normal conditions of use is not discussed. The authors determined the composition of the CP mixture but only the main products are quantified: "The CP mixture obtained from a 0.5 mM β-carotene stock solution contained β-carotene (0.16 mM), apo-15'-carotenal (0.08 mM), apo-12'-carotenal (0.12 mM) and apo-8'-carotenal (0.006 mM) and a number of products which could not be identified by HPLC". Thus 13.4% was not identified. Using GC-MS, the authors determine semi quantitatively some other components : "Related to all peaks detected during GC-MS analysis was a peak area of ~4.8% accounting for β-cyclocitral, 0.1% for ionene, 9.9% for β-ionone, 1.9% for β-ionone-5,6-epoxide and 4.5% for dihydroactinidiolide. Furthermore, 4-oxo-β-ionone was detected in trace amounts." But some components of the CP are non-identified. The use of NaClO may produce chlorinated derivatives that are generally more mutagenic than the same non-chlorinated products. Concerning the cell type used in this assay system, the Panel noted that the incidence of spontaneous aberrations observed with the control cultures was unusually high: 7.90±0.90% for the frequency of micronuclei and 1.20±0.03 aberrations per metaphase. This shows genomic instability of this cell system.

The Panel identified more pitfalls in this study (see Discussion), which is, therefore, considered of limited validity for the evaluation of the genotoxicity of β-carotene and its cleavage products.

In a follow up study, Alija et al. (2006) investigated the genotoxic action of 0.01 - 10 µM of a β-carotene cleavage product mixture in a rat primary hepatocyte assay in the presence or absence of DMNQ (2,3-dimethoxy-1,4-naphtoquinone) or hypoxia/reoxygenation (Hy/re) induced oxidative stress. The mitotic indices, the percentage of necrotic and apoptotic cells, the percentages of micronucleated cells, chromosomal aberrations and sister chromatid exchanges were measured.

In combination with DMNQ (40 µM), the cleavage product mixture (CP) induced a significant increase in micronuclei (at 0.01 and 1 µM), increased chromosomal aberrations (at 1 µM) and induced SCE (at 1 µM). Cytotoxic effects were observed at 40 µM DMNQ in combination with 10 µM of the cleavage product mixture. In combination with hypoxia/reoxygenation, the cleavage product mixture induced a significant increase in micronuclei (at 0.01, 1 and 10 µM), increased CAs (at 0.01, 1 and 10 µM), and induced SCEs (at 1 and 10 µM). No cytotoxicity was observed. The Panel noted that this study, which concerns the enhancement by CP of the genotoxic effects of the free radical-generator DMNQ or hypoxia/reoxygenation, has limited relevance for the assessment of the genotoxic potential of CP *per se*.

Kalariya et al. (2009), studied the genotoxic effects of the β-carotene breakdown product β-apo-8'-carotenal in Human Retinal Pigment Epithelial Cells (ARPE-19) using the Comet assay. They concluded that their results suggest that β-apo-8'-carotenal, when applied at partially toxic doses, is genotoxic inducing DNA strand breaks prevented by high levels of glutathione (GSH). The authors stated that the mechanism of genotoxicity of β-apo-8'-carotenal.

However, for the Kalariya study, the same limitations hold as for the Alija et al. (2004; 2005) studies about the representativeness of the degradation products because the same method was used to produce them. The same group showed (Kalariya et al., 2008) that these degradation products induced cell toxicity and apoptosis in the same range of concentrations that was tested in the study with ARPE-19 cells. To which extent ghost cells are taken into account is not described. Therefore the Panel noted that the comets could be, at least partly, a consequence of the effects of cytotoxicity and/or apoptosis.

Overall the Panel concluded that the genotoxicity studies of the β-carotene breakdown products in primary rat hepatocytes and in ARPE-19 cells provide very limited evidence of genotoxicity.

In another in vitro study by Marques et al. (2004), calf thymus DNA was allowed to react with β-apo-8'-carotenal, β-carotene and retinal at 37°C for 72 hours in the presence and absence of 50 mM H_2O_2 in neutral (pH 7.4) and basic (pH 9.4) conditions. 1,N^2-etheno-2'-deoxyguanosine formation was measured. This adduct was also formed in all control incubations (DNA only, in the absence and presence of H_2O_2 in neutral and basic conditions). 1,N^2-etheno-2'-deoxyguanosine formation was significantly increased at both pHs in the presence of β-apo-8'-carotenal, β-carotene and retinal, compared with control incubations. Also, in the presence of H_2O_2, adduct formation was increased by all test compounds. In addition, significant increases in 8-oxo-7,8-dihydro-2'-deoxyguanosine were observed when all three compounds were incubated in the presence of H_2O_2. 1,N^2-etheno-2'-deoxyguanosine has been proven to be mutagenic in *E. coli* uvrA$^-$ (Langouët et al., 1998).

In an in vitro study by Yeh and Wu (2006), it was found that β-apo-8'-carotenal induced DNA strand breaks, lipid peroxidation and expression of CYP1A2 in A549 cells (human alveolar epithelial cells). Furthermore, both β-apo-8'-carotenal and β-carotene significantly enhanced DNA strand breaks and CYP1A2 expression, induced by benzo[a]pyrene at 20 µM. However, β-carotene at 2 µM significantly suppressed BaP-induced strand breaks. DNA damage was found to be associated with expression of CYP since the effects were diminished in the presence of 1-aminobenzotriazole, a CYP inhibitor.

Additional studies on genotoxicity have been provided upon a public call for data, which are described in more detail hereafter.

A *Salmonella typhimurium/Escherichia coli* reverse mutation assay (standard plate test) with β-apo-8'-carotenal was conducted according OECD guidelines and under GLP (BASF, 1998a). The strains used were TA 100, TA 1537, TA 98 and *E. coli* WP2 uvrA, concentrations tested were 20 µg- 5,000 µg/plate (all tester strains) and 20 µg - 6,000 µg/plate (TA 100), and tests were performed in the absence and presence of S9. Precipitation of the test substance was found from about 100 µg/plate onward. A weakly bacteriotoxic effect was occasionally observed depending on the strain and test conditions from about 2,500 µg/plate onward.

No increase in the number of revertants was observed for all strains except for TA 100. For TA100 without S9 mix a slight increase in the number of his+ revertants was observed depending on the test conditions from about 500 µg - 2,500 µg/ plate onward up to 4,000 µg - 6,000 µg/plate (factor 1.9 - 4.1). The authors concluded that β-apo-8'-carotenal is weakly mutagenic in the *Salmonella typhimurium* coli reverse mutation assay under the experimental conditions chosen.

β-Apo-8'-carotenal 10% WS/N was assayed for mutagenicity in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) in the absence and in the presence of metabolic activation (S9) (Loget and Johnson, 2006). β-Apo-8'-carotenal 10% WS/N consists of free flowing particles (beadlets), containing β-apo-8'-carotenal dispersed in a cornstarch-coated matrix of gelatin, sucrose and corn oil. An initial toxicity range finding experiment was carried out in strain TA100 only in the absence of S9, using final concentrations of β-apo-8'-carotenal 10% WS/N at 0.0889, 0.4446, 2.223, 11.12, 55.58 and 277.9 µg/plate. The highest concentration was

limited by the maximum achievable solubility of the test article. No evidence of toxicity was observed. No reproducible increases in revertant numbers were observed in any of the strains in the absence or presence of S9. It was concluded that β-apo-8'-carotenal 10% WS/N did not induce mutation in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) at concentrations up to 277.9 µg/plate. The Panel noted that the concentrations of β-apo-8'-carotenal active ingredient tested in this study were relatively low.

A mixture of β-apo-8'-carotenal (95.5%) plus crocetindial (0.47%) was tested in an in vitro genotoxicity assay using Chinese hamster ovary (CHO) cells (Loget and Whitwell, 2006) in two experiments. Treatment covering a broad range of doses up to 5000 µg/ml, both in the absence and presence of metabolic activation (S9). The test article was formulated as a suspension in 0.5% methyl cellulose. In experiment 1, treatment in the absence and presence of S9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+17). The test article concentrations for chromosome analysis were selected by evaluating the effect of β-apo-8'-carotenal on population doubling. Chromosome aberrations were analysed at three or four concentrations, the highest concentrations chosen for analysis being 200.0 µg/ml in the absence of S9 and 800.0 µg/ml in the presence of S9, inducing approximately 69% and 74% reduction in population doubling respectively. In experiment 2, treatment in the absence of S9 was continuous for 20 hours (20+0). Treatment in the presence of S9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+17). Chromosome aberrations were analysed at three dose levels the highest concentrations chosen for analysis being 50.00 µg/ml (20+0 –S9), and 125.0 µg/ml (3+17 +S9), inducing approximately 49% and 46% reduction in population doubling, respectively.

Treatment of cultures with β-apo-8'-carotenal plus crocetindial in the presence of S9 (experiments 1 and 2) resulted in frequencies of cells with structural chromosome aberrations that were similar to those seen in concurrent vehicle controls for the majority of concentrations analysed. However, increased frequencies of cells with aberrations were observed and associated with cultures inducing high cytotoxicity (greater than 50%) but not at concentrations inducing moderate to low cytotoxicity. The increases observed were therefore considered by the authors to be of questionable biological relevance.

Treatment of cultures with β-apo-8'-carotenal plus crocetindial in the absence of S9 (20+0 hour and 3+17 hour treatments), resulted in frequencies of structural chromosome aberrations that were similar to those seen in concurrent vehicle controls for all but a single concentration analysed. With the exception of one replicate culture at the highest concentration analysed following 3+17 hour treatment (200 µg/ml, a concentration inducing 69% cytotoxicity), all treated cultures exhibited aberrant cell frequencies that fell within historical vehicle control (normal) values. The increase observed was therefore considered by the authors to be of questionable biological relevance.

With the exception of sporadic increases in endoreduplicated and polyploid cells (single cultures at 400.0 and 600.0 µg/ml respectively following 3+17 hour +S9 treatment in experiment 1), no increases in numerical aberrations, (exceeding historical vehicle control values), were observed in cultures treated with β-apo-8'-carotenal plus crocetindial. The authors concluded that β-apo-8'-carotenal plus crocetindial showed evidence of inducing structural chromosome aberrations in cultured Chinese hamster ovary (CHO) cells when tested in excess of its limit of cytotoxicity (50%) in both the absence and presence of metabolic activation (S9). These increases were sporadic and in all instances associated with high cytotoxicity (greater than 50% as assessed by population doubling data). No such increases were observed in cultures inducing less than 50% cytotoxicity, or at any concentration analysed following continuous 20+0 hour –S9 treatment. Therefore, the increases observed were considered of questionable biological relevance as they were observed only at cytotoxic (or highly cytotoxic) concentrations. The Panel noted that this study has limited relevance for evaluating the genotoxicity of β-apo-8'-carotenal given the combined exposure with crocetindial.

The new studies submitted to EFSA also included an in vivo rat bone marrow micronucleus test (Lodget and Beevers, 2006). β-Apo-8'-carotenal 10% WS/N was assayed in vivo in a rat bone marrow micronucleus test at three dose levels. β-Apo-8'-carotenal 10% WS/N consists of free flowing particles (beadlets), containing β-apo-8'-carotenal dispersed in a cornstarch-coated matrix of gelatin,

sucrose and corn oil. The choice of dose levels was based on an initial toxicity range-finding study in which β-apo-8'-carotenal 10% WS/N, formulated in purified water (water for injection) was administered to rats via oral gavage. The test article was administered once daily on two consecutive days to a group of three male and three female rats at a dose of 800 mg β-apo-8'-carotenal active ingredient/kg bw/day. This dose was the maximum practicable dose based on the formulation used and a dose volume of 20 ml/kg bw. Observations were made over a 2 day period following the second administration and signs of toxicity recorded. No clinical signs of toxicity were observed, thus confirming that 800 mg β-apo-8'-carotenal active ingredient/kg bw/day was well tolerated and therefore a suitable maximum dose for the micronucleus test. Two further doses of 200 and 400 mg/kg/day were chosen. As there were no gender-specific differences in toxicity, male animals only were used in the micronucleus test.

In the micronucleus test, β-apo-8'-carotenal 10% WS/N, formulated as described above, was administered at 200, 400 and 800 mg β-apo-8'-carotenal active ingredient/kg bw/day to groups of six male rats. Doses were administered once daily for two consecutive days and rats killed 24 hours after the second administration. No clinical signs of toxicity were observed in any animal receiving β-apo-8'-carotenal 10% WS/N. The negative (vehicle) control in the study was water for injection. In addition, a placebo group was dosed with empty beadlets at a dose equivalent to the maximum test article dose (800 mg/kg/day). Groups of six male rats were treated with vehicle or placebo once daily on two consecutive days via oral gavage. The animals were killed and sampled 24 hours after the second administration. Cyclophosphamide (CPA), the positive control, was dissolved in saline and administered via oral gavage as a single dose of 30 mg/kg bw to a group of six male rats which were killed after 24 hours. Negative (vehicle) control rats exhibited an acceptable group mean frequency of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE; ratio expressed as %PCE) and the frequencies of micronucleated PCE fell within the historical negative control (normal) data. Positive control animals exhibited increased numbers of micronucleated PCE such that the frequency in the positive control group was significantly greater than in concurrent controls. Placebo treated rats resulted in %PCE and micronucleated PCE (MN PCE) frequencies that were consistent with data from the concurrent vehicle control group, thus confirming that the beadlets used in preparation of the test article had no adverse effect on the assay system and did not induce micronuclei under the conditions tested.

Groups of rats treated with β-apo-8'-carotenal 10% WS/N exhibited %PCE and MN PCE values that were similar to vehicle controls and consistent with the laboratory's historical control range. There were no statistically significant increases in MN PCE frequency in any of the test article treated groups. Analysis of plasma confirmed that rats dosed with β-apo-8'-carotenal 10% WS/N were systemically exposed to β-apo-8'-carotenal and three metabolites.

It was concluded by the authors that β-apo-8'-carotenal 10% WS/N did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of rats treated up to 800 mg/kg/day (the maximum practicable dose for this study). The Panel agreed with this conclusion.

Overall the Panel concluded that the available in vitro genotoxicity studies and the in vivo micronucleus study with β-apo-8'-carotenal do not give reason for concern with respect to genotoxicity. A few in vitro studies with β-carotene cleavage products provide limited evidence of DNA damaging activity and covalent binding to DNA. In this respect the Panel noted that such results may reflect a pro-oxidant effect, common to other antioxidants, which is elicited under specific in vitro conditions which may not occur in vivo.

3.2.4. Chronic toxicity and carcinogenicity

JECFA describes a single long-term study conducted with the ethyl ester of β-apo-8'-carotenoic acid. In this study 15 male rats were fed β-apo-8'-carotenoic acid ethyl ester at 1% of their diet (equivalent to 500 mg/kg bw/day) for 2 years. Two control groups received either the basic diet or 7500 International Units (IU) of vitamin A/animal/day. No effects were noted compared to animals

receiving the basic diet regarding general health, mortality, weight gain, or fertility. In the vitamin A control group some of these parameters were affected (no further details given) (Anonymous, 1966).

Upon a public call for data results from chronic studies performed in the 1960's became available for evaluation (Schärer, 1960; Schärer, 1961; Schärer and Studer, 1961a; Schärer and Studer, 1961b; Schärer and Studer, 1961c; Schärer and Studer, 1961d; Schärer and Studer, 1961e; Schärer and Studer, 1962a; Schärer and Studer, 1962b; Schärer and Studer, 1962c; Schärer, 1963; Schärer, 1965).

A summary of these chronic toxicity studies with β-apo-8'-carotenal was also provided (Schärer and Studer 1961b). β-Apo-8'-carotenal was administered at 0.1% in the diet to the first generation of Wistar rats and their offspring (second generation) for a period of 2 years and to a third generation for a period of 1 year. The average intake of β-apo-8'-carotenal was about 40 mg/kg bw/day. The number of animals per group amounted to 20 males and females in the control and treatment groups of the first generation and the rats in this treatment group received 0.1% β-apo-8'-carotenal in the diet for 104 weeks. From the offspring of this first generation 14 respectively 15 males and 15 respectively 15 females were included in the control and treatment groups of the second generation of which the treatment group also received 0.1% β-apo-8'-carotenal in the diet for 104 weeks. From the offspring of this second generation 5 males and 12 females were included in the control group of the third generation and 12 males and 11 females were included in the treatment group of the third generation which received 0.1% β-apo-8'-carotenal in the diet for 52 weeks

The results were as follows:

1) The number of spontaneous deaths was greater among controls (25 out of 122 animals) than among the treated animals (18 out of 147 animals).

2) The average weight of the animals treated with 0.1% β-apo-8'-carotenal in both the 1 year and the 2 year experiments were slightly below those of the respective controls but still within the permissible range.

3) Results of the haematology test were not influenced by addition of 0.1% β-apo-8'-carotenal to the diet.

4) The treated animals exhibited orange-yellow discolouration of the body fat and yellow to ochre discolouration of the liver.

5) The absolute weights of the liver, kidneys and heart did not reveal any important differences between treated and untreated rats. Some of the testes of the treated animals were lighter but their weights varied greatly; thus it was concluded by the authors that the weight of the gonads of rats given β-apo-8'-carotenal did not differ significantly from the controls.

6) Histopathological examination of sections stained for fat revealed golden-yellow to yellow-brown, iron-free pigment granules in the liver and kidneys of treated animals which were absent in the controls. The interstitial changes in the kidneys as well as the tubular distensions and calcium incrustations at the medulla cortex margin were less pronounced in the treated animals than in the controls. The other organs (lungs, heart, spleen, bone marrow, stomach, duodenum, jejumen, pancreas, ovaries, skeletal musculature, thyroid and skin) did not reveal any differences when examined microscopically. Degenerative changes in the epithelium of the seminiferous tubules were slightly, but not statistically significantly more frequent ($p=0.17$) in the animals treated with β-apo-8'-carotenal than in the controls.

7) The spontaneous tumours were distributed evenly over both groups (11/147 treated rats and 10/122 untreated rats) and consequently the authors concluded that their occurrence was not connected with the administration of β-apo-8'-carotenal.

8) The reproductive power and the average number of animals per litter were approximately the same in the treated and untreated rats.

Based on these results the Panel considered that there were no adverse effects in this chronic toxicity study at the single dose level tested amounting to about 40 mg/kg bw/day.

3.2.5. Reproductive and developmental toxicity

JECFA describes two multi-generation studies with β-apo-8'-carotenal.

Three generations of rats received diets containing 0, 1000, 2000 or 5000 mg/kg diet β-apo-8'-carotenal (equivalent to 0, 50, 100 and 250 mg/kg bw/day, respectively) for 2 years.

No adverse effects were observed in any generation (no further details) (Anonymous, 1966).

The second study examined four generations of rats (20-40/group/sex) fed diets containing 0, 0.1, 0.2, 0.5 or 1% β-apo-8'-carotenoic acid methyl ester (equivalent to 0, 50, 100, 250 and 500 mg/kg bw/day, respectively) for 52 - 104 weeks.

No compound-related differences were observed with regard to food consumption, general health, or mortality (no further details) (Anonymous, 1966).

Additional studies on developmental toxicity have been provided upon a public call for data which are described in more detail hereafter.

In a range-finding study (Loget and Marsden, 2006) information was obtained for the selection of appropriate dose levels for a subsequent embryo toxicity study with β-apo-8'-carotenal 10% WS/N following administration from implantation to the day of caesarean section. The study was performed according to OECD guidelines. Three groups each consisting of 6 mated female SD rats were given β-apo-8'-carotenal 10% WS/N beadlets by admixture in powdered diet from days 6 to 20 of gestation inclusive at the concentrations of 2064, 10344 and 51720 mg/kg diet (groups 3, 4 and 5 respectively) corresponding to β-apo-8'-carotenal active ingredient target doses of 20, 100 and 500 mg β-apo-8'-carotenal/kg bw/day. A fourth group of 6 mated rats received placebo WS/N beadlets over the same period and served as a control (group 2). A second control (group 1) of 6 mated rats received basic powdered diet only over the same period. The total beadlet concentration (β-apo-8'-carotenal 10% WS/N and/or placebo WS/N) was equalised in groups 2 to 5.

The authors concluded that within the context of this study, dietary concentrations of β-apo-8'-carotenal 10% WS/N up to 51720 mg/kg diet (equivalent to a target dose of 500 mg/kg bw/day of the β-apo-8'-carotenal active ingredient β-apo-8'-carotenal) are considered to be NOAELs for both maternal and embryo-fetal toxicity.

Consistent with the nature of the test item (orange-red colouring agent), the only findings related to treatment were orange coloured integuments, faeces, tissues and organs in all β-apo-8'-carotenal 10% WS/N treated groups. All doses could be employed in a subsequent embryo-fetal development study.

In this subsequent study the effect of β-apo-8'-carotenal 10% WS/N on embryonic and fetal development of the rat was investigated when administered by the oral route (dietary admixture) from implantation to the day of caesarean section on day 20 of gestation (Loget et al. 2006). The study was performed according to OECD guidelines and under GLP. Three groups each consisting of 25 mated female SD rats were given β-apo-8'-carotenal 10% WS/N beadlets by admixture in powdered diet from days 6 to 20 of gestation inclusive at dose levels of 2064, 10344 and 51720 mg/kg diet (groups 3, 4 and 5 respectively) corresponding to β-apo-8'-carotenal active ingredient target doses of 20, 100 and 500 mg β-apo-8'-carotenal/kg bw/day. A fourth group of 25 mated rats received placebo WS/N beadlets over the same period and served as a control (group 2). A second control (group 1) of 25 mated rats received basic powdered diet only over the same period. The total beadlet concentration (β-apo-8'-carotenal 10% WS/N and/or placebo WS/N) was equalised in groups 2 to 5. Clinical condition, body weights and food consumption were monitored throughout the study. Surviving females were killed on day 20 of gestation for examination of their uterine contents, including examination of the placentae. At necropsy, the females were examined macroscopically and fetuses were weighed, sexed and examined for external abnormalities. Half of the fetuses were examined internally prior to processing for skeletal examination. There was no skeletal malformation noted in any group. The

remaining fetuses were preserved for fixed-visceral examination by the modified Wilson-Barrow technique. Blood sampling was performed on day 20 of gestation whilst the animals still had access to the diets. The liver of the first 5 surviving animals per group was sampled and frozen at necropsy. Plasma and liver samples were collected for bioanalysis.

There were two unscheduled deaths in the 500 mg/kg bw/day group, neither of which was clearly attributable to treatment. Consistent with the nature of test item, orange stained fur and/or orange coloured faeces and/or orange coloured integuments were noted amongst the β-apo-8'-carotenal treated animals. The placebo group was not affected. There was a slight reduction in mean body weight gain and food consumption in the 100 and 500 mg/kg bw/day groups compared with the vehicle control group. The other groups were not affected.

Overall mean intake of the β-apo-8'-carotenal active ingredient, β-apo-8'-carotenal, was consistent with the target dose (within 1%) in each group during the treatment period (days 6 to 20 of gestation).

There were no treatment-related macroscopic abnormalities noted in any group at the terminal necropsy examinations. However, consistent with the nature of the test item, essentially all β-apo-8'-carotenal 10% WS/N treated animals had orange coloured organs and tissues.

Consistent with an effect on mean fetal weight (see below), gravid uterus weight was slightly lower (6%) in all beadlet treated groups (including the placebo group) than in the vehicle control group with the exception of the 500 mg/kg bw/day group. The absence of a similar finding in the high dose group was due to an incidentally slightly greater mean litter size compared with the other groups.

There were 24, 24, 22, 25 and 19 pregnant females at the terminal caesarean sections in groups 1 to 5 respectively, all of which had viable fetuses. There was no adverse effect of treatment on embryo-fetal survival in any group. Mean live litter size in all beadlet treated groups (with or without the test item) was comparable with, or slightly superior to (particularly in group 5), that in the vehicle control group.

Mean fetal weight tended to be slightly lower than in the vehicle control group in each of the beadlet treated groups (with or without the test item). This finding suggested an association with the reduced nutritional content of the test diets due to the beadlet content rather than an effect of the test item.

There was one malformed fetus in each of groups 1, 2, 3 and 5, and two in group 4. Neither the incidence, nor the types of malformation detected, suggested any association with β-apo-8'-carotenal 10% WS/N.

There was no β-apo-8'-carotenal or any of the three metabolites detected in the plasma or liver samples in either of the control groups. All three treated groups were exposed to β-apo-8'-carotenal and the three metabolites in the plasma and liver. There was some evidence of a dose proportional increase in exposure (at least for β-apo-8'-carotenal levels) between the low and mid doses. The increase was then under proportional between the mid and high dose groups suggesting that levels were becoming saturated. Liver levels of β-apo-8'-carotenal and the three metabolites were greater than the equivalent plasma values in each group, particularly for β-apo-8'-carotenal and the β-apo-8'-carotenol and β-apo-8'-carotenoic metabolites.

The authors concluded that within the context of this study, dietary concentrations of 2064, 10344 and 51720 mg/kg diet of β-apo-8'-carotenal 10% WS/N (equivalent to target doses of 20, 100 and 500 mg/kg bw/day of the β-apo-8'-carotenal active ingredient β-apo-8'-carotenal) were associated with slight reductions in mean body weight gain and food consumption at the two higher doses. Other treatment-related findings in all β-apo-8'-carotenal 10% WS/N treated groups, such as orange coloured integuments, faeces, tissues and organs, were consistent with the nature of the test item (orange-red colouring agent).

The NOAEL for maternal toxicity was therefore considered by the authors to be the dietary concentration of 2064 mg/kg diet (20 mg β-apo-8'-carotenal/kg bw/day).

The Panel noted that the reduction in mean body weight gain was observed during the first 9 days of treatment (days 6 to 15 of gestation) in the 100 and 500 mg/kg bw/day groups compared with the vehicle control group and that this effect attained statistical significance only between days 6 and 11

of gestation. As a consequence, net mean body weight gain during the treatment period (days 6 to 20 of gestation) was slightly but not statistically significantly lower in these groups. The Panel therefore considered the slight reduction in mean body weight and food intake not as adverse and concluded that the NOAEL of this study was 500 mg/kg bw/day.

Evidence of an embryo-fetal effect was restricted to slightly lower mean fetal weight in all beadlet treated groups (with or without the test item) compared with the vehicle control. This finding suggested an association with the reduced nutritional content of the test diets due to the beadlet content rather than an effect of the test item. The NOAEL for embryo-fetal toxicity was therefore considered to be the dietary concentration of 51720 mg/kg diet (500 mg β-apo-8'-carotenal /kg bw/day). Overall the Panel concluded that there was no evidence of developmental toxicity of β-apo-8'-carotenal 10% WS/N.

3.2.6. Allergenicity, hypersensitivity and intolerance

In a study with a group of 135 persons diagnosed with urticaria or atopic dermatitis, oral exposure to 100 mg β-apo-8'-carotenal and 100 mg β-carotene induced one positive and one equivocal reaction. One positive reaction was also noted after exposure to a placebo. In a group of 123 persons with contact dermatitis, no response was observed (BIBRA, 1996).

As no cases of intolerance/allergenicity have been reported after oral exposure to the compound, it appears that at the current levels of exposure the incidence is very low, if any.

4. Discussion

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following a public call for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

β-Apo-8'-carotenal (E 160e) is allowed as a food additive in the EU and has been previously evaluated by JECFA in 1974 and the SCF in 1975. The SCF and JECFA both established an ADI of 0-5 mg/kg bw/day. JECFA defined the ADI for the sum of the carotenoids β-apo-8'-carotenal, β-carotene, β-carotenoic acid methyl ester and β-carotenoic acid ethyl ester, while the SCF used the same definition but omitted β-carotenoic acid methyl ester. However, more recently, the SCF withdrew the entire group ADI based on a recent evaluation of β-carotene (SCF, 2000). The SCF decided that there was insufficient scientific basis, either from human or experimental studies, on which to set a new ADI for β-carotene and related carotenoids, but was nonetheless of the opinion that currently permitted food additive uses of β-carotene and related carotenoids are temporarily acceptable from a health point of view at the estimated levels of intake (SCF, 2000). Although it is not mentioned explicitly in the SCF opinion, the Panel considered it reasonable to assume that the term related to carotenoids referred to β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester, since these were the compounds included in the group ADI defined by the SCF in 1975.

At present only β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester are specifically defined in Commission Directive 2008/128/EC and JECFA. According to specifications, β-apo-8'-carotenal has to comprise at least 96% of the final product in commercial β-apo-8'-carotenal products. The sum of all subsidiary colouring matters is less than 3% and these subsidiary colouring matters are related carotenoids, mainly β-carotene. The Panel noted that the specifications should be updated to more clearly define the purity of the material. The Panel noted that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$ 10 mg/kg. The Panel also noted that specifications should be extended to include maximum residue limits for residual solvents.

The Panel concluded that the available data indicate that after oral administration absorption of β-apo-8'-carotenal and/or its metabolites is at least 15% (JECFA 1974a,b; Thommen, 1962; Brubacher et al., 1960; Wiss and Thommen, 1963; Bagdon et al., 1960; Rümbeli et al. 2007). The absorbed β-apo-8'-carotenal is metabolically converted to β-apo-8'-carotenoic acid as well as to its ethyl and methyl

esters in rats (Wiss and Thommen, 1963) and β-apo-8'-carotenol (Rümbeli et al. 2007; Edwards et al. 2007; Loget et al. 2006). In rats and monkeys, β-apo-8'-carotenal accumulates in the liver together with β-apo-8'-carotenoic acid and vitamin A (rats) and an unidentified carotenoic acid (monkeys) (Thommen, 1962; Brubacher et al., 1960; Tiews 1963). In monkeys, accumulation of orange material has also been found in the body fat (Tiews, 1963). In dogs, β-apo-8'-carotenal accumulated in serum, and vitamin A levels were increased in the kidney and pigmentation was observed in adipose tissue, kidney and adrenal cortex (Bagdon et al., 1962). In rats, conversion of β-apo-8'-carotenal to vitamin A in the gut is low (4%) compared to that for β-carotene (Wiss and Thommen, 1963; Glover, 1960). β-Apo-8'-carotenal is excreted in the urine in dogs (Bagdon et al., 1960) predominantly as an (undefined) ester of β-apo-8'-carotenoic acid. In human infants the ester of β-apo-8'-carotenal was rapidly eliminated from the blood (Kűbler, 1963).

The Panel noted that the SCF (2000) has questioned the suitability of rodents as a test species for evaluating the bioavailability and effects of β-carotene in human, since rodents were considered to convert β-carotene to vitamin A much more efficiently than humans. Most laboratory animals were reported to degrade β-carotene in their intestines and absorb almost no β-carotene intact, due to high dioxygenase activity converting β-carotene to retinal, which, according to SCF, is in contrast to humans where β-carotene is mainly (20-75%) absorbed intact. Therefore rodent studies were considered to lack relevance for human risk assessment, and the Panel considered that such limitations may also apply to β-apo-8'-carotenal evaluated in the present opinion. Therefore, the Panel carefully evaluated the ADME characteristics for β-apo-8'-carotenal in both rodent and human focusing on possible differences.

An overview of the major results from an ADME study with β-apo-8'-carotenal in rats (Rümbeli et al., 2007) compared to those from a human study (Zeng et al., 1992) was made. According to the metabolite pattern in plasma the uptake and metabolism of β-apo-8'-carotenal seems qualitatively similar in rats and humans. Comparison of the plasma kinetic data from the rat with the data from the human study indicates also quantitative similarities between the two species.

Based on the metabolite pattern in blood plasma and based on the plasma kinetics the Panel concluded that for β-apo-8'-carotenal rats are a suitable model for humans concerning uptake and systemic exposure to β-apo-8'-carotenal and metabolites.

A direct comparison of the formation of vitamin A from β-apo-8'-carotenal in rats and humans is more difficult, since the experimental setup of the two studies was not identical. Results obtained reveal that in rats as well as in humans vitamin A is formed from β-apo-8'-carotenal. From the existing data a quantitative comparison can not be made.

No acute oral toxicity of β-apo-8'-carotenal was seen at relatively high doses (JECFA, 1975a, b; Bächthold, 1972; Bächthold, 1975; Bächthold, 1976; Bächthold, 1973; Bächthold, 1977; Bächthold, 1980; BASF 1972; Bächthold, 1977; Loget O and Arcelin G, 2006a; Loget and Arcelin, 2006b) and acute toxicity is considered to be of little relevance for the safety evaluation of these compounds as food colours.

Several in vitro genotoxicity studies (Alija et al., 2004; 2005; 2006; Marques et al., 2004; Kalariya et al., 2009; Yeh and Wu, 2006) were performed to further investigate the genotoxicity of β-carotene cleavage products.

A β-carotene cleavage products mixture and β-apo-8'-carotenal were reported to induce increases in MN, CAs and SCEs (Alija et al., 2004; 2005; 2006) in primary rat hepatocytes. In this respect, however, the Panel noted that there is limited experience with cytogenetic assays in primary rat hepatocytes, which show a very high spontaneous incidence of both MN and CAs. Moreover, the increase in the frequency of MN and CAs observed in presence of β-carotene cleavage products, and of micronuclei in presence of apo-8'-carotenal, were not clearly dose-related over a 104-fold concentration range. The statistically significant increases in the frequency of MN were only 20 and 11% (at 0.1 and 1 μM respectively) over control incidence, which is within the range of experimental variation for the end-points studied, and thus has limited or no biological significance. The frequencies

of CAs in presence of apo-8'-carotenal show an apparent treatment-related increase, but data are unreliable since they are based on only 20 metaphases/culture. The increase in SCEs observed in presence of both β-carotene cleavage products and apo-8'-carotenal is more credible, but the biological significance of this indicative assay in relation to genotoxicity is indirect.

Kalariya et al. (2009), studied the genotoxic effects of the β-carotene breakdown product β-apo-8'-carotenal in Human Retinal Pigment Epithelial Cells (ARPE-19) using the Comet assay. They concluded that their results suggest that breakdown products of dietary carotenoids could be genotoxic in ARPE-19 cells. The authors also stated that the mechanism of genotoxicity of β-apo-8'-carotenalwas not elucidated..

The Panel concluded that the genotoxicity studies with β-apo-8'-carotenal, β-carotene and a β-carotene cleavage product in primary rat hepatocytes reported by Alija et al. (2004; 2005; 2006) and in ARPE-19 cells by Kalariya et al. (2009), provide very limited evidence of genotoxicity.

In in vitro studies it was found that β-apo8'-carotenal induced DNA strand breaks in A549 cells (Yeh and Wu, 2006), strand breakage in supercoiled DNA (Zhang and Omaye, 2001), and etheno-adduct to calf thymus DNA (Marques et al., 2004).

The Panel also noted that a few in vitro studies with β-carotene cleavage products provide limited evidence of DNA damaging activity and covalent binding to DNA, and that such results may reflect a pro-oxidant effect, common to other antioxidants, which is elicited under specific in vitro conditions which may not occur in vivo.

Additional studies on genotoxicity provided upon a public call for data revealed generally negative results in Salmonella typhimurium/Escherichia coli reverse mutation assays (BASF, 1998; Loget and Johnson, 2006), an in vitro genotoxicity assay using Chinese hamster ovary (CHO) cells (Loget and Whitwell, 2006) and an in vivo rat bone marrow micronucleus test (Lodget and Beevers, 2006).

Overall the Panel concluded that the available in vitro genotoxicity studies and the in vivo micronucleus study with β-apo-8'-carotenal do not give reason for concern with respect to genotoxicity.

Upon a public call for data two subchronic toxicity studies in rats performed according to OECD guidelines and under GLP became available for evaluation.

These included a study in which four groups of 5 male and 5 female Sprague Dawley rats were dosed continuously by diet for at least 4 consecutive weeks with β-apo-8'-carotenal 10% WS/N, at levels of 0, 20, 100 and 500 mg β-apo-8'-carotenal active ingredient/kg bw/day (Loget and Morgan 2006). Increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were noted in all treated groups along with increases in creatinine and total bilirubin levels in treated female animals. Increases in liver weight were also noted in all treated female groups. There were no other in-life findings that could be attributed to administration with β-apo-8'-carotenal 10% WS/N. There was no histopathological correlation with necropsy findings. When considering histopathological changes, there were minimal eosinophilic droplets in the kidneys of animals treated at 500 mg /kg bw/day. This finding may be of equivocal toxicological significance. The authors indicated that under the conditions of the study, a NOAEL in both sexes was considered to be 100 mg/kg bw/day. The Panel noted that effects on AST and ALT activities were also observed in the new 13 week study described below but appeared to be reversible upon a 4 weeks recovery phase included in this new study. Thus the Panel concluded that the NOAEL of this 28 week toxicity study was 100 mg/kg bw/day.

The new studies that became available following a public call for data also included a 13 week toxicity study on β-apo-8'-carotenal 10% WS/N in rats incorporating a neurotoxicity screen with administration by the diet with a 4 week recovery period (Edwards et al., 2007). The study design was in compliance with OECD guideline 408 and the study was performed in accordance with GLP. Five groups of 10 male and 10 female Sprague-Dawley rats were dosed continuously by diet for 13 consecutive weeks at levels of 0, 0 (placebo), 10, 30 and 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. Dietary administration with β-apo-8'-carotenal 10% WS/N for 13 weeks resulted in the expected orange/red coloured faeces in all treated animals and orange coloured skin in

animals treated at 30 mg β-apo-8'-carotenal active ingredient/kg bw/day and above. In males at 100 mg β-apo-8'-carotenal active ingredient /kg bw/day the discoloured skin lasted for 2 weeks after the completion of treatment. There were no signs of neurotoxicity, body weight or food consumption effects that could be attributed to administration of β-apo-8'-carotenal 10% WS/N. Statistically significant increases in white blood cells and some of the associated parameters were seen in males receiving 30 and 100 mg β-apo-8'-carotenal active ingredients/kg bw/day. Minor increases were seen in AST and ALT levels in females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day. These differences were not evident after the recovery phase.

Histopathologically, administration of β-apo-8'-carotenal 10% WS/N in the diet was associated with:

- eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above,

- multinucleate hepatocytes in the liver of females at 30 mg β-apo-8'-carotenal active ingredient/kg bw/day and above,

- increased numbers of inflammatory cell foci in the liver of females at 100 mg β-apo-8'-carotenal active ingredient/kg bw/day.

The Panel closely evaluated the data on the eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and above. From these data the Panel concluded that 10 mg/kg bw/day is a lowest observed adverse effect level (LOAEL) for this effect.

In rats, testicular weights were significantly decreased after being fed 500 mg/kg bw/day β-apo-8'-carotenal or β-apo-8'-carotenoic acid *methyl* ester (Anonymous, 1962; 1966) (or possible *ethyl* ester, discrepancy between JECFA and TemaNord evaluations).

Following administration of β-apo-8'-carotenal in rats granular pigment deposition in the liver and kidneys was noted (Brubacher et al., 1960; Schärer et al. 1961; Loget and Morgan 2006; Edwards et al. 2007) and in dogs pigmentation was found in adipose tissue, kidney and adrenal cortex (Bagdon 1961; Bagdon et al., 1962). Besides pigment deposition, a marked increase in the vitamin A level in the kidney was noted in dogs. As none of the latter findings appear to have been accompanied by any signs of pathology, these effects are considered to be of no toxicological concern.

Two studies on reproductive and developmental toxicity have been conducted well before the introduction of (OECD) GLP guidelines, both in rats (Anonymous, 1966). Although on the basis of these two studies, there appears to be no direct reason for concern, it should be considered that the available studies were conducted well before the introduction of (OECD) GLP guidelines, and that in addition, the studies have been described in very little detail in an evaluation conducted over three decades ago.

Upon a public call for data two additional studies on reproductive and developmental toxicity became available for evaluation; a range-finding study (Loget and Marsden, 2006) and a subsequent study investigating the effects of β-apo-8'-carotenal 10% WS/N on embryonic and fetal development of the rat when administered by the oral route (dietary admixture) (Loget et al., 2006). These studies were performed according to OECD guidelines and under GLP. Within the context of this study, dietary concentrations of 2064, 10344 and 51720 mg/kg diet of β-apo-8'-carotenal 10% WS/N (equivalent to target doses of 20, 100 and 500 mg β-apo-8'-carotenal/kg bw/day) were associated with slight reductions in mean body weight gain and food consumption at the two higher doses. Other treatment-related findings in all β-apo-8'-carotenal 10% WS/N treated groups, such as orange coloured integuments, faeces, tissues and organs, were consistent with the nature of the test item (orange-red colouring agent).

Based on reduction in mean body weight gain the NOAEL for maternal toxicity was considered by the authors to be the dietary concentration of 2064 mg/kg diet (20 mg β-apo-8'-carotenal/kg bw/day).

The Panel noted that the reduction in mean body weight gain was observed during the first 9 days of treatment (days 6 to 15 of gestation) in the 100 and 500 mg/kg bw/day groups compared with the

vehicle control group and that this effect attained statistical significance only between days 6 and 11 of gestation. As a consequence, net mean body weight gain during the treatment period (days 6 to 20 of gestation) was slightly but not statistically significantly lower in these groups. The Panel therefore considered the slight reduction in mean body weight and food intake not as adverse and concluded that the NOAEL of this study was 500 mg/kg bw/day.

Long term carcinogenicity studies on β-apo-8'-carotenal were made available to the Panel upon a public call for data and were performed well before the introduction of (OECD) GLP guidelines. A summary of these chronic toxicity studies with β-apo-8'-carotenal was also provided (Schärer and Studer 1961b). β-Apo-8'-carotenal was administered at 0.1% in the diet to the first generation of Wistar rats and their offspring (second generation) for a period of 2 years. The average intake of β-apo-8'-carotenal was about 40 mg/kg bw/day.

Histopathological examination of sections stained for fat revealed golden-yellow to yellow-brown, iron-free pigment granules in the liver and kidneys of treated animals which were absent in the controls. The interstitial changes in the kidneys as well as the tubular distensions and calcium incrustations at the medulla cortex margin were less marked in the treated animals than in the controls. The other organs (lungs, heart, spleen, bone marrow, stomach, duodenum, jejumen, pancreas, ovaries, skeletal musculature, thyroid and skin) did not reveal any differences when examined microscopically. Degenerative changes in the epithelium of the seminiferous tubules were slightly, but not statistically significantly more frequent (p=0.17) in the animals treated with β-apo-8'-carotenal than in the controls.

The spontaneous tumours were distributed evenly over both groups (11/147 treated rats and 10/122 untreated rats) and consequently the authors concluded that their occurrence was not connected with the application of β-apo-8'-carotenal.

The reproductive performance and the average number of animals per litter were approximately the same in the treated and untreated rats.

Based on these results the Panel considered that there were no adverse effects in this chronic toxicity study at the single dose level tested amounting to about 40 mg/kg bw/day. Overall, the Panel concluded that the present database on β-apo-8'-carotenal provides a basis to revise the ADI of 5 mg/kg bw/day.

The Panel concluded that based on the LOAEL of 10 mg/kg bw/day from the 13 week study in rats showing an increased incidence of eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and an uncertainty factor of 200, an ADI for β-apo-8'-carotenal of 0.05 mg/kg bw/day can be established. The Panel considered an uncertainty factor of 200 to derive the ADI from the LOAEL sufficient, given the fact that the increase in the eosinophilic droplets in the kidneys at the LOAEL was minimal.

The Panel considered whether this carotenoid could be included in a group ADI with β-carotene.

However, since the re-evaluation of β-carotene concluded that no ADI for β-carotene could be established the Panel concluded that a group ADI including β-apo-8'-carotenal and β-carotene cannot be established.

Exposure estimates based on Tier 2 using maximum permitted levels would result in exposures to β-apo-8´-carotenal of 0.9 mg/kg bw/day for adults on average and of 3.3 mg/kg bw/day at the 95th percentile. For children, Tier 2 estimates would result in exposures to the colour in the range of 0.5-3.4 mg/kg bw/day on average and in the range of 1.2-7.2 mg/kg bw/day at the 95th/97.5th percentile.

It was indicated by food industry however that the colour is relatively rarely used due to its comparability to β-carotene. Based on this information and the maximum reported use levels provided, Tier 3 estimates lead to exposure to β-apo-8´-carotenal of adults of 0.05 mg/kg bw/day on average and of 0.19 mg/kg bw/day at the 97.5th percentile with non-alcoholic flavoured drinks being the main contributor (92%). Exposure estimates at Tier 3 for children were calculated across European countries in the range of 0.02-0.22 mg/kg bw/day on average and in the range of 0.09-0.71 at the

95th/97.5th percentile. Main contributors for children´s exposure to β-apo-8´-carotenal were non-alcoholic flavoured drinks (50-91%) and fine bakery wares (11-50%).

Exposure estimates at Tier 3 even at the reported maximum use levels in only few food categories would lead to exposure of adults on average at the level of the ADI of 0.05 mg/kg bw and to exceedance of the ADI by adults at the 95th percentile and by children both on average and at the 95th/97-5th percentile for all European countries.

CONCLUSIONS

β-Apo-8'-carotenal (E 160e) is authorised as a food additive in the EU and has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1974 and the EU Scientific Committee for Food (SCF) in 1975 and 2000. Both committees have established an ADI of 0-5 mg/kg bw/day, which was withdrawn by SCF in 2000.

The Panel concluded that based on the LOAEL of 10 mg/kg bw/day from the 13 week study in rats showing an increased incidence of eosinophilic droplets in the kidneys of both sexes at 10 mg β-apo-8'-carotenal active ingredient/kg bw/day and an uncertainty factor of 200, an ADI for β-apo-8'-carotenal of 0.05 mg/kg bw/day can be established. The Panel considered an uncertainty factor of 200 to derive the ADI from the LOAEL sufficient, given the fact that the increase in the eosinophilic droplets in the kidneys at the LOAEL was minimal.

The Panel concluded that a group ADI including β-apo-8'-carotenal and β-carotene cannot be established.

Exposure estimates for β-apo-8'-carotenal at Tier 2 using maximum permitted levels in all food categories where the use of the additive is authorised would lead to exposures of both adults and children exceeding the ADI of 0.05 mg/kg bw at average level and at the 95th/97.5th percentile.

Exposure estimates at Tier 3 even at the reported maximum use levels in only few food categories would lead to exposure of adults on average at the level of the ADI of 0.05 mg/kg bw and to exceedance of the ADI of adults at the 95th percentile and of children both on average and at the 95th/97-5th percentile for all European countries.

The Panel noted that the specifications should be updated to more clearly define the purity of the material. The Panel noted that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$ 10 mg/kg.

The Panel noted that specifications should be extended to include maximum residue limits for residual solvents.

DOCUMENTATION PROVIDED TO EFSA

1. Pre-evaluation document prepared by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.
2. Bächthold, 1972. Prüfung oral in 5-tage-Toxizitätsversuch an Ratten. 26 December 1972 (B-0004434/101648). DSM Nutritional Products.
3. Bächthold, 1973. Akute Toxizitätsversuche mit Apocarotinal und dessen Vorstufen. 26 Februar 1973 (B-0004531/101709). DSM Nutritional Products.
4. Bächthold, 1975. 5-Tage Toxizitätsversuch an Ratten. 10 November 1975 (B-0006297/109892). DSM Nutritional Products.
5. Bächthold, 1976. 5-Tage Toxizitätsversuch an Ratten oral und intraperitonäal. 12. April 1976 (B-0006545/102751). DSM Nutritional Products.
6. Bächthold, 1977. Akute Toxizitätsversuche mit Apocarotinal und einige Vorstufen einer neuen Synthese. 31. Oktober 1977 (B-0007513/81083). DSM Nutritional Products.
7. Bächthold, 1980. Acute Toxicity of Apocarotenal and some intermediates of the synthesis. 14. July 1980 (B-0090297/90297). DSM Nutritional Products.
8. Bagdon RE, 1964. 13-week rat toxicity studies on β-apo-8'-carotenal and degraded β-apo-8'-carotenal (RCR 16519) – DSM Nutritional Products.
9. Bagdon RE, 1961. The Toxicity and Metabolism Studies of β-Apo-8`-Carotenal in Dogs, 14. März 1961 (RCR 14674) – DSM Nutritional Products.
10. BASF. 1972. Prüfung der akuten Toxizität von C30-Aldehyd an der Ratte, report from 30.08.1972.
11. BASF, 1998. *Salmonella typhimurium*/*Escherichia coli* reverse mutation assay (standard plate test) with C30-Aldehyd, Project No.: 40M0306/974142.
12. BASF, 1998. Primary skin irritation study with C30-aldehyd in rabbits, Project No.: 18H0306/979040.
13. Edwards J, Evers R, Perry C, Shearer J, Schierle J, Decker-Ramanzina N, 2007. 13-week toxicity study incorporating neurotoxicity screen in rats with administration by the diet with a 4 week recovery period (2007) Charles River Laboratories Study No: 457761 – DSM Nutritional Products.
14. Loget O and Morgan G, 2006. Apocarotenal 10% WS/N 4 week toxicity study in rats administration by diet. 5th April 2006 (2500226) DSM Nutritional Products.
15. Loget O and Arcelin G, 2006a Apocarotenal 10% WS/N: Acute Oral Toxicity Study in Rats. 29. March 2006 (2500228) DSM Nutritional Products.
16. Loget O and Arcelin G, 2006b Apocarotenal crystalline: Acute Oral Toxicity Study in Rats. 16 June 2006 (2500274) DSM Nutritional Products.
17. Loget O and Johnson M, 2006. Apocarotenal 10% WS/N: reverse mutation in five histidine-requiring strains of *Salmonella typhimurium* 29 March 2006 (RDR 2500227). DSM Nutritional Products.
18. Loget O and Whitwell J, 2006. Apocarotenal + Crotetindial: Induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. 7 July 2006 (RDR 2500278). DSM Nutritional Products.
19. Loget O and Beevers C, 2006. Apocarotenal 10% WS/N: Induction of micronuclei in the bone marrow of treated rats. 25 July 2006 (2500309). DSM Nutritional Products.

20. Loget O, Schierle J, Goessl R, and Marsden E, 2006 Apocarotenal 10% WS/N – dose range-finding study by the oral route (dietary admixture) in the pregnant rat (Segment II). 29 March 2006 (RDR 2500025) – DSM Nutritional Products.

21. Loget O and Marsden E, 2006, Apocarotenal 10% WS/N - Developmental toxicity study by the oral route (dietary admixture) in the rat (Segment II) 23 August 2006 (2500209) – DSM Nutritional Products.

22. NATCOL contribution concerning the intake of β-Apo-8'-carotenal, 8 April 2011.

23. NATCOL contribution concerning the manufacturing process of β-Apo-8'-carotenal, 31 March 2011.

24. Perry C and Shearer J, 2008. 13-week toxicity study incorporating neurotoxicity screen in rats with administration by the diet with a 4 week recovery period. Report Amendment 1. Charles River Laboratories Study No: 457761. (Report Number 26909 DSM Report Number 2500412.) DSM Nutritional Products.

25. Rümbeli R, Ringenbach F and Elste V, 2007. Plasma Kinetics and Characterization of Metabolites after Single Oral Administration of 14C-b-Apocarotenal as a Simulated Beadlet Formulation to Male Rats Unpublished report 6 March 2007(RDR 2500426) – DSM Nutritional Products.

26. Schärer K, Studer F and Thommen H, 1961. β-Apo-8'-carotenal and β-apo-8'-carotinic acid (C30) methylester. Comparative subacute toxicity in administration of very high daily doses. 14 November 1961 (RCR 32352, Translation)/DSM Nutritional Products.

27. Schärer, 1960. Stand der chronischen Verträglichkeitsprüfung bei oraler Verabreichung an Ratten, 27. Januar 1960 (RCR 52080) – DSM Nutritional Products

28. Schärer, 1961. Chronische Verträglichkeit bei einjähriger Verabreichung an Ratten, 17. März 1961 (RCR77259 / 32345) – DSM Nutritional Products.

29. Schärer and Studer, 1961a. Zusammenfassender Rapport über die Chronische Verträglichkeitsversuche an Ratten, 12. Januar 1961 (RCR 32342) – DSM Nutritional Products

30. Schärer and Studer, 1961 b. Report Summarizing Chronic Toxicity Studies in Rats, 12. January 1961 (RCR 32342, Translation) – DSM Nutritional Products.

31. Schärer and Studer, 1961c, Fertilität männlicher Ratten bei langedauernder Verabreichung von grossen Dosen des Präp. 4-1259, 17. November 1961 (RCR 32353) – DSM Nutritional Products.

32. Schärer and Studer, 1961d. Fertility of male rats during prolonged administration of large doses of Ro. 4-1259, November 1961 (RCR 32353) – DSM Nutritional Products.

33. Schärer and Studer, 1961e. Chronic Toxicity Study of Ro 4-1259 Administered as Dietary Supplement in Concentrations of 0.2% and 0.5% (for 69 weeks), 26. Oktober 1961 (RCR 32350, Translation) – DSM Nutritional Products.

34. Schärer and Studer, 1962a. Chronische Verträglichkeitsversuche mit β-Apo-8`-Carotenal, 22. November 1962 (RCR 32370) – DSM Nutritional Products

35. Schärer and Studer, 1962b. Fertilität männlicher Ratten und allgemeiner Versuchsverlauf bei 34-wöchiger Verabreichung von 100 und 500 mg/kg/Arbeitstag der Präp. 4-1259, 4-2000 und β-Carotin, 28. Februar 1962 (RCR 32357) – DSM Nutritional Products.

36. Schärer und Studer, 1962c, Untersuchungen über die Ursachen in den chronischen Verträglichkeitsversuchen mit Präp. 4-1259 und 4-2000 vermehrt beobachteten Hodenschädigungen, 7. März 1962 (RCR 32358) – DSM Nutritional Products.

37. Schärer, 1963, Fertilität männlicher Ratten bei 1% iger von Ro 4-1259, Ro 4-2000 und Ro 4-2463 in einer Vit. E-reichen Diät, 22. Juli 1963 (RCR 32379) – DSM Nutritional Products.

38. Schärer, 1965. Fertilität und Hodenbefunde nach 1 ½ jähriger Verabreichung von RO 4-1259, Ro 4-2000 und Ro 2463 in einer Vit. E-angereicherten Diät an Ratten, 23. Dezember 1965 (RCR 68114) – DSM Nutritional Products.

REFERENCES

Anonymous, 1966. Hoffmann-La Roche, unpublished report submitted to WHO.

Anonymous, 1962. Hoffmann-La Roche, unpublished report submitted to WHO.

Alija AJ, Bresgen N, Sommerburg O, Langhans CD, Siems W and Eckl PM, 2005. Cyto- and genotoxic potential of β-carotene and cleavage products under oxidative stress. Biofactors 24(1-4), 159-163.

Alija AJ, Bresgen N, Sommerburg O, Siems W and Eckl PM, 2004. Cytotoxic and genotoxic effects of β-carotene breakdown products on primary rat hepatocytes. Carcinogenesis 25(5), 827-831.

Alija AJ, Bresgen N, Sommerburg O, Langhans CD, Siems W and Eckl PM, 2006. β-Carotene breakdown products enhance genotoxic effects of oxidative stress in primary rat hepatocytes. Carcinogenesis 27, 1128-1133.

Astorg P, Gradelet S, Leclerc J and Siess M-H, 1997. Effects of provitamin A or non-provitamin A on liver xenobiotic-metabolising enzymes in mice. Nutrition and Cancer 27, 245-249 (as referred to by SCF, 2000).

Astorg P, Gradelet S, Leclerc J, Canivenc M-C and Siess M-H, 1994. Effects of ß-carotene and canthaxanthin on liver xenobiotic-metabolising enzymes in the rat. Food and Chemical Toxicology 32, 735-742 (as referred to by SCF, 2000).

ATBC Study group (The Alpha-Tocopherol, β-carotene Cancer Prevention Study Group), 1994. The effects of vitamin E and β-carotene on the incidence of lung cancer and other cancers in male smokers. New England Journal of Medicine 330, 1029-1035.

Azuine MA, Goswami UC, Kayal JJ and Bhide SV, 1992. Antimutagenic and anticarcinogenic effects of carotenoids and dietary palm oil. Nutrition and Cancer 17, 287-295

Bagdon RE, Impellizzeri C and Osadca M, 1962. Studies on the toxicity and metabolism of β-apo-8'-carotenal in dogs. Toxicol. Appl. Pharmacol. 4, 444-456 (as referred to by JECFA, 1975).

Bagdon RE, Zbinden G and Studer A, 1960. Chronic toxicity studies of β-carotene. Toxicology and Applied Pharmacology 2, 225-236.

BIBRA, 1996. The British Industrial Biological Research Association working group. Toxicity profiles, β-apo-8'-carotenal, ethyl-β-apo-8'-carotenal, ethyl-β-apo-8'-carotenoate and methyl β-apo-8'-carotenoate (as referred to by TemaNord, 2002).

Brubacher G, Gloor U and Wiss O, 1960. Zum Stoffwechsel von β-Apo-8'-carotenal (C_{30}) Chimia 14, 19-20 (as referred to by JECFA, 1975).

EFSA 2009 Safety of use of colouring agents in animal nutrition1 Part III: β-apo-8' carotenal, ethyl ester of β-apo-8'-carotenoic acid, lutein, zeaxanthin and concluding remarks Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (Question No EFSA-Q-2003-060) Adopted on 12 May 2009. The EFSA Journal (2009) 1098, 1-48.

European Commission, 1997. Food Science and Techniques. Reports on Tasks for Scientific Cooperation (SCOOP). Report of Experts participating in task 4.2. Report on the Methodologies for the Monitoring of Food Additive Intake across the European Union. Directorate General Industry. December 1997.

Finch S, 1998. National diet and nutrition survey people aged 65 years and over. The Stationery Office. ISBN: 0112430198 (as referred to by Tennant, 2007).

Glover J, 1960. The conversion of β-carotene into vitamin A. Vitam. Horm. 18, 371-386 (as referred to by JECFA, 1975).

Gradelet S, Leclerc J, Siess M-H and Astorg PO, 1996. β-Apo-8'-carotenal, but not β-carotene, is a strong inducer of liver cytochromes P4501A1 and 1A2 in rat. Xenobiotica 26, 909-919.

Gregory J, 2000. National Diet and Nutrition Survey: young people aged 4 to 18 years. Findings: Volume 1. London: The Stationery Office.

Gregory JR, Collins DL, Davies PSW, Hughes JM and Clarke PC, 1995. National Diet and Nutrition Survey; Children aged 1.5 to 4.5 years. HMSO, London.

Huybrechts I, Sioen I, Boon PE, Ruprich J, Lafay L, Turrini A, Amiano P, Hirvonen T, De Neve M, Arcella D, Moschandreas J, Westerlund A, Ribas-Barba L, Hilbig A, Papoutsou S, Christensen T, Oltarzewski M, Virtanen S, Rehurkova I, Azpiri M, Sette S, Kersting M, Walkiewicz A, Serra-Majem L, Volatier JL, Trolle E, Tornaritis M, Busk L, Kafatos A, Fabiansson S, De Henauw S and Van Klaveren J, 2011. Dietary Exposure Assessments for Children in Europe (the EXPOCHI 159 project): rationale, methods and design. Archives of Public Health. Archives of Public Health, 69-4.

Ishidate M, Harnois MC and Sofuni T, 1988. A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. Mutation Research 195, 151-213.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2011. Prepared at the 74th JECFA and published in FAO Monographs 11.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1975. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. 18th Report. WHO Food Additives Series, 6.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1974. Toxicological evaluation of some food colours, thickening agents, and certain other substances. 18th Report. WHO Technical Report Series, 1974, No. 557. FAO Nutrition Meetings Report Series, 1974, No. 54.

Kalariya NM, Ramana KV, Srivastava SK, van Kuijk FJ, 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Experimental Eye Research 34(9), 737-747.

Kalariya NM, Ramana KV, Srivastava SK and van Kujik FJ, 2008. Carotenoid derived aldehydes-induced oxidative stress causes apoptotic cell death in human retinal pigment epithelial cells. Experimental Eye Research 86(1), 70-80.

Kübler W, 1963. Wiss. Veröff. Dtsch. Gesellsch. Ernährung. 9, 222 (as referred to by JECFA, 1974b).

Langouët S, Mican AN, Müller M, Fink SP, Marnett JL, Muhle SA and Guengerich FP, 1998. Misincorporation of nucleotides opposite five-membered exocyclic ring guanine derivatives by *Escherichia coli* polymerases in vitro and in vivo: 1,N-2-ethenoguanine, 5,6,7,9-tetrahydro-9-oxoimidazo[1,2-a]purine, and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-a]purine. Biochemistry 37, 5184-5193 (as referred to by Marques et al., 2004).

Marques SA, Loureiro AP, Gomes OF, Garcia CC, Di Mascio P and Medeiros MH, 2004. Induction of 1,N^2-etheno-2'-deoxyguanosine in DNA exposed to β-carotene oxidation products. FEBS Letters 560 (1-3), 125-130.

Minguez-Mosquera M, Hornero-Mendez D and Garrido-Fernandez J, 1995. Detection of bixin, lycopene, canthaxanthin and beta-apo-8'carotenal in products derived from red pepper. Journal of AOAC International 78, 491-496.

Oliver J and Palou A, 2000. Chromatographic determination of carotenoids in foods. Journal of Chromatography A 881, 543-555.

Rauscher R, Edenharder R and Platt KL, 1998. In vitro antimutagenic and in vivo anticlastogenic effects of carotenoids and solvent extracts from fruits and vegetables rich in carotenoids. Mutation Research 413, 129-142.

SCF (Scientific Committee for Food), 2000. Reports of the Scientific Committee for Food. Opinion on the safety of use of β-carotene from all dietary sources. Report no. SCF/CS/ADD/COL/159 Final Opinion expressed in 2000.

SCF (Scientific Committee for Food), 1975. Reports from the Scientific Committee for Food (1st series), opinion expressed in 1975, pp. 17-23.

Scotter MJ and Castle L, 2004. Chemical interactions between additives in foodstuffs: a review. Food Additives & Contaminants 21(2), 93-124.

Scotter M, Castle L, Croucher J and Olivier L, 2003. Method development and analysis of retail foods and beverages for carotenoid food colouring materials E160a and E160e. 23, 115-126.

TemaNord, 2002. Food additives in Europe 2000; Status of safety assessments of food additives presently permitted in the EU. TemaNord 560, 184-187.

Tennant DR, 2008. Screening potential intakes of colour additives used in non-alcoholic beverages, Food and Chemical Toxicology 46, 1985-1993. ,

Tennant D, 2007. Screening potential intakes of natural food colours. Report provided for the Natural Food Colours Association, NATCOL. July, 38 pp.

Tennant D, 2006. Screening of colour intakes from non-alcoholic beverages. Report prepared for the Union of European beverages associations UNESDA. December, 57 pp.

Thommen H, 1962. Die Naturwissenschafen 22, 517-518.

Williams GM and Iatropoulos MJ, 2002. Alteration of Liver Cell Function and Proliferation: Differentiation Between Adaptation and Toxicity, Toxicologic Pathology, 30, 41–53.

Wiss O and Thommen H, 1963, Deutsche Gesellschaft für Ernährung, 9, 179.

Wood JD, 1963. The hypocholesterolemic activity of ss-apo-8'-carotenal. Canadian Journal of Biochemistry. 41, 1663-1665.

Yeh SL and Wu SH, 2006. Effects of quercetin on β-apo-8'-carotenal-induced DNA damage and cytochrome P1A2 expression in A549 cells. Chemico-Biological Interactions 163(3), 199-206.

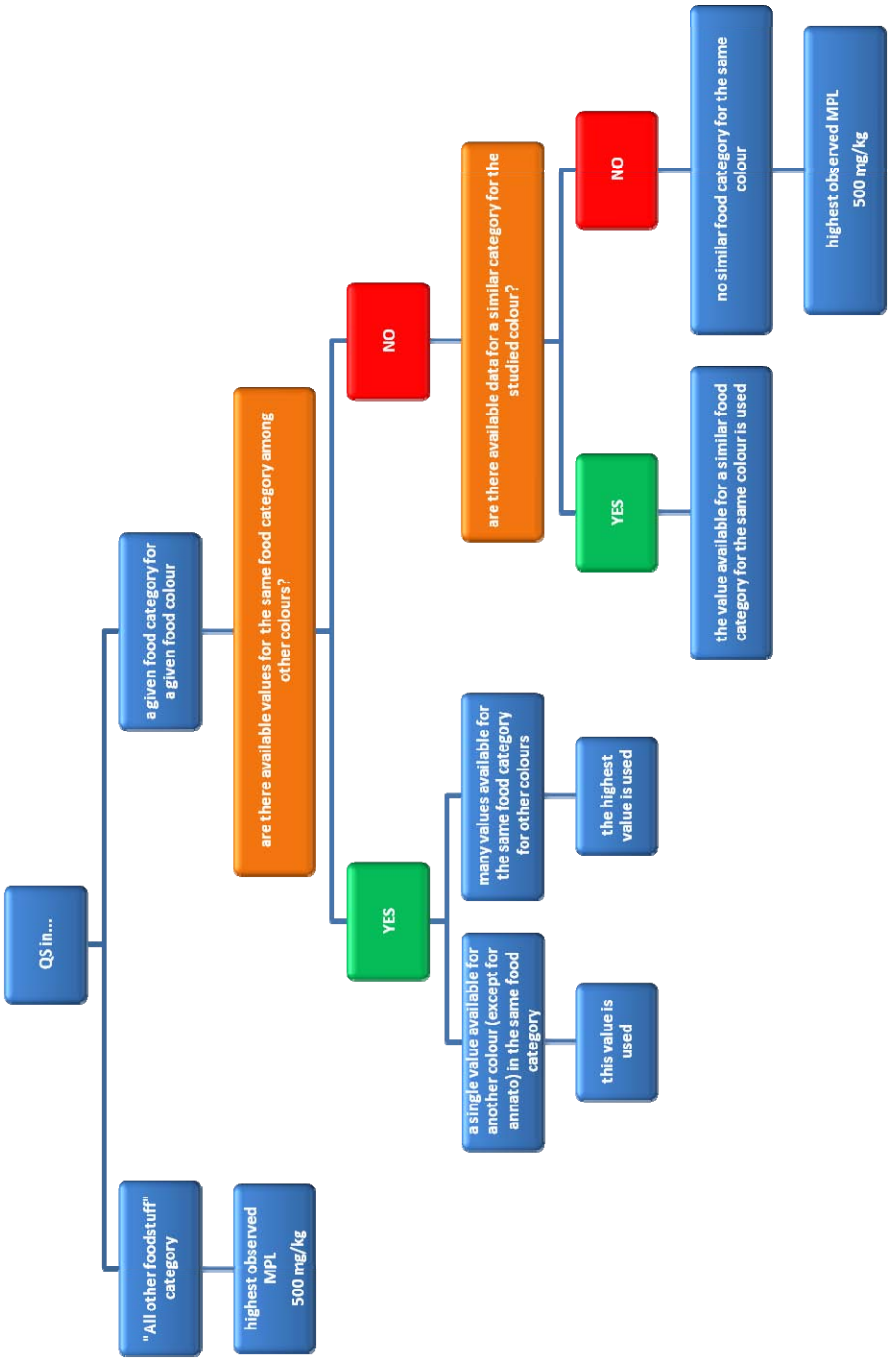
Zeng S, Furr HC and Olson JA, 1992. Metabolism of carotenoid analogs in humans. The American Journal of Clinical Nutrition, 56, 433-439.

ANNEX A

RULES DEFINED BY THE PANEL TO DEAL WITH QUANTUM SATIS (QS) AUTHORISATION, USAGE DATA OR OBSERVED ANALYTICAL DATA FOR ALL REGULATED FOOD ADDITIVES TO BE RE-EVALUATED

Figure 1: Rules defined by the Panel to deal with usage data or observed analytical data for all regulated food additives to be re-evaluated and procedures for estimating intakes using these rules.

Rules defined by the Panel to deal with *quantum satis* (QS) authorisation.



GLOSSARY / ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism and Excretion
ALT	Alanine Transaminas
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
AST	Aspartate Transaminase
BMD	Benchmark Dose
CA	Chromosomal aberration
CAS	Chemical Abstracts Service
CEN	Committee for Standardization
CHO	Chinese Hamster Ovary
CP	Cleavage Product
CPA	Cyclophosphamide
CRL	European Union Reference Laboratory for GM Food and Feed formerly known as Community Reference Laboratory
DMNQ	2,3-dimethoxy-1,4-naphtoquinone
DNA	Deoxyribonucleic acid
EC	European Commission
EU	European Union
EFSA	European Food Safety Authority
EINECS	Existing Commercial chemical Substances
EU	European Union
EXPOCHI	Individual food consumption data and exposure assessment studies for children
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practise
GSH	Glutathione
HPLC	High-performance liquid chromatography
ISO	International Organization for Standardization
IU	International Units
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50% i.e. dose that causes death among 50% of treated animals
LOAEL	Lowest-Observed-Adverse-Effect Level
LOD	Limit of Detection
LOQ	Limit of Quantification
MN	Micronuclei

MNH	Multinucleated Hepatocytes
MPL	Maximum Permitted Level
NCE	Normochromatic Erythrocytes
NDNS	UK National Dietary and Nutrition Survey
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
SCE	Chromatid Exchange
SCF	Scientific Committee on Food
SCOOP	A scientific cooperation (SCOOP) task involves coordination amongst Member States to provide pooled data from across the EU on particular issues of concern regarding food safety
TLC	Thin Layer Chromatography
US EPA	US Environmental Protection Agency
UV	Ultraviolet
WHO/FAO	World Health Organization/Food and Agriculture Organization

European Food Safety Authority

EFSA Journal 2012; 10(3):2593

SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of mixed carotenes (E 160a (i)) and beta-carotene (E 160a (ii)) as a food additive[1]

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Additives and Nutrient Sources added to Food provides a scientific opinion re-evaluating the safety of mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] when used as food colouring substances. Mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] are authorised as food additives in the EU and have been evaluated previously by the JECFA the latest in 2001 and by the SCF in 1997 and 2000. Both Committees established an Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day. In this opinion the mixed carotenes are defined according to the Commission Directive 2008/128/EC and consist of two groups of substances: plant carotenes and algal carotenes. β-Carotene comprises (synthetic) β-carotene and β-carotene obtained by fermentation of the fungus *Blakeslea trispora.* The Panel noted (i) that the specifications of mixed carotenes are inadequate and need to be updated, (ii) that most toxicological studies have been performed with rodents, although rodents, in contrast to humans, very efficiently convert β-carotene to vitamin A. The Panel concluded that based on the presently available dataset, no ADIs for mixed carotenes and β-carotene can be established and that the use of (synthetic) β-carotene and mixed β-carotenes obtained from palm fruit oil, carrots and algae as food colour is not of safety concern, provided the intake from this use as a food additive and as food supplement, is not more than the amount likely to be ingested from the regular consumption of the foods in which they occur naturally (5-10 mg/day). This would ascertain that the exposure to β-carotene from these uses would remain below 15 mg/day, the level of supplemental intake of β-carotene for which epidemiological studies did not reveal any increased cancer risk. Furthermore, the Panel could not conclude on the safety in use of mixed carotenes [E 160a (i)]

KEY WORDS

Mixed carotenes, plant/vegetable β-carotene, algal β-carotene, synthetic β-carotene, β-carotene derived from *Blakeslea trispora*, E 160a (i), E 160a (ii), CAS Registry Number 7235-40-7, food colour.

---

[1] On request from the European Commission, Question Nos EFSA-Q-2011-00354 and EFSA-Q-2011-00431 adopted on 16 February 2012.

[2] Panel members: F. Aguilar, R. Crebelli, B. Dusemund, P. Galtier, J. Gilbert, D.M. Gott, U. Gundert-Remy, J. König, C. Lambré, J-C. Leblanc, A. Mortensen, P. Mosesso, D. Parent-Massin, I.M.C.M. Rietjens, I. Stankovic, P. Tobback, D. H Waalkens-Berendsen, R.A. Woutersen, M. C. Wright. Correspondence: ans@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank the members of the Working Group B on Food Additives and Nutrient Sources added to Food: F. Aguilar, R. M. Bakker, Crebelli, B. Dusemund, J. Gilbert, D. Gott, T. Hallas-Møller, J. König, D. Marzin, I. Meyland, A. Mortensen, I. Pratt, D. H. Waalkens-Berendsen and R. A. Woutersen for the preparatory work on this scientific opinion.

Suggested citation: EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Scientific Opinion on the re-evaluation of Mixed Carotenes (E 160a (i)) and beta-Carotene (E 160a (ii)) as a food additive. EFSA Journal 2012;10(3):2593. [67 pp.] doi:10.2903/j.efsa.2012.2593. Available online: www.efsa.europa.eu/efsajournal.htm

SUMMARY

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion re-evaluating the safety of mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] when used as food colour.

Mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] are authorised as food additives in the European Union (EU) and have been evaluated previously by the Joint FAO/WHO/Expert Committee on Food Additives (JECFA) in 1975 (β-carotene), 1993 (carotenes from natural sources) and 2001 (β-carotene derived from *Blakeslea trispora*), and by the EU Scientific Committee for Food (SCF) in the EU in 1975, 1997 (β-carotene derived from *Dunaliella salina*) and 2000 (β-carotene derived from *Blakeslea trispora*). The SCF and JECFA both established an Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day.

JECFA defined the ADI for the sum of the carotenoids β-apo-8'-carotenal, β-carotene, β-carotenoic acid methyl ester and β-carotenoic acid ethyl ester. However, in 2000 the SCF withdrew the entire group ADI based on a new evaluation of β-carotene. The SCF decided that there was insufficient scientific basis, either from human or experimental studies, to set a new ADI for β-carotene and related carotenoids, but was nonetheless of the opinion that currently permitted food additive uses of β-carotene and related carotenoids are temporarily acceptable from a health point of view at the estimated levels of intake.

According to the rough intake estimate by the SCF, intake of β-carotene and related carotenoids as additives is about 1-2 mg/person/day, in addition to an average of 2-5 mg/person/day consumed through natural food sources. The total intake was consequently considered to be 3-7 mg/person/day or up to 10 mg/person/day depending on seasonal and regional variations.

If indeed 10 mg/person/day were the maximum level of intake, daily intake would be about 167 µg/kg bw/day for a 60 kg adult and 667 µg/kg bw/day for a 15 kg child. Based on these data, it may be concluded that the group ADI, as set by JECFA, is unlikely to be exceeded.

In this opinion the mixed carotenes [E 160a (i)] are defined according to the Commission Directive 2008/128/EC and consist of two groups of substances: plant carotenes and algal carotenes. Plant carotenes are substances obtained by solvent extraction from natural strains of edible plants such as carrots and palm fruit oils. The Panel considered edible plants as materials of plant origin for which it can be sufficiently demonstrated that they hitherto have been consumed by humans. Further, the Panel noted, based on information obtained from the Natural Food Colours Association (NATCOL), that plant carotenes obtained from other plants such as grass, alfalfa (lucerne) and nettle are presently not commercially used to obtain mixed carotenes. The main colouring principle consists of carotenoids, of which β-carotene accounts for the major part. α-, γ-Carotene and other pigments may be present. Besides the colour pigments, the substance may contain oils, fats and waxes naturally occurring in the source material. The specification as defined by the Commission Directive 2008/128/EC lay down that the content of carotene calculated as β-carotene should not be less than 5% in mixed carotenes from edible plants. Mixed carotenes from natural strains of algae should contain not less than 20% carotenes (calculated as β-carotene).

The Panel noted that specifications on the purity of mixed carotenes define only 20% of the commercial material as carotenes. The actual content of β-carotene is not specified. A full quantitative and qualitative characterisation of mixed carotenes from carrots, palm fruit oils and algae is not available. Therefore, the Panel noted that the specifications for mixed carotenes are inadequate and need to be updated to define the amount of food colour and the material not accounted for.

β-Carotene [E 160a (ii)] comprises synthetic β-carotene and β-carotene obtained by a fermentation process using a culture of the fungus *Blakeslea trispora*. The specification as defined by the Commission Directive 2008/128/EC for synthetic β-carotene and β-carotene obtained from *Blakeslea*

trispora define that these substances predominantly consist of the all *trans* isomer of β-carotene with an assay of not less than 96% total colouring matters (expressed as beta-carotene).

The Panel noted that synthetic β-carotene and β-carotene from *Blakeslea trispora* are highly pure, crystalline substances of one chemical entity, β-carotene, predominantly composed of all-*trans*-β-carotene. The assay value of not less than 96% (expressed as β-carotene) is linked to the method given in specifications for the determination of the purity.

The Panel noted that the extent of absorption of β-carotene in man reported in the literature varies between 10 and 90%, and inter- and intra-individual variability in apparent absorption is rather high. In man, high concentrations of β-carotene have been found in the adrenals and testes, but the major storage sites for carotenoids are the liver and adipose tissue. In man, roughly 20-75% of β-carotene is absorbed intact. In mammalian tissues, β-carotene is cleaved mainly at the central double bond, yielding two molecules of retinal, which is further reduced to retinol or further oxidized to retinoic acid. A wide range of (intestinal) cleavage rates have been demonstrated among species. Rats, mice, hamsters and rabbits have very active dioxygenase enzymes converting β-carotene to retinal in the intestine and as a consequence have (very) low serum β-carotene levels. Based on the kinetics of β-carotene, ferrets, the pre-ruminant calf, and the Mongolian gerbil, have been proposed as more useful test species, since particularly ferrets mimic the absorption and distribution of β-carotene in humans. Furthermore, supplementation of ferrets feed with β-carotene has been shown to increase β-carotene concentrations in serum, liver, adipose, and other tissues relevant for comparison with humans.

The Panel noted that although the data on kinetics indicate that rodents, in contrast to humans, very efficiently convert β-carotene to vitamin A, most toxicological studies have been performed with rats and mice. Therefore, the Panel concluded that the relevance of these studies for humans may be questionable since rodents appear to be no suitable models for evaluating the bioavailability and effects of β-carotene in human.

Furthermore, the Panel noted that mixed carotenes [E 160a (i)] obtained from algae (*Dunaliella salina, or Dunaliella bardawil*) using edible oil (soy bean oil or olive oil), natural mixed carotene complexes prepared by solvent extraction from palm fruit oil (*Elaeis guineensis*) or from carrots (*Daucus carota*) are commercially available, but toxicological studies have only been performed with mixed carotenes obtained from algae.

No acute oral toxicity of (synthetic) β-carotene [E 160a (ii)] was observed at relatively high doses.

A 90 day feeding study in F344 rats revealed a No-Observed-Adverse-Effect Level (NOAEL) of 696 mg/kg bw/day for males and 2879 mg/kg bw/day for females based on growth suppression.

In rats given synthetic β-carotene at dose levels of 0, 250, 500, or 1000 mg/kg bw/day for 90 days, the only effect observed was discolouration of the liver and/or adipose tissue in some females of all dose groups. This effect disappeared after a non-treatment period. No treatment-related abnormalities were seen regarding clinical signs, haematology, clinical chemistry, organ weights, or gross and histopathological examination.

In a study with ferrets fed synthetic β-carotene at doses of 0.16 or 2.4 mg/kg bw/day for six months, increases in the concentration of β-carotene in both plasma (up to 21-fold) and lung tissue (up to 300-fold) were found. All animals fed the high dose of β-carotene showed localised proliferation of alveolar cells (type II pneumocytes), alveolar macrophages, and keratinised squamous metaplasia of alveolar wall epithelium.

The Panel considered none of the effects observed in the reproductive and developmental studies to be of toxicological significance. The four-generation rat study, in which no adverse effects were noted up

to 50 mg/kg bw/day (ß-carotene 96% purity), was used for calculation of the JECFA group ADI. In more recent studies, higher doses were used that did not cause adverse effects.

In vitro genotoxicity data are available for mixed (algal) carotenes (*Dunaliella bardawil)* and β-carotene derived from *Blakeslea trispora*. No mutagenic effect was observed in bacterial reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli* and in a chromosomal aberration assay in Chinese hamster ovary cells.

Synthetic β-carotene was not mutagenic in the bacterial reverse mutation assay using the *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 in both the absence and the presence of the S9-mix, and did neither induce chromosomal aberrations in vitro nor chromosomal aberrations or micronuclei in mouse bone marrow in vivo.

Negative results for the induction of sister chromatid exchanges, chromosomal aberrations or micronuclei in vitro and in vivo are reported from limited antimutagenicity studies with β-carotene.

A β-carotene cleavage products mixture and β-apo-8'-carotenal were reported to induce increases in Micronuclei (MN), Chromosomal Aberrations (CAs) and Sister Chromatid Exchanges (SCEs) in primary rat hepatocytes. In this respect, however, the Panel noted that there is limited experience with cytogenetic assays in primary rat hepatocytes, which show a very high spontaneous incidence of both MN and CAs. Moreover, the increase in the frequency of MN and CAs observed in presence of β-carotene cleavage products, and of micronuclei in presence of apo-8'-carotenal, were not clearly dose-related over a 10^4-fold concentration range. The statistically significant increases in the frequency of MN were only 20 and 11% (at 0.1 and 1 μM respectively) over control incidence, which was within the range of experimental variation for the end-points studied, and thus has limited or no biological significance. The frequencies of CAs in presence of β-apo-8'-carotenal show an apparent treatment-related increase, but data are unreliable since they are based on only 20 metaphases/culture. The increase in SCEs observed in presence of both β-carotene cleavage products and β-apo-8'-carotenal is more credible, but the biological significance of this indicative assay in relation to genotoxicity is indirect.

The genotoxic effects of the β-carotene breakdown product β-apo-8'-carotenal was also studied in Human Retinal Pigment Epithelial Cells (ARPE-19) using the Comet assay. The results suggested that breakdown products of dietary carotenoids could be genotoxic in ARPE-19 cells. The authors stated that the mechanism of genotoxicity of β-apo-8'-carotenal, i.e. whether direct via DNA damage and apoptosis, or indirect through plasma membrane damage and necrosis, could not be disclosed.

The Panel, however, noted that the β-carotene breakdown products used in these studies where not representative of β-carotene breakdown products under physiological conditions.

The Panel therefore concluded that the genotoxicity studies with β-apo-8'-carotenal, β-carotene and a β-carotene cleavage product in primary rat hepatocytes reported by Alija et al. (2004; 2005; 2006) and in ARPE-19 cells by Kalariya et al. (2009), provided very limited evidence of genotoxicity.

The Panel noted further that in previous evaluations many studies on β-carotene have been described that have focussed on a broad range of genotoxicity endpoints in different in vitro and in vivo test systems. In none of these studies were any signs of genotoxicity noted.

Therefore, the Panel concluded that β-carotene is not of concern with respect to genotoxicity.

The Panel also noted that a few in vitro studies with β-carotene cleavage products provided limited evidence of DNA damaging activity and covalent binding to DNA, and that such results may reflect a pro-oxidant effect, common to other antioxidants, which is elicited under specific in vitro conditions which may not occur in vivo.

In previous evaluations by the SCF some chronic studies with synthetic β-carotene were described in mice, rats, and dogs. The main effects were observed in the livers of mice and dogs. In mice, cells lining the sinusoids of the liver were found to be vacuolated, and in dogs irregular pale-orange foci on the surface of the liver and perisinusoidal fat storage cells were noted. As in dogs no degenerative changes in the liver were obvious, the authors considered the presence of these cells indicative of storage of vitamin A rather than of a toxic effect. In addition to these findings, weight gain reduction was noted in rats from 100 mg/kg bw/day, but this was not accompanied with any sign of organ toxicity.

Three studies in hamsters and two studies in ferrets have been reported in which animals were exposed to a combination of β-carotene and cigarette smoke (constituents). One study in hamsters showed an inhibitory effect of β-carotene on cigarette smoke-induced respiratory tract tumourigenesis. In the other two hamster studies, β-carotene caused increases in overall respiratory tract tumour incidence and preneoplastic and neoplastic changes in the larynx, trachea and lung induced by cigarette smoke (constituents). In ferrets fed β-carotene in the diet, increased cell proliferation and squamous metaplasia of alveolar epithelium was observed which was further enhanced in the animals also exposed to cigarette smoke.

It was found that a high dose of β-carotene (equivalent to 30 mg/day in humans), in contrast to a low dose (equivalent to 6 mg/day in humans), induced alveolar cell proliferation and keratinized squamous metaplasia in the lung tissue of all ferrets with or without smoke exposure. In ferrets given the low-dose of β-carotene alone, no pathological changes were observed. These experiments were performed with an unstable, non-protected synthetic β-carotene, whereas similar experiments performed with a protected (combination with DL-α tocopherol and ascorbic acid) form of synthetic β-carotene did not show any histopathological changes in the lungs of ferrets.

The Panel noted that the limited number of studies performed with hamsters and ferrets demonstrated equivocal results.

The differences observed between the various studies may be related to the different formulations of β-carotene used. Antioxidants have important effects on the stability, metabolism and action of β-carotene. β-Carotene can be converted to retinoids in mammals by two possible pathways: a central cleavage resulting in retinal and also an eccentric cleavage resulting in a number of β-apo-carotenals and carotenoic acids (Fuster et al., 2008). The formation of oxidative eccentric metabolites of β-carotene can occur through an enzymatic mechanism or through a simple oxidative process. Liu et al. (2000) found that production of individual β-apo-carotenals was significantly decreased in the presence of α-tocopherol and ascorbic acid, indicating that the presence of antioxidants could have a stabilizing effect on the unoxidized form of β-carotene, but in their absence, high levels of oxidized cleavage products may appear.

Based on the two human studies on synthetic β-carotene and a combined synthetic β-carotene and retinol supplementation dealing with the relation of β-carotene with cardiovascular disease, it appears that β-carotene supplementation increased the risk of cardiovascular death, especially among men who had a history of myocardial infarction. On the other hand, from another epidemiological study, dietary synthetic β-carotene intake was found to decrease the risk of myocardial infarction in elderly who were free of myocardial infarcts at baseline. The Panel concluded that, based on the limited data available, it is not possible to come to a decisive conclusion on this equivocal issue.

In the past, high serum β-carotene levels have been associated with a decrease in the incidence of cancer, including lung cancer, in humans. However, the Alpha Tocopherol Beta Carotene Prevention Study (ATBC trial) and the Beta CARotene and Retinol Efficacy (CARET) trial unexpectedly revealed that heavy smokers and asbestos workers (CARET only) receiving long-time β-carotene supplementation (ATBC) or β-carotene + retinol supplementation (CARET) at doses well below the previously established group ADI of 5 mg/kg bw/day had increased rather than decreased incidences

of lung cancer. Besides increased lung cancer incidence, increased stomach cancer mortality was seen in subjects receiving β-carotene supplementation in combination with a mixture of vitamins and minerals. It was commented that due to the combined exposure, the effects could not be ascribed to β-carotene only. It should be noted that in the CARET trial combined supplementation of β-carotene and Vitamin A was tested, and therefore the effects cannot be solely attributed to either of these compounds.

Druesne-Pecollo et al. (2010) performed a systematic review and meta-analysis of 9 randomized controlled trials investigating β-carotene supplementation and cancer risk. They found absence of any protective effect associated with β-carotene supplementation with regard to primary cancer risk. However, their results indicated an increased risk of lung and stomach cancers in smokers and asbestos workers supplemented with β-carotene at dose levels equal or above 20 mg/day. The authors, however, noted several significant caveats over the interpretation of their findings.

The negative effects observed in heavy smokers in the ATBC and CARET studies were not seen in any other intervention study, included in an extensive review of the scientific literature published since 2000 (DSM, 2009) Therefore, the authors concluded that the increased lung cancer incidence in β-carotene supplemented smokers is most probably specific to individuals who chronically smoke more than 20 cigarettes per day.

The SCF discussed the unexpected findings of the ATBC and CARET trials and posed several possible explanations for these findings.

One possible explanation for the increased incidence of lung cancer, observed in β-carotene supplemented groups, is that interaction between smoke and β-carotene may have caused the enhanced lung cancer incidence in these trials. β-Carotene may stimulate phase I bioactivating enzymes which may result in an increased formation of genotoxic metabolites of (among others) cigarette smoke constituents. Another theory supposes that the observed β-carotene-enhanced lung tumourigenesis may be caused by interference with normal retinoid signalling through the formation of reactive oxidative cleavage products of β-carotene. In addition to the retinoid pathway, a more recent study provided evidence for cell type-specific growth-stimulating effects of β-carotene on human pulmonary adenocarcinoma cells and their normal cells of origin via the cAMP/PKA-CREB and -ERK pathways. As pulmonary adenocarcinoma is the leading type of lung cancer, the authors suggested that the growth promoting effects of β-carotene on these cancer cells may indeed have contributed to enhance lung tumour incidence in the ATBC and CARET trials. Another hypothesis of how β-carotene may enhance lung tumourigenesis, is by the pro-oxidant activity of oxidative metabolites of β-carotene formed due to a combination of reactive oxygen species derived from tobacco smoke and the large rise in β-carotene concentrations in lung tissue. The free radical atmosphere in the lungs of cigarette smokers enhances β-carotene oxidation and the formation of eccentric cleavage oxidative metabolites. These metabolites might cause diminished retinoid signalling by down-regulating RARβ expression and the retinoic acid level in lung tissue and by up-regulating AP-1 and, therefore, accelerating lung tumourigenesis.

Taking into account the following observations:

i. The limited database on long-term, reproductive and developmental toxicity of mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)],

ii. That most of the available studies were in rodents which did not appear to be suitable models for human risk assessment of β-carotene,

iii. The equivocal results of in vitro genotoxicity studies with β-apo-8'-carotenal (a metabolite of β-carotene) and with a mixture of β-carotene [E 160 a (ii)] cleavage products,

iv. The equivocal results of the long-term studies with β-carotene [E 160a (ii)] in combination with cigarette smoke or its components in hamsters and ferrets,

v. The observations in the epidemiological (ATBC and CARET) studies, indicating that long time β-carotene [E 160a (ii)] supplementation, either with or without retinol, at dose levels of respectively 20 and 30 mg caused an increase in lung cancer incidence in heavy smokers,

the Panel concluded that ADIs for mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] could not be established.

The Panel noted that it was not possible to identify a NOAEL from the non-rodent data using a margin of safety approach. However, the Panel also noted that epidemiological studies reported no increased cancer incidence at supplemental dose levels varying from 6-15 mg/day for about 5 up to 7 years (Druesne-Pecollo et al., 2010).

The Panel further noted that the specifications for mixed carotenes are inadequate and need to be updated to define the amount of food colour and the material not accounted for.

The Panel noted that there is a considerable discrepancy between data on β-carotene usage reported by NATCOL and those originally reported by CIAA in 2009. Following further correspondence with CIAA and NATCOL, the Panel considered the data reported by NATCOL to better reflect the actual situation on use although they do not take into account all food categories. Calculations by the Panel on food consumption data provided for UK adults using the typical use levels of β-carotene as a food additive provided by NATCOL indicate a mean exposure of β-carotene of 0.06 mg/kg bw/day and an exposure at the 97.5th percentile of 0.11 mg/kg bw/day. The same scenarios would result for children in an average exposure in the range of 0.03-0.22 mg/kg bw/day and at the 97.5th percentile in the range of 0.09-0.43 mg/kg bw/day across European Member States.

The Panel also considered the typical exposure to β-carotene from the regular diet as relevant for the assessment. Data has therefore been collected from the European Nutrition and Health Report 2009 (Elmadfa, 2009) which provides intake estimates for β-carotene from 9 European countries (Czech Republic, Denmark, Finland, Germany, Ireland, Italy, Norway, Poland, and Sweden) based on different methods of dietary intake assessment for children aged 4-6 years, and from 13 countries (Austria, Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, Lithuania, Poland, Spain, Sweden, and The United Kingdom) for adults.

If 10 mg/day is considered the maximum level of β-carotene intake from the diet, the total daily intake of β-carotene would be about 0.167 mg/kg bw/day for a 60 kg adult and 0.667 mg/kg bw/day for a 15 kg child. The Panel noted that due to lack of data this exposure assessment did not include the use of colouring food containing β-carotene.

Based hereon the Panel concluded that:

The use of a) mixed carotenes [E 160a (i)] obtained from algae (*Dunaliella salina* or *Dunaliella bardawil*) by extraction with edible oil (soy bean or olive oil) or by solvent (acetone, methanol or hexane) extraction from carrots (*Daucus carota*) or palm fruit oil (*Elaeis guineensis*) and of β-carotene [E 160a (ii)] as food colours are not of safety concern, provided that the estimated combined intake from their use as a food additive and as food supplement, is not more than the amount likely to be ingested as a result of the regular consumption of the foods in which they occur naturally (5-10 mg/day). This would ascertain that the exposure to β-carotene from the use as a food additive and as food supplement would remain below 15 mg/day, the level of supplemental intake of β-carotene for which epidemiological studies did not reveal any increased cancer risk.

The Panel could not conclude on the safety in use of mixed carotenes [E 160a (i)] obtained by solvent extraction from other sources such as e.g. grass, alfalfa/lucerne or nettle in the absence of information on the safety and the quantitative and qualitative characterisation of each of the extracts, including minor components, and current manufacturing methods for each source.

TABLE OF CONTENTS

Abstract 1
Summary 2
Table of Contents 8
Background as provided by the European Commission....9
Terms of reference as provided by the European Commission....9
Assessment....10
1. Introduction 10
2. Technical data....10
2.1. Identity of the substance 10
2.2. Specifications....12
2.3. Manufacturing process....15
2.3.1. Mixed carotenes [E160a (i)]....15
2.3.2. β-Carotene [E160a (ii)]....15
2.4. Methods of analysis in food....16
2.5. Reaction and fate in food....16
2.6. Case of need and proposed uses....16
2.7. Information on existing authorisations and evaluations....19
2.8. Exposure 21
2.8.1. Crude estimates (Budget Method)....22
2.8.2. Refined estimates....22
2.8.3. Exposure from dietary sources 23
2.8.4. Exposure from food supplements....24
2.8.5. Expsoure from colouring food....24
3. Biological and toxicological data 24
3.1. Absorption, distribution, metabolism and excretion....25
3.1.1. Animal data 25
3.1.2. Human studies 26
Studies with β-carotene unspecified....26
Studies with mixed carotenes [E 160a (i)] 27
3.2. Toxicological data....29
3.2.1. Acute oral toxicity 29
3.2.2. Short-term and subchronic toxicity 30
Studies with mixed carotenes [E160a (i)] 30
Studies with β-carotenes [E 160a (ii)]....30
Studies with mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] 31
3.2.3. Genotoxicity 32
3.2.4. Chronic toxicity and carcinogenicity....36
3.2.5. Reproductive and developmental toxicity 38
Mixed-carotenes [E 160a (i)] 38
β-Carotene [E 160a (ii)] 38
3.2.6. Human studies 39
3.2.7. Sensitivity, allergenicity and intolerance....43
3.2.8. Other studies....43
4. Discussion....44
Conclusions 51
Documentation provided to EFSA 53
References 54
Annex A 64
Glossary/Abbreviations....66

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 1333/2008[4] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union. In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under Regulation (EU) No 257/2010[5]. This Regulation also foresees that food additives are re-evaluated whenever necessary in light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[6] of 2001. The report "Food additives in Europe 2000[7]" submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with the highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of the adoption of Regulation (EU) 257/2010 the 2003 Terms of Reference are replaced by those below.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedure and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives

[4] OJ L 354, 31.12.2008, p. 16.
[5] OJ L 80, 26.03.2010, p19
[6] COM(2001) 542 final.
[7] Food Additives in Europe 2000, Status of safety assessments of food additives presently permitted in the EU, Nordic Council of Ministers. TemaNord 2002:560.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)], when used as a food colour.

Mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] are authorised as food additives in the European Union (EU) and have been evaluated previously by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1975 (β-carotene), 1993 (carotenes from natural sources) and 2001 (β-carotene derived from *Blakeslea trispora*), and by the EU Scientific Committee for Food (SCF) in 1975, 1997 (β-carotene derived from *Dunaliella salina*) and 2000 (β-carotene derived from *Blakeslea trispora*).

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following a public call for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

2. Technical data

2.1. Identity of the substance

β-Carotene is a member of the carotenoids, which are coloured fat-soluble compounds naturally present in many fruits, grains, oil and vegetables. The term "carotenoids" is the generic name for a class of hydrocarbons consisting of *carotenes* (non-oxygenated hydrocarbon forms) and *xanthophylls* (oxygenated hydrocarbon forms). The main chain of the carotenoid molecule consists of eight isoprenoid units joined in such a manner that the arrangement of the isoprenoid units is reversed at the centre of the molecule.

Rules for the nomenclature of carotenoids (semi-synthetic names) have been published by the International Union of Pure and Applied Chemistry (IUPAC) and IUPAC International Union of Biochemists (IUB) Commissions on Nomenclature (1975). For most common carotenoids, trivial names are normally used.

Mixed carotenes [E 160a (i)]

In this opinion the mixed carotenes are defined according to the Commission Directive 2008/128/EC[8].

Mixed carotenes are obtained by solvent extraction from natural strains of edible plants and from algae. The Panel considered edible plants as materials of plant origin for which it can be sufficiently demonstrated that they have hitherto been consumed, whether regularly or occasionally, by humans.

Currently, carrots (*Daucus carota*), palm fruit oil (*Elaeis guineensis*) and the algae *Dunaliella salina,* (incl. *Dunaliella bardawil*) are sources for mixed carotenes. Based on information obtained from the Natural Food Colours Association (NATCOL), the Panel noted that grass, alfalfa (lucerne) and nettle are presently not commercially used to obtain mixed carotenes. The Panel further noted that algae according to the definition are not considered to be edible plants

The main colouring principle consists of carotenoids of which β-carotene accounts for the major part. α-, γ-carotene and other pigments may be present. Besides the pigments, the substance may contain oils, fats and waxes naturally occurring in the source material.

[8] Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. OJ L 6, 10.1.2009, p. 20–63.

A CAS Registry Number is not available. The EINECS number is 230-636-6 and its colour index number is 75130.

Synonym: CI Food Orange 5.

Algal carotenes are produced from natural strains of *Dunaliella salina* or *Dunaliella bardawil*. The main colouring principle consists of carotenoids of which *trans* and *cis* β-carotene account for the major part. α-Carotene, lutein, zeaxanthin and β-cryptoxanthin may be present. Besides the colour pigments, this substance may contain long-chain alkanes, fatty acids, natural terpenes, sterols, fatty alcohols and triglycerides.

A CAS Registry Number is not available. The colour index number is 75130.

Synonym: CI Food Orange 5.

β-carotene [E 160a (ii)]

Two sources are described:

(Synthetic) β-carotene with the chemical name (all-E)-1,1'-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18-diyl)bis[2,6,6-trimethylcyclohexene]. The CAS Registry Number is 7235-40-7. The molecular formula is $C_{40}H_{56}$, its molecular weight is 536.88 g/mol and its structural formula is shown in the Figure 1 below.

Synthetic β-carotene forms a dark red to brownish-red crystals or it is a crystalline powder. The compound is practically insoluble in water and in ethanol (JECFA, 2006; ChemIDplus, 2008).

Synonyms are: β,β-carotene, β-carotene, all-*trans*-β-carotene, CI Food Orange 5.

β-Carotene from *Blakeslea trispora* is obtained by a fermentation process using a mixed culture of the two sexual mating types (+) and (-) of natural strains of the fungus *Blakeslea trispora*. The crystallised product consists mainly of *trans* β-carotene. Approximately 3% of the product consists of mixed carotenoids other than β-carotene.

Both categories of substances have the EINECS number 230-636-6 and as colour index number 40800.

The structural formula of β-carotene is presented in Figure 1.

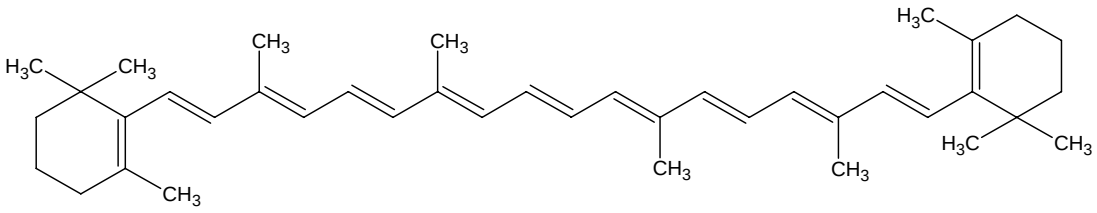


Figure 1: Structural formula of β-carotene

2.2. Specifications

Specifications of mixed carotenes [E 160a (i)] and β-carotene [E160a (ii)] have been defined in Directive 2008/128/EC and by JECFA (JECFA, 2006) (Tables 1 and 2).

Mixed carotenes [E 160a (i)]

Table 1: Specifications for assay and purity of mixed carotenes [E160a (i)] according to Commission Directive 2008/128/EC and JECFA (JECFA, 2006)

Purity		Commission Directive 2008/128/EC	JECFA (2006)
Assay	(plant carotenes)	≥ 5%	Not less than declared
	(algal carotenes)	≥ 20%	Not less than declared
Acetone	(plant carotenes)	≤ 50 mg/kg (singly or in combination)	≤ 50 mg/kg (singly or in combination)
Methanol	(plant carotenes)		
Propan-2-ol	(plant carotenes)		
Hexane	(plant carotenes)		
Ethanol	(plant carotenes)		
Methyl ethyl ketone	(plant carotenes)		-
Dichloromethane	(plant carotenes)	≤ 10 mg/kg	-
Natural tocopherols in edible oil	(algal carotenes)	≤ 0.3%	≤ 0.5%
Lead		≤ 5 mg/kg	≤ 2 mg/kg

The Panel has been informed by NATCOL that commercially available mixed carotenes are presently restricted to those obtained from edible plants such as palm fruit oil (*Elaeis guineensis*), carrots (*Daucus carota*) and from algae (*Dunaliella salina* and the variety *Dunaliella bardawil*).

Mixed carotenes from plants

Mixed carotenes from palm fruit oil (*Elaeis guineensis*) usually contain 20-30% concentrate in reddish vegetable oil suspension. These mixtures contain mainly α- and β-carotene and also minor amounts of γ-carotene and lycopene. A typical chemical composition of a 20% preparation is as follows: alpha-carotene, 6.5 g/100 g oil; beta-carotene, 13.5 g/100 g oil; gamma-carotene 0.1 g/100 g oil; lycopene, 0.1 g/100 g oil; tocopherol, 0.3 g/100 g oil. Geometrical isomerisation: 34% *cis* and 66% *trans* form. Besides the colour pigments, the mixed carotenes may contain oils, fats and waxes naturally occurring in the source material (70-80% mono-, di-, and triglycerides). The content of residual solvents (methanol, hexane and acetone only are presently used according to information from NATCOL) should be less than 50 mg/kg (singly or in combination).

Mixed carotenes from carrots *(Daucus carota)* should contain not less than 5% of carotenes calculated as β-carotene (generally 10-15%). The main colouring principles are α- and β-carotene, of which β-carotene accounts for the major part. Minor amounts of other pigments, as well as α- and γ-carotene may be present. Besides the colour pigments the product may contain oils, fats and waxes naturally occurring in the source material. However, the Panel noted that a full qualitative and quantitative characterization is not available. The products supplied to the food industry may be formulated with appropriate carriers of food grade quality. The content of residual solvents (presently only acetone, methanol and hexane are used according to information obtained from NATCOL) should be less than 50 mg/kg (singly or in combination). The amount of lead is less than 5 mg/kg (Commission Directive 2008/128/EC) or 2 mg/kg (JECFA, 2006).

Mixed carotenes from) algae (Commission Directive 2008/128/EC): Mixed carotenes from natural strains of algae (*Dunaliella salina* including the variety *Dunaliella bardawil)* are extracted using an essential oil. The preparation is a 20 to 30% suspension in edible oil (soy bean oil or olive oil). The ratio of *trans-cis* isomers is in the range of 70 to 30. Algal carotenes contain less than 0.3%

(Commission Directive 2008/128/EC) or less than 0.5% (JECFA, 2006) natural tocopherols in edible oil and less than 5 mg/kg (Commission Directive 2008/128/EC) or less than 2 mg/kg (JECFA, 2006) lead.

The EU specifications differ for the use of methyl ethyl ketone and dichloromethane. The Panel has been informed by NATCOL that presently dichloromethane, but not methyl ethyl ketone, is used for the preparation of (synthetic) β-carotene, but not for the preparation of mixed carotenes.

The Panel noted that specifications on the purity of mixed carotenes define only 5–30% of the commercial material as carotenes. The actual content of β-carotene is not specified. From the definition it is unclear what the remainder of the product consists of.

The Panel noted that the JECFA specification for lead is < 2 mg/kg, whereas the EC specification is < 5 mg/kg.

The Panel further noted that grass, alfalfa (lucerne) and nettle are presently not commercially used to obtain mixed carotenes.

β-Carotene [E 160a (ii)]

Synthetic β-carotene is specifically defined in Commission Directive 2008/128/EC and by JECFA (JECFA, 2011). Specifications of synthetic β-carotene apply predominantly to the all-*trans* isomer of β-carotene (purity 96%) together with minor amounts of other carotenoids. Diluted and stabilised preparations may have different *trans-cis* isomer ratios (Commission Directive 2008/128/EC).

β-Carotene from *Blakeslea trispora*: The crystallised product consists mainly of *trans* β-carotene. Because of the natural process, approximately 3% of the crystallised product consists of mixed carotenoids, which is specific for the product (Commission Directive 2008/128/EC).

The β-carotene content in synthetic β-carotene and β-carotene from *Blakeslea trispora* is not less than 96% of total colouring matters (expressed as β-carotene).

In the various applications in the preparation of food products synthetic, stabilized β-carotene oil dispersions are used. These dispersions are prepared by combining β-carotene and vegetable oil. DL-α-tocopherol is added to the mixture for stabilisation. Water dispersible forms are prepared from carotene bound in a base composed of proteins and glucose syrup. The powder contains DL-α-tocopherol, of ascorbic stearic ester as antioxidant and calcium phosphate to increase fluidity. Other forms of solid β-carotene may contain microcrystals of β-carotene dried by spray drying and bound to carriers composed of glucose and gelatin containing antioxidants E 304 and E 307.

Table 2: Specifications for assay and purity of β-carotene [E 160a (ii)] according to Commission Directive 2008/128/EC and JECFA (JECFA, 2011)

Purity	**Commission Directive 2008/128/EC**	**JECFA (2011)**
Assay	≥ 96% colouring matters (expressed as β-carotene)	≥ 96% colouring matters (expressed as β-carotene)
Sulphated ash	≤ 0.2%	≤ 0.1%
Subsidiary colouring matters	Carotenoids other than beta-carotene: not more than 3.0 % of total colouring matters	Carotenoids other than beta-carotene: not more than 3.0 % of total colouring matters
Ethyl acetate (*Blakeslea trispora*)	≤ 0.8% singly or in combination	≤ 0.8% singly or in combination-
Ethanol (*Blakeslea trispora*)		
Isopropanol (*Blakeslea trispora*)	-	≤ 0.1%
Isobutyl acetate (*Blakeslea trispora*)	-	≤ 1.0%
Mycotoxins: (*Blakeslea trispora*)		
Aflatoxin B1	absent	-
Trichothecene (T2)		-
Ochratoxin		-
Zearalenone		-
Microbiology: (*Blakeslea trispora*)		
Moulds	≤ 100/g	-
Yeasts	≤ 100/g	-
Salmonella	absent in 25g	-
Escherichia coli	absent in 25g	-
Arsenic	≤ 3 mg/kg	≤ 3 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg
Heavy metals	-	-

The Panel noted that synthetic β-carotene and β-carotene from *Blakeslea trispora* are highly pure, crystalline substances of one chemical entity, β-carotene, predominantly composed of all-*trans*-β-carotene. The assay value of not less than 96% (expressed as β-carotene) is linked to the method given in the specifications for the determination of the purity.

The specified method which is accepted internationally for this purpose is based on spectrophotometric determination of the absorbance of a solution of the sample at the *trans*-β-carotene maximum wavelength (455 nm) and the use of the specific absorbance value (2500 $M^{-1}cm^{-1}$) of *trans*-β-carotene in the calculation. This method determines not only β-carotene but also subsidiary colouring matters and β-carotene *cis*-isomers and expresses them all quantitatively as β-carotene. As the *cis*-isomers however have a specific absorbance lower than all-*trans*-β-carotene, the presence of roughly up to 10% of *cis*-isomers in β-carotene results in an under-determination of the total β-carotene content because the specific absorbance values of *cis*-isomers at 455 nm are 10 - 30% lower than the specific absorbance of all-*trans*-β-carotene.

Aluminium lake: According to the information provided by NATCOL, only a few aluminium lake versions of natural colours are technically possible (carmine, curcumin, chlorophyllin, copper chlorophyllin), and thus the aluminium lake of carotene is apparently not technically possible.

The Panel noted that β-carotene is susceptible to autoxidation, photodegradation and thermodegradation giving rise to cleavage and degradation products (Boon et al., 2010). Therefore, regarding the vitamin use, instructions are given to store β-carotene in a light resistant container sealed under nitrogen from light and kept below a temperature of 15 ºC (Ph. Eur. 6, 2008; The Merck Index, 2006). Even under mild conditions (30°C, in the dark) autoxidation of β-carotene may occur and

epoxides and carbonyl compounds are formed which undergo further oxidation reactions to produce secondary short chain carbonyl compounds (Mordi et al., 1991; Boon et al., 2010) (see section 2.5). The Panel noted that, since some of the β-carotene degradation products might be of toxicological relevance, suitable storage conditions should be chosen for β-carotene and β-carotene containing preparations used as a food colour. Furthermore the Panel concluded that specifications should address possible undesired impurities such as β-ionone, dihydroactinidiolide and β-damascone which can be formed due to oxidative degradation of β-carotene as a result of unsuitable storage conditions of the food additive (Boon et al., 2010; Mordi et al., 1991).

2.3. Manufacturing process

2.3.1. Mixed carotenes [E160a (i)]

Mixed carotenes from palm fruit oil (Elaies guineensis):

Natural mixed carotene complexes, usually 20% concentrate in reddish vegetable oil suspension, are prepared from palm fruits by solvent extract with acetone, methanol and/or hexane. These mixtures contain mainly β- and α-carotenes and they have also a small percentage of γ-carotene and lycopene.

Mixed carotenes from carrots (Daucus carota):

Mixed carotenes can also be obtained by solvent extraction of carrots. The solvents used for the extraction are acetone, methanol and/or hexane. The main products are solutions or suspensions in food grade vegetable/plant oil. Besides the colour pigment the solvent extract may contain oils, fats and waxes naturally occurring in the source material (Commission Directive 2008/128/EC).

Mixed carotenes from algae (Dunaliella salina, including the variety Dunaliella bardawil).

Produced from natural strains of the algae *Dunaliella salina* (or the variety *Dunaliella bardawil)*, -Carotene is extracted using an edible oil (soy bean oil or olive oil). The preparation contains 20-30% carotenoids (calculated as β-carotene) as suspension in edible oil. The ratio of *trans-cis*-isomers is in the range of 50/50 – 71/29 (no further details provided) (Commission Directive 2008/128/EC).

It has been determined that mixed carotenes produced from *Dunaliella bardawil* are identical to mixed carotenes produced from *Dunaliella salina* (Olmos et al., 2009).

The Panel noted that mixed carotenes [E 160a (i)] as defined by the Commission Directive 2008/128/EC may be obtained from carrots, plant fruit oils and algae as well as from other sources such as grass, alfalfa/lucerne and nettle. The Panel further noted based on information from NATCOL that grass, alfalfa (lucerne) and nettle are presently not commercially used to obtain mixed carotenes.

2.3.2. β-Carotene [E160a (ii)]

Chemically, synthetic β-carotene is synthesised by the so called “C15+C10+C15” strategy. The whole process is a double Wittig condensation of a symmetrical C10 dialdehyde as the central C10 building block and two equivalents of C15 phosphonium salt (Ernst, 2002). A second approach is based on a Grignard reaction: the C19+C2+C19 strategy (Hansgeorg 2002, Wong, 1989). Water dispersible forms are also prepared. These are soluble in water and prepared from β-carotene bound in a base composed of proteins and glucose syrup. The powder contains DL-α tocopherol, stearic ester of ascorbic acid as antioxidant and calcium phosphate to increase fluidity. Other forms of β-carotene may contain microcrystals of β-carotene prepared by spray drying and bound to carriers composed of glucose and gelatin containing antioxidants E 304 and E 307. Solvents used are: dichloromethane, hexane, methanol, methylcyclohexane, toluene, acetone, ethanol, ethyl acetate, heptane, isobutylalcohol, isopropylalcohol.

β-Carotene from Blakeslea trispora

β-Carotene can also be obtained by a fermentation process using a mixed culture of the two sexual mating types (+) and (-) of natural strains of the fungus *Blakeslea trispora*. The β-carotene is extracted from the biomass with ethyl acetate or isobutyl acetate followed by isopropyl alcohol, and is then crystallised (Commission Directive 2008/128/EC).

2.4. Methods of analysis in food

Several methods for the determination of β-carotene in foods are described in literature, of which high performance liquid chromatography (HPLC) with UV-VIS detection appears to be most commonly employed (Weissenberg et al., 1997; Scotter, 2011).

2.5. Reaction and fate in food

Information on the reaction and fate in food can be derived from a review on the chemical interactions between additives in foodstuffs (Scotter and Castle, 2004; and references therein).

The main causes of carotenoid degradation in food are various oxidative reactions mainly involving oxygen, hydroperoxides and peroxy-radicals. A report has been made on the formation of volatile aromatic hydrocarbons and non-volatile colourless carotene-like compounds as (anaerobic) thermal degradation products of (principally) β-carotene (Scotter and Castle, 2004). Further, coloured degradation products may arise from the degradation of *trans*-β-carotene during extrusion cooking. Reversible stereoisomerization was considered important in the formation of both non-oxidised volatiles and oxidation products. Since many of the non-volatile degradation products of β-carotene possess chemically reactive groups such as carbonyls, they are likely to react with amino acids and/or their degradation products during the thermal processing of foods.

Boon et al. (2010) and Mordi et al., (1991) described autoxidation, photodegradation and thermodegradation as factors influencing the stability of β-carotene in food. The autoxidation processes under mild conditions (30°C, in the dark) first result e.g. in the production of epoxides (e.g. 5,6-epoxy-β-carotene, 5,6,5',6'-diepoxy-β-carotene, 5,8-epoxy-β-carotene and 5,8,5',8'-diepoxy-β-carotene) and carbonyl compounds (e.g. retinal, β-apo-13-carotenone, beta-ionone, β-apo-14'-carotenal, β-ionylidene acetaldehyde), followed by further oxidation reactions of these compounds to produce β-apo-12'-carotenal and short chain mono- and di-oxygenated compounds including, β-cyclocitral, 2,2,6-timethylcyclohexanone, dihydroactinidiolide, 5,6-epoxy-β-ionone and 5,8-epoxy-β-ionone.

2.6. Case of need and proposed uses

Permitted use levels of β-carotene have been defined in Directive 94/36/EC[9] on colours for use in foodstuffs (Table 3).

Carotenes as a food colour are permitted *quantum satis* in all foodstuffs except sausages, pâtés and terrines in which only 20 mg/kg is allowed, and except those foodstuffs in which the use of colours is prohibited or restricted to food colours other than carotenes (Commission Directive 94/36/EC). Table 3 summarises those beverages and foodstuffs that are permitted to contain β-carotene as set by Directive 94/36/EC.

[9] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. OJ L 273, 10.9.94, p.13.

Table 3: Maximum Permitted Levels of β-carotene in beverages and foodstuffs according to the European Parliament and Council Directive 94/36/EC.

Beverages	**Maximum Permitted Level** mg/l
Non-alcoholic flavoured drinks	qs
Liquid food supplements/dietary integrators	qs
Spirituous beverages	qs
Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails Fruit wines, cider and perry	qs
Foodstuffs	**Maximum Permitted Level** mg/kg
Complete formulae for weight control intended to replace total daily food intake or an individual meal	qs
Complete formulae and nutritional supplements for use under medical supervision	qs
Soups	qs
Flavoured processed cheese	qs
Fish paste and crustaceans paste Smoked fish Meat and fish analogues based on vegetable proteins	qs
Savoury snack products and savoury coated nuts	qs
Desserts including flavoured milk products	qs
Edible ices	qs
Candied fruit and vegetables, Mostarda di frutta Preserves of red fruits	qs
Extruded or expanded savoury snack products	qs
Fine bakery wares	qs
Pre-cooked crustaceans	qs
Mustard	qs
Solid food supplements/dietary integrators	qs
Confectionery	qs
Fish roe	qs
Salmon substitutes Surimi	qs
Sauces, seasonings, pickles, relishes, chutney and piccalilli	qs
Decorations and coatings	qs
Edible cheese rind	qs
Edible casings	qs
sausages, pâtés and terrines	20

2.6.1. Actual levels of use of β-carotene

In order to refine the exposure assessment for children and adults to food colours, the Panel has defined some rules to identify maximum reported use levels based on data reported by industries or analytical data from others sources. The rules followed to deal with *quantum satis* (QS) authorisation, with usage data or observed analytical data, for all regulated colours re-evaluated by the Panel, are given in Annex A, Figure 2. Table 4 summarises the maximum reported use levels of β-carotene in beverages and foodstuffs used for the refined exposure assessment. They have been defined by applying the rules reported in Annex A, Figure 1 to the data available to EFSA.

Table 4: Outcome of usage survey for β-carotene

Beverages	Data provided by	Reported range of typical[10] use levels (lowest-highest)	Maximum Reported use levels	Levels used for exposure assessment at Tier 3
Non-alcoholic flavoured drinks	Tennant et al., 2004	2.0-5.0		5
	CIAA, 2009	1-25	94	94
Foodstuffs				
Confectionery	Tennant et al., 2004	0-31		31
	CIAA , 2009	5-38	200	200
Decorations and coatings	Tennant et al., 2004	0-50		50
Fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafers)	Tennant et al., 2004	0-40		40
	CIAA, 2009	1.5-384	1300	1300
Edible ices	Tennant et al., 2004	1.0-70		70
	CIAA, 2009	5-163	518	518
Flavoured processed cheese	Tennant et al., 2004	0.6-3.0		3
	CIAA, 2009	30-100	400	400
Desserts inc. flavoured milk products	Tennant et al., 2004	4.0		4
	CIAA, 2009**	0.5-300	500	500
Sauces, seasonings, pickles, relishes, chutney, piccalilli	Tennant et al., 2004	3-10		10
	CIAA, 2009	0.4-150	350	350
Mustard	Tennant et al., 2004	5.0		5
	CIAA, 2009	10	25	25
Snacks: dry, savoury potato, cereal or starch-based snack products	Tennant et al., 2004	4.0		4
	CIAA ,2009	70	200	200
Other savoury snack products and savoury coated nuts	Tennant et al., 2004	4.1		4.1
	CIAA, 2009	70	200	200
Edible cheese rind and edible chasings	Tennant et al., 2004	12.0		12
Soups	Tennant et al., 2004	7.0		7
	CIAA ,2009	0.02-90	304	304
Spirituous beverages (inc. products less than 15% alcohol by volume)	Tennant et al., 2004	0-2.5		2.5
	CIAA, 2009	0-5	30	30
Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails as mentioned in Regulation 1601/91, except any mentioned in Schedule 2 or 3	Tennant et al., 2004	0-2.5		2.5
Fruit wines (still or sparkling), cider (except cidre bouche) and perry	Tennant et al., 2004	0-2.5		2.5
Butter (including reduced-fat butter and concentrated butter	Tennant et al., 2004	2.0-3.6		3.6
Margarine, minarine, other fat emulsions, and fats essentially free from water	Tennant et al., 2004	3-10		10
	CIAA, 2009	1-50	330	330
Ripened Orange, Yellow and broken-white cheese; unflavoured processed cheese	Tennant et al., 2004	3.0		3
Extruded, puffed and/or fruit-flavoured breakfast cereals	Tennant et al., 2004	10-50		50
	CIAA, 2009	50-100	330	330
Jams, jellies and marmalades as mentioned in Directive 79/693/EEC and other similar fruit preparations including low-calorie	Tennant et al., 2004	5.0		5
	CIAA, 2009	1-100	330	330

[10] The terms "typical use level" and "normal use level" are assumed to represent the same meaning. Extreme use level" and "maximum use level" are also assumed to represent the same meaning. Range given is based on the information received from different industries members for different food products that fall into the same food category.

products				
Complete formulae and nutritional supplements	CIAA, 2009	4.4	750	750
Meat and fish products	CIAA, 2009	7	7	7
Liquid egg products	CIAA, 2009	50	50	50
Milk and dairy based drinks	CIAA, 2009	0.18-50	90	90
Fruit and vegetables, canned, frozen, etc.	CIAA ,2009	20	50	50
Dairy products	CIAA, 2009	6-30	850	850
Processed potato products	CIAA, 2009	125	125	125
Liquid dough	CIAA, 2009	100	400	400
Milk and cream analogues	CIAA, 2009	2	2	2
Complete formulae for weight control intended to replace total daily food intake or an individual meal	CIAA, 2009	4	8	8

* All dosages related to complete food items not to the specifically coloured fraction.
** CIAA data excluding dairy and cereal based desserts

The Panel noted that there is a considerable discrepancy between data on β-carotene usage reported by NATCOL and those originally reported by CIAA in 2009. Following exchanges with CIAA in 2011 it could be deducted that the original data reported by CIAA probably refer to colour preparations than to the colouring principle.

2.7. Information on existing authorisations and evaluations

Mixed carotenes and β-carotene are authorised as a food additive in the EU under Directive 94/36/EC.

Mixed carotenes and β-carotene have been evaluated previously by JECFA in 1975 (β-carotene), 1993 (carotenes from natural sources) and 2001 (β-carotene derived from *Blakeslea trispora*), and by the SCF in 1975, 1997 (β-carotene derived from *Dunaliella salina*) and 2000 (β-carotene derived from *Blakeslea trispora*). An overview of the JECFA and the SCF evaluations of mixed carotenes and β-carotene is provided in Table 5 and described below.

Mixed carotenes and β-carotene has also been evaluated by TemaNord (TemaNord, 2002). This report concluded that according to new published studies, this food colour should be re-evaluated. Data on specifications and maximum permitted levels have been defined in EU legislation (in particular Directives 2008/128/EC and 94/36/EC).

Based on the JECFA evaluation of 1975, a group Acceptable Daily Intake (ADI) of 0-5 mg/kg bw/day was established for the sum of the carotenoids β-carotene, β-apo-8'-carotenal, and β-apo-8'-carotenoic acid methyl and ethyl ester. Results from a four-generation rat study with β-carotene (Bagdon et al., 1960) were used for calculation of the group ADI. In this study, no adverse effects were seen at 50 mg β-carotene/kg bw/day, and a safety factor of 10 instead of 100 was used because of the natural occurrence of carotenoids in the human diet and the low toxicity of carotenoids in animal studies.

In 1975, the SCF endorsed the ADI established by JECFA of 0-5 mg/kg bw/day as the sum of the carotenoids β-carotene, β-apo-8'-carotenal and β-apo-8'-carotenoic acid methyl and ethyl ester. The SCF in an accompanying comment (1975) mentioned that only the ethyl ester was listed in the Community Directive and that this was the only compound considered. Consequently, the ADI was expressed as the sum of β-carotene, β-apo-8'-carotenal and the ethyl ester of β-apo-8'-carotenoic acid alone.

When, however, β-carotene was re-evaluated (SCF, 2000b), the SCF decided to withdraw the group ADI of 0-5 mg/kg bw for β-apo-8´-carotenal, β-carotene and the ethyl ester of β-apo-8'-carotenoic acid. The ADI was withdrawn for two reasons. First, the ADI was based on rodent studies, and since rodents were considered to convert β-carotene to vitamin A much more efficiently than humans, these

studies were considered to lack relevance for human risk assessment. The second reason was the adverse findings observed in human smokers receiving β-carotene supplements at 20 mg/person/day or more, amounts that are much lower than the previously established ADI. The SCF considered the scientific basis to be insufficient to set a new ADI.

As,, there were no indications that daily intakes of about 1-2 mg β-carotene and/or related carotenoids, as food additives, were harmful in the context of the overall dietary intake of these substances, and , in addition, the scientific basis was considered to be insufficient to set a new ADI, the Committee decided that currently permitted food additive uses of β-carotene and related carotenoids would be temporarily acceptable (SCF, 2000b).

Mixed carotenes [E160a (i)]

Carotenes from natural sources were most recently evaluated by JECFA in 1993. JECFA considered the data inadequate to establish an ADI for the dehydrated algal carotene preparations or for the vegetable oil extracts of *Dunaliella salina*. However, JECFA concluded that there was no objection to the use of vegetable extracts as colouring agents, provided that the level of use did not exceed the level normally present in vegetables; no numerical ADI was allocated.

For mixed α-,β-,γ-carotenes from natural foods, the SCF did not establish an ADI but felt able to accept the use of mixed carotenes prepared from natural foods as a food colour for food (SCF, 1975). In addition, the SCF (1997) considered the use of a dispersion of mixed carotenes produced by the alga *Dunaliella salina*, acceptable as a food additive.

(Synthetic) β-carotene [E160 a (ii)]

The ADI for synthetic β-carotene was extended by JECFA in 2001 to also include β-carotene from *Blakeslea trispora*. It is not completely clear from the JECFA documentation, but probably this current group ADI applies to synthetic β-carotene and β-carotene from *Blakeslea trispora* as well as to the other three carotenoids β-apo-8'-carotenal, β-apo-8'-carotenoic acid methyl and ethyl ester, but most probably does not include the mixed carotenes.

In 2000, the SCF concluded that β-carotene produced by co-fermentation of *Blakeslea trispora* DS 30627 and DS 30628 is equivalent to the chemically synthesised material used as food colorant and is therefore acceptable for use as a colouring agent for foodstuffs (SCF, 2000a).

Table 5: Overview of the JECFA and SCF authorisations of mixed carotenes and β-carotene

	SCF	JECFA
	Mixed carotenes (E 160a (i))	
Plant carotenes (from natural foods)	Acceptable for use as food colour, no Acceptable Daily Intake (ADI) was established (SCF, 1975).	Acceptable for use as food colour, provided that use as food colour did not exceed the level normally present in vegetables. No ADI was established (JECFA, 1993).
Algal carotenes (from *Dunaliella salina*)	Acceptable for use as food colour, no ADI was established (SCF, 1997).	No ADI was established (JECFA, 1993)
	β-carotene (E 160a (ii))	
β-carotene (synthetic and derived from *Blakeslea trispora*)	Group ADI of 0 – 5 mg/kg bw/day for (synthetic) β-carotene, β-apo-8'-carotenal and the ethyl ester of β-apo-8'-carotenoic acid established (SCF, 1975). β-carotene from *Blakeslea trispora* is considered equivalent to chemically synthesised β-carotene and is included in the group ADI (SCF, 2000a). Group ADI withdrawn in 2000, however, currently permitted food additive uses of β-carotene and related carotenes are temporarily acceptable (SCF, 2000b). SCF was not able to set an Tolerable Upper Intake Level (UL) for β-carotene	Group ADI of 0 – 5 mg/kg bw/day for (synthetic) β-carotene, β-apo-8'-carotenal and the methyl and ethyl ester of β-apo-8'-carotenoic acid established (JECFA, 1975). β-carotene from *Blakeslea trispora* is also included in the group ADI (JECFA, 2001).

In humans, the major known function of carotenoids is to serve as an important precursor for vitamin A. β-Carotene is the principal provitamin A. In 2000, the SCF adopted an opinion on the Tolerable Upper Intake Level (UL) of β-carotene in which the SCF concluded that there is insufficient scientific basis to set a precise figure for an UL of isolated β-carotene (SCF, 2000c).

The Panel noted that it is not clear which carotenoids are included in the group ADI from JECFA and SCF and which are not.

2.8. Exposure

The Panel followed the principles of the stepwise approach, which were used in the report of the scientific cooperation (SCOOP) Task 4.2 (EC, 1997), to estimate intakes of food additives. For each successive Tier, this involved a further refinement of intake estimates. The approach progresses from the conservative estimates that form the first Tier of screening, to more realistic estimates that form the Second and Third Tiers. In the tiered approach, Tier 1 is based on theoretical food consumption data and maximum permitted use levels (MPLs) for additives as permitted by relevant Community legislation. The Second and Third tiers refer to assessment at the level of individual Member States, combining national data on food consumption with the maximum permitted usage levels for the additive (Tier 2) and with its maximum use level (Tier 3).

2.8.1. Crude estimates (Budget Method)

As mixed carotenes and beta-carotene are authorised at quantum satis in almost all foodstuffs, the Panel could not estimate the dietary exposure using the Budget method (Tier 1).

2.8.2. Refined estimates

As with the exception of sausages, patés and terrines all applications are authorised *quantum satis*. It was not possible to perform Tier 2 calculations.

Tier 3

Concentration data made available to the Panel by the industry were used to calculate the dietary exposure to beta-carotene coming from mixed carotenes (Tier 3). The Panel noted that there is a considerable discrepancy between data on β-carotene usage reported by NATCOL and those reported by CIAA in 2009 (Table 4). Following exchanges with CIAA in 2011 it was deduced that the original data reported by CIAA probably referred to colour preparations rather than to the colouring principle itself. In line with this suggestion, the Panel considered that the CIAA original data are unlikely to reflect usages of pure β-carotene as a food colour. However, , the Panel calculated exposure based on both datasets provided, in particular since CIAA reported use levels for a broader range of food categories than did NATCOL. A generally valid conversion factor could not be derived from the data submitted to EFSA due to wide variation in the ratio of CIAA data to NATCOL data for the individual food categories.

The Panel noted that its estimates could be considered as being conservative as it is assumed that all processed foods and beverages contain mixed carotenes and beta-carotene added at maximum reported use levels.

Data from the EXPOCHI countries and the UK data were used by the Panel to calculate from summary statistics (average all population and 95^{th}/97.5^{th} percentile for consumers only) the mean and high level exposure to mixed carotenes and β-carotene based on the food categorisation of EU food colours and using maximum permitted levels and maximum reported use levels. High level exposure (95^{th}/97.5^{th} percentile of consumers only) was based on the assumption that an individual might be a high level consumer of one food category and would be an average consumer of the others. This approach has been tested several times by the Panel in re-evaluation of food colours and has shown reasonable correlation with high level total intakes when using the raw individual food consumption data. Therefore, this approach was preferred for the calculation in order to avoid excessively conservative estimates.

When considering the maximum reported use levels from Table 4 as provided by CIAA, estimates reported for the UK adult population give a mean dietary exposure to mixed carotenes and beta-carotene of 1.5 mg/bw/day and 3.2 mg/bw/day for high level consumers (mean consumption plus intake at the 97.5^{th} percentile of 'non-alcoholic flavoured drinks'). The main contributors (>10%) to the total anticipated mean exposure to mixed carotenes and β-carotene were non-alcoholic beverages (35%), soups (13%), and extruded breakfast cereals (14%).

When considering the maximum reported use levels from Table 4 as provided by CIAA, the mean dietary exposure of European children (aged 1-14 years and weighing 16-54 kg), ranged from 2.2-11.4 mg/kg bw/day, and from 4.5-21.3 mg/kg bw/day at the 95^{th}/97.5^{th} percentile. The main contributors to the total anticipated mean exposure to mixed carotenes and beta-carotene (>10% in all countries), were non alcoholic flavoured drinks (10-40%), fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (11-40%), desserts (including flavoured milk products) (12-47%), edible ices (11%), soups (19-22%), and sauces and seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney, piccalilli) (24%).

When considering the maximum reported use levels from Table 4 as provided by Tennant, estimates reported for the UK adult population give a mean dietary exposure to mixed carotenes and β-carotene of 0.06 mg/bw/day and 0.11 mg/bw/day for high level consumers (mean consumption plus intake at the 97.5th percentile of 'non-alcoholic flavoured drinks'). The main contributors (>10%) to the total anticipated mean exposure to carotenes were non-alcoholic beverages (26%), fine bakery wares (31%), and desserts (including flavoured milk products) (15%).

When considering the maximum reported use levels from Table 4 as provided by Tennant, the mean dietary exposure of European children (aged 1-14 years and weighing 16-54 kg), ranged from 0.03 to 0.22 mg/kg bw/day, and from 0.09 to 0.43 mg/kg bw/day at the 95th/97.5th percentile. The main contributors to the total anticipated mean exposure to mixed carotenes and β-carotene (>10% in all countries), were non alcoholic flavoured drinks (10-27%), fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (12-48%), desserts (including flavoured milk products) (12-43%), milk and dairy based drinks (11-29%), processed potato products (11-16%), and sauces and seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney, piccalilli) (13%).

Table 6: Summary of anticipated exposure to mixed carotenes and β-carotene using the tiered approach (EC, 2001) in children and the adult population

	Adults (UK population) (>18 years old)	**Children* (UK & EXPOCHI population)** (1-14 years old, 16-54[1] kg body weight)
	mg/kg bw/day	mg/kg bw/day
Tier 3. Maximum reported use levels (data from CIAA 2009)		
• Mean exposure	1.5	2.2-11.4
• Exposure 95th*or 97.5th percentile**	3.2	4.5-21.3
Tier 3. Maximum reported use levels (data from NATCOL (Tennant et al. 2004)		
• Mean exposure	0.06	0.03-0.22
• Exposure 95th*or 97.5th percentile**	0.11	0.09-0.43

* For EU children, consumption figures for 95th percentile intake estimates have been calculated by EFSA from the raw EXPOCHI data.

** For UK, estimates are based on the UNESDA report which gives the 97.5th percentile intake from beverages plus *per capita* average from the rest of diet (Tennant, 2007).

[1] Including the Cypriot children where the reported body weight was 54 kg for 11-14 years old.

2.8.3. Exposure from dietary sources

The Panel also considered the typical exposure to β-carotene from the regular diet as relevant for the assessment. Data have therefore been collected from the European Nutrition and Health Report 2009 (Elmadfa, 2009) which provides intake estimates for β-carotene from 9 European countries (Czech Republic, Denmark, Finland, Germany, Ireland, Italy, Norway, Poland, and Sweden) based on different methods of dietary intake assessment for children aged 4-6 years, and from 13 countries (Austria, Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, Lithuania, Poland, Spain, Sweden, and The United Kingdom) for adults. As indicated in Table 7, the range of β-carotene exposure from the diet is 1.1-3.9 mg/day for children at an age of 4-6 years and 1.4-5.6 mg/day for adults.Although not clearly indicated, the data reported in Table 7 are considered to only include β-carotene from natural sources and not from its use as food colour. Based on a body weight of 15 kg for children and of 60 kg for adults, exposure from the natural dietary sources would therefore be in the range of 0.07-0.25 mg/kg bw/day for children and in the range of 0.02-0.09 mg/kg bw/day for adults.

Table 7: Beta-carotene exposure from the diet (mg/day)

Country	Children 4-6 years		Adults	
	Males, mean ± SD (n)	Females, mean ± SD (n)	Males, mean ± SD (n)	Females, mean ± SD (n)
Austria	2.8 ± 2.6 (641)	2.8 ± 2.6 (446)	n.a.	n.a.
Czech Republic	3.8 ± 2.7 (117)	3.4 ± 2.9 (117)	3.5 ± 3.5 (1283)	4.6 ± 4.5 (1486)
Denmark	1.6 ± 1.5 (671)	1.6 ± 1.4 (656)	n.a.	n.a.
Denmark*	3.7 ± 2.6 (81)	3.9 ± 3.2 (78)	3.7 ± 3.5 (1569)	5.1 ± 4.9 (1785)
Finland	2.4 ± 2.1 (102)	2.4 ± 1.6 (102)	5.3 ± 4.2 (4912)	5.6 ± 4.0 (6016)
Germany	n.a.	n.a.	2.2 ± 1.9 (473)	2.3 ± 2.0 (706)
Hungary	2.0 ± 1.6 (72)	1.4 ± 1.2 (72)	2.5 ± 2.1 (662)	2.3 ± 1.6 (717)
Ireland	2.0 ± 1.3 (21)	2.0 ± 1.3 (21)	3.1 ± 1.9 (660)	3.1 ± 1.9 (801)
Italy	n.a.	n.a.	3.5 ± 7.0 (849)	3.0 ± 8.1 (1087)
Lithuania	2.4 ± 3.4 (82)	2.7 ± 3.2 (82)	3.6 ± 3.4 (1106)	2.8 ± 3.2 (1334)
Poland	n.a.	n.a.	1.4 ± 0.5 (706)	1.4 ± 0.6 (875)
Spain	1.2 ± 1.1 (302)	1.1 ± 0.9 (302)	1.7 ± 3.3 (517)	1.8 ± 1.2 (575)
Sweden	n.a.	n.a.	2.1 ± 2.1 (603)	2.1 ± 2.3 (1125)
United Kingdom	n.a.	n.a.	2.5 ± 2.5 (119) 1.7 ± 1.7 (152) 1.9 ± 2.1 (143) 2.3 ± 2.5 (189)	2.0 ± 1.8 (295) 2.0 ± 2.7 (349) 2.0 ± 2.1 (245) 2.5 ± 2.3 (236)
All countries (min-max)	1.2–3.8	1.1–3.9	1.4–5.3	1.4–5.6

* Pedersen et al. 2010

2.8.4. Exposure from food supplements

The Panel noted, that β-carotene is also widely used in vitamin and mineral food supplements at levels resulting in intakes ranging from 0.4 mg to 20 mg/day (EVM 2003). Only very limited information is available on the intake of β-carotene from supplemental use, but in view of the amount used for supplements it is likely that this may substantially contribute to the overall exposure.

2.8.5. Expsoure from colouring food

The Panel noted that due to lack of data this exposure assessment did not include the use of colouring food[11] containing β-carotene.

3. Biological and toxicological data

Mixed carotenes and β-carotene have been previously evaluated by JECFA in 1975 (β-carotene), 1993 (carotenes from natural sources) and 2001 (β-carotene derived from *Blakeslea trispora)* and by the SCF in 1975, 1997 (β-carotene derived from *Dunaliella salina*) and 2000 (β-carotene derived from *Blakeslea trispora*). Additional data have been taken from a report released by the Nordic Council of Ministers (TemaNord, 2002) who took into account the relevant literature published until 2000. The present opinion briefly reports the major studies evaluated in these reports and describes additional newly reported literature data in some more detail. In most of the studies evaluated the identity of the test material is not specified.

[11] Colouring foods are not defined by the EU legislation. The Commission is currently working on the interpretation of the function class "colour" in particular when a colouring ingredient extracted from a source material is to be considered a food colour.

3.1. Absorption, distribution, metabolism and excretion

3.1.1. Animal data

The JECFA (1973, 1983) evaluations succinctly describe the following studies on the toxicokinetic aspects of β-carotenes.

Studies with both mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)]

Male weanling rats (CD strain) were fed *ad libitum* a retinol-deficient diet for 60 days (Ben-Amotz et al., 1988). Afterwards, groups of depleted rats (six per group) were fed a retinol-deficient diet supplemented with: synthetic all-*trans* β-carotene at 12, 29 or 48 mg/kg diet (equivalent to 600, 1450, or 2400 µg/kg bw/day respectively), dry algae providing 29, 58 or 112 mg β-carotene/kg diet (equivalent to 1450, 2900, or 5600 µg/kg bw/day respectively), an algal-oil-extract providing 16 mg β-carotene/kg diet (equivalent to 800 µg/kg bw/day respectively), or retinol at 7.5 mg/kg diet (equivalent to 3750 µg/kg bw/day respectively). A control group received no supplementation. After seven days repletion, livers were taken for analysis for retinol, retinol isomers and β-carotene.

All liver extracts, except those of the retinol-deficient rats, contained all-*trans* retinol. Livers from rats fed the dried algae and the algae-oil-extract contained 9-*cis* retinol in addition to the all-*trans* isomer. The percentage conversion of β-carotene to retinol was similar in all β-carotene treated groups except for the highest dietary β-carotene level (from dried algae) which showed lower conversion. In the β-carotene treated groups the β-carotene to retinol ratio in the liver ranged from about 1:2 to 1:3 on a µg/g wet weight basis (Ben-Amotz et al., 1988).

In a 45-day study (Ghazi et al., 1992) with Sprague-Dawley rats (21 days old) animals received diets containing: spray dried *Dunaliella salina* (0.61% β-carotene), *Dunaliella salina*-oil-extract (25% β-carotene), oleoresin of carrots (11.5% β-carotene), and synthetic β-carotene (100%). The percentages of β-carotene in the diets were equal and the control diet contained 0.036% β-carotene.

The bioavailability of β-carotene from dried algae and algae-oil-extract was higher (actual numbers not available) than that of β-carotene from oleoresin of carrots or synthetic β-carotene. These results may be due to the presence of extra lipids in both the dried algae (8.6% lipid) and algae-oil-extract. (Ghazi et al., 1992).

Groups of 1-day-old chicks (white Leghorn; unknown number) received a retinol-deficient diet, or similar diets supplemented with: 8.04 mg retinol/kg diet, 30 mg synthetic β-carotene/kg diet, or freeze-dried *Dunaliella bardawil* providing 30 mg β-carotene/kg diet (further containing 200 mg NaCl/kg diet). In a second experiment, four similar groups of 1-day old chicks were used except that the freeze-dried algae preparation was replaced with a similar concentration of drum-dried algae, containing 34 g β-carotene/kg (further containing 260 g NaCl, 180 g glycerol, 5.5 g chlorophyll, 210 g protein, 170 g carbohydrate and 120 g lipid/kg). In a third experiment three groups of 1-day-old chicks (15 per group) received either a retinol-deficient diet, a diet supplemented with 0.58 g freeze-dried algae/kg diet, or a diet supplemented with 1 g drum-dried algae/kg diet (no details on duration of exposures) (Ben-Amotz et al., 1986).

Serum and liver concentrations of retinol were normal in all cases except for the chicks receiving retinol-deficient diets without supplements. The serum of chicks fed the diets supplemented with algae contained lutein but no β-carotene, although the β-carotene to lutein ratio in the algae exceeded 15:1 (Ben-Amotz et al., 1986).

In another experiment, two groups of three egg-laying hens received a diet containing 150 g maize-meal/kg (control), or the same diet supplemented with 4 g freeze-dried algae providing 200 mg β-

carotene/kg diet (algal preparation containing 50 g β-carotene and 300 g NaCl/kg) (Ben-Amotz et al., 1986). Eggs from these hens showed an enhanced yolk colour attributable to lutein. No β-carotene was present in the egg yolk (Ben-Amotz et al., 1986).

In the SCF evaluation of 2000 the following additional animal studies have been described.

The main site of carotenoid metabolism in rodents is the intestinal mucosa. In several mammals (including man), β-carotene is also metabolised in peripheral tissues such as lung, kidney, liver and fat (Redlich et al., 1996; Wang et al., 1992).

In mammalian tissues (mainly in the gut and liver), β-carotene is cleaved mainly at the central double bond (C15-15'). This yields two molecules of retinal (which is further reduced to retinol or further oxidised to retinoic acid). In vitro cleavage also occurs at excentric double bonds to form apo-carotenals. The relevance of excentric cleavage in vivo is however controversial (Woutersen et al., 1999; Wang et al., 1992, 1999; Krinsky et al., 1990).

Most laboratory animals break down β-carotene in their intestine. When dioxygenase cleavage is very active, almost no β-carotene will be absorbed intact. Rodents have low serum carotenoid levels (about 1/1000 of human levels) that are not related to dietary intake due to very active cleavage of β-carotene by dioxygenase to retinal. In man, roughly 20-75% of the β-carotene is absorbed intact (Rock, 1997: Wang et al., 1992).

Studies in hamsters showed that β-carotene serum concentrations remained low in animals given dietary β-carotene supplementation, although retinol levels increased, indicating that hamsters are also efficiently converters of β-carotene to retinol (IARC, 1998). Rabbits do not appear to absorb β-carotene well and these animals when fed a carotenoid-rich diet showed no carotenoids in the blood and only small increases in liver vitamin A concentrations (IARC, 1998). Strict carnivores obtain a diet rich in pre-formed vitamin A and thus do not depend on provision via carotenoids in the diet. Cats, for example, lack the enzyme, β-carotene-15, 15'-dioxygenase and, thus, have a requirement for pre-formed vitamin A in the diet (Bauernfeind et al., 1981).

Ferrets (Gugger et al., 1992; Rock, 1997; Wang et al., 1992; 1999; White et al., 1993b), the pre-ruminant calf (Poor et al., 1992) and the Mongolian gerbil (Krinsky et al., 1990; Mathews-Roth, 1993) have been proposed as useful models for human β-carotene absorption and cleavage, as these animals also absorb and release intact β-carotene from the enterocyte. Although serum β-carotene levels are normally very low in these animals, dietary supplementation has been shown to increase concentrations to levels similar to those detected in human serum, and also to increase levels in the liver, adipose and other tissues (Gugger et al., 1992; Ribaya-Mercado et al., 1989, 1992, 1993; Wang et al., 1999; White et al., 1993a; 1993b).

3.1.2. Human studies

In addition to the above described animal studies, the JECFA evaluations describe a range of studies conducted in humans.

Studies with β-carotene unspecified

After ingestion of β-carotene (unspecified), humans excreted 30-90% in the faeces and only small amounts appeared in the serum. Concomitant intake of fat did not improve absorption. Excessive doses of β-carotene depress the vitamin A activity of the absorbed fraction. When β-carotene is dissolved in oil, absorption ranged between 10-41% for adults and 50-80% for children (Fraps and Meinke, 1945; Bernhard, 1963*).

* Reference only given in an earlier evaluation of β-carotene (JECFA, 1966)

In another study, adults and babies receiving a dose of 20 mg carotene (unspecified) absorbed 11 and 2.6% respectively (no further details) (Kübler, 1963).

Fifteen human subjects received daily doses of 60 mg β-carotene for three months. Serum carotene levels rose from 128 µg/100 ml to a maximum of 308 µg/100 ml after one month. Vitamin A levels remained unchanged and no clinical signs of hypervitaminosis A were seen (Greenberg et al., 1959).

Wagner (1962) found that vitamin E is necessary to prevent enzymatic destruction of β-carotene and that bile acids are necessary for absorption of β-carotene (no further details). Diseases of the gastrointestinal tract, liver and kidneys, diabetes mellitus, and phosphor poisoning reduced the conversion of β-carotene to vitamin A. In myxoedema (medical condition related to severe prolonged hypothyroidism) conversion of β-carotene to vitamin A is completely blocked. The conversion capacity of β-carotene to vitamin A in infants and small children is smaller than that in adults (Wagner, 1962).

In infants a single administration of 20 mg of β-carotene in milk produced a rise in the levels of β-carotene (peak at 24 hours) and vitamin A ester in blood. Practically no change is produced in the level of retinol in blood (Auckland, 1952).

Studies with mixed carotenes [E 160a (i)]

Human volunteers (12 male and 20 female), received a low-carotene diet for 10 days, after which the subjects were randomly assigned to five groups. Two groups received capsules containing vegetable oil extracted from *Dunaliella salina* providing 8 or 24 mg β-carotene and 1.1 or 3.2 mg α-carotene respectively. Two further groups received quantities of carrots providing similar amounts of β-carotene and 6.3 and 18.9 mg α-carotene respectively. A fifth group received placebo capsules. The subjects received the treatment for seven days and then underwent another depletion phase of seven days (Jensen et al., 1985).

Treatments with both oil-extracted *Dunaliella salina* and carrots led to an increase in serum concentrations of α- and β-carotenes, with the higher dose treatments being less efficient per mg carotene consumed. The oil-extracted *Dunaliella salina* was most efficient in raising the serum carotene values per mg carotenes fed. This is consistent with other reports showing that carotenes are better absorbed from oily solutions than from vegetable matrices (Jensen et al., 1985).

Seventeen volunteers received a low-carotene diet for 10 days, after which the subjects were randomized into three groups based on β-carotene levels on day six. On day 11, following a low-carotene breakfast, group one consumed three carrots, group two consumed one carrot, and the third group consumed three placebo capsules. Blood α- and β-carotene levels were determined before treatment and at 1, 2, 3, 4, 5, 7 and 24 hours after treatment (Jensen et al., 1986).

Consumption of three carrots gave significantly greater peak α- and β-carotene levels in serum five hours after treatment compared with consumption of one carrot or three placebo capsules. Furthermore, the results suggested that the best condition for drawing blood samples to assess the serum carotene status of adults is at fasting state, and that significant alterations in serum concentrations of β-carotene can occur within five hours of a carotene-rich meal (Jensen et al., 1986).

In another study, 16 volunteers were given a low-carotene diet for 10 days after which they were randomly divided into three groups. The first group received 24 mg β-carotene per day extracted from *Dunaliella salina* (all-*trans* β-carotene and 9-*cis* β-carotene in approximately equal amounts). The second group was given quantities of fresh carrots providing 24 mg β-carotene per day (predominantly *transtrans* β-carotene). A third group received single doses of avocado oil (control). The subjects received the treatment for seven days.

Trans β-carotene to *cis* β-carotene ratios showed that *trans* β-carotene was the predominate serum isomer before and during all treatments. In the *Dunaliella salina* and carrot groups, serum *trans* β-carotene concentrations were significantly increased; in the carrot and placebo groups *cis* β-carotene concentrations were increased, both in comparison to the start of the study. In the *Dunaliella salina* and carrot groups the *trans* β-carotene increments strongly exceeded *cis* β-carotene increments which demonstrated that, when approximately equivalent amounts of both isomers are ingested by humans, *trans* β-carotene absorption is favoured over *cis* β-carotene absorption (Jensen et al., 1987).

In a study, ten volunteers received a low-carotene diet for two weeks followed by a 10 day dosing (capsule form) of a powdered algal preparation providing approximately 135 mg β-carotene. Serum carotene was measured on days 0, 7 and 10 of treatment. There was considerable inter-individual variation in response with regard to both absolute values (not specified) and treatment-dependent increases in serum carotene concentration. In six of the subjects the serum levels of carotene continued to rise between days 7 and 10, while in three others there was a slight fall in this period. A single subject showed virtually no response to treatment. No adverse effects due to ingestion of the preparation were reported (Cyanotech, 1988).

The SCF (2000b) gives some additional information on the toxicokinetics of β-carotene which is described below briefly.

The extent of human absorption for β-carotene reported in the literature varies between 10 and 90%, and inter- and intra-individual variability in apparent absorption determined by plasma response is high. The general mechanism of intestinal β-carotene absorption in mammals is by passive diffusion of mixed micelles. Human absorption of β-carotene appears to be linear up to intakes of 20-30 mg, but becomes limited at higher intakes depending on the formulation or food matrix, the amount and type of co-ingested fat, and the presence of bile acids. In humans, high concentrations of β-carotene have been found in the adrenals and testes, but the major storage sites for carotenoids are the liver and adipose tissue (Woutersen et al., 1999). Interactions between carotenoids may decrease absorption. Competitive inhibition may occur at the level of micellar incorporation, intestinal uptake and/or lymphatic transport. Simultaneous ingestion of various carotenoids may induce an anti-oxidant sparing effect in the intestinal tract, resulting in increased levels of uptake of protected carotenoids. Even in the presence of large amounts of β-carotene, chylomicrons preferentially take up xanthophylls rather than β-carotene from the intestinal lumen (Gärtner et al., 1996)

About 15% of β-carotene is cleaved into retinal in the intestine of poorly nourished humans. As this percentage decreases when the β-carotene content of the diet increases, even large quantities of β-carotene do not produce enough retinol to cause hypervitaminosis (Omenn, 1998).

Carotenoids are known to exist in different forms (*cis*- and *trans*-isomers) which may be interconverted by light, thermal energy or chemical reactions. In human serum, most of the β-carotene is present as the all-*trans* isomer, even after significant intakes of the 9-*cis* isomer over long periods, whereas the liver and adrenal tissue contained more of the 9-*cis* and 13-*cis* isomers of β-carotene (Woutersen et al., 1999; Rock, 1997).

Carotenoids are transported in association with lipoproteins. After absorption in the enterocyte, β-carotene (and retinyl esters formed in the enterocyte) are incorporated into chylomicrons, before they are secreted into the intestinal lymph and move into the blood stream. In the fasted state about 75% of the β-carotene is bound to Low Density Lipoprotein (LDL) and about 25% to High Density Lipoprotein (HDL) and Very Low Density Lipoprotein (VLDL). Tissue distribution of β-carotene roughly parallels LDL receptor density on the plasma membranes of the cells. Liver and adipose tissue are the main sites of carotenoid deposition. Circulating carotenoid concentrations are found to be lower in smokers in comparison to non smokers, due in part to the depletion of these compounds by components of cigarette smoke (Woutersen et al., 1999)

The Panel concluded that although the mechanism of intestinal β-carotene absorption and metabolism appears to be comparable in animals and humans, a wide range of absorption and intestinal cleavage rates has been quantitatively demonstrated among species (Woutersen et al., 1999; and references therein). In rodents and rabbits dioxygenase cleavage is very active, hence almost no β-carotene is absorbed intact, whereas in man almost 20-75% is absorbed intact and distributed to the liver, kidneys and fat (Redlich et al., 1996; Rock, 1997; Wang et al., 1992). Therefore, it may be concluded that rodents are not suitable models for evaluating the bioavailability and effects of β-carotene in humans, also taking into account the extent of efficient biliary elimination of β-carotene in humans. The ferret (Gugger et al., 1992; Rock, 1997; Wang et al., 1992, 1999; White et al., 1993b), pre-ruminant calf (Poor et al., 1992) and the Mongolian gerbil (Krinsky et al., 1990; Mathews-Roth, 1993) are considered more suitable animal models to study the effects of β-carotene as these animals also absorb and release intact β-carotene from the enterocyte into plasma and liver, kidney fat. Normally, serum β-carotene levels are low in these animals, but after dietary supplementation serum β-carotene levels increase to concentrations similar to those detected in human serum (Gugger et al., 1992; Ribaya-Mercado et al., 1989, 1992, 1993; Wang et al., 1999; White et al., 1993a, 1993b).

3.2. Toxicological data

3.2.1. Acute oral toxicity

In mice, the LD_{50} of dried *Dunaliella bardawil* diluted in a carboxylmethyl cellulose sodium (CMC-Na) solution (single oral intubation no details on β-carotene content are given) was >10 000 mg/kg bw (Aruga, 1987).

In another mouse study, the LD_{50} of a preparation containing 30% β-carotene was found to be > 20000 mg/kg bw (SCF, 1999).

In rats given dried *Dunaliella* (by gavage; no details on β-carotene content) no mortalities or overt signs of toxicity were noted after a single dose of 5000 mg/kg bw (Lock, 1985).

In a study with Wistar rats (2/sex) animals were given a single oral dose of 2000 mg/kg of β-carotene from *Blakeslea trispora* dissolved in propylene glycol and were observed and weighed daily for eight days following administration. Minimal weight loss was observed and gross macroscopy showed no adverse effects. No deaths occurred (Kluifthoof, 2001). JECFA noted that only means and standard deviations were provided, which, in view of the small size of the study, appeared inappropriate.

In Sprague-Dawley rats, no acute toxicity (mortality, health or behaviour) was noted after exposure to a single oral dose of 5000 mg β-carotene/kg bw (SCF, 2000b).

In another acute toxicity study in rats (Wistar), a single oral dose (gavage) of 2000 mg β-carotene/kg bw did not induce effects on mortality, health or behaviour. At autopsy no gross lesions were observed (Woutersen et al., 1999).

In a single acute oral toxicity test in dogs, the LD_{50} of β-carotene was found to be >8000 mg/kg bw (Nieman et al., 1954[*]).

No new studies on acute toxicity have been published since the previous evaluations.

The Panel concluded that no acute oral toxicity occurs at relatively high doses of β-carotene.

[*] In an earlier evaluation of β-carotene (JECFA, 1966) this study is ascribed to Bagdon et al. 1960)

3.2.2. Short-term and subchronic toxicity

JECFA describes several short-term and subchronic toxicity studies with β-carotene.

The Panel noted that many of the studies used for the JECFA and SCF evaluations were performed before or around the mid-seventies. At that time the first Good Laboratory Practice (GLP) guidelines were implemented. It is unclear whether the studies described in the previous evaluations comply with the (OECD) GLP guidelines. In addition, TemaNord does not report on the GLP compliance of the more recent studies used for their evaluation.

Studies with mixed carotenes [E160a (i)]

In a short-term study in rats, groups of animals (5/group/sex) were orally administered a 0.5% CMC-Na solution containing spray-dried *Dunaliella bardawil* at doses of 0, 0.5 or 2.5 g/kg bw/day (no detail on β-carotene content) for 28 days (Furuhashi, 1989).

In the males of the high-dose group a significant increase in the relative weight of the kidneys was noted, in comparison to controls. In addition, a few animals in the high-dose group showed slight histopathological changes in the kidneys and thymus (no further details). No effects were noted with regard to food consumption, body weight gain, urinalysis, ophthalmoscopy, haematology, or serum biochemistry. Despite the effects observed in the kidneys, the authors suggested a No-Observed-Adverse-Effect Level (NOAEL) of 2.5 g *Dunaliella bardawil*/kg bw/day (Furuhashi, 1989).

In a 45-day study, Sprague-Dawley rats (21 days old; sex not given) animals received diets containing: spray dried *Dunaliella salina* (0.61% β-carotene), *Dunaliella salina*-oil-extract (24.83% β-carotene), oleoresin of carrots (11.5% β-carotene), and synthetic β-carotene (100%). The diets contained equivalent percentages of β-carotene, and the control diet contained 0.036% β-carotene. The rats on both types of *Dunaliella salina* diets gained weight more rapidly than those receiving either control diet or the diet containing synthetic β-carotene. Gross observations at autopsy revealed no alterations in the rats (Ghazi et al., 1992). The Panel considered this finding not relevant for the risk assessment of β-carotene.

Kuroiwa et al. (2006) performed a 90-day toxicity study with D*unaliella* carotene (*Dunaliella bardawil* or *Dunaliella salina*) in F344 rats (10/group/sex) at dose levels of 0 (control), 0.63%, 1.25%, 2.5% and 5% in powdered basal diet. The average daily intakes of *Dunaliella* carotene were 352, 696, 1420 and 2750 mg/kg bw/day respectively for males and 370, 748, 1444 and 2879 mg/kg bw/day for females. No mortality or treatment-related clinical signs were observed throughout the experimental period in any of the groups. Body weight gain was slightly but significantly ($p<0.05$) reduced from week 5 to the end of the study in 2.5% and 5% males. Based on growth suppression, NOAELs were determined to be 1.25% (696 mg/kg bw/day) for males and 5% (2879 mg/kg bw/day) for females.

Two groups of weanling Sprague-Dawley rats (10/group/sex) were given diets containing 0 or 10% algal β-carotene powder (no detail on β-carotene content) for 12 weeks. No significant differences in food intake or body weight gain were observed compared to controls. No treatment-related differences were observed in organ weights (heart, lungs, liver, kidneys, spleen, gonads and adrenals), or clinical biochemical parameters (serum glucose, alkaline phosphatase, uric acid, Blood Urea Nitrogen (BUN), triglycerides, and cholesterol). In a single treated male aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were elevated. No histopathological examination was performed (Majnarich, 1988).

Studies with β-carotenes [E 160a (ii)]

Groups of rats (Wistar; 5/sex/group) were fed a diet containing 0.2, 1, or 5% β-carotene (equivalent to 0.1, 0.5 or 2.5 g/kg bw/day) derived from *Blakeslea trispora* for 28 days. Other groups were fed either the powdered diet only, or a different sample of β-carotene from *Blakeslea trispora*, or a commercial β-carotene sample (Kluifthoof, 2001). No significant adverse effects were observed with regard to

body weight, dietary intake, haematological and biochemical parameters, or histopathological examination of the organs (Kluifthoof, 2001).

In the first study, groups of Sprague-Dawley rats (both sexes; no detail on group size) were fed synthetic β-carotene at dose levels of 0, 250, 375, or 500 mg/kg bw/day for four weeks. Relative liver and kidney weights were significantly increased in the top-dose group for both sexes. This effect disappeared within two weeks after treatment was terminated. In addition, no treatment-related abnormalities were observed with regard to clinical signs, haematology, clinical chemistry, or urinalysis (Merkle et al., 1980).

In a sub-chronic toxicity study, groups of rats (Wistar) received synthetic β-carotene at dose levels of 0, 250, 500, or 1000 mg/kg bw/day for 90 days. The only effect observed was discolouration of the liver and/or adipose tissue in some females of all dose groups. This effect disappeared after a non-treatment period. No treatment-related abnormalities were seen regarding clinical signs, haematology, clinical chemistry, organ weights, or gross and histopathological examination (Buser and Arceo, 1995).

Finally, in addition to the above mentioned studies, the JECFA (1975) evaluation describes some short-term studies on hypercarotenemia and hypervitaminosis A. Hypercarotenemia (or carotenodermia) has no toxicological symptoms other than yellowish discolouration of the skin. When excess intake of β-carotene is discontinued hypercarotenemia disappears (Abrahamson and Abrahamson, 1962; Nieman et al., 1954).

In another experiment, no evidence of hypervitaminosis A or liver damage (or any other deleterious effects) was observed when 20 mg/kg bw of β-carotene was fed to 30 young rats on a vitamin A-deficient diet for 6 to 11 months (JECFA, 1975).

Studies with mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)]

In a subchronic study in mice, animals received a vitamin A-deficient synthetic standard diet containing spray dried *Dunaliella bardawil* or synthetic all-*trans* β-carotene. The concentration of β-carotene in both test diets was 0.55 mg/kg diet (equivalent to approximately 0.08 mg/kg bw/day). Mammary growth and endocrine parameters were examined. Consumption of the *Dunaliella bardawil*-containing diet showed no deleterious effects on mammary gland and uterine growth or mammatrophic hormone secretion in mice between 20 and 120 days of age. These results were similar to previously-observed results in aged and mammary tumour-bearing mice. Puberty and body growth were accelerated by *Dunaliella bardawil* compared to the synthetic all-*trans* β-carotene (Nagasawa et al., 1989).

3.2.2.1 New literature

Studies with mixed carotenes [E 160a (i)]

The effect of algal β-carotene was investigated in a short-term toxicity study in rats after they received oral doses (not further specified) of 500 and 1000 mg/kg bw/day. No signs of toxicity were noted (Shiomi and Koike, 2000).

Studies with β-carotene [160a (ii)]

Jonker (1997) examined the sub-acute oral toxicity of synthetic β-carotene by administering the compound at dietary concentrations of 0, 0.2, 1.0 and 5.0% to Wistar rats for 28 days. All groups consisted of 5 males and 5 females. None of the rats died intercurrently. Apart form the orange discolouration of the fur of animals kept on diets containing 1.0 or 5.0% β-carotene, clinical observations did not reveal any treatment-related changes. There were no significant differences in body weight, food consumption, haematology, clinical chemistry, absolute and relative organ weights.

Apart form the orange discolouration of the gastrointestinal content and the fur (1.0 and 5.0% groups), gross and histopathological examinations revealed no abnormalities attributable to the ingestion of the test substance. Based on these observations it was concluded that the NOAEL was 5.0% in the diet corresponding to 3809 mg/kg bw/day for males and 4175 mg/kg bw/day for females.

In another subchronic toxicity study with β-carotene (from *Blakeslea trispora*), rats (10/sex/group) received the compound at dietary levels of 0, 0.2, 1 or 5% (equal to average intakes of 118, 581, and 3127 mg/kg bw/day for males, and 126, 663, and 3362 mg/kg bw/day for females, respectively) for 90 days. During clinical observations, red colouring of fur (1 and 5%) and faeces (all doses) was observed. No treatment-related adverse effects were noted with regard to body weight, food and water consumption, urinalysis, ophthalmology, haematology, serum biochemistry, or organ weights. Histopathological examination revealed sporadic spontaneous lesions which were not treatment-related and were common to this strain of rats. Based on these findings, the NOAEL was identified by the authors at 3127 and 3362 mg/kg bw/day, the highest doses tested, for males and females respectively (Nabae et al., 2005)

3.2.3. Genotoxicity

3.2.3.1 In vitro studies

Mixed carotenes [E160a (i)]

The mutagenic potency of *Dunaliella bardawil* was studied in an bacterial reverse mutation assay with *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2 uvrA), at concentrations of 313, 625, 1250, 2500 and 5000 µg *Dunaliella bardawil* paste/plate, with and without metabolic activation. No mutagenic effect was observed in any of the strains tested (Aruga, 1988).

A commercial corn oil-extract of *Dunaliella salina* (EK 87-0048 B-CAT; concentration tested unknown) was not mutagenic in the bacterial reverse mutation assay with *Salmonella typhimurium* (TA 98, TA100, TA1535, TA1537, and TA1538) with or without metabolic activation (Jagannath, 1987). The same product was not active in an in vitro unscheduled DNA synthesis assay using rat primary hepatocytes (Cifone, 1987). The same product also gave negative results in a hypoxanthine-guanine phosphoribosyltransferase (HGPRT) forward mutation assay in cultured Chinese hamster ovary cells, with or without metabolic activation. A dose-related cytotoxicity was noted at concentrations above 2.0 µl/ml without metabolic activation and above 10.0 µl/ml with metabolic activation (Young, 1987).

β-Carotene [E 160a (ii)]

No mutagenic activity was observed in an study with β-carotene derived from *Blakeslea trispora* when tested in two independent experiments with *Salmonella typhimurium* (TA1535, TA1537, TA98, and TA100) at five (non-specified) concentrations with and without metabolic activation (Kluifthoof, 2001).

In the same study, no clastogenic effects of β-carotene derived from *Blakeslea trispora* were recorded in two independent assays in cultured Chinese hamster ovary cells with serial dilutions from 0.1 to 25 mg/ml with and without exogenous metabolic activation (Kluifthoof, 2001).

In vitro studies have shown that synthetic β-carotene was not mutagenic in the bacterial reverse mutation assay using the *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 in both the absence and the presence of S9-mix (van Delft, 1996; Schüpbach, 1979), and that it was not clastogenic in Chinese hamster ovary cells (50, 100 and 200 µg/ml, with and without metabolic activation) (De Vogel and van Delf, 1996).

Synthetic β-carotene was also shown not to affect the frequencies of sister chromatid exchanges (SCEs) in cultured Balb/c mouse mammary gland cells (Manohararan and Banerjee, 1985), chromosomal aberrations (CAs) or micronuclei in Chinese hamster ovary cells (Cozzi et al., 1997; Salvadori et al., 1994; Stich and Dunn, 1986, De Vogel and van Delft, 1996), or micronuclei in metabolically competent HepG2 human hepatoma cells (Salvadori et al., 1993) in in vitro antimutagenicity studies. The Panel noted however that such studies have limited relevance in view of the very low concentrations of β-carotene applied.

It has been reported that relatively high concentrations of synthetic β-carotene enhanced hydrogen peroxide-induced DNA damage in human hepatocellular HepG2 and HT29 cell lines (Lowe et al., 1999; Woods et al., 1999). In HT29 cells β-carotene protected against oxidative damage at low concentrations but rapidly lost this capacity at higher doses (Lowe et al., 1999).

In an in vitro study in human lymphocytes, a statistically significant increase in micronucleus formation was observed in blood cultures incubated 16-18 hours in the presence of synthetic all-*trans* β-carotene (1-30 µg/ml). In the same study, natural β-carotene (containing 9-*cis* β-carotene) had no effect on micronucleus formation (Xue et al., 1998). The Panel noted that the study was based on a protocol ("nuclear anomaly test") that was not validated, and different from the current in vitro micronucleus test; moreover, the reported increase of micronuclei (0.5‰ vs 2.0‰ in control and high dose, respectively) is in the range of the spontaneous incidence of micronuclei in human lymphocytes. Thus no firm conclusion on the genotoxicity of synthetic all-*trans* β-carotene can be drawn from this in vitro study.

3.2.3.2 In vivo studies

β-Carotene [E 160a (ii)]

In BALB/c mice receiving synthetic β-carotene in distilled water or corn oil by gavage at concentrations of 200 mg/kg bw/day for five days, no induction of CAs was observed in bone marrow cells (Salvadori et al., 1992a, 1992b).

In a study on the anti-clastogenic activity of synthetic β-carotene, male Swiss Albino mice received the substance orally, dissolved in oil, at doses of 2.7 and 27 mg/kg bw/day for seven days. Significant increases in both structural CAs and micronuclei were observed at the highest dose (Mukherjee et al., 1991). However, these results were not confirmed in a subsequent study by the same research group (Agarwal et al., 1993).

In an in vivo study, the anti-mutagenic potential of β-carotene was investigated. In this study, the carcinogen N-ethyl-N-nitrosourea was used and anti-mutagenicity was observed after administering synthetic β-carotene (0.15% in drinking water) to N-ethyl-N-nitrosourea treated rats in the drinking water (at 0.15%) for up to eight weeks (Aidoo et al., 1995).

The Panel noted that the study in mice showed negative results

β-Carotene (not specified)

In an in vivo mouse micronucleus assay in adult ICR mice, a commercial carotene extract (not further specified) did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes (no further details) (Ivett, 1987).

No increase in micronuclei was observed in bone-marrow cells of Swiss Albino mice given daily doses of 2.5 mg β-carotene (of unspecified origin) in drinking-water for 15 days (Lahiri et al., 1993) or after two-fold oral application of 58.5, 117 and 234 mg synthetic β-carotene/kg bw, 30 and 6 hours prior to sacrifice of the mice (Gallandre, 1979).

In hybrid B6C3F1 mice no increase in micronuclei was observed in bone-marrow cells after being fed 100 mg/kg β-carotene (unspecified) in the diet for one week (Raj and Katz, 1985).

The Panel concluded that in vivo genotoxicity studies did not show any genotoxic effect of β-carotene.

3.2.3.3 New literature

In an bacterial reverse mutation assay with *Salmonella typhimurium* (strains TA100, TA98, TA135) and *Escherichia coli* (strain WP2uvrA) algal β-carotene was found to be non-mutagenic both with and without metabolic activation (no further details) (Shiome and Koike, 2000; based on abstract).

Three publications by Alija et al. (2004; 2005; 2006) are available, which describe experiments investigating the genotoxic potential of β-carotene and its oxidation products, among which β-apo-8'-carotenal.

Alija et al. (2004; 2005) investigated the genotoxic potential of β-apo-8'-carotenal, β-carotene, and a β-carotene cleavage product (CP) mixture in primary rat hepatocytes at concentrations of 0, 0.01, 0.1, 1, 5 or 10 µM. The endpoints tested were: the mitotic indices, percentage of necrotic and apoptotic cells, cells with micronuclei (MN), chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs). A statistically significant increase of micronuclei and chromosomal aberrations was observed when cells were treated with CP or apo-8'-carotenal at concentrations from 0.1 to 10 µM. In the same experimental conditions, a dose related increase in sister chromatid exchanges, which attained statistical significance at the top dose, was also observed. In the same study, β-carotene (0.01 – 10 µM) induced neither significant cytotoxic nor genotoxic effects.

The Panel noted that the CP mixture is produced by degradation using NaClO. The similarity of the degradation products obtained by this method and those produced under normal conditions of use is not discussed. The authors determined the composition of the CP mixture but only the main products are quantified: "*The CP mixture obtained from a 0.5 mM β-carotene stock solution contained β-carotene (0.16 mM), β-apo15'-carotenal (0.08 mM), β-apo12'-carotenal (0.12 mM) and β-apo8'-carotenal (0.006 mM) and a number of products which could not be identified by HPLC*". Thus 13.4% was not identified. Using GC-MS, the authors determined semi-quantitatively some other components: "*Related to all peaks detected during GC--MS analysis was a peak area of ~4.8% accounting for β-cyclocitral, 0.1% for ionene, 9.9% for β-ionone, 1.9% for β-ionone-5,6-epoxide and 4.5% for dihydroactinidiolide. Furthermore, 4-oxo-β-ionone was detected in trace amounts*." But some components of the CP are non-identified. The use of NaClO may produce chlorinated derivatives that are generally more mutagenic than the same non-chlorinated products. Concerning the cell type used in this assay system, the Panel noted that the incidence of spontaneous aberrations observed in the control cultures is unusually high: 7.90±0.90% for the frequency of micronuclei and 1.20±0.03 aberration per metaphase. This points to genomic instability in this cell system.

The Panel identified more pitfalls in this study (see Discussion), which is therefore considered of limited validity for the evaluation of the genotoxicity of β-carotene and its cleavage products.

In a follow up study, Alija et al. (2006) investigated the genotoxic action of 0.01 - 10 µM of a β-carotene cleavage product mixture in a rat primary hepatocyte assay in the presence or absence of DMNQ (2,3-dimethoxy-1,4-naphthoquinone) or hypoxia/reoxygenation induced oxidative stress. The mitotic indices, the percentage of necrotic and apoptotic cells, the percentages of MN, CAs and SCEs were measured.

In combination with DMNQ (40 µM), the cleavage product mixture (CP) induced a significant increase in MN (at 0.01 and 1 µM), increased CAs (at 1 µM) and induced SCEs (at 1 µM). Cytotoxic effects were observed at 40 µM DMNQ in combination with 10 µM of the cleavage product mixture. In combination with hypoxia/reoxygenation, the cleavage product mixture induced a significant increase in MN (at 0.01, 1 and 10 µM), increased CAs (at 0.01, 1 and 10 µM), and induced SCEs (at 1

and 10 µM). No cytotoxicity was observed. The Panel noted that this study, which concerns the enhancement by CP of the genotoxic effects of the free radical-generator DMNQ or hypoxia/reoxygenation, has limited relevance for the assessment of the genotoxic potential of CP *per se*.

Kalariya et al. (2009), studied the genotoxic effects of the β-carotene breakdown product β-apo-8'-carotenal in Human Retinal Pigment Epithelial Cells (ARPE-19) using the Comet assay. They concluded that their results suggest that β-apo-8'-carotenal, when applied at partially toxic doses, is genotoxic, inducing DNA strand breaks prevented by high levels of glutathione (GSH). The authors stated that the mechanism of genotoxicity of β-apo-8'-carotenal was not elucidated.

However, for the Kalariya study, the same limitations hold as for the Alija et al. (2004; 2005) studies about the representativeness of the degradation products, because the same method was used to produce them. The same group showed (Kalariya et al., 2008) that these degradation products induced cell toxicity and apoptosis in the same range of concentrations that was tested in the study with ARPE-19 cells. To which extent ghost cells are taken into account is not described. Therefore the Panel noted that the comets could be, at least partly, a consequence of the effects of cytotoxicity and/or apoptosis.

The Panel further noted that products used in these studies are not representative of the β-carotene breakdown products under physiological conditions.

Overall, the Panel concluded that the genotoxicity studies of the β-carotene breakdown products in primary rat hepatocytes and in ARPE-19 cells provide very limited evidence of genotoxicity.

In another in vitro study by Marques et al. (2004), calf thymus DNA was allowed to react with β-apo-8'-carotenal, β-carotene and retinal at 37°C for 72 hours in the presence or absence of 50 mM H_2O_2 in neutral (pH 7.4) and basic (pH 9.4) conditions. 1,N^2-etheno-2'-deoxyguanosine formation was measured. This adduct was also formed in all control incubations (DNA only, in the absence and presence of H_2O_2 in neutral and basic conditions). 1,N^2-etheno-2'-deoxyguanosine formation was significantly increased at both pHs in the presence of β-apo-8'-carotenal, β-carotene and retinal, compared with control incubations. Also, in the presence of H_2O_2, adduct formation was increased by all test compounds. In addition, significant increases in 8-oxo-7,8-dihydro-2'-deoxyguanosine were observed when all three compounds were incubated in the presence of H_2O_2. 1,N^2-etheno-2'-deoxyguanosine has been proven to be mutagenic in *E. coli* uvrA$^-$ (Langouët et al., 1998).

In an in vitro study by Yeh and Wu (2006), it was found that β-apo-8'-carotenal induced DNA strand breaks, lipid peroxidation and expression of CYP1A2 in A549 cells (human alveolar epithelial cells). Furthermore, both β-apo-8'-carotenal and β-carotene significantly enhanced DNA strand breaks and CYP1A2 expression, induced by benzo[a]pyrene at 20 µM. However, β-carotene at 2 µM significantly suppressed BaP-induced strand breaks. DNA damage was found to be associated with expression of CYP since the effects were diminished in the presence of 1-aminobenzotriazole, a CYP inhibitor.

Zhang and Omaye (2001) have investigated the relationship between β-carotene (0.1, 0.2, 0.4, 0.8, and 1.6 µM) and induced (supercoiled plasmid) DNA strand breakage under different O_2 tensions (2.0, 20.0, and 101 kPa). Under 2.0 kPa (15 torr), β-carotene provided a dose-dependent protection against 2,2'-azobis(2-amidinopropane)dihydrochloride) (AAPH)-induced DNA strand breaks. Under 20.0 kPa (150 torr) of oxygen tension, the anti-oxidant effect of β-carotene was diminished at ≥ 0.8 µM. Also, at 20.0 kPa (150 torr), a pro-oxidant effect was found at ≥ 0.8 µM β-carotene, producing more single- and double-strand breaks. At 101 kPa (760 torr) the pro-oxidant effect of 0.8 µM β-carotene caused DNA to completely breakdown to circular and linear forms. The researchers concluded that β-carotene causes concentration-dependent DNA breakdown at high O_2 tension.

The Panel concluded that in previous evaluations many studies on β-carotene have been described that have focussed on a broad range of genotoxicity endpoints in different in vitro and in vivo test systems.

In none of these studies were any signs of genotoxicity noted. Therefore, the Panel concluded that β-carotene is not of concern with respect to genotoxicity.

A few in vitro studies with β-carotene cleavage products provide limited evidence of DNA damaging activity and covalent binding to DNA. In this respect the Panel noted that such results may reflect a pro-oxidant effect, common to other antioxidants, which is elicited under specific in vitro conditions which may not occur in vivo.

3.2.4. Chronic toxicity and carcinogenicity

β-Carotene [E 160a (ii)]

In a mouse study, animals (100/sex/group) were fed diets containing synthetic β-carotene at levels of 0, 100, 250, 500, or 1000 mg/kg bw/day for 104 weeks. Cells lining the sinusoids of the liver were found to be vacuolated in a number of animals (no further details). Discolouration of faeces (red), stomach contents and adipose tissue (orange), and fur and skin (yellow) was also noted. No change in tumour incidence was observed (Buser and Hummler, 1983).

In a study in rats, four groups of animals (60/sex/group) were given diets containing synthetic β-carotene at doses of 0, 100, 250, 500, or 1000 mg/kg bw/day for 114 weeks (females) or 116 weeks (males). Weight-gain reduction was noted at all doses but was not accompanied with any sign of organ toxicity. Macroscopic observations at autopsy revealed yellow-orange fur and adipose tissue. Furthermore, no treatment-related effects were noted with respect to haematology, blood chemistry, urinalysis, ophthalmoscopy, histopathology, or tumour incidence (Buser and Hummler, 1983). The Panel noted that the Busre and Hummler study was not available for the evaluation

In a dog study, groups of animals (8/sex/group) were treated with synthetic β-carotene at doses of 0, 50, 100, or 250 mg/kg bw/day. Per group, three animals were treated for 104 weeks, three animals for 88 weeks followed by a 16 week recovery, and two animals were autopsied after 52 weeks of treatment. No adverse effects were found with regard to food and water consumption, haematology, clinical chemistry, urinalysis, ophthalmoscopy, or organ weights (Buser and Hummler, 1983). According to the SCF (2000c) evaluation a dramatic weight loss was observed after withdrawal of β-carotene treatment in the recovery period (no further details). Macroscopic and histopathological examinations revealed irregular pale-orange foci on the surface of the liver in the treated animals, which at microscopical examination appeared to be perisinusoidal fat storage cells. The authors considered the presence of these cells an indication of storage of vitamin A rather than a reflection of a toxic effect.

The SCF (2000b) evaluation briefly described a large number of animal studies to assess the potential inhibitory effects of β-carotene on experimentally induced carcinogenesis. Although the possible beneficial effects of β-carotene are beyond the scope of this evaluation, the findings are listed below without providing study details.

In association with β-carotene supplementation, cancer preventive effects have been reported for the following tumours in the animals indicated (IARC, 1998; Moreno et al., 1991; 1995; Temple and Basu, 1987; Shivapurkar et al., 1995; Yamamoto et al., 1994; Alabaster et al., 1995; Appel and Woutersen, 1996; Appel et al., 1991; Azuine et al., 1992; Mathews-Roth et al., 1991; Alam and Alam, 1987) in skin (mice), liver (rats), colon (rats, mice), pancreas (rats, hamsters), forestomach (mice), bladder (mice), salivary gland (rats) and adenocarcinomas and nephroblastomas (rats). Other studies (Mathews-Roth and Krinsky, 1987; Steinel et al., 1990; Astorg et al., 1996, 1997; Colacchio and Memoli, 1986; Jones et al., 1989; Appel et al., 1996; Pedrick et al., 1990; Colacchio et al., 1989; Alam et al., 1984, 1988) have shown no effect of β-carotene supplementation on the incidence of tumours of the skin (mice), liver (mice, rats), colon (rats), pancreas (rats, hamsters), glandular stomach (rats), bladder (mice, rats), small intestine (rats), and salivary gland (rats).

The SCF (2000b, c) evaluations more specifically focussed on the inhibitory and/or stimulatory effect of β-carotene on experimentally-induced tumourigenesis in the respiratory tract. Although these studies are not necessarily long-term studies, they do address carcinogenicity and, therefore, are discussed in this section. The majority of studies have shown no or equivocal (inhibitory or stimulatory) effects of β-carotene on experimentally-induced respiratory tract tumourigenesis in mice or hamsters (Murakoshi et al., 1992; Moon, 1994; Nishino, 1995; Yun et al., 1995).

In a study in hamsters, an inhibitory effect of β-carotene on cigarette smoke-induced respiratory tract tumourigenesis was observed. In this study, male animals (30/group) were exposed to dietary supplementation with β-carotene at levels of 0, 0.005, 0.05, or 0.25% (equivalent to approximately 0, 5, 50, or 250 mg/kg bw/day) in combination with cigarette smoke for 12 weeks. The incidence and multiplicity of papillomas in the group administered 0.5% β-carotene in their diet was statistically significantly lower than in controls. Moreover, β-carotene statistically significantly reduced both the incidence and multiplicity of hyperplasias in a dose-dependent manner (Furukawa et al., 1999[*]).

In another study, Syrian Golden hamsters (40/sex in the test groups; 60/sex in control group) received a diet containing β-carotene at a dose level of 0 or (approximately) 5.6 mg/kg bw/day for 374 days (females) or 429 days (males). Beginning at day 30, all animals received eight intra-tracheal instillations of 8 mg benzo[a]pyrene (a typical cigarette-smoke carcinogen) during a 16-week period. Although a slight increase in overall respiratory tract tumour incidence was noted in both male and female β-carotene-treated groups, these findings were not statistically significant. However, increases in some further specified respiratory tract tumour incidences epidermoid papillomas in the trachea, bronchi and larynx, and overall incidence of tracheal tumours) were statistically significant. The development of pre-neoplastic changes in the respiratory tract was not affected by β-carotene supplementation (Beems, 1987).

Groups of Syrian golden hamsters (50 males/group) were fed a diet containing 0 or 1% (w/w) (approximately 990 mg/kg bw/day) β-carotene. After one month, all animals received 10 intra-tracheal instillations of 8 mg B[a]P in 12 weeks. The incidences of pre-neoplastic and neoplastic changes (hyperplasia, squamous metaplasia, papilloma, adenoma, squamous-cell carcinoma and adenocarcinoma) in the larynx, trachea and lungs were almost twice as high in the β-carotene/B[a]P group (37%) compared to the B[a]P treated controls (21%), although the difference was not statistically significant (Wolterbeek et al., 1995).

In a study in ferrets, four groups of animals (six males/group) were fed a pure crystalline (all-*trans*) β-carotene at dose levels of approximately 0.16 (based on β-carotene present in the basal diet) or 2.4 mg/kg bw/day (which includes the β-carotene present in the diet) for six months. In addition, animals were either exposed or not to cigarette smoke (smoke from 10 cigarettes in a chamber; 30 minutes, twice daily). Plasma β-carotene increased 17-22-fold in the high dose group compared to the basal diet group. Histopathological evaluation revealed that all animals fed the high dose of β-carotene in the diet, even those not exposed to cigarette smoke, showed increased cell proliferation and squamous metaplasia of alveolar epithelium. These findings were further enhanced in the animals also exposed to cigarette smoke, whereas animals exposed to cigarette smoke alone did not show these changes (Wang et al., 1999; Liu et al., 2000).

These experiments were performed with an unstable, non-protected synthetic β-carotene, whereas similar experiments performed with a protected form (beadlets) of synthetic β-carotene did not show any histopathological changes in the lungs of ferrets (Fuster et al., 2008; Kim et al., 2006).

Fuster et al. (2008) treated ferrets for 6 months with β-carotene (0.8 and 3.2 mg/kg bw/day) using a stabilized water soluble formulation (beadlets) containing β-carotene, DL-α-tocopherol and ascorbyl

[*] SCF refers to a study by Gocke (1994) which appears to be erroneous.

palmitate. They found no higher expression of cell proliferation markers or of squamous metaplasia in the lungs of animals treated with these beadlets.

Kim et al. (2006) found that a combination of β-carotene, α-tocopherol and ascorbate protected against 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinogenesis in smoke-exposed ferrets; moreover, there were no lesions observed in the lungs of the group supplemented with the combined β-carotene, α-tocopherol and ascorbic acid only.

The Panel noted that the limited number of studies performed with hamsters and ferrets demonstrated equivocal results, which might be related to the instability of β-carotene.

3.2.5. Reproductive and developmental toxicity

Mixed-carotenes [E 160a (i)]

In a one-year multigeneration study, (F0-F3) groups of rats (10 males and 20 females/group) were raised on diets containing 0, 5 or 10% dried *Dunaliella bardawil* (equivalent to 0, 2500, or 5000 mg/kg bw/day). As β-carotene content of the dried algae was 2%, β-carotene intake levels were 0, 50, or 100 mg/kg bw/day respectively (Mokady et al., 1989). No significant differences between exposed rats and controls were detected in any generation with regard to growth, general appearance, behaviour, survival, or reproductive performance (frequency of pregnancies, litter sizes, and pup weights and survival) In treated males, gross and microscopic examination of the internal organs revealed a slight increase in weight of the kidneys and a slightly higher frequency of metaplasia of the renal pelvis epithelium with nephrocalcinosis in the renal papillae. These effects have been attributed to a relatively high salt content of the algae. In addition, an increased frequency of bronchopneumonia was noted which might be due to the powdery nature of the algal diet. Overall, these effects were not considered by the authors to be of major toxicological significance (Mokady et al., 1989).

β-Carotene [E 160a (ii)]

In a multigeneration study, four generations of rats received synthetic β-carotene as a 0 or 0.1% dietary admixture (equivalent to 0 or 50 mg/kg bw/day) for 110 weeks. The only change attributable to β-carotene treatment was storage of (probably) vitamin A in the Kupffer cells of rats of the first and second generations. In comparison to control, no adverse effects were observed with respect to body weight gain, growth, food consumption, haematological determinations, gross and microscopic pathology, or reproductive performance (Bagdon et al., 1960).

In a 3-generation study, rats received synthetic β-carotene in the diet at dose levels of 0, 100, 250, 500, and 1000 mg/kg bw/day. Treatment did not affect reproductive function (mating performance, pregnancy rate and duration, litter size, pup mortality, litter and pup weight), or development of offspring. Faecal pellets, fur and extremities were discoloured in β-carotene-treated animals (Woutersen et al., 1999).

In an embryotoxicity/teratogenicity study, rats were given daily oral doses of 0, 18, 36, or 180 mg synthetic β-carotene per 100 g (presumably body weight) from gestational days (GDs) 9 to 12. The rats were sacrificed on day 21 of pregnancy. In the low- and high-dose groups some fetal resorptions were observed. The incidence of deformity of the sternum was increased in fetuses in the β-carotene-treated groups compared to control. However, severity and incidence of these effects were not dose-dependent (Komatsu, 1971).

In a study with rats, animals received synthetic β-carotene at dietary doses of 0, 250, 500, or 1000 mg kg bw/day from GDs 7 to 16. No embryotoxic or teratogenic effects were observed. In maternal animals a slight reduction in body weight gain was observed at the highest dose (Kistler, 1981).

Rabbits were given synthetic β-carotene at oral doses of 0, 100, 200, or 400 mg/kg bw/day from GDs 7 to 19. No embryotoxic or teratogenic effects were noted (Kistler, 1982).

3.2.6. Human studies

β-Carotene [E 160a (ii)]

The SCF (2000c) evaluation describes several epidemiological studies that focus on the relationship of β-carotene exposure and the incidence of cardiovascular disease, cancer in general, cancer in the respiratory tract, and other diseases. For convenience, these topics have been subdivided accordingly in this section.

In a study by Klipstein-Grobusch et al. (1999) (Rotterdam-1999-Study in the elderly), the relation of dietary β-carotene intake to the risk of myocardial infarction in an elderly population was investigated. A total of 4802 participants (aged 55-95) free of myocardial infarction at baseline, were followed for four years.

At the highest tertile of β-carotene intake, the relative risk of myocardial infarction was 0.55 (95% Confidence Interval 0.34-0.83) compared with the lowest tertile.

Data from the Alpha Tocopherol Beta Carotene (ATBC) cancer prevention study and the beta CARotene and RETinol (CARET) study strongly suggest a possible harmful role of dietary β-carotene supplementation in cardiovascular diseases.

In one study, male smokers (five or more cigarettes per day at entry) were randomly assigned to one of the four following treatments: α-tocopherol (50 mg/day), β-carotene (20 mg/day), both α-tocopherol (50 mg/day) and β-carotene (20 mg/day) and placebo for five to eight years (median six years). Cardiovascular death increased by 11% in men taking β-carotene. When the analysis was restricted to 1862 participants who had previously had a myocardial infarction, the relative risk for fatal coronary heart disease increased by 75%, and the relative risk for fatal myocardial infarction increased by 244% (ATBC Study group, 1994).

In the CARET study, an increased number of deaths from cardiovascular disease (26%) was seen in a study among male smokers, former smokers (male and female) and male workers exposed to asbestos taking both supplemental β-carotene (30 mg/day) and retinol (25 000 IU, in the form of retinyl palmitate/day) for (mean) four years (Omenn et al., 1996b; Omenn, 1998).

In a study in patients previously diagnosed with skin cancer, supplementation with 50 mg β-carotene/day for five years had no effect on the occurrence of new basal-cell or squamous-cell carcinoma. However, a 12-year latency period for these cancers diminished the relevance of these results (Greenberg et al., 1990).

In a study with patients who had a prior history of adenomas, intake of β-carotene (25 mg/day), with or without vitamin C (1 g/day) and α-tocopherol (400 mg/day) for 5-8 years, had no effect on the occurrence of colorectal adenomas (Greenberg et al., 1994).

In a case-control study in women attending a breast clinic, the relationship between concentrations of carotenoids and retinoids in breast adipose tissue and risk of breast cancer was investigated. An inverse association between some (retinyl palmitate, β-carotene, lycopene, and lutein/zeaxanthin) but not all of the measured nutrients was found (no further details) (Zhang et al., 1997).

In a case-control study, 820 women with histologically confirmed breast cancer were compared with 1548 control women. Among post-menopausal women no association was found between any of the micronutrients evaluated and risk of breast cancer. Among pre-menopausal women, a significant

inverse relationship was noted between β-carotene intake from food and breast cancer (Bohlke et al., 1999).

In a placebo controlled cohort study (Tyler asbestos cohort), 755 asbestos workers received 50 mg/day of β-carotene (equal to approximately 0.7 mg/kg bw/day for a 70 kg adult) together with 25 000 IU retinol daily. There was no difference in the two groups with regards to criteria for sputum atypia (which may precede the diagnosis of lung cancer) (McLarty, 1992).

In a trial designed to test the effect of aspirin on cardiovascular disease incidence, 22 071 male physicians (50% never-smokers, 11% current smokers) were given β-carotene at doses of 50 mg on alternate days (equal to approximately 0.7 mg/kg bw/2 days for a 70 kg adult) and followed for a mean of 12.5 years; during the trial the β-carotene treatment group had 4-fold higher mean serum β-carotene concentrations. Overall mortality increased by 2%, whereas slight decreases were noted with regard to (all) malignant neoplasms (2%) and lung cancer (7%) (statistical significance not given) (Steering Committee of the Physicians' Health Study Research Group, 1989).

In a study with complex factorial design conducted in Linxian (China), 3318 residents diagnosed with oesophageal dysplasia received daily a single supplement containing 26 vitamins and minerals plus a capsule with 15 mg β-carotene (equal to approximately 0.2 mg/kg bw/day for a 70 kg adult) for six years. Controls received placebos. After six years, relative risks for deaths from cancer, in comparison to control, were decreased in the supplemented group by 8% for oesophagus plus gastric cardia, by 16% for oesophagus alone, by 4% for cancer mortality, and by 7% for total mortality. Only stomach cancer mortality was increased by 18% (Li et al., 1993) compared to control.

Blot et al. (1993) also conducted a study with a complex factorial design in Linxian (China). A total of 29 584 adults (presumed to be vitamin- and mineral deficient) were given daily supplementation with a combination of 15 mg β-carotene (equal to approximately 0.2 mg/kg bw/day for a 70 kg adult), 50 μg selenium, and 30 mg α-tocopherol for over five years. The effects were compared with three other combinations of vitamins/minerals and placebos.

After the 5-year intervention period, the β-carotene/selenium/α-tocopherol combination resulted in a 13% reduction in cancer deaths which was due primarily to a reduced incidence of gastric cancer. The relative risk of death from lung cancer was decreased by 45% (statistical power was limited due to only 31 lung cancer deaths).

In the ATBC study, 29 133 male smokers, with a smoking history of 1 pack/day on average for 36 years, received daily doses of 20 mg β-carotene (indicated to be approximately 0.3 mg/kg bw/day for a 70 kg adult) for five to eight years (median 6 years) years. The control group received a placebo. After two years of treatment, median serum β-carotene levels had increased 17.5-fold in the β-carotene treatment groups. Subjects receiving β-carotene had significantly higher lung cancer incidence (18%) and mortality (8%) compared to controls (ATBC Study group, 1994).

In the CARET study (Omenn et al., 1996a; 1996b), 18 314 participants (14 254 smokers and former smokers (45% male) and 4060 asbestos-exposed males) received daily doses of 30 mg β-carotene (indicated to be approximately 0.4 mg/kg bw/day for a 70 kg adult) and 25 000 IU vitamin A. The control group received a placebo. After five years of treatment, median serum β-carotene levels had increased 12-fold in the β-carotene treatment groups. After a mean 4-years follow up, lung cancer incidence was significantly increased (28%), and more overall deaths were observed (17%) in smokers and asbestos workers treated with β-carotene and vitamin A, compared to controls (Omenn et al., 1996). Treatment had no statistically significant effect on the risk of mesothelioma (14 incidences in treatment group and 9 incidences in control group) and other cancers. The SCF (2000b) evaluation comments that in this study it is not possible to distinguish between effects from β-carotene and vitamin A, since the two compounds were administered in combination.

In humans, β-carotene has been used to treat patients with erythropoietic protoporphyria. In these patients years-long treatment with β-carotene at doses of 20-180 mg/day induced no toxic effects (no details) or abnormally elevated blood vitamin A concentrations (Mathews-Roth, 1993; Meyers et al., 1996).

Dietary intake of carotenoids has been suggested to reduce the risk of age-related macular degeneration (Seddon et al., 1994; Cooper et al., 1999).

β-Carotene has been studied for a possible role in the prevention of senile cataracts (an ocular condition potentially related to oxidation). However the available results are inconsistent (IARC, 1998).

Finally, although data are limited, carotenoids have been suggested to be of benefit for several other health outcomes such us ageing, impaired cognition, rheumatoid arthritis and cystic fibrosis (IARC, 1998).

The SCF concluded: *"that the general assumption is confirmed that individuals who eat more fruits and vegetables, rich in carotenoids, and/or have high levels of serum ß-carotene, have a lower risk for cancer and cardiovascular diseases. However, a possibility could be that ß-carotene may be only a marker of the intake of other beneficial substances in fruits and vegetables, or perhaps other life-style habits. Actually (see below) no clinical trial of ß-carotene as a single agent, has shown a reduction in the risk of cancer at any specific site. On the contrary there is evidence of an increase in the risk for lung cancer among smokers and asbestos workers receiving ß-carotene supplements at high doses, which resulted in blood concentrations an average of 10-15 times higher than normal.*

In summary there was no effect of ß-carotene supplementation on total cancer, on total mortality, or on heart disease. Neither was an effect on lung cancer observed, but due to the lower number of cases, the power of the statistical analysis underlying this conclusion is rather weak (SCF, 2000)."

3.2.6.1 New literature

β-Carotene [E 160a (ii)]

Baron et al. (2003) studied the effect of β-carotene supplementation on colorectal adenoma recurrence among 864 subjects who had had an adenoma removed and were polyp-free at time of entry into the study. Subjects received 25 mg β-carotene or placebo and were followed with colonoscopy for adenoma recurrence one year and four years after the qualifying endoscopy (two other groups received either vitamins C and E or β-carotene in combination with vitamins C plus E, but further details were not provided).

Among subjects who neither smoked cigarettes nor drank alcohol, β-carotene was associated with a marked decrease in the risk of one or more recurrent adenomas (Relative Risk (RR) = 0.56, 95% CI = 0.35 to 0.89). For participants who both smoked cigarettes and also drank more than one alcoholic drink per day, β-carotene doubled the risk of adenoma recurrence (RR = 2.07, 95% CI = 1.39 to 3.08; for difference from non-smoker/non-drinker RR, $P < 0.001$). For smokers who did not drink, the relative risk was 1.36 and significantly higher than for non-smoker/non-drinkers. However, for smokers who had ≤ 1 drink/day, the RR was 0.89 and not significantly different from participants who neither smoked nor drank. For participants who did not smoke but drank alcohol, the RR was 1.10 for participants who consumed ≤ 1 alcoholic drink/day and 1.09 for participants who consumed > 1 drink/day. Baron et al. (2003) concluded that alcohol intake and cigarette smoking may modify the effect of β-carotene supplementation on the risk of colorectal adenoma recurrence.

Graffin et al. (2002) reported a case of β-carotene-induced liver fibrosis. A 66-year-old woman, an alcohol drinker, had been treated with β-carotene during 30 years for a pigmentary retinopathy (total dose 165 g, which would correspond to about 14 mg/day). This woman was presented with anicteric

cholestasis of two years duration. The liver biopsy showed a pronounced portal fibrosis. The blood level of vitamin A was 0.43 g/l (normal range 0.5-0.8 g/l). Amelioration was observed within six months after β-carotene therapy was stopped and ursodeoxychloric acid supplementation was given. Subsequently, the patient remained stable for three years (despite continued alcohol use).

Graffin et al. (2002) postulated that the combined exposure to alcohol and β-carotene was involved in the pathogenesis of the observed liver fibrosis. Further they stated that ethanol, while promoting a deficiency of β-carotene also enhances its toxicity. The authors enforce this conclusion by referring to a review (Leo et al., 1999) in which hepatotoxicity was associated with interference of alcohol on β-carotene conversion to retinol.

DSM (2009) conducted an extensive review of the scientific literature, published since 2000. This review identified 9 randomized controlled trials, 7 follow-up publications related to trials performed before 2000, 34 observational studies and 11 meta-analyses. Of the 9 high quality intervention studies, 8 included cancer as an endpoint. Across these studies approximately 11 000 current smokers received β-carotene doses of at least 20 mg/day and in some cases up to 50 mg/day for periods of time similar or longer than those used in the ATBC or CARET studies. Furthermore, approximately 10 000 ever smokers and 2500 current smokers took between 6 and 15 mg β-carotene per day. The negative effects observed in heavy smokers in the ATBC and CARET studies were not seen in any other intervention study. Therefore, the authors concluded that the increased lung cancer incidence in β-carotene supplemented smokers has been demonstrated to be specific to individuals who chronically smoke more than 20 cigarettes per day.

Druesne-Pecollo et al. (2010) performed a systematic review and meta-analysis of randomized controlled trials (RCT) investigating β-carotene supplementation and cancer risk. A total of 352 abstracts or full-text manuscripts were identified and reviewed independently by 2 investigators using the following inclusion criteria: original research article, RCT design, intervention consisting of beta-carotene supplementation (alone or in combination with other antioxidants), with primary cancer as outcome, reporting the relative risk and 95% confidence interval of cancers at the end of the intervention (except for the Women's Health Study, which provided data 2 years after the end of the intervention). Among the 352 publications, 31 were identified as potentially appropriate articles describing data from trials on beta-carotene supplementation and the incidence of various cancer sites. Eighteen of these articles were excluded as they provided duplicate information. Thirteen publications providing data from 9 RCTs were included in the meta-analyses. The meta-analysis of data from these studies, included 180,702 subjects and 1,852 cases of lung cancer, gave a significant overall increased RR of 1.13 (95% CI, 1.04–1.24) in subjects supplemented with beta-carotene compared to those receiving placebo. Compared to corresponding placebo groups, the risk of lung cancer was significantly increased in subjects supplemented with beta-carotene in combination with other antioxidants (RR of 1.16; 95% CI, 1.04–1.29) or with doses of 20 mg/day and above (RR, 1.16; 95% CI, 1.06–1.27). Significantly increased overall RR were also found for subjects supplemented with beta-carotene in populations exclusively composed of smokers or asbestos workers (RR, 1.20; 95% CI, 1.07–1.34) as well as in populations with a majority of men (RR, 1.14; 95% CI, 1.04–1.25) compared to the control groups. No significant effect of beta-carotene supplementation was observed in the other subgroup analyses.

They found absence of any protective effect associated with β-carotene supplementation with regard to primary cancer risk. However, their analyses indicated an increased risk of lung and stomach cancers in individuals supplemented with β-carotene at dose levels equal to or greater than 20 mg/day as well as in smokers and asbestos workers supplemented with β-carotene. A statistically significant interaction was found between beta-carotene intake and smoking status. The authors identified four limitations arising from the available data as a reason certain results should be considered with caution.

1. in most RCTs subjects were supplemented with beta-carotene in combination with other antioxidants.
2. the number of RCTs was limited for some meta-analyses.
3. meta-analyses conducted in subgroups also have some limitations. For example, the results obtained in the case of lung and stomach cancers suggest that the daily dose of beta-carotene supplementation as well as the exposure to tobacco or asbestos influence the risk of lung cancer in individuals supplemented with beta-carotene compared to controls. Nevertheless, the results of the meta-analyses do not allow conclusions on which of these criteria is the most important.
4. Fourth, the interpretation of the results of the meta-analyses should take into account the specificities of the available RCTs.

The authors noted that the VITAL cohort study has shown that long-term use of beta-carotene supplements for years was associated with a significant increase in lung cancer risk after adjustments for age, gender and smoking.

3.2.7. Sensitivity, allergenicity and intolerance

β-Carotene [E 160a (ii)]

The TemaNord (2002) report described an additional study in 135 patients with urticaria or atopic dermatitis which were given oral doses of 100 mg β-carotene together with 100 mg β-apo-carotenal. One patient reacted positively, while in another patient the response was equivocal. In a group of 123 contact dermatitis patients no response was noted. In both groups a false positive response was also noted (no further details) (BIBRA, 1996).

No new literature on this subject has become available since these evaluations.

3.2.8. Other studies

In a study on the effect of β-carotene on cytochrome P450 (CYP-catalysed) reactions in the liver, kidney, lung and intestine, rats (3/sex/group) were given doses of 0, 250, or 500 mg β-carotene/kg bw (gavage) once or repeatedly for 5 days. Both individual specific substrates for different CYPs and testosterone (as multi-bioprobe) were used. Furthermore, Reactive Oxygen Species (ROS) production by subcellular preparations from the various tissues was measured by EPR (Electron Paramagnetic Resonance) spectroscopy coupled with the spin-probe technique (Paolini et al., 2001).

No differences in body- and organ-weights among the different groups were observed. β-Carotene was able to induce a number of CYP isoforms in all tissues. The most affected were CYP3A1/2 (all organs tested), CYP2E1 (all organs but the lung), CYP1A1/2 (all organs tested), and CYP2B1/2 (liver only). In addition, marked ROS generation (up to 33-fold increase in the liver) was recorded in the various organs (liver > lung > intestine > kidney). CYP and ROS induction were correlated with the concentration of β-carotene accumulated in the various tissues (the liver being most affected).

In an in vitro study by Ni et al. (2001), the toxicity of β-carotene and acetaldehyde alone and in combination, was investigated in HepG2 cells using the mitochondrial reduction function test (MTT) and lactate dehydrogenase (LDH) leakage test.

Mitochondrial MTT reduction was inhibited by β-carotene at very low concentrations (0.05 – 4.5 µM) and in a dose-dependent manner; exposure of cells to acetaldehyde only (single dose) also inhibited MTT reduction. Combined exposure resulted in an additive effect. Acetaldehyde increased LDH leakage from the HepG2 cells into the medium, whereas β-carotene by itself did not show such an effect, but it exacerbated the toxicity of acetaldehyde when exposures were combined. In addition, acetaldehyde and β-carotene inhibited each other's clearance from the medium, which according to Ni et al. (2001) suggests that competitive inhibition of a common metabolic pathway (possibly via aldehyde dehydrogenase) may exist.

In an in vitro study by Al-Wadei et al. (2006), 20 nM of β-carotene caused a significant increase in intracellular cyclic adenosine monophosphate (cAMP) and activated protein kinase A (PKA), as well as increased phosphorylation of extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) and Cyclic AMP Response Element Binding protein (CREB) in a human Pulmonary Adeno Carcinoma (PAC) cell line and immortalised human small airway epithelial cell line. Furthermore, exposure of cells to 20 nM of β-carotene significantly stimulated proliferation of both cell lines at all doses.

4. Discussion

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following a public call for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

In this opinion, the nomenclature and categorisation as described in Commission Directive 2008/128/EC and JECFA (JECFA, 2006) is used, with "*mixed carotenes*" (E 160a (i)) defined as being extracted from either edible plants or other sources (vegetable oils, algae and non edible plants such a grass, lucerne and nettle) and "*β-carotene*" (E 160a (ii)) defined as being chemically synthesised or obtained by extraction from the fungus *Blakeslea trispora*. Mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] are authorised as food additives in the EU and have been evaluated previously by JECFA in 1975 (β-carotene), 1993 (carotenes from natural sources) and 2001 (β-carotene derived from *Blakeslea trispora*), and by the SCF in 1975, 1997 (β-carotene derived from *Dunaliella salina*) and 2000 (β-carotene derived from *Blakeslea trispora*).

Carotenes from natural sources (both vegetable and algal) were evaluated by JECFA in 1993. JECFA considered the data inadequate to establish an ADI for the dehydrated algal carotene preparations or for the vegetable oil extracts of *Dunaliella salina*.

The JECFA (1993) evaluation concluded that no relevant toxicological data for vegetable β-carotene had been submitted and therefore no ADI was established. Nonetheless, JECFA concluded that the use of vegetable extracts as colouring agents was acceptable, provided that the level of use did not exceed the level normally present in vegetables (no numerical ADI allocated) (JECFA, 1993).

For mixed α-,β-,γ- carotenes from natural foods, the SCF did not establish an ADI but felt able to accept the use of mixed carotenes prepared from natural foods as a food colour in food (SCF, 1975). In 1997, the SCF considered the use of a dispersion of β-carotene produced by the alga *Dunaliella salina* growing in large saline lakes in South Australia, acceptable as a food additive.

In 2000, the SCF concluded that β-carotene produced by co-fermentation of *Blakeslea trispora* DS 30627 and DS 30628 is equivalent to the chemically synthesized material used as food colourant and is, therefore, acceptable for use as a colouring agent for foodstuffs (SCF, 2000a).

Mixed carotenes are obtained by solvent extraction from natural strains of (edible) plants. The Panel considered edible plants as materials from plant origin for which it can be sufficiently demonstrated that they have hitherto been consumed by humans.

Currently, carrots (*Daucus carota*), palm fruit oil (*Elaeis guineensis*) and the algae *Dunaliella salina* (incl. *Dunaliella bardawil*) are used as sources for mixed carotenes. Based on information obtained from NATCOL, the Panel noted that grass, alfalfa (lucerne) and nettle are presently not commercially used to obtain mixed carotenes.

Although several HPLC and spectrophotometric methods for analysis of mixed carotenes [E160a (i)] and/or β-carotenes [E160a (ii)] are described in the literature, there are no validated methods for their analysis in foods that might be used for official purposes. Specifications on the purity of mixed carotenes [E 160a (i)] define not less than 5.0% of the commercial product as carotenes when

extracted from edible plants, and not less than 20% when extracted from algae. The actual content of β-carotene in these substances is not specified. From the definition, it is unclear what the remainder of the product exactly consists of. The Panel noted that synthetic β-carotene and β-carotene from *Blakeslea trispora* are highly pure, crystalline substances of one chemical entity, β-carotene, predominantly composed of all-trans-β-carotene. The assay value of not less than 96% (expressed as β-carotene) is linked to the method given in the specifications for the determination of the purity.

In addition, the Panel noted that the specifications of mixed carotenes [E 160a (i)] for the quantities of heavy metals differ from comparable specifications for food colours from natural sources.

The Panel concluded that the specifications of the mixed beta-carotenes [E 160a (i)] obtained from edible plant materials such as carrots and palm fruit oil or from algae are inadequate and need to be updated to define the amount of colouring matters and the material not accounted for.

The Panel noted that β-carotene is susceptible to autoxidation, photodegradation and thermodegradation giving rise to cleavage and degradation products (Boon et al., 2010). Since some of the β-carotene degradation products might be of toxicological relevance, suitable storage conditions should be chosen for β-carotene and β-carotene containing preparations used as a food colour. Furthermore the Panel concluded that specifications should address possible undesired impurities such as β-ionone, dihydroactinidiolide and β-damascone which can be formed due to oxidative degradation of β-carotene as a result of unsuitable storage conditions of the food additive (Boon et al., 2010; Mordi et al., 1991).

Furthermore, the Panel noted that apart from mixed carotenes obtained from algae (*Dunaliella salina or Dunaliella bardawil*) using edible oil (soy bean oil or olive oil), natural mixed carotene complexes prepared by solvent extraction from palm fruit oil (*Elaeis* guineensis) and mixed carotenes obtained by solvent extraction of carrots (*Daucus carota*) are commercially available, but toxicity studies have only been performed with mixed carotenes obtained from algae.

Human absorption of β-carotene varies from 10-90%. The general mechanism of intestinal absorption of β-carotene in mammals is by passive diffusion of mixed micelles. In humans, after intake of β-carotene, 30-90% is excreted in the faeces and only small amounts appear in the serum. Upon intakes of 20-30 mg β-carotene, absorption is linear, whereas at higher intakes absorption becomes limited depending on the food matrix, the amount and type of co-ingested fat and the presence of bile acids. In addition, based on the percentages absorbed and eliminated via the faeces, considerable biliary elimination may be anticipated, which may appear to be dose-dependent. Inter- and intra-species absorption rates differ significantly. Interactions between carotenoids may decrease absorption. Competitive inhibition may occur at the level of micellar incorporation, intestinal uptake and/or lymphatic transport. Simultaneous ingestion of various carotenoids may induce an anti-oxidant sparing effect in the intestinal tract, resulting in increased levels of uptake of protected carotenoids. Even in the presence of large amounts of β-carotene, chylomicrons preferentially take up xanthophylls rather than β-carotene from the intestinal lumen (Gärtner et al., 1996).

After absorption in the enterocyte, β-carotene is incorporated into chylomicrons and reaches the liver via the portal system and to a minor extent also via the lymphatics. In human serum, β-carotene is mainly present in its all-*trans* form, even after significant intake of the 9-*cis* isomer for long periods. However, accumulation of *cis*-isomers in tissues has been reported. Tissue distribution of β-carotene roughly parallels LDL receptor density on the plasma membranes of cells. Storage of β-carotene takes place predominantly in the liver.

The main site of β-carotene metabolism is the intestinal mucosa, although metabolism also occurs in peripheral tissues such as the lung, kidney, liver, and fat. β-Carotene is cleaved mainly at the central double bond yielding two molecules of retinal. Retinal is subsequently converted to retinol (= vitamin A). In poorly nourished humans, about 15% of β-carotene is cleaved to retinal in the intestine. This

percentage decreases when the β-carotene content of the diet increases, and therefore vitamin A levels do not reflect β-carotene intake. The conversion capacity of β-carotene to vitamin A of (young) children is smaller than that of adults.

A wide range of (intestinal) cleavage rates has been demonstrated among species. Rats, mice, hamsters and rabbits have very active dioxygenase enzymes converting β-carotene to retinal in the intestine and as a consequence have (very) low serum β-carotene levels. Based on the kinetics of β-carotene, ferrets, the pre-ruminant calf, and the Mongolian gerbil, have been proposed as more useful test species, since particularly ferrets mimic the absorption and tissue distribution of β-carotene in humans (Gugger et al., 1992; Rock, 1997; Wang et al., 1992, 1999; White et al., 1993b; Poor et al., 1992; Krinsky et al., 1990; Matthews-Roth, 1993). Furthermore, supplementation of ferrets feed with β-carotene has been shown to increase β-carotene concentrations in serum, liver, adipose, and other tissues relevant for comparison with humans (Gugger et al., 1992; Ribaya-Mercado et al., 1989, 1992, 1993; White et al., 1993a and b).

In F344 rats no effects of diets containing algal β-carotene were observed on mortality, clinical observations, organ weights or histopathology. Based on growth suppression, the NOAEL was 696 mg/kg bw/day for males and 2879 mg/kg bw/day for females (Kuroiwa et al., 2006).

In a study with ferrets (Wang et al., 1999), four groups of animals (six males/group) were fed a pure crystalline synthetic (all-*trans*) β-carotene at dose levels of approximately 0.16 (based on β-carotene present in the basal diet) or 2.4 mg/kg bw/day (which includes the β-carotene present in the diet) for six months. Plasma β-carotene increased 17-22-fold and β-carotene in the lung tissue up to 300-fold in the 2.4 mg/kg bw/day treated group, compared to the basal diet group. All animals treated with the high dose of β-carotene, including those not exposed to cigarette smoke, showed localised proliferation of alveolar cells (type II pneumocytes), alveolar macrophages, and keratinised squamous metaplasia of epithelium of alveolar walls (Wang et al., 1999).

The Panel considered none of the effects observed in the reproductive and developmental studies to be of toxicological significance (Mokaday et al., 1989; Komatsu, 1971; Kistler, 1981 and 1982). The four-generation rat study by Bagdon et al. (1960), in which no adverse effects were noted up to 50 mg/kg bw/day (beta-carotene 96% purity), was used for the establishment of the group ADI by JECFA..

In vitro studies with commercial mixed carotene extracts from *Dunaliella*, encompassing a range of genotoxicity endpoints, did not show any signs of genotoxicity (Aruga, 1988; Jagannath, 1987; Cifone, 1987; Young, 1987). No mutagenic effect was observed either with β-carotene derived from *Blakeslea trispora* in a bacterial reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli* and in a chromosomal aberration assay in Chinese hamster ovary cells (Kluifthoof, 2001).

Synthetic β-carotene was not mutagenic in the bacterial reverse mutation assay (van Delft, 1996) and did not induce chromosomal aberration in vitro (De Vogel and van Delft, 1996) nor chromosomal aberrations (Salvadori et al., 1992a; 1992b) or micronuclei (Gallandre, 1979; Raj & Katz, 1985) in mouse bone marrow in vivo.

Negative results for the induction of SCEs, CAs or MN were also reported from in vitro and in vivo antimutagenicity studies with synthetic β-carotene (Agarwal et al., 1993; Aidoo et al., 1995; Manoharan and Banerjee, 1985; Cozzi et al., 1997; Salvadori et al., 1993 and 1994; Stich and Dunn, 1986). However, the Panel noted that these studies have limited relevance for the assessment of genotoxicity of β-carotene, given the low doses applied.

Several in vitro genotoxicity studies were performed to further investigate the genotoxicity of β-carotene cleavage products (Alija et al., 2004; 2005; 2006; Marques et al., 2004; Kalariya et al., 2009; Yeh and Wu, 2006).

A β-carotene cleavage product mixture and β-apo-8'-carotenal were reported to induce increases in MN, CAs and SCEs in primary rat hepatocytes (Alija et al., 2004; 2005; 2006). In this respect, however, the Panel noted that there is limited experience with cytogenetic assays in primary rat hepatocytes, which show a very high spontaneous incidence of both MN and CAs. Moreover, the increase in the frequency of MN and CAs observed in presence of β-carotene cleavage products, and of micronuclei in presence of β-apo-8'-carotenal, were not clearly dose-related over a 10^4-fold concentration range. The statistically significant increases in the frequency of MN were only 20 and 11% (at 0.1 and 1 μM respectively) over control incidence, which was within the range of experimental variation for the end-points studied, and thus has limited or no biological significance. The frequencies of CAs in the presence of β-apo-8'-carotenal show an apparent treatment-related increase, but data are unreliable since they are based on only 20 metaphases/culture. The increase in SCEs observed in presence of both β-carotene cleavage products and β-apo-8'-carotenal is more credible, but the biological significance of this indicative assay in relation to genotoxicity is indirect.

Kalariya et al. (2009), studied the genotoxic effects of the β-carotene breakdown product β-apo-8'-carotenal in Human Retinal Pigment Epithelial Cells (ARPE-19) using the Comet assay. They concluded that their results suggest that breakdown products of dietary carotenoids could be genotoxic in ARPE-19 cells. The authors also stated that the mechanism of genotoxicity of β-apo-8'-carotenal, i.e. whether direct via DNA damage and apoptosis, or indirect through plasma membrane damage and necrosis, could not be disclosed.

The Panel however noted that the β-carotene breakdown products used in these studies where not representative of β-carotene breakdown products formed under physiological conditions.

The Panel therefore concluded that the genotoxicity studies with β-apo-8'-carotenal, β-carotene and a β-carotene cleavage product in primary rat hepatocytes reported by Alija et al. (2004; 2005; 2006) and in ARPE-19 cells by Kalariya et al. (2009), provide very limited evidence of genotoxicity.

In in vitro studies it was found that β-apo 8'-carotenal induced DNA strand breaks in A549 cells (Yeh and Wu, 2006), strand breakage in supercoiled DNA (Zhang and Omaye, 2001), and etheno-adduct to calf thymus DNA (Marques et al., 2004).

The Panel noted further that in previous evaluations many studies on β-carotene have been described that have focussed on a broad range of genotoxicity endpoints in different in vitro and in vivo test systems. In none of these studies were any signs of genotoxicity noted. Therefore, the Panel concluded that β-carotene is not of concern with respect to genotoxicity.

The Panel also noted that a few in vitro studies with β-carotene cleavage products provide limited evidence of DNA damaging activity and covalent binding to DNA, and that such results may reflect a pro-oxidant effect, common to other antioxidants, which is elicited under specific in vitro conditions which may not occur in vivo.

In previous evaluations by SCF several chronic toxicity studies with (synthetic) β-carotene [E 160a (ii)] were described in mice, rats, and dogs. The main effects were observed in the livers of mice and dogs. In mice, cells lining the sinusoids of the liver were found to be vacuolated, and in dogs irregular pale-orange foci on the surface of the liver and perisinusoidal fat storage cells were noted. As in dogs no degenerative changes in the liver were obvious, the authors considered the presence of these cells indicative of storage of vitamin A rather than of a toxic effect. In addition to these findings, weight gain reduction was noted in rats from 100 mg/kg bw/day (beta-carotene 96% purity), but this was not accompanied with any sign of organ toxicity.

Three studies in hamsters (Beems, 1987; Wolterbeek et al., 1995; Furukawa et al., 1999) and two studies in ferrets (Wang et al., 1999; Liu et al., 2000) have been reported in which animals were exposed to a combination of β-carotene and cigarette smoke (constituents). One study in hamsters

showed an inhibitory effect of β-carotene on cigarette smoke-induced respiratory tract tumourigenesis. In the other two hamster studies, increases in overall respiratory tract tumour incidence and preneoplastic and neoplastic changes in the larynx, trachea and lung were observed. In ferrets fed β-carotene in the diet, increased cell proliferation and squamous metaplasia of alveolar epithelium was observed, which was further enhanced in the animals also exposed to cigarette smoke.

Liu et al. (2000), compared the effects of physiological (low dose) versus pharmacological (high dose) β-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets. In contrast to the physiological dose (equivalent to 6 mg/day in humans), the pharmacological supplement (equivalent to 30 mg/day in humans) induced alveolar cell proliferation and keratinized squamous metaplasia in the lung tissue of all ferrets with or without smoke exposure. In ferrets given the low dose of β-carotene alone, no pathological changes were observed. These experiments were performed with an unstable, non-protected synthetic β-carotene, whereas similar experiments performed with a protected (combination with DL-α tocopherol and ascorbic acid) form of synthetic β-carotene did not show any histopathological changes in the lungs of ferrets (Kim et al., 2006; Fuster et al., 2008).

The Panel noted that although the data on kinetics indicate that rodents, in contrast to humans, efficiently convert β-carotene to vitamin A, most toxicological studies have been performed with mice or rats. Therefore, the Panel concluded that the relevance of these studies for humans may be questionable since rodents appear to be no suitable models to study the effects of β-carotene

The Panel noted that the limited number of mechanistic studies performed with hamsters and ferrets demonstrated equivocal results.

According to the Panel the discrepancies observed between the various studies in rats and ferrets may be related to the differences in the stability of β-carotene in the different formulations tested. Antioxidants have important effects on the stability, metabolism and action of β-carotene. Furthermore, β-carotene can be converted to retinoids in mammals by two possible pathways: a central cleavage resulting in retinal and also an eccentric cleavage resulting in a number of β-apo-carotenals and carotenoic acids (Fuster et al., 2008). The formation of oxidative metabolites of β-carotene can occur through an enzymatic mechanism or through a simple oxidative process. Liu et al. (2000) found that production of individual β-apo-carotenals was significantly decreased in the presence of α-tocopherol and ascorbic acid, indicating that the presence of antioxidants could have a stabilizing effect on the unoxidized form of β-carotene, but in their absence, high levels of oxidized cleavage products may appear.

Based on the two human studies on β-carotene and a combined β-carotene and retinol supplementation dealing with the relation of β-carotene with cardiovascular disease, it appears that β-carotene supplementation increased the risk of cardiovascular death, especially among men who had a history of myocardial infarction (ATBC Study group, 1994; Omenn et al., 1996; Omenn, 1998). On the other hand, from another epidemiological study, dietary β-carotene intake was found to decrease the risk of myocardial infarction in the elderly who were free of myocardial infarcts at baseline. The Panel concluded that, based on the limited data available, it is not possible to come to a decisive conclusion on this equivocal issue.

In the past, high serum β-carotene levels have been associated with a decrease in the incidence of cancer, including lung cancer, in humans (Mayne, 1996, Ziegler et al., 1996). However, the ATBC and CARET trials (ATBC Study group, 1994; Omenn et al., 1996; Omenn, 1998) unexpectedly revealed that heavy smokers and asbestos workers (CARET only) receiving long-time β-carotene supplementation (ATBC) or β-carotene + retinol supplementation (CARET) at doses well below the previously established group ADI of 5 mg/kg bw/day, had increased rather than decreased incidences of lung cancer. Besides lung cancer incidence, increased stomach cancer mortality was seen in subjects receiving β-carotene supplementation in combination with a mixture of vitamins and minerals

(Li et al., 1993). The authors commented that due to the combined exposure, the effects could not be ascribed to β-carotene only.

Baron et al. (2003) studied the effect of β-carotene supplementation on colorectal adenoma recurrence among 864 subjects who had had an adenoma removed and were polyp-free at time of entry into the study. Subjects received 25 mg β-carotene or placebo and were followed with colonoscopy for adenoma recurrence one year and four years after the qualifying endoscopy. The authors concluded that alcohol intake and cigarette smoking may modify the inhibitory effect of β-carotene supplementation on the risk of colorectal adenoma recurrence. Moreover, based on a single case report, it has been postulated by the authors that combined exposure to alcohol and β-carotene was involved in the pathogenesis of liver fibrosis. It was stated that ethanol, although promoting β-carotene deficiency, enhances β-carotene toxicity. The Panel noted that data on this subject may at this time be considered too scarce to draw conclusions.

Recently, DSM (2009) conducted an extensive review of the scientific literature, published since 2000. This review identified 9 randomized controlled trials, 7 follow-up publications related to trials performed before 2000, 34 observational studies and 11 meta-analyses.

The negative effects observed in heavy smokers in the ATBC and CARET studies were not seen in any other intervention study. Therefore, the authors concluded that the increased lung cancer incidence in β-carotene supplemented smokers may be specific to individuals who chronically smoke more than 20 cigarettes per day.

Druesne-Pecollo et al. (2010) performed a systematic review and meta-analysis of 9 randomized controlled trials investigating β-carotene supplementation and cancer risk. They found absence of any protective effect associated with β-carotene supplementation with regard to primary cancer risk. However, their results indicated an increased risk of lung and stomach cancers in smokers and asbestos workers supplemented with β-carotene equal or above 20 mg/day.

The SCF (2000b) and Woutersen et al. (1999) discussed the unexpected findings of the ATBC and CARET trials and posed several possible explanations for these findings. It should be noted that in the CARET trial combined supplementation of β-carotene and vitamin A was tested, and therefore the effects cannot be solely attributed to either of these compounds.

One possible explanation for the increased incidence of lung cancer observed in β-carotene supplemented groups, is that interaction between smoke and β-carotene may have caused the enhanced lung cancer incidence in these trials. β-Carotene may induce phase I bioactivating enzymes which may result in an increased formation of genotoxic metabolites of (among others) cigarette smoke constituents (SCF, 2000b). Another study suggests that the observed β-carotene-enhanced lung tumourigenesis may be caused by interference with normal retinoid signalling through the formation of reactive oxidative cleavage products of β-carotene (SCF, 2000b). In addition to the retinoid pathway, a more recent study (Al-Wadei et al., 2006) provided evidence for cell type-specific growth-stimulating effects of β-carotene on human pulmonary adenocarcinoma cells and their normal cells of origin via the cAMP/PKA-CREB and -ERK pathways. As pulmonary adenocarcinoma is the leading type of lung cancer, the authors suggested that the growth promoting effects of β-carotene on these cancer cells may indeed have contributed to enhance lung tumour incidence in the ATBC and CARET trials. Another hypothesis of how β-carotene may enhance lung tumourigenesis, is by the pro-oxidant activity of oxidative metabolites of β-carotene formed due to a combination of reactive oxygen species derived from tobacco smoke, the large rise in β-carotene concentrations in lung tissue, the proliferation of alveolar (type II) pneumocytes, alveolar macrophages and keratinised squamous epithelium (Wang et al., 1999; SCF, 2000b; Paolini et al., 2001). The free radical atmosphere in the lungs of cigarette smokers enhances β-carotene oxidation and the formation of eccentric cleavage oxidative metabolites. These metabolites might cause diminished retinoid signalling by down-regulating RARβ expression

and the retinoic acid level in lung tissue and by up-regulating AP-1 and, thereby, accelerating lung tumourigenesis (Russell, 2004; Liu et al., 2003; 2004).

Taking into account the following observations:

i. The limited database on long-term, reproductive and developmental toxicity of mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)],

ii. That most of the available studies were in rodents which did not appear to be suitable models for human risk assessment of β-carotene,

iii. The equivocal results of in vitro genotoxicity studies with a mixture of β-carotene [E 160 a (ii)] cleavage products,

iv. The equivocal results of the long-term studies with β-carotene [E 160a (ii)] in combination with cigarette smoke or its components in hamsters and ferrets,

v. The observations in the epidemiological (ATBC and CARET) studies, indicating that long time β-carotene [E 160a (ii)] supplementation, either with or without retinol, at dose levels of respectively 20 and 30 mg caused an increase in lung cancer incidence in heavy smokers,

the Panel concluded that, based on the data presently available, ADIs for mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] could not be established.

The Panel concluded that it was not possible to identify a NOAEL from the non-rodent data using a margin of safety approach. However, the Panel also noted that epidemiological studies reported no increased cancer incidence at supplemental dose levels varying from 6-15 mg/day for about 5 up to 7 years (Druesne-Pecollo et al., 2010).

According to the approximate intake estimate by the SCF (SCF, 2000b), intake of β-carotene and related carotenoids as food additives is about 1-2 mg/day in addition to an average 2-5 mg/day consumed through natural food sources. Consequently, the total intake was considered to be 3-7 mg/day or up to 10 mg/day depending on seasonal and regional variations.

The Panel noted that there is a considerable discrepancy between data on β-carotene usage reported by NATCOL and those originally reported by CIAA in 2009. This discrepancy was identified by the working group and exchanges took place between EFSA, CIAA and NATCOL. In May 2011 CIAA after discussion with NATCOL informed EFSA that it should rely on the information provided by NATCOL. In line with this suggestion, the Panel considered that the original CIAA data were unlikely to reflect usages of pure β-carotene as a food colour. However, the Panel calculated exposure based on both data sets provided, in particular since CIAA reported use levels for a broader range of food categories than did NATCOL. The Panel noted that clarification is urgently needed on the specifications of the colour preparation used by the food industry, indicating both the exact amount of β-carotene in these preparations and the amount of the remainder. The Panel noted that the exposure to β-carotene from its use as a food colour derived by using CIAA data would drastically exceed the exposure from natural sources in the diet. In contrast, calculations by the Panel on food consumption data provided for UK adults using the typical use levels of β-carotene as a food additive provided by NATCOL (Tennant 2004) indicate a mean exposure of β-carotene of 0.06 mg/kg bw/day and an exposure at the 97.5th percentile of 0.11 mg/kg bw/day. The same scenarios would result for children in an average exposure in the range of 0.03-0.22 mg/kg bw/day and at the 97.5th percentile in the range of 0.09-0.43 mg/kg bw/day across European Member States.

The Panel also considered the typical exposure to β-carotene from the regular diet as relevant for the assessment. Data have therefore been collected from the European Nutrition and Health Report 2009 (Elmadfa, 2009) which provides intake estimates for β-carotene from 9 European countries (Czech Republic, Denmark, Finland, Germany, Ireland, Italy, Norway, Poland, and Sweden) based on different methods of dietary intake assessment for children aged 4-6 years, and from 13 countries

(Austria, Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, Lithuania, Poland, Spain, Sweden, and The United Kingdom) for adults. The range of average β-carotene exposure from the diet (natural sources thus excluding supplements and additives) appeared to be 1.1-3.8 mg/day for children at an age of 4-6 years and 1.4-5.6 mg/day for adults which is equivalent to 0.07-0.25 mg/kg bw/day for children and 0.02-0.09 mg/kg bw/day for adults. No data were available for high level consumers, but in view of the large standard deviations reported for the average intakes, it can be assumed that the dietary β-carotene exposure from the diet is considerably higher at high percentiles reaching 10 mg/day.

The Panel noted that the estimates of exposure to β-carotene from its use as food colour are close to the range of exposure estimates from natural sources in the diet based on the data reported by NATCOL and would drastically exceed natural sources based on the data reported by CIAA. The Panel considered the data reported by NATCOL to better reflect the actual situation on use although they do not take into account all food categories.

Dietary intake of beta-carotene has been estimated for European adults ranging from 1.4 ± 0.5 mg/day for male adults in Poland up to 5.6 ± 4.0 mg/day for female adults in Finland. Assuming a normal distribution of intake, this would result in dietary intake ranging from 2.4 up to 13.6 mg/day at the 97.5th percentile. For children, average intakes have been reported in a range of 1.1 to 3.8 mg/day with standard deviations of intake from 0.9 to 3.4 mg/day. Consequently, 97.5th percentile intakes for children may be in the range of 2.9 to 10.3 mg/day. If 10 mg/day is considered the maximum level of β-carotene intake from the diet, the total daily intake (from the diet and β-carotene supplements) of β-carotene would be about 0.167 mg/kg bw/day for a 60 kg adult and 0.667 mg/kg bw/day for a 15 kg child. The Panel noted that due to lack of data this exposure assessment did not include the use of colouring food containing β-carotene.

Based hereon the Panel concluded that:

The use of a) mixed carotenes [E 160a (i)] obtained from algae (*Dunaliella salina* or *Dunaliella bardawil*) by extraction with edible oil (soy bean or olive oil) or by solvent (acetone, methanol or hexane) extraction from carrots (*Daucus carota*) or palm fruit oil (*Elaeis guineensis*) and of b) β-carotene [E 160a (ii)] as food colours are not of safety concern, provided that the combined estimated intake from their use as a food additive and as food supplement, is not more than the amount likely to be ingested as a result of the regular consumption of the foods in which they occur naturally (5-10 mg/day). This would ascertain that the exposure to β-carotene from the use as a food additive and as food supplement would remain below 15 mg/day, the level of supplemental intake of β-carotene for which epidemiological studies did not reveal any increased cancer risk. The Panel could not conclude on the safety in use of mixed carotenes [E 160a (i)] obtained by solvent extraction from other sources such as e.g. grass, alfalfa/lucerne or nettle in the absence of information on the safety and the quantitative and qualitative characterisation of each of the extracts, including minor components, and current manufacturing methods for each source.

CONCLUSIONS

The specifications for mixed carotenes are inadequate and need to be updated to define the amount of food colour and the material not accounted for.

The Panel concluded that on the basis of the presently available database, no ADIs for mixed carotenes [E 160a (i)] and β-carotene [E 160a (ii)] can be established. Further, the Panel concluded that the use of mixed carotenes [E 160a (i)] obtained by extraction from palm fruit oil, carrots and algae and of (synthetic) carotenes [E 160a (ii)] as food colour is not of safety concern, provided that the estimated intake from their use as a food additive and as food supplement is not more than the amount likely to be ingested as a result of the regular consumption of the foods in which they occur naturally (5-10 mg/day). This would ascertain that the exposure to β-carotene from the use as a food

additive and as food supplement would remain below 15 mg/day, the level of supplemental intake of β-carotene for which epidemiological studies did not reveal any increased cancer risk.

Finally, the Panel could not conclude on the safety in use of mixed carotenes [E 160a (i)] obtained by solvent extraction from other sources such as e.g. grass, alfalfa/lucerne or nettle in the absence of information on the safety and the quantitative and qualitative characterisation of each of these extracts, including minor components, and current manufacturing methods for each source.

DOCUMENTATION PROVIDED TO EFSA

1. Pre-evaluation document on Mixed carotenes (E 160a (i)) and β-carotene (E 160a (ii)), prepared by the Dutch National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands, June 2008.
2. DSM Nutritional Products 2009. Safety of β-carotene with particular references to smokers. Basel, Switzerland.
3. Saqual on behalf of stakeholder's contribution concerning mutagenicity studies, December, 2011.
4. Saqual on behalf of stakeholder's contribution concerning algal carotenes, 2 December, 2011.
5. Saqual on behalf of stakeholder's contribution concerning stability and specifications of β-carotenes 3 October, 2011.
6. Saqual on behalf of stakeholder's contribution concerning stability and specifications of β-carotenes 28 September, 2011.
7. CIAA response concerning further clarification on usage data of natural colours 25 May 2011.
8. CIAA response on behalf of stakeholder's contribution concerning an EFSA request on usage data of natural colours, 18 April , 2011.
9. Saqual on behalf of stakeholder's contribution concerning updated intake assessment of mixed carotenes and β-carotene, 8 April, 2011.
10. Saqual on behalf of stakeholder's contribution concerning smokers and the toxicity of β-carotene, 4 April, 2011.
11. Saqual on behalf of stakeholder's contribution concerning extraction, manufacturing and specification of mixed carotenes and β-carotene, 8 March, 2011.
12. Saqual on behalf of stakeholder's contribution concerning full study reports, 8 and 28 September, 2010.
13. CIAA response on behalf of stakeholder's contribution concerning on usage data of natural colours (collected in 2009), 12 March, 2010.
14. NATCOL contribution concerning β-carotene obtained from both *Dunaliella salina* and from *Dunaliella bardawil*, 26 October 2009.
15. NATCOL contribution concerning the solvents and extraction of mixed carotenes, 22 October 2009.
16. Saqual on behalf of stakeholder's contribution concerning synthesis of carotenoids, 2 October 2009.
17. NATCOL contribution concerning the manufacturing, use and composition of mixed carotenes and β-carotene, 22 September and 1 October 2009.
18. NATCOL contribution concerning available studies on β-carotene, 30 March 2007.
19. NATCOL contribution concerning available studies on mixed carotenes, 30 March 2007.

REFERENCES

Abrahamson IA Sr and Abrahamson IA Jr, 1962. Hypercarotenemia. Archives of Ophthalmology 68, 4-7.

Agarwal K, Mukherjee A and Sharma, A, 1993. In vivo cytogenetic studies on male mice exposed to Ponceau 4R and β-carotene. Cytobios 74, 23-28.

Aidoo A, Lyn-Cook LE, Lensing S, Bishop ME and Wamer W, 1995. In vivo antimutagenic activity of in rat spleen. Carcinogenesis 16, 2237-2241. Abstract only.

Alabaster O, Tang Z, Frost A, Shivapurkar N, 1995. Effect of beta-carotene and wheat bran fiber on colonic aberrant crypt and tumor formation in rats exposed to azoxymethane and high dietary fat. Carcinogenesis 16 (1), 127-32.

Alam BS, Alam SQ and Weir JC, 1988. Effects of excess vitamin A and canthaxanthin on salivary gland tumors. Nutrition and Cancer 11, 233-241.

Alam BS, Alam SQ, 1987. The effect of different levels of dietary beta-carotene on DMBA-induced salivary gland tumors. Nutrition and Cancer 9 (2-3), 93-101.

Alam SQ, Alam BS, Chen TW, 1984. Activities of fatty acid desaturases and fatty acid composition of liver microsomes in rats fed beta-carotene and 13-*cis*-retinoic acid. Biochimica et Biophysica Acta-BBA 792 (2), 110-7.

Alija AJ, Bresgen N, Sommerburg O, Langhans CD, Siems W and Eckl PM, 2006. Beta-carotene breakdown products enhance genotoxic effects of oxidative stress in primary rat hepatocytes. Carcinogenesis 27(6), 1128-1133.

Alija AJ, Bresgen N, Sommerburg O, Langhans CD, Siems W and Eckl PM, 2005. Cyto- and genotoxic potential of β-carotene and cleavage products under oxidative stress. Biofactors 24(1-4), 159-163.

Alija AJ, Bresgen N, Sommerburg O, Siems W and Eckl PM, 2004. Cytotoxic and genotoxic effects of beta-carotene breakdown products on primary rat hepatocytes. Carcinogenesis 25(5), 827-831.

Al-Wadei HA, Takahashi T and Schuller HM, 2006. Growth stimulation of human pulmonary adenocarcinoma cells and small airway epithelial cells by beta-carotene via activation of cAMP, PKA, CREB and ERK1/2. International Journal of Cancer 118(6), 1370-1380.

Appel MJ, Woutersen RA, 1996. Effects of dietary beta-carotene and selenium on initiation and promotion of pancreatic carcinogenesis in azaserine-treated rats. Carcinogenesis 17 (7), 1411-6.

Appel MF, van Garderen-Hoetmer, Woutersen RA, 1991. Lack of inhibitory effects of p-carotene, vitamin C, vitamin E and selenium on development of ductular adenocarcinomas in exocrine pancreas of hamster. Cancer Letters 103, 157-162.

Aruga F, 1988. Mutagenicity test of *Dunaliella bardawil* paste with *Salmonella typhimurium* and *Escherichia coli*. Nihon Bioresearch Center Inc. Hashima, Gifu, Japan, as submitted to WHO by Nikken Sohonsha Corporation, Hashim-City, Japan.

Aruga F, 1987. Acute oral toxicity study on *Dunaliella bardawil* spray dried powder in mice. Nihon Bioresearch Center Inc. Hashima, Gifu, Japan, as submitted to WHO by Nikken Sohonsha Corporation, Hashim-City, Japan.

Astorg P, Gradelet S, Bergès R, Suschetet M, 1996. No evidence for an inhibitory effect of beta-carotene or of canthaxanthin on the initiation of liver preneoplastic foci by diethylnitrosamine in the rat. Nutrition and Cancer 25(1): 27-34.

Astorg P, 1997. Food carotenoids and cancer prevention: An overview of current research. Trends in Food Science & Technology, December 1997, Vol. 8, 406-413.

ATBC Study group (The Alpha-Tocopherol, β-carotene Cancer Prevention Study Group), 1994. The effects of vitamin E and β-carotene on the incidence of lung cancer and other cancers in male smokers. New England Journal of Medicine. 330, 1029-1356.

Auckland G, 1952. A case of carotinaemia. British Medical Journal 2, 267-268.

Azuine MA, Goswami UC, Kayal JJ, Bhide SV, 1992. Antimutagenic and anticarcinogenic effects of carotenoids and dietary palm oil. Nutrition and Cancer 17 (3), 287-95.

Bagdon RE, Zbinden G and Studer A, 1960. Chronic toxicity studies of beta-carotene. Toxicology and Applied Pharmacology 2, 223-236.

Baron JA, Cole BF, Mott L, Haile R, Grau M, Church TR, Beck GJ and Greenberg ER, 2003. Neoplastic and antineoplastic effects of beta-carotene on colorectal adenoma recurrence: results of a randomized trial. Journal of the National Cancer Institute 95 (10), 717-722.

Bauernfeind JC, Adams CR and Marusich WL, 1981. Carotenes and other vitamin A precursors in animal feed. In: Carotenoids as colorants and vitamin A precursors, ed. J.C. Bauernfeind, New York, Academic Press, pp. 563-743.

Beems RB, 1987. The effect of β-carotene on BP-induced respiratory tract tumors in hamsters. Nutrition and Cancer 10, 197-204.

Ben-Amotz A, Mokady S and Avron M, 1988. The β-carotene-rich alga *Dunaliella bardawil* as a source of retinol in a rat diet. British Journal of Nutrition 59, 442-449.

Ben-Amotz A, Edelstein S and Avron M, 1986. Use of the β-carotene rich alga *Dunaliella bardawil* as a source of retinol. British Poultry Science 27, 613-619.

Bernhard K, 1963. Wiss. Veröff. Dtsch. Gesellsch. Ernährung, 9, 169.

BIBRA, 1996. British Industrial Biological Research Association working group. Toxicity profiles, beta-apo-8'-carotenal, ethyl-beta-apo-8'-carotenal, ethyl-beta-apo-8'-carotenoat and methyl beta-apo-8'-carotenoate.

Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S et al., 1993. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. Journal of the National Cancer Institute 85, 1483-91.

Bohlke K, Spiegelma D, Trichopoulou A, Katsouyanni K and Trichopoulos D, 1999. Vitamins A, C and E and the risk of breast cancer: results from a case-control study in Greece. British Journal of Cancer 79, 23-29.

Boon CS, McClements DJ, Weiss J and Decker E, 2010. Factors Influencing the Chemical Stability of Carotenoids in Foods. Critical Reviews in Food Science and Nutrition, Volume 50, 515-532.

Buser SM and Arceo RG, 1995. Subchronic (13-week) oral study with β-carotene as a feed admixture in the rat. Unpublished Report No. B-161'158.

Buser S and Hummler H, 1983. The Effect of Beta-Carotene in a combined Tumorigenicity and Toxicity Study in Rats. Unpublished Report No. B-104 701.

ChemIDplus Advanced (via Internet, 2008). Accessible via: http://chem.sis.nlm.nih.gov/chemidplus/

Cifone MA, 1987. Mutagenicity test on EK 87-0048 B-CAT in the rat primary hepatocyte unscheduled DNA synthesis assay. Unpublished report of Hazleton Laboratories America Inc. Submitted to WHO by Eastman Kodak Co., Rochester, NY, USA.

Colacchio TA, Memoli VA, Hildebrandt L, 1989. Antioxidants vs. carotenoids. Inhibitors or promoters of experimental colorectal cancers. Archives of Surgery 124(2), 217-21.

Colacchio TA and Memoli VA, 1986. Chemoprevention of colorectal neoplasms. Ascorbic acid and beta-carotene. Archives of Surgery 121(12), 1421-4.

Cooper DA, Eldridge AL, Peters JC, 1999. Dietary carotenoids and certain cancers, heart disease, and age-related macular degeneration: a review of recent research. Nutrition Rewiew 57, 201-214.

Cozzi R, Ricordi R, Aglitti T, Gatta V, Perticone P and De Salvia R, 1997. Ascorbic acid and β-carotene as modulators of oxidative damage. Carcinogenesis 18, 223-228.

Cyanotech, 1988. Ten-day Konatene (TM) feeding study: effects on serum beta-carotene levels. Unpublished summary report submitted to WHO by Cyanotech Corporation, Woodinville, Washington, USA.

Druesne-Pecollo N, Latino-Martel P, Norat T, Barrandon E, Bertrais S, Galan P and Hercberg S, 2010. Beta-carotene supplementation and cancer risk: a systematic review and metaanalysis of randomized controlled trials. International Journal of Cancer 127, 172-184.

European Commission, 2001. Commission of the European Communities (COM). 542 final. Report from the Commission on dietary food additive intake in the European Union. Brussels. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2001:0542:FIN:EN:PDF

European Commission, 1997. Food Science and Techniques. Reports on Tasks for Scientific Cooperation (SCOOP). Report of Experts participating in task 4.2. Report on the Methodologies for the Monitoring of Food Additive Intake across the European Union. Directorate General Industry. December 1997.

Elmadfa I, 2009. European Nutrition and Health Report 2009. Forum of Nutrition Vol 62, Karger, Basel.

Ernst, H. 2002. Recent advances in industrial carotenoid synthesis. Pure Applied Chemistry Vol. 74, 11, 2213-2226.

Expert Group on Vitamins and Minerals (EVN). Safe upper levels of vitamins and minerals. Food Standards Agency, United Kingdom, 2003.

Fraps GS and Meinke WW, 1945. Archives of Biochemistry 6, 323.

Furuhashi T, 1989. Twenty-eight-day oral subacute toxicity study on Dunaliella bardawil. Nihon Bioresearch Center Inc., Hashima, Gifu, Japan, as submitted to WHO by Nikken Sohonsha Corporation, Hashima-City, Japan.

Furukawa F, Nishikawa A, Kasahara K, Lee IS, Wakabayashi K, Takahashi M and Hirose M, 1999. Inhibition by β-carotene of upper respiratory tumorigenesis in hamsters receiving diethylnitrosamine followed by cigarette smoke exposure. Japanese Journal of Cancer Research 90, 154-161.

Fuster A, Picó C, Sánchez J, Oliver P, Zingaretti MC, Murano I, Morroni M, Hoeller U, Goralczyk R, Cinti S, Palou A, 2008. Effects of 6-month daily supplementation with oral beta-carotene in combination or not with benzo[a]pyrene on cell-cycle markers in the lung of ferrets. Journal of Nutritional Biochemistry 19, 295–304.

Gallandre F, 1979. Mutagenicity studies with Ro 01-8300 in Mammalian Systems. Unpublished Report N. B-90-155.

Gärtner C, Stahl W, Sies H, 1996. Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to beta-carotene in the human. International Journal for Vitamin and Nutrition Research 66(2), 119-25.

Ghazi A, De Lumen B and Oswald WJ, 1992. Comparative bioavailability of beta-carotene from Dunaliella salina, Dunaliella salina extract, carrot extract and synthetic beta-carotene. Report submitted to WHO by Microbio Resources, Inc., San Diego, CA USA.

Graffin B, Genty I, Cretel E, Rodolphe J and Durand JM, 2002. Case report – Carotene-induced hepatic fibrosis. Digestive Diseases Science 47, 793.

Greenberg ER, Baron JA, Tosteson TD, Freeman DH Jr, Beck GJ et al., 1994. A clinical trial of antioxidant vitamins to prevent colorectal adenoma. New England Journal of Medicine 331, 141-147.

Greenberg ER, Baron JA, Stukel TA, Stevens MM, Mandel JS et al., 1990. A clinical trial of β-carotene to prevent basal-cell and squamous-cell cancers of the skin. New England Journal of Medicine 323, 789-795.

Greenberg R, Cornbleet T and Joffay AI, 1959. Accumulating and excretion of vitamin a-like fluorescent material by sebaceous glands after the oral feeding of various carotenoids. Journal of Investigative Dermatology 32, 599-604.

Gregory JR, Foster K, Tyler H and Wiseman M, 1990. The Dietary and Nutritional Survey of British Adults, London, Her Majesty's Stationery Office.

Gugger ET, Bierer TL, Henze TM, White WS and Erdman JW, 1992. β-Carotene uptake and tissue distribution in ferrets (*Mustela putorius furo*). The Journal of Nutrition 122, 115-119.

Hansgeorg E, 2002. Recent advances in industrial carotenoid synthesis. Pure Applied Chemistry, 74, 11, 2213–2226.

IARC, 1998. International Agency for Research on Cancer; Working group on the Evaluation of Cancer Preventive Agents. IARC: Handbooks of cancer prevention, vol. 2: carotenoids. IARC press, Lyon, France.

Ivett JL, 1987. Mutagenicity test on EK 87-0047, corn oil control and EK 87-0048, B-CAT in the in vivo mouse micronucleus assay. Unpublished report of Hazleton Laboratories America Inc. submitted to WHO by Eastman Kodak Co., Rochester, NY, USA.

Jagannath DR, 1987. Mutagenicity test on EK 87-0048 B-CAT in the Ames Salmonella/microsome reverse mutation assay. Unpublished report of Hazleton Laboratories America Inc. submitted to WHO by Eastman Kodak Co., Rochester, NY, USA.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2006. Combined compendium of food additive specifications - all specifications monographs from the 1st to the 65th meeting (1956-2005). FAO JECFA Monographs Series, No. 1 Volume 1-3, 2006.

JECFA, 2001. WHO/FAO Joint Expert Committee on Food Additives. Safety evaluation of certain food additives and contaminants. WHO Food additives series, 48.

JECFA, 1993. WHO/FAO Joint Expert Committee on Food Additives. Toxicological evaluation of certain food additives and contaminants. WHO Food additives series, 32.

JECFA, 1975. WHO/FAO Joint Expert Committee on Food Additives. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents and certain food additives. WHO Food additives series, 6.

Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH and Scala J, 1987. Observations on the effects of ingesting *cis*- and *trans*-beta-carotene isomers on human serum concentrations. Nutrition Reports International 35, 413-422.

Jensen CD, Spiller GA, Pattison TS, Whittam JH and Scala J, 1986. Acute effects of dietary carotenes on serum alpha and beta carotene in humans. Nutrition Reports International 33, 117-122.

Jensen CD, Pattison TS, Spiller GA, Whittam JH and Scala J, 1985. Repletion and depletion of serum alpha and beta carotene in humans with carrots and an algae-derived supplement. Acta Vitaminologica et Enzymologica 7, 189-198.

Jones RC, Sugie S, Braley J, Weisburger JH, 1989. Dietary beta-carotene in rat models of gastrointestinal cancer. Journal of Nutrition 119(3), 508-14.

Jonker, 1997. Sub-acute oral toxicity study with beta-carotene in rats. TNO report V96.889

Kalariya NM, Ramana KV, Srivastava SK, van Kuijk FJ, 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Current Eye Research 34(9):737-47.

Kim Y, Liu XS, Liu C, Smith DE, Russell RM, Wang XD, 2006. Induction of pulmonary neoplasia in the smoke-exposed ferret by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK): a model for human lung cancer. Cancer Letters 234(2), 209-19.

Kistler A, 1982. Embryotoxicity study in rabbits with oral administration of Ro 01-8300, beta-carotene. Phase II – Teratological study. Unpublished Report Nr. B-46 351.

Kistler A, 1981. Embryotoxicity study in rats with oral administration of Ro 01-8300, beta-carotene. Phase II – Teratological study with postnatal evaluation. Unpublished Report Nr. B-94 683.

Klipstein-Grobusch K, Geleijnse JM, den Breeijen JH, Boeing H, Hofman A, Grobbee DE and Witteman JC, 1999. Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam study. American Journal of Clinical Nutrition 69, 261-266.

Kluifthoof JD, 2001. Unpublished data submitted to WHO by DSM Food Specialties.

Komatsu S, 1971. Teratogenic effects of vitamin A: effect of β-carotene. Shika Gakuho 71, 2067-1074.

Krinsky NI, Mathews-Roth MM, Welankiwar S, Sehgal PK, Lausen NCG, Russett M, 1990. The metabolism of [14-C]β-carotene and the presence of other carotenoids in rats and monkeys. Journal of Nutrition 120, 81-87.

Kübler W, 1963. Wiss. Veröff. Dtsch. Gesellsch. Ernährung. 9, 222.

Kuroiwa Y, Nishikawa A, Imazawa T, Kitamura Y, Kanki K, Ishii Y, Umemura T and Hirose M, 2006. A subchronic toxicity study of Dunaliella carotene in F344 rats. Food and Chemical Toxicology 44(1), 138-145.

Lahiri M, Maru GB and Bhide SV, 1993. Effect of plant phenols, β-carotene and a-tocopherol on benzo[a]pyrene-induced DNA damage in mouse forestomach mucosa (target organ) and bone marrow polychromatic erythrocytes (non-target organ). Mutation Research 303, 97-100.

Langouët S, Mican AN, Müller M, Fink S P, Marnett JL, Muhle SA and Guengerich FP, 1998. Biochemistry 37, 5184-5193.

Leo MA and Lieber CS, 1999. Alcohol, vitamin A, and beta-carotene: adverse interactions, including hepatotoxicity and carcinogenicity. American Journal of Clinical Nutrition 69(6), 1071-1085.

Li JY, Taylor PR, Li B, Dawsey S, Wang GQ et al., 1993. Nutrition intervention trials in Linxian, China: multiple vitamin/mineral supplementation, cancer incidence and disease specific mortality among adults with esophageal dysplasia. Journal of the National Cancer Institute 85, 1492-1498.

Liu C, Russell RM, Wang XD, 2004. Alpha-tocopherol and ascorbic acid decrease the production of beta-apo-carotenals and increase the formation of retinoids from beta-carotene in the lung tissues of cigarette smoke-exposed ferrets in vitro. Journal of Nutrition 134(2), 426-30.

Liu C, Russell RM, Wang XD, 2003. Exposing ferrets to cigarette smoke and a pharmacological dose of beta-carotene supplementation enhance in vitro retinoic acid catabolism in lungs via induction of cytochrome P450 enzymes. Journal of Nutrition 133(1), 173-9.

Liu C, Wang XD, Bronson RT, Smith DE, Krinsky NI, Russell RM, 2000. Effects of physiological versus pharmacological beta-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets. Carcinogenesis 21(12), 2245-53.

Lock S, 1985. Fourteen days oral rat testing using dried Dunaliella cells. BioMed No. 4476. Unpublished summary report of Biomed Research Laboratories Inc. submitted to WHO by Cyanotech Corporation, Woodinville, Washington, USA.

Lowe G.M, Booth LA, Young AJ and Bilton RF, 1999. Lycopene and beta carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses. Free Radical Research 30, 141-151.

Majnarich JJ, 1988. Subchronic oral toxicity (12 week) study of algal beta carotene fed to male and female Sprague-Dawley rats. Unpublished report of Biomed Research Laboratories Inc. submitted to WHO by Cyanotech Corporation, Woodinville, Washington, USA.

Manoharan K and Banerjee MR, 1985. Beta-carotene reduces sister chromatid exchanges induced by chemical carcinogens in mouse mammary cells in organ culture. Cell Biology International Report 9, 783-789.

Marques SA, Loureiro AP, Gomes OF, Garcia CC, Di Mascio P and Medeiros MH, 2004. Induction of 1,N^2-etheno-2'-deoxyguanosine in DNA exposed to β-carotene oxidation products. FEBS Letters 560 (1-3), 125-130.

Mathews-Roth MM, 1993. Carotenoids in erythropoietic protoporphyria and other photosensitivity diseases. Annals of the New York Academy of Sciences 691, 127-138.

Mathews-Roth MM, Lausen N, Drouin G, Richter A, Krinsky NI, 1991. Effects of carotenoid administration on bladder cancer prevention. Oncology 48 (3), 177-9.

Mathews-Roth MM, Krinsky NI, 1987. Carotenoids affect development of UV-B induced skin cancer. Photochemistry and Photobiology 46 (4), 507-9.

Mayne ST, 1996. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J. 10, 690-701.

McLarty JW, 1992. An intervention trial in high risk asbestos exposed persons. Advances in Experimental Medicine and Biology 320, 141-149.

Merck Index, 2006.14th Edition. Published by Merck Research Laboratories, Division of Merck & Co. Inc., Whitehouse Station, NJ, USA.

Merkle J, Kirsch P, Dèckardt K, Freisberg KO, Hempel K-J, 1980. Bericht über die Prüfung der Toxizität von beta-carotin-Trockenpulver im 4-Wochen-Fütterungsversuch an der ratte. BASF-report 25698n 5M 7107.

Meyers DG, Maloley RA and Weeks D, 1996. Safety of antioxidant vitamins. Archives of Internal Medicine. 156, 925-935.

Mokady S, Abramovici A and Cogan U, 1989. The safety evaluation of Dunaliella bardawil as a potential food supplement. Food and Chemical Toxicology 27, 221-226.

Moon RC, 1994. Chemoprevention of respiratory tract neoplasia in the hamster by oltipraz, alone and in combination. International Journal of Oncology 4, 661-667.

Mordi RC, Walton JC, Burton GW, Hughes L, Ingold KU and Lindsay DA, 1991. Exploratory study of β-carotene autoxidation. Tetrahedron Letters, Vol. 32, 33, 4203-4206.

Moreno FS, Wu TS, Penteado MV, Rizzi MB, Jordão Júnior AA, Almeida-Muradian LB, Dagli ML, 1995. A comparison of beta-carotene and vitamin A effects on a hepatocarcinogenesis model. International Journal for Vitamin and Nutrition Research 65 (2), 87-94.

Moreno FS, Rizzi MB, Dagli ML, Penteado MV, 1991. Inhibitory effects of beta-carotene on preneoplastic lesions induced in Wistar rats by the resistant hepatocyte model. Carcinogenesis.12 (10), 1817-22.

Mukherjee A, Agarwal K, Aguilar MA and Sharma A, 1991. Anticlastogenic activity of β-carotene against cyclophosphamide in mice in vivo. Mutation Research 263, 41-46.

Murakoshi M, Nishino H, Satomi Y, Takayasu J, Hasegawa T, Tokuda H, Iwahima A, Okuzumi J, Okabe H, Kitano H and Iwasaki R, 1992. Potent preventive action of a-carotene against

carcinogenesis: spontaneous liver carcinogenesis and promoting stage of lung and skin carcinogenesis in mice are suppressed more effectively by a-carotene than by β-carotene. Cancer Research 52, 6583-6587.

Nabae K, Ichihara T, Hagiwara A, Hirota T, Toda Y, Tamano S, Nishino M, Ogasawara T, Sasaki Y, Nakamura M and Shirai T, 2005. A 90-day oral toxicity study of beta-carotene derived from Blakeslea trispora, a natural food colorant, in F344 rats. Food and Chemical Toxicology 43(7), 1127-1133.

Nagasawa H, Fuji Y, Yamamoto K, Konoshi R and Ben-Amotz A, 1989. No deleterious side-effects on mammary growth and endocrine parameters of chronic ingestion of beta-carotene-rich alga Dunaliella bardawil in virgin mice in comparison with synthetic all- *trans* beta-carotene. The Cancer Journal 2, 391-394.

Ni R, Leo MA, Zhao J and Lieber CS, 2001. Toxicity of beta-carotene and its exacerbation by acetaldehyde in HepG2 cells. Alcohol and Alcoholism 36(4), 281-285.

Nieman C and Obbink HJ, 1954. The biochemistry and pathology of hypervitaminosis A. Vitamins and Hormones 12, 69-99.

Nishino H, 1995. Cancer chemoprevention by natural carotenoids and their related compounds. Journal of Cellular Biochemistry - Supplement 22, 231-235.

Olmedilla B, Granado F, Blanco I, Rojas-Hidalgo E, 1994. Seasonal and sex related variations in six serum carotenoids, retinol, and α-tocopherol. The American Journal of Clinical Nutrition 60, 106-110.

Olmos J, Ochoa L, Paniagua-Michel J, Contrera R, 2009. DNA fingerprinting differentiation between β-carotene hyperproducer strains of Dunaliella from around the world. Saline Systems 5.

Omenn GS, 1998. Chemoprevention of lung cancer: the rise and demise of β-carotene. Annual Review of Public Health 19, 73-99.

Omenn GS, Goodman GE, Thornquist M, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S and Hammar S, 1996a. Effects of a combination of β-carotene and vitamin A on lung cancer incidence, total mortality, and cardiovascular mortality in smokers and asbestos-exposed workers. The New England Journal of Medicine 334, 1150-1155.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Cherniack MG, Brodkint CA and Hammar S, 1996b. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficiency trial. Journal of the National Cancer Institute 88, 1550-1559.

Paolini M, Antelli A, Pozzetti L, Spetlova D, Perocco P, Valgimigli L, Pedulli GF and Cantelli-Forti G, 2001. Induction of cytochrome P450 enzymes and over-generation of oxygen radicals in beta-carotene supplemented rats. Carcinogenesis 22(9), 1483-1495.

Pedersen AN, Fagt S, Velsing Groth M, Christensen T, Biltoft-Jensen A, Matthiessen J, Andersen NL, Kørup K, Hartkopp H, Hess Ygil K, Hinsch HJ, Saxholt E, Trolle E, 2010. Danskernes kostvaner 2003-2008. Danmarks Tekniske Universitet, Søborg, Denmark.

Pedrick MS, Turton JA, Hicks RM, 1990. The incidence of bladder cancer in carcinogen-treated rats is not substantially reduced by β-carotene (BC). Eight Eur. Fat Soluble Vitamins Group Meeting (Abstract) p. 189.

Ph. Eur., 2008. European Pharmacopoeia, 6th edition, Govi Verlag, Eschborn, Germany.

Poor CL, Bierer TL, Merchen NR, Fahey GC, Murphy M, Erdman JW, 1992. Evaluation of the preruminant calf as a model for the study of human carotenoid metabolism. Journal of Nutrition 122, 262-268.

Raj AS and Katz M, 1985. Beta-carotene as an inhibitor of benzo[a]pyrene and mitomycin induced chromosome breaks in bone marrow of mice. Canadian Journal of Genetic Cytololgy 27, 668-602.

Redlich CA, Grauer JN, van Bennekum AM, Clever SL, Ponn RB, Blaner WS, 1996. Characterization of carotenoid, vitamin A, and a-tocopherol levels in human lung tissue and pulmonary macrophages. American Journal of Respiratory and Critical Care Medicine. 154, 1436-1443.

Ribaya-Mercado JD, Lopez-Miranda J, Ordovas JM, Blanco MC, Fox JG, Russell RM, 1993. Distribution of b-carotene and vitamin A in lipoprotein fractions of ferret serum. New York Academy of Sciences 691, 232-237.

Ribaya-Mercado JD, Fox JG, Rosenblad WD, Blanco MC, Russell RM, 1992. Beta-carotene, retinol and retinyl ester concentrations in serum and selected tissues of ferrets fed beta-carotene. Journal of Nutrition 122, 1898-1903.

Ribaya-Mercado JD, Holmgren SC, Fox JG, Russell RM, 1989. Dietary beta-carotene absorption and metabolism in ferrets and rats. Journal of Nutrition 119, 665-668.

Rock CL, 1997. Carotenoids: biology and treatment. Pharmacology & Therapeutics 75, 185-197.

Russel RM, 2004. The Enigma of β-Carotene in Carcinogenesis: What Can Be Learned from Animal Studies. Journal of Nutrition 134, 262S-268S.

Salvadori DMF, Ribeiro LR and Natarajan AT, 1994. Effect of β-carotene on clastogenic effects of mitomycin C, methyl methanesulphonate and bleomycin in Chinese hamster ovary cells. Mutagenesis 9, 53-57.

Salvadori DMF, Ribeiro LR and Natarajan AT, 1993. The anticlastogenicity of β-carotene evaluated on human hepatoma cells. Mutation Research 303, 151-156.

Salvadori DMF, Ribeiro LR, Oliveira MD, Pereira CA and Becak W, 1992a. β-Carotene as a modulator of chromosomal aberrations induced in mouse bone marrow cells. Environmental and Molecular Mutagenesis 20, 206-210.

Salvadori DMF, Ribeiro LR, Oliveira MD, Pereira CA and Becak W, 1992b. The protective effect of β-carotene on genotoxicity induced by cyclophosphamide. Mutation Research 265, 237-244.

SCF, 2000a. Opinion of the Scientific Committee on Food on β-carotene from *Blakeslea trispora* – Correction. (Adopted on 22 June 2000, and corrected on 7 September 2000) SCF/CS/ADD/COL 158 Final – correction.

SCF, 2000b. Reports of the Scientific Committee for Food. Opinion on the safety of use of beta-carotene from all dietary sources. Report no. SCF/CS/ADD/COL/159 Final. Opinion adopted by the SCF on 7 September 2000. http://ec.europa.eu/food/fs/sc/scf/out71_en.pdf

SCF, 2000c. Opinion of the Scientific Committee on Food on the Tolerable Upper Intake level of beta-Carotene (expressed on 19 October 2000) SCF/CS/NUT/UPPLEV/37 Final 28 November 2000.

SCF, 1999. Reports of the Scientific Committee for Food (43rd opinion). Opinion on a request for the use of algal beta-carotene as a food colour, pp 34-36. Opinion expressed 1997.

SCF, 1997. Minutes of the 107th meeting of the Scientific Committee for Food held on 12-13 June 1997 in Brussels. European Commission. Document XXIV/1270/97-EN. Brussels, 30 June 1997.

SCF, 1993. Reports of the Scientific Committee for Food (31st series). Nutrient and energy intakes for the European Community. Commission of the European Communities, Luxembourg, 1993.

SCF, 1975. Report of the Scientific Committee for Food (1st opinion). Opinion on the revision of the Directive on colouring matters authorized for use in foodstuffs intended for human consumption, p. 17.

Schüpbach, 1979. Mutagenicity evaluation of Ro 01-8300 (ß-Carotene) in the Ames *Salmonella*/mammalian liver microsome plate test. Unpublished report 25. June 1979 (B-0091706 / 71489).

Scotter MJ, 2011. Methods for the determination of European Union-permitted added natural colours in foods: a review. Part A. Food Additives and Contaminants March 2011, 1–70.

Scotter MJ and Castle L, 2004. Chemical interactions between additives in foodstuffs: a review. Food Additives & Contaminants 21(2), 93-124.

Seddon JM, Ajani UA, Sperduto RD et al., 1994. Dietary carotenoids, vitamins A, C and E, and advanced age-related macular degeneration. JAMA 272, 1413-1420.

Steering Committee of the Physicians' Health Study Research Group, 1989. Final report on the aspirin component of the ongoing Physicians' Study. New England Journal of Medicine 321, 129-135.

Steinel HH, Baker RS, 1990. Effects of beta-carotene on chemically-induced skin tumors in HRA/Skh hairless mice. Cancer Letters 51 (2), 163-8.

Stich HF and Dunn BP, 1986. Relationship between cellular levels of β-carotene and sensitivity to genotoxic agents. International Journal of Cancer 38, 713-717.

Shiomi T and Koike T, 2000. Safety evaluation tests of beta-carotene extracted from Dunaliella. Eiyogaku Zasshi 58, 219-224.

Shivapurkar N, Tang Z, Frost A, Alabaster O, 1995. Inhibition of progression of aberrant crypt foci and colon tumor development by vitamin E and beta-carotene in rats on a high-risk diet. Cancer Letters 91 (1), 125-32.

TemaNord, 2002. Food additives in Europe 2000; Status of safety assessments of food additives presently permitted in the EU. TemaNord 2002, 560, 145-148.

Temple NJ, Basu TK, 1987. Protective effect of beta-carotene against colon tumors in mice. Journal of the National Cancer Institute 78 (6), 1211-4.

Tennant, 2007. Screening potential intakes of natural food colours. Report provided for the Natural Food Colours Association (NATCOL).

Tennant DR, Gedrich K, Godfrey D and Davidson J, 2004. Intakes of beta-carotene from its use as a food additive, fortificant and dietary supplement in France, Germany and the UK. British Food Journal 106, 436-456.

van Delft JHM, 1996. Ames test with β-carotene. TNO Report V 96.827. TNO Nutrition and Food Research Institute.

de Vogel N and van Delft JHM, 1996. Chromosome aberration test with β-carotene in cultured Chinese hamster ovary cells. TNO Report V 96.704. TNO Nutrition and Food Research Institute.

Wagner K, 1962. [On the problem of carotene pseudoicterus]. Wien. Klin. Wochenschr. 74, 909-913.

Wang XD, Liu C, Bronson RT, Smith DE, Krinsky NI and Russell RM, 1999. Retinoid signalling and activator protein 1 expression in ferrets given β-carotene supplements and exposed to tobacco smoke Journal of the National Cancer Institute 91, 60-66.

Wang XD, Krinsky NI, Marini RP, Tang G, Yu J, Hurley R, Fox JG and Russell RM, 1992. Intestinal uptake and lymphatic absorption of β-carotene in ferrets: a model for human β-carotene metabolism. American Journal of Physiology 263, G480-486.

Weissenberg M, Levy A, Schaeffler I, Managen E and Barzilai M, 1997. Rapid isocratic HPLC analysis of beta-carotene in red peppers (*Capsicum annuum* L.) and food preparations. Chromatographia 46, 399-403.

White WS, Peck KM, Bierer TL, Gugger ET, Erdman JW Jr, 1993a. Interactions of oral b-carotene and canthaxanthin in ferrets. Journal of Nutrition 123, 1405-1413.

White WS, Peck KM, Ulman EA, Erdman JW Jr, 1993b. The ferret as a model for evaluation of the bioavailabilities of all-*trans*-b-carotene and its isomers. Journal of Nutrition 123, 1129-1139.

Wolterbeek AP, Schoever EJ, Bruyntjes JP, Rutten AA, Feron VJ, 1995. Benzo[a]pyrene-induced respiratory tract cancer in hamsters fed a diet rich in β-carotene. A histomorphological study. Journal of Environmental Pathology, Toxicology and Oncology 14, 35-43.

Wong DWS, 1989. Mechanism and theory in food chemistry. Chap. 4, Colorants, pp. 153-158; An AVI Book; Van Nostrand Reinhold Intl. Co. Ltd.

Woods JA, Bilton RF and Young AJ, 1999. β-Carotene enhances hydrogen peroxide-induced DNA damage in human hepatocellular HepG2 cells. FEBS Letters 449, 255-258.

Woutersen RA, Wolterbeek A, Appel MJ, van den Berg H, 1999. Safety evaluation of synthetic β-carotene. (TNO report, V 97.221. TNO Nutrition and Food Research Institute. 1998). Critical Reviews in Toxicology 29, 515-542.

Xue KX, Wu JZ, Ma GJ, Yuan S, Qin HL, 1998. Comparative studies on genotoxicity and antigenotoxicity of natural and synthetic β-carotene stereoisomers. Mutation Research 418, 73-78.

Yamamoto I, Maruyama H, Moriguchi M, 1994. Effect of beta-carotene, sodium ascorbate and cellulose on 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats. Cancer Letters 86 (1), 5-9.

Yeh SL and Wu SH, 2006. Effects of quercetin on beta-apo-8'-carotenal-induced DNA damage and cytochrome P1A2 expression in A549 cells. Chemico-Biological Interaction 163(3), 199-206.

Young RR, 1987. Mutagenicity test on EK 87-0048 B-CAT in the CHO/HGPRT forward mutation assay. Unpublished report of Hazleton Laboratories America Inc. submitted to WHO by Eastman Kodak Co., Rochester, NY, USA.

Yun T-K, Kim S-H, Lee Y-S, 1995. Trial of a new medium-term model using benzo[a]pyrene induced lung tumour in newborn mice. Anticancer Research 15, 839-846.

Zbinden J and Studer A, 1958. Tierexperimentelle Untersuchungen uber die chronische Verträglichkeit vor β-carotin, Lycopin, 7,7-dihydro-β-carotin und Bixin. Z. Lebensm. Unters. Forsch. 108, 113-134.

Zhang P and Omaye ST, 2001. DNA strand breakage and oxygen tension: effects of beta-carotene, alpha-tocopherol and ascorbic acid. Food and Chemical Toxicology 39(3), 239-246.

Zhang S, Tang G, Russell RM, Mayzel KA, Stampfer MJ, Willett WC and Hunter DJ, 1997. Measurements of retinoids and carotenoids in breast adipose tissue and a comparison of concentrations in breast cancer cases and control subjects. American Journal of Clinical Nutrition. 66, 626-632.

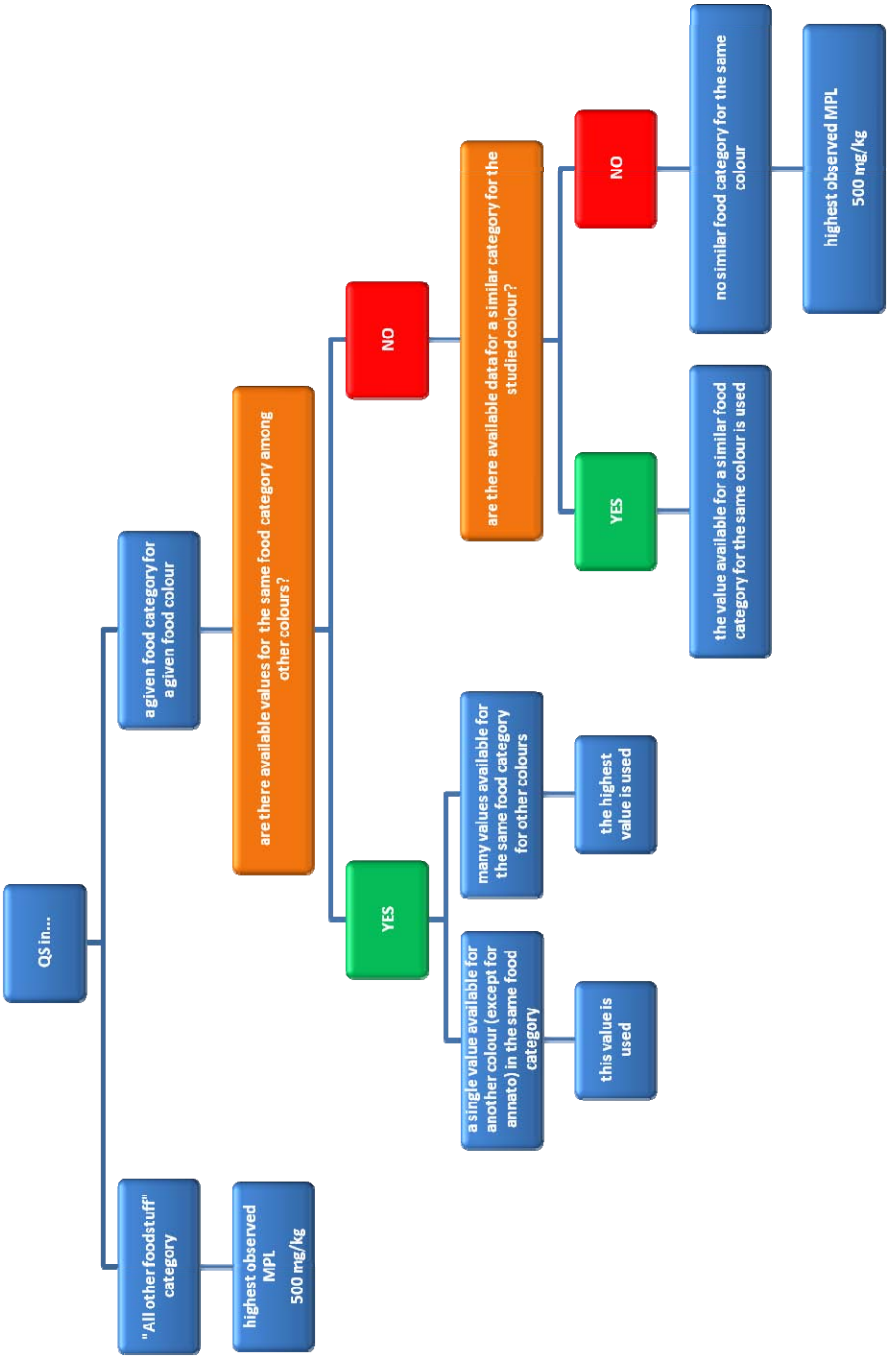
Ziegler RG, Mayne ST and Svanson CA, 1996. Nutrition and lung cancer. Cancer causes and control 7(1) 157-177.

ANNEX A

RULES DEFINED BY THE PANEL TO DEAL WITH QUANTUM SATIS (QS) AUTHORISATION, USAGE DATA OR OBSERVED ANALYTICAL DATA FOR ALL REGULATED FOOD ADDITIVES TO BE RE-EVALUATED

Figure 1: Rules defined by the Panel to deal with usage data or observed analytical data for all regulated food additives to be re-evaluated and procedures for estimating intakes using these rules.

Rules defined by the Panel to deal with *quantum satis* (QS) authorisation.



GLOSSARY/ABBREVIATIONS

AAPH	2,2'-Azobis(2-amidinopropane)dihydrochloride
ADI	Acceptable Daily Intake
ALAT	Alanine aminotransferase
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
ARPE-19	Human Retinal Pigment Epithelial Cells
ASAT	Aspartate aminotransferase
ATBC	Alpha-Tocopherol, β-carotene Cancer Prevention Study Group
B[a]P	Benzo[a]pyrene
BUN	Blood Urea Nitrogen
CAs	Chromosomal aberrations
CARET	β-Carotene and Retinol Efficacy Trial
CAS	Chemical Abstracts Service
CIAA	FoodDrinkEurope until June 2011 known as Confederation of the Food and Drink Industries of the EU
CMC-Na	Carboxylmethyl cellulose sodium
CP	Cleavage products
CREB	Cyclic AMP Response Element Binding protein
CYP	Cytochrome P450
DMNQ	2,3-Dimethoxy-1,4-naphthoquinone
DNA	Deoxyribonucleic acid
EC	European Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial chemical Substances
EPR	Electron Paramagnetic Resonance
EU	European Union
FAO/WHO	Food and Agriculture Organization/World Health Organization
GD	Gestational days
GLP	Good Laboratory Practice
GSH	Glutathione
HDL	High Density Lipoprotein
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPLC	High Pressure Liquid Chromatography
IARC	The International Agency for Research on Cancer
IUB	International Union of Biochemists

IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50% i.e. dose that causes death among 50% of treated animals
LDH	Lactate dehydrogenase
LDL	Low Density Lipoprotein
MN	Micronuclei
MPL	Permitted Use Levels
MTT	Mitochondrial reduction function test
NATCOL	Natural Food Colours Association
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organisation for Economic Co-operation and Development
QS	*Quantum Satis*
PAC	Pulmonary Adenocarcinoma
RCT	Randomized Controlled Trials
PKA	Protein kinase A
ROS	Reactive Oxygen Species
RR	Relative Risk
SCEs	Sister chromatid exchanges
SCF	Scientific Committee on Food
SCOOP	A scientific cooperation (SCOOP) task involves coordination amongst Member States to provide pooled data from across the EU on particular issues of concern regarding food safety
UL	Tolerable Upper Intake Level
VLDL	Very Low Density Lipoprotein