

**Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to**

***tertiary-Butylhydroquinone (TBHQ)***

Question number EFSA-Q-2003-141

**Adopted on 12 July 2004**

**Summary**

The Scientific Panel **on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC)** has been asked to advise on the safety in use of TBHQ (*tertiary-butylhydroquinone*) in fats and oils used for human consumption. TBHQ is intended to be used at a level of up to 200 mg/kg fat or oil.

TBHQ has already been evaluated by the former Scientific Committee on Food (SCF) which concluded in 1987 that there was still a need for additional data. The SCF requested an adequate carcinogenicity study and an *in vivo* mutagenicity study of germ cell effects. At that time, the SCF was of the opinion that TBHQ was not acceptable for use as an antioxidant to food and did not establish an acceptable daily intake (ADI). Since then additional data on carcinogenicity and mutagenicity have become available.

Based on the data reviewed the Panel concluded that TBHQ is not carcinogenic and that further genotoxicity studies were unnecessary. The Panel considers the dog as the most sensitive species and allocated an ADI of 0-0.7 mg/kg bw based on a no-observed-adverse-effect-level (NOAEL) of 72 mg/kg bw per day in dogs to which a 100-fold safety factor was applied.

Considering that not all fat intake in high fat eaters will come from refined oils, conservative intake estimates suggest that exposure in adults and children who are high fat eaters would

not exceed the ADI. However, if TBHQ were allowed to be used in infant formulae and follow on formulae at the maximum amount requested, exposure in infants and children would exceed the ADI.

The formation of 2-*tertiary*-butyl-p-benzoquinone (TBBQ) is likely to be due to the antioxidant action of TBHQ and this compound would probably be reduced back to TBHQ to a certain extent when ingested. The formation of TBBQ from the use of TBHQ in food is not considered to be a matter of concern.

### Keywords

*tertiary*-Butylhydroquinone, TBHQ, CAS Registry Number 1948-33-0, other CAS Numbers 123477-69-0, 140627-33-4, 29863-17-0, 68816-56-8, Food Additive, Antioxidant

### Background

The Scientific Committee on Food (SCF) previously expressed its opinion on TBHQ in December 1981 when it drew attention to the lack of adequate data on which to assess the genotoxic and carcinogenic potential of this antioxidant. Subsequently, in its opinion in December 1987, the SCF restated the need for an adequate carcinogenicity study and requested an *in vivo* mutagenicity study of germ cell effects. At that time, the Committee was of the opinion that TBHQ was not acceptable for use as an antioxidant in food and did not establish an ADI (CEC, 1989).

TBHQ has also been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at several meetings between 1975 and 1997. In reviewing long-term toxicity studies in rats, mice and dogs and reproductive toxicity studies in rats, JECFA concluded at its forty-ninth meeting that TBHQ was not carcinogenic in rats and mice and that the most sensitive species was the dog. An ADI of 0-0.7 mg/kg bw was allocated based on a NOEL of 70 mg/kg bw per day to which a 100-fold safety factor was applied (WHO, 1998).

TBHQ has already been used as food additive in several countries outside the EU including Australia, Brazil, China, and United States, from which intake data are available (WHO, 1999).

## Terms of reference

The Commission asks EFSA to issue an opinion on the safety in use of TBHQ (*tertiary*-butylhydroquinone) in fats and oils used for human consumption.

## Assessment

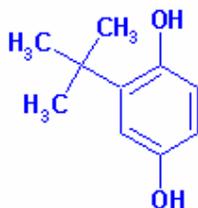
### Chemistry

TBHQ is a solid antioxidant that is readily soluble at use levels in fats and oils and in a number of food-grade solvents, but is practically insoluble in water.

Its chemical name in IUPAC nomenclature is tert-butyl-1,4-benzenediol. The name in CAS nomenclature is 1,4-benzenediol, 2-(1,1-dimethylethyl)-. In addition there are some synonyms.

CAS Registry Number is 1948-33-0, other CAS Numbers are 123477-69-0, 140627-33-4, 29863-17-0, 68816-56-8.

The structural formula is:



### Specification

According to the petitioner TBHQ meets all of the requirements listed below and the specification for TBHQ antioxidant as set forth in Food Chemical Codex (FCC), 3<sup>rd</sup> Edition, 1981.

The purity criteria indicated by the petitioner are as follows: TBHQ (99.0 % minimum), *tertiary-butyl-p-benzoquinone* (0.2 % maximum), *2,5-di-tertiary-butylhydroquinone* (0.2 % maximum), hydroquinone (0.1 % maximum), heavy metals (as Pb, 5 ppm maximum), toluene (not more than 0.0025 %). Melting point range 126.5 – 128.5°C.

### **Manufacturing Process**

TBHQ is manufactured by chemical synthesis. Different manufacturing processes are described by the petitioner.

### **Methods of Analysis**

Methods to detect and quantify the substance, evaluate its purity, and measure its physicochemical characteristics are given by the petitioner.

### **Stability and fate in food during heating and storage**

Studies demonstrate that phenolic antioxidants like TBHQ exhibit significant decomposition at elevated temperatures giving rise to a number of breakdown products which may undergo further decomposition. At elevated temperatures both evaporation and decomposition result in a loss of antioxidative activity. According to the petitioner, under normal conditions of storing and cooking the main decomposition product is *2-tertiary-butyl-p-benzoquinone* (TBBQ). This finding is supported by experiments heating TBHQ at frying temperatures (180°C or above). Heating was carried out with TBHQ either in pure form or dissolved in vegetable oil or in fatty foods, at different concentrations. The formation of TBBQ is in accordance with the antioxidative action of TBHQ.

Pure TBHQ was heated to 175-185°C for differing times from 1 to 100 hours either singly or repeatedly. The amount of unaltered volatile and non-volatile TBHQ remaining decreased over time and accounted for between 10 to 50% of its original concentration. Using GC/MS 20 to 30 decomposition products were identified. The main decomposition product was TBBQ accounting for around 30% of the original material. Quantitative data on the other

decomposition products were incomplete, but where measured concentrations were less than 1%. These decomposition products included hydroquinone, dimerized compounds with ether linkages and some higher molecular weight products. (Hamama and Nawar, 1991, Kim and Pratt, 1990)

Two studies used, ring-labelled [<sup>14</sup>C]-TBHQ to examine and compare its decomposition with both labelled butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in deep-fat frying and cookie baking (Warner *et al.*, 1986) or labelled BHA in heated vegetable oil at 180-190°C for 4.5 hours (Lin *et al.*, 1981). High performance liquid chromatographic (HPLC) analysis of volatiles and non-volatiles confirmed the complexity of the products derived from the parent antioxidants but these were not fully characterised or quantified. Overall, the heat treatment resulted in virtually total decomposition (ca. 95-99%) of the added TBHQ and there was some evidence for decomposition of TBHQ in lard at room temperature.

The stability of TBHQ and some other antioxidants in fatty preparations and stock acetonitrile solutions stored under different conditions was studied by Irache *et al.* (1993). Degradation was adequately described by a first-order kinetic process for all antioxidants examined. The presence of degradation products in stock solutions was indicated by changes in its appearance and was accelerated by the presence of light. Based on results obtained with TLC and HPLC, TBBQ was suggested as possible degradation product of TBHQ but degradation products were not quantified nor definitively identified.

Additionally, the petitioner summarised the results of three unpublished reports of studies on the effect of heating differing concentrations of TBHQ in cottonseed oil or a 50:50 blend of cottonseed and soybean oil at 185-190°C for up to 48 hours. However, these study reports were not available for evaluation. TBHQ losses in these studies could have resulted from degradation or interaction with the food in addition to volatilization.

### **Case of need and proposed uses**

According to the petitioner TBHQ is especially effective for highly unsaturated vegetable oils and many animal fats and carries advantage over other previously approved antioxidants in its ability to extend the storage stability of vegetable oils.

TBHQ is intended to be used at a level of up to 200 mg/kg fat or oil.

## Exposure

Potential human exposure to TBHQ through the diet was therefore estimated by considering that all fats consumed would contain TBHQ at 200 mg/kg. In order to provide conservative estimates of exposure, high fat intakes were considered in different population groups: adults, children and infants. Exposure in infants and children requires specific consideration since both their energy intake per kg body and the percent energy from fat of their diet is higher than that of adults.

Within a research project aimed at identifying food consumption patterns leading to high fat intake, high fat eaters had been identified in a standardized way (subjects present in the highest quartile of percent energy from fat) on the basis of food consumption databanks deriving from food surveys conducted on representative samples of adults (Institute of European Food Studies, 1999). Comparable data were made available for ten European countries or regions (Belgium, Denmark, Finland, Germany, Crete, Ireland, Italy, The Netherlands, Portugal and Spain). Mean percentage of energy from fat in high fat eaters varied from 33% in Portugal to 53% in Crete (average of the 10 countries: 44%) whereas their mean total fat intake varied from 86 g in Portugal to 194 g in Belgium (average 122 g). Fat intake in the Belgium sample may be considered a worse case estimate due to the short duration of the survey (24 h recall): in this case observed intake estimates in the highest quantiles overestimate high intakes in the long term. European adults who are high fat eaters and for which all fats would contain TBHQ at 200 mg / kg would therefore be exposed on average to 24.4 mg of this additive. A worse case estimate is 38.8 mg of TBHQ (Belgium survey). Considering a 60 kg body weight, this exposure would be equivalent on average to 0.4 mg/kg with a worse case of 0.6 mg /kg (Belgium survey).

According to European Recommended Dietary Allowances, average energy requirement is approximately 100 kcal / kg bw /day in infants aged 6 months to 3 years of age (CEC, 1993). Exposure was assessed for an infant diet in which all fats would contain TBHQ. The maximum lipid content established by the European legislation for infant formulae was

considered: 6.5 g /100 kcal, equivalent to 58,5% energy from fat (CEC, 1991). TBHQ exposure in an infant consuming a normocaloric diet based on a high fat formulae would be 1.3 mg /kg body weight. If TBHQ was to be used in fats for infant formulae, this exposure could be reached on a regular basis since all the energy requirement of infants may be covered by formulae and infants tend to consume always the same formulae.

Potential exposure was assessed in a child aged 10 years. According to European Recommended Dietary Allowances, at this age average body weight is 30 kg and average energy requirement is 68 kcal / kg body weight (CEC, 1993). In order to assess potential exposure in a child with average energy intake consuming a diet high in fats, it was assumed that 50% of his energy requirement was covered by fats. This assumption leads to a conservative (but not unrealistic) estimate of 3.8 g /kg body weight of fat i.e. 0.75 mg TBHQ /kg body weight.

Intake estimates provided by the petitioner are not here presented since they were based on either per capita national availability of oil or mean energy from fat and therefore did not allow to assess potential exposure in high fat eaters. Previous intake estimates had also been performed within the frame of a JECFA evaluation however they were based on differing assumptions (TBHQ as only antioxidant present in foods and all foods contain either the national maximum permitted level or the highest level of use in the draft General Standard on Food Additives of 1000 mg/kg) in relation to the concentration of TBHQ in fats (WHO, 1999).

### **Toxicological studies**

The SCF, in its previous opinions, requested adequate data on the carcinogenic and genotoxic potential of TBHQ, therefore the present opinion mainly focuses on the results of carcinogenicity and genotoxicity studies that have become available since the SCF opinion. In addition, studies are reviewed which may be important for establishing an ADI. All other toxicological data are summarised briefly. More details on the toxicological data can be found in the monograph published by JECFA (WHO, 1998).

### Kinetic studies

In several studies TBHQ was shown to be well absorbed and rapidly excreted, mainly in the urine. Main metabolites are the 4-O-sulphate conjugate and the 4-O-glucuronide. Excretion seems to be essentially complete after 2 - 4 days.

Tissue levels found in liver, kidney, brain, and fat of rats receiving  $^{14}\text{C}$ -TBHQ at a concentration of 0.029 % in diet (equal to 5.7 mg  $^{14}\text{C}$ -TBHQ / kg bw / day) for 17 days were in the range of 0.06 - 0.56 mg TBHQ equivalents / g wet tissue (Astill *et al.*, 1967a). For humans, the recovery of TBHQ from urine was dependent on the vehicle used and the manner of ingestion. When a mixture of TBHQ in corn oil and graham cracker crumbs as a single dose, equivalent to about 125 mg TBHQ, was received by two male adult volunteers, the recovery of the dose as metabolites in the urine was nearly complete. This was accompanied by a serum level of 31 - 37 mg TBHQ / litre at 3 hours, which declined to a level of 15 - 17 mg / litre at 25 hours (Astill *et al.*, 1967c). Fetal exposure was measured in a reproductive toxicity study in which pregnant rats were given a single dose of 40 mg  $^{14}\text{C}$ -TBHQ / kg bw about one day before sacrifice. In the fetuses, total radioactivity equivalent to 3.2  $\mu\text{g}$   $^{14}\text{C}$ -TBHQ was measured at 7.6 hours after dosing. This was equivalent to 1.5 mg TBHQ equivalents / kg bw of fetus (with an average fetal weight of 2.2 g). The authors extrapolated these results to possible human exposures and suggested that at an intake of 0.1 mg TBHQ / kg bw / day, the human fetus would be exposed to the order of 1 % of the daily intake in the form of unchanged TBHQ and probably higher levels of the sulphate and glucuronide conjugates of TBHQ (Astill and Walton, 1968).

In rats, dogs and humans the main metabolites of TBHQ found are the 4-O-sulphate conjugate and the 4-O-glucuronide (Astill *et al.*, 1967a; 1968; 1967c). Following intraperitoneal administration to rats, three glutathione conjugates of TBHQ were identified in the bile of male rats, and sulphur-containing metabolites of TBHQ were detected in the urine (Peters *et al.*, 1996a). *In vitro*, none of the major GST isoenzymes were required for the formation of glutathione conjugates from TBHQ (van Ommen *et al.*, 1992). These glutathione conjugates were toxic to the kidney and bladder when administered intravenously to male rats (Peters *et al.*, 1996b). As part of an investigation into the metabolism of BHA, two metabolites, 2-*tert*-butyl-5-methylthiohydroquinone and 2-*tert*-butyl-6-methylthiohydroquinone, were identified in the urine of male rats after intraperitoneal administration of BHA as well as after

administration of TBHQ (Tajima *et al.*, 1991). The authors suggested that these metabolites resulted from the metabolic conversion of glutathione conjugates of a quinone or semiquinone form of TBHQ.

Redox cycling of TBHQ with the corresponding quinone, 2-*tertiary*-butyl-p-benzoquinone (TBBQ), was accompanied by the production of reactive oxygen species, including superoxide anion, hydrogen peroxide, and hydroxyl radical. This was shown with several microsome preparations from rat liver and forestomach (Kahl *et al.*, 1989; Phillips *et al.*, 1989; Bergmann *et al.*, 1992), and was catalyzed by prostaglandin H synthase and lipoxygenase *in vivo* and *in vitro* (Schilderman *et al.*, 1993a). TBHQ, but not BHA and TBBQ, also produced hydrogen peroxide in the absence of S9 mix, however, this could only be demonstrated at concentrations above 200  $\mu\text{mol/l}$  (Phillips *et al.*, 1989). *In vitro*, the glutathione conjugates of TBHQ showed an increased redox cycling activity (oxygen consumption in the presence of a reducing agent) compared with unconjugated TBHQ (van Ommen *et al.*, 1992).

#### Effects on subcellular and cellular systems

In numerous *in vitro* studies, TBHQ was shown to induce the activity of several enzymes of the Ah gene battery including UDP-glucuronosyl-S-transferase (UGT1\*06), glutathione-S-transferase, NAD(P)H:quinone reductase (NAD(P)H:menadione oxidoreductase), cytosolic aldehyde dehydrogenase (ALDH3c), as well as  $\gamma$ -glutamylcysteine synthetase and GSH synthetase, whereas CYP1A1 mRNA levels were unaffected (Prochaska, 1987 and 1994; Twerdok and Trush, 1990; Liu *et al.*, 1994; Shertzer *et al.*, 1995; Vasiliou *et al.*, 1995). The induction of activities of Phase II enzymes in the Ah gene battery seems to be mediated by regulatory elements EpRE (Electrophilic Response Element) and ARE (Antioxidant Responsive Elements) and independent of the Ah receptor. Induction of hepatic glutathione-S-transferase activity (2-fold) was also demonstrated *in vivo* following short-term administration of TBHQ in female mice while UDP-glucuronyl transferase activity was reduced by one third (Rahimtula *et al.*, 1982). In feeding studies on rats and dogs, no or only minor changes in enzyme activities of glucose-6-phosphatase (G-6-Pase), p-nitroanisole demethylase (pNaD) and aniline hydroxylase (AHase) were observed (Tischer and Walton, 1968).

In a study on the role of NAD(P)H:quinone oxidoreductase (NQO1) in the progression of neuronal cell death, the activity of NQO1 was measured *in vitro* in primary cultures of rat cortex after treatment with ethylcholine aziridinium, inducing mainly apoptotic cell death, or oxygen-glucose deprivation, which combines features of apoptotic and necrotic cell death (Kapinya *et al.*, 2003). Neuronal damage was exaggerated after chemical induction of NQO1 activity by TBHQ. The authors suggested that the activity of NQO1 is a deteriorating rather than a protective factor in neuronal cell death.

2,5-Di(*tertiary*-butyl)-1,4-benzohydroquinone (di-TBHQ), structurally related to TBHQ, has been shown to inhibit the calcium ATPase of the sarco-endoplasmic reticulum which is involved in the refilling of  $[Ca^{2+}]_i$  stores. In an *in vitro* study on rodent dorsal root ganglion, hippocampal, and motor neurons, di-TBHQ blocked the  $Ca^{2+}$  current at concentrations used to inhibit sarco-endoplasmic ATPase in all cell types tested ( $IC_{50} = 35 \mu M$ ) (Scamps *et al.*, 2000).

TBHQ, TBBQ, and BHA were all cytotoxic to human lymphocytes in a dose-dependent manner. TBHQ at 0.1 mM (17 mg/l) reduced the viability of lymphocytes after 50 hours of incubation to 72 %. Only a small percentage of the dose of TBHQ administered could be recovered, indicating a considerable binding to macromolecules (Schilderman *et al.*, 1995). For comparison, the serum level resulting from an intake of 125 mg TBHQ by human volunteers was in the range of 31 - 37 mg/l at 3 hours, which declined to a level of 15 - 17 mg/l at 25 hours (Astill *et al.*, 1967c). However, TBHQ (as well as TBBQ and BHA) induced a dose-dependent increase in cell proliferation of phytohaemagglutinin-stimulated human lymphocytes, 50  $\mu M$  being the optimal dose. TBHQ and TBBQ at 50  $\mu M$  increased 7-hydroxy-8-oxo-2'-deoxyguanosine formation in lymphocytes by 680 and 320 %, whereas BHA was not capable of inducing oxidative DNA damage to a significant degree (Schilderman *et al.*, 1995).

GSH levels of mouse hepatoma and rat lung epithelial cells were shown to be elevated by TBHQ treatment *in vitro* (Liu *et al.*, 1994; Shertzer *et al.*, 1995; Liu *et al.*, 1996b), whereas in some other studies, the exposure of HepG2 cells and freshly isolated rat hepatocytes to 0.5 mM TBHQ resulted in a depletion of intracellular GSH, protein thiol and ATP levels and a

time-dependent cell death (Nakagawa and Moldeus, 1992; Nakagawa *et al.*, 1994; Nakagawa, 1996; Pinkus *et al.*, 1996).

A 50 % inhibition of prostaglandin (PGE<sub>1</sub> and PGE<sub>2</sub>) biosynthesis was observed using microsomal preparations of bovine seminal vesicles at TBHQ concentrations of 5.5 and 6.1 µmol/l, respectively (Boehme and Branen, 1977).

#### Acute toxicity studies

The acute oral LD<sub>50</sub> in guinea pigs, rats and mice was in the range of approximately 700 to 1040 mg/kg bw. In dogs, the LD<sub>50</sub> could not be defined since the material was consistently regurgitated at doses below that which may cause lethal effects (Terhaar *et al.*, 1968a). TBHQ introduced into rabbit eyes at an amount of 10 mg produced moderate to severe injury (Valentine and Montgomery, 1988). In an inhalation study on rats exposed to TBHQ aerosols for 4 hours at concentrations of 1100 to 4200 mg/m<sup>3</sup>, a NOEL could not be determined. Deaths occurred at concentrations of 2900 and 4200 mg/m<sup>3</sup> (1/6 and 2/6 rats, respectively) (Valentine and Montgomery, 1988).

#### Subchronic studies

In a 13-week toxicity study in mice, which were fed diets containing 2500 to 40 000 mg TBHQ / kg, the NOEL was 5000 mg/kg (equal to 870 mg/kg bw per day) based on decreased body weight gain and increased incidences of mucosal hyperplasia of the forestomach and inflammation of both the nose and skin at higher doses (NTP, 1995).

When rats were fed diets incorporating TBHQ in unheated and heated oil at up to 250 mg TBHQ / kg diet for 6 month, only negligible effects were observed. The study is described by JECFA (WHO, 1998) as follows. Eight groups, each of 30 SD rats (equally divided by sex), were fed four levels of TBHQ in unheated oil and at the same level in heated oil (one hour to raise temperature to 190°C followed by 3 hours at 190°C) for 6 months. The levels of TBHQ in the oils were 0, 0.02, 0.1 and 0.5%. Oils were incorporated at a 5% level into a standard diet resulting in final dietary levels of 0, 10, 50 and 250 mg/kg, respectively. Body weight and gross feed consumption were recorded weekly for the first two nights and thereafter

fortnightly. General appearance and behaviour were observed during the test period. Haemograms and urinalysis were conducted on the 0.5% unheated and heated and control at 1, 5 and 6 months. Haemograms consisted of haemoglobin, haematocrit, WBC and differentials and protein determination. Urinalysis consisted of pH, specific gravity, occult blood, albumin, reducing sugar and microscopic examinations of the sediment. Aspartate aminotransferase (AST) and alkaline phosphatase (AP) were determined for the high and control groups at 3 and 6 months. At autopsy, liver, kidney, heart, spleen, lung, brain and testes weights were determined. About 20 organs were examined microscopically: lung, heart, tongue, oesophagus, stomach, small and large intestines, liver, kidney, urinary bladder, pituitary gland, adrenal, pancreas, thyroid, gonads, spleen, bone marrow, cerebrum, cerebellum and eye.

Three deaths occurred during the test period, but these were not compound-related. Male rats on the 0.5% TBHQ/unheated fat showed a slight depression in weight gain and those in the 0.02% TBHQ/unheated fat a significant increase in weight gain over control. These effects were not observed in female rats on diets containing heated fats. Female rats in all groups showed similar weight gains to controls. Food intake of test groups was comparable or better than controls. Haematological tests gave similar values for test and control groups with the exception of the 0.5% TBHQ/unheated fat male group at three month, where the WBC was slightly elevated. This effect was not noted at six month. Urinalysis, AST and AP values of test and control groups were comparable and within normal limits. Organ/body weight ratios indicated a slight increase in ratios for testes and livers of the male rats from 0.5% TBHQ/heated oil group and liver weight ratio of the female rats of the 0.5% and 0.2% heated oil group. According to JECFA, these minor differences appeared to be related to heated versus unheated fat rather than a compound effect. Histological studies did not reveal any compound-related effects (Terhaar and Krasavage, 1968b).

In a 13-week study in rats fed diets containing 0, 2500, 5000 or 10 000 mg/kg TBHQ, high-dose males gained 9 % less body weight than controls. Hair discoloration was observed in all exposed groups except the low-dose females. Serum bile acids were significantly increased in the 5000 and 10 000 mg/kg groups. An increased incidence of hyperplasia of the nasal respiratory epithelium was observed in mid-dose males and high-dose males and females. Atrophy of the splenic red pulp was observed in 8/10 and 10/10 mid- and high-dose females, respectively. In addition, there was a dose-related decrease in the incidence of renal

mineralization in exposed females. An increased incidence of splenic pigmentation was observed in females at all dose levels. Similar effects were observed in the long-term study, but the biological significance was considered minimal by the authors, because other changes corroborating anaemia were not observed (NTP, 1997a).

#### Special studies on organ toxicity

To compare the possible effect of TBHQ on the lung with the known effects of BHT, TBHQ was given to mice by a single i.p. injection of up to 500 mg / kg bw. TBHQ treatment resulted in mortality at 125 mg/kg bw and higher doses, but did not lead to any treatment-related lung lesions within 5 days. In contrast injection of BHT produced pulmonary congestion and discoloration of the lungs at all dose levels (300 to 1230 mg / kg bw), with lesions which were manifested histologically as hyperplasia of pneumocytes, hypertrophy, and general disorganization of cellular components (Krasavage and O'Donoghue, 1984).

Several studies were undertaken to examine forestomach cell proliferation. Hyperplasia of the basal cell layer in the forestomach epithelium of rats and an increase of <sup>3</sup>H-thymidine-labeling index were observed after feeding diets containing 1 % TBHQ for 9 days (Nera *et al.*, 1984). In rats fed diets containing 2 % TBHQ for 28 days, TBHQ was shown to induce brownish discolorations and mild hyperplasia of the forestomach mucosa with focally increased hyperplasia of basal cells (Altmann *et al.* 1985, 1986).

In further studies, rats received 2 % dietary TBHQ alone or in combination with 0.3 % sodium nitrite (a promoter of forestomach carcinogenesis) in the drinking water for 4 weeks. In the absent of sodium nitrite, TBHQ resulted in a significant increase in the thickness of the forestomach mucosa in the pre-fundic or mid-regions compared to controls. Co-administration of TBHQ with sodium nitrite resulted in an increase (more than 10 times) in the height of the forestomach mucosa compared to those receiving TBHQ, sodium nitrite or basal diet alone. Slight increases were also observed in glandular stomach and oesophagus. Effects on mucosa thickness were accompanied by increases of 5-brom-2'-deoxyuridine labelling indices (Kawabe *et al.*, 1994; Yoshida *et al.*, 1994).

However, in hamsters fed a diet containing 0.5 % TBHQ for 20 weeks, TBHQ did not induce hyperplasia or tumourous lesions of the forestomach, the glandular stomach or the urinary bladder, and did not increase the labelling index in the tissues investigated (Hirose *et al.*, 1986).

Some studies were undertaken to examine effects on kidney and urinary bladder. In rats fed a diet containing 2 % TBHQ for 4 to 8 weeks, TBHQ caused morphological surface alterations of bladder epithelia, an elevation of DNA synthesis in the urothelium, an increase in urinary pH, and a decrease in potassium and phosphate contents as well as in osmolality (Shibata *et al.*, 1989). Three GSH conjugates of TBHQ (5-GSyl-TBHQ, 6-GSyl-TBHQ, and 3,6-bis-GSyl-TBHQ) produced increases in the urinary excretion of  $\gamma$ -glutamyl transpeptidase, AP, LDH and glucose (2-, 2-, 22- and 11-fold increases, respectively) and caused severe haemorrhaging of the bladder when administered intravenously at a dose of 400  $\mu$ mol/kg to male rats (Peters *et al.*, 1996b).

#### Long-term toxicity / carcinogenicity studies

Groups of 55 male and 55 female albino rats (A&C Farms, Altamont, N.Y.) were fed diets containing TBHQ at concentrations of 0, 0.016, 0.05, 0.16 and 0.5 % for a period of 20 months. Data recorded throughout the study included general appearance and behaviour, weight gain, feed consumption, mortality, haematological parameters and urine analysis. At 6 and 12 months, approximately 20 rats (10 per sex) of each group were sacrificed and at 20 months all surviving animals were sacrificed. At the time of necropsy, organ weights were determined for lung, liver, kidney, heart, adrenal, testes, spleen and brain. Histopathological studies were made on these and some other organs.

No adverse changes in appearance and behaviour of the rats were observed during the test period. Mortalities occurred with equal frequency in all groups and were particularly heavy during the 12- to 20-month period. However, deaths did not appear to be compound-related. Growth rate, food intake and feed efficiency were comparable for all groups during the experimental period. Urinalysis and haematological and biochemical tests of dosed and control groups were similar and within normal limits. Although there were some decreases in the absolute organ weights of spleen and brain of males of the 0.05 and 0.16 % groups at 20 months, these were not significantly different from the controls when expressed as

organ/body weight ratios. No compound-related gross or microscopic lesions were detected (Terhaar *et al.*, 1968).

Groups of 60 female F344/N rats ( $F_0$  generation) were fed diets containing 0, 1250, 2500 or 5000 ppm TBHQ beginning two weeks prior to cohabitation and continuing until  $F_1$  pups were weaned. Male  $F_0$  rats used as breeders were not considered to be part of the study. Following weaning, groups of 68-70 male and 68-70 female  $F_1$  rats were fed diets containing the same levels of TBHQ as those given to their respective dams for approximately 30 months or until the survival rate of the exposure group was less than 20 %. The *ad libitum* access at these exposure levels resulted in average daily doses of 50, 110, and 225 mg/kg bw in males or 60, 120, and 240 mg/kg bw in females. Food consumption, body weights and clinical findings were recorded periodically throughout the study. An interim sacrifice of 10 male and 10 female rats of each exposure group was conducted at 3 months. At this time, complete gross and microscopic evaluations were conducted, the weights of the right kidney, liver and right testis were recorded and a standard set of haematological parameters was determined. A complete necropsy, including gross and microscopic evaluations, was performed where possible on rats dying during the study and all animals surviving to study termination.

Survival of females in the 5000 ppm group was significantly ( $p < 0.05$ ) greater than that of the control group. Although feed consumption by exposed groups was generally similar to that of the appropriate control, body weights of 5000 ppm groups were generally about 10 % lower than those of the control groups. The only clinical observation attributed to TBHQ was hair discoloration in all exposed groups. The incidence of haemosiderin pigmentation of the spleen increased dose-related in females (24/60, 27/60, 33/57, 41/60 in the 0, 1250, 2500 and 5000 ppm groups, respectively), but was significantly different ( $P \leq 0.01$ ) from the control group only at the high dose and was unaffected by TBHQ treatment in males. Since other changes corroborating an anemia were not observed, the biological significance was considered minimal by the authors. The incidences of cysts (2/60, 3/60, 7/58, 11/60) and suppurative inflammation (9/60, 8/60, 9/58, 20/60) in the kidney of male rats were increased. In female rats, the incidence of chronic kidney inflammation was increased (1/60, 1/60, 3/57, 5/60) while there was no change in the incidence of suppurative inflammation. TBHQ treatment was associated with a decreased incidence of mammary fibroadenoma in females, adenoma in the pars distalis of the pituitary gland in males, and adenoma of the

adrenal cortex in females. The authors suggested that these decreases may have been related to decreased body weights.

The NOEL for the long-term toxicity of TBHQ was 2500 mg/kg diet (equal to 110 mg/kg bw per day) based on an increased incidence of cysts and suppurative inflammation in the kidneys of males and an increased incidence of chronic inflammation of the kidneys of females at higher doses. Under the conditions of the study, there was no evidence of carcinogenic activity of TBHQ in male or female F344/N rats (NTP 1995, 1997a).

Groups of 60 male and 60 female B6C3F<sub>1</sub> mice were fed diets containing 0, 1250, 2500 or 5000 ppm TBHQ for 104 weeks. The *ad libitum* access at these exposure levels resulted in average estimated daily doses of 130, 290, or 600 mg/kg bw per day in males or 150, 300, or 680 mg/kg bw per day in females. Food consumption, body weights and clinical findings were recorded periodically throughout the study. An interim necropsy of up to 10 mice in each exposure group was conducted at 15 months. At this time, complete gross and microscopic evaluations were conducted, the weights of the right kidney, liver and right testis were recorded, and a standard set of haematological parameters was determined. A complete necropsy, including gross and microscopic examination, was performed on all mice at termination of the study and where possible, on animals dying during the study. All organs and tissues were examined for grossly visible lesions, and all major tissues were examined microscopically.

Survival of all exposed groups of males and females was similar to that of the control group. Although feed consumption by exposed groups was generally similar to that of the appropriate control, body weights of 5000 ppm groups were generally about 10 % lower than those of the control groups. There were no clinical findings in exposed groups considered to be related to TBHQ treatment. At the 15-month interim evaluation, males in the 5000 ppm group had a significantly ( $p \leq 0.05$ ) higher level of reticulocytes than controls ( $0.47 \times 10^6/\mu\text{l}$  vs.  $0.21 \times 10^6/\mu\text{l}$ ). There were no other differences in haematological parameters. Although the absolute liver weights of all exposed male and female groups were generally higher than those of the control groups, statistically only the relative liver weight of high dose females was significantly ( $p \leq 0.01$ ) different from that of the controls. At the end of the study, females in the 1250 ppm group had significantly higher incidences of hepatocellular adenomas and hepatocellular adenomas or carcinomas (combined). However, the incidence of hepatocellular adenomas or carcinomas (combined) was in the range of historical control

values, and the incidences of hepatocellular neoplasms were lower in high-dose males and females than in controls. The incidences of follicular cell adenoma in the thyroids of exposed females were higher than in controls (1/51, 3/51, 2/50 and 5/54). However, the differences were not statistically significant, and the incidences did not exceed the historical control range for 2-year NTP feed studies (0 % to 9 %). Incidences of follicular cell hyperplasia in exposed groups of females were greater than those in controls at the end of the 2-year study (12/51, 19/51, 24/50 and 24/54), although, the severity of the hyperplasia in exposed groups was similar to that in the controls. This lesion was not observed at the 15-month interim evaluation. The authors of the study report considered these marginal increases were not related to TBHQ exposure. Under the conditions of the study, there was no evidence of carcinogenic activity of TBHQ in male or female B6C3F<sub>1</sub> mice (NTP, 1995, 1997a).

Carcinogenicity of TBHQ was additionally evaluated under different NTP feeding protocols. For the evaluation under defined NTP conditions, data have been obtained from the NTP study in F344/N rats described above which was conducted under the *ad libitum* feeding protocol normally used:

- The *ad libitum* feeding protocol is described above. Briefly, up to 70 males and 70 females were used per group. At 3 months, 10 males and 10 females per group were used for an interim evaluation. After termination, 60 males and 60 females of the control and the high-dose (5000 ppm) group, respectively, were used for comparison of tumour incidences.

For the evaluation of carcinogenicity under dietary restriction conditions, data have been obtained from additional investigations using two dietary restriction protocols:

- In a weight-matching protocol, unexposed groups of 70 males and 70 females were fed in such a way so that their mean body weight matched that of the high-dose (5000 ppm) *ad libitum* group. Corrections for body weight changes, if necessary, were made weekly for the first thirteen weeks and monthly thereafter. 10 males and 10 females served for a 3-month interim evaluation. After termination, 60 males and 60 females were used for comparison of tumour incidences.
- In a feed restriction protocol, groups of 70 males and 70 females, one control and one dosed as the high-dose group (5000 ppm) were offered identical quantities of feed. The amounts given were limited so that the control group would attain body weights of approximately 85 % that of the *ad libitum* fed controls. 10 males and 10 females per group

served for a 3-month interim evaluation. The remaining 60 males and 60 females per group were used for comparison of tumour incidences.

In each protocol, the exposure began two weeks prior to cohabitation and continued until weaning of pups. Following weaning, groups of F<sub>1</sub> rats were fed diets containing the same levels of TBHQ as those given to their respective dams. The animals were observed twice daily for signs of morbidity, mortality, or toxicity. Necropsy of F<sub>1</sub> rats was performed on animals dying during the study and all animals surviving after a dosing period of 122 weeks for males and 128 weeks for females (in the *ad libitum* feeding protocol and in the weight-matching protocol). Under the feed restriction protocol, the animals were evaluated for carcinogenic effects after a dosing period of 130 weeks or at the time when the survival in any group reached 20 %. For each animal, 42 tissues were collected for routine histopathological examination, plus any tissue with grossly visible lesions. The data were evaluated by comparing tumour incidences of control and high-dose *ad libitum* groups, by comparing tumour incidence in the weight matched-control with that of the *ad libitum* high-dose group, and by comparing tumour incidences of control and high-dose feed restricted groups.

Although feed intake by exposed groups was generally similar to that of the *ad libitum* fed controls, mean body weights of the high-dose *ad libitum* fed male and female rats at 1 year were depressed by 8 % and 10 % relative to the *ad libitum* fed controls. The body weights of weight-matched control male and female rats were similar to that of the *ad libitum* fed high-dose groups. Under the feed restriction protocol at 1 year, the mean body weights of exposed rats were similar to that of the appropriate controls and were depressed by about 20 % compared to the *ad libitum* controls. Survival rates of *ad libitum* fed rats were 13 % (control) and 23 % (high-dose) for males and 17 % (control) and 28 % (high-dose) for females. Survival rates for weight matched controls were 20 % for males and 37 % for females. Survival rates for feed restricted control and exposed groups were 17 % and 37 % for males and 30 % and 40 % for females.

Under the *ad libitum* feeding protocol, as it is used in the NTP bioassay, no increase in tumour incidence that could be attributed to TBHQ was found in rats of either sex. When the weight matched unexposed rats were used as controls, statistically significant ( $p < 0.05$ ) increased incidences of preputial and clitoral gland tumours (adenoma or carcinoma combined) were observed in exposed rats (preputial gland tumours in males: 0 % (control) vs. 13 % (high-dose); clitoral gland tumours in females: 10 % (control) vs. 23 % (high-dose)). These different tumour incidences of control and high-dose groups resulted from a decrease

of the tumour incidence of the weight matched controls (males: 0%, females: 10%) compared to the ad libitum controls (males: 12 %; females: 21 %). Under the feed restriction protocol, there was a significant ( $p < 0.05$ ) increase in the incidence of clitoral gland tumours in females (8 % in the control group vs. 25 % in the high-dose group) whereas TBHQ had negligible effects on the incidence of preputial gland tumours in males (Abdo and Kari, 1996; NTP, 1997b). These differences in neoplasm incidences are considered by the authors an uncertain finding for several reasons.

Groups of 4 beagle dogs per sex were fed diets containing 500, 1580 or 5000 mg/kg TBHQ ad libitum 1 hour per day, 6 days per week (equal to 21/22, 72/73, 260/220, mg/kg bw per day in males/females, respectively, as assessed by JECFA (WHO, 1998)) for up to 117 weeks. On termination of the study, all animals were examined for gross and pathologic changes. The liver, kidneys, spleen, heart, brain, lungs, gonads, adrenals, thyroid, and pituitary of all dogs were weighed, and at least 24 organs and tissues from control and high-dose animals were collected for gross and microscopic pathology. Samples of liver and kidney tissues were prepared for electron microscopy.

Appearance and behaviour were judged to be normal, and physical examinations revealed no compound-related effects. Growth, food consumption and weight gain were similar for all groups of animals. Urinalysis and biochemical tests showed no compound-related effects. Haematological tests showed variable effects in the high-dose group. These included slightly lower erythrocyte counts and slightly lower haemoglobin and haematocrit levels in high-dose male and female dogs compared to controls, while reticulocyte counts during week 99 showed elevations in the high-dose animals. As these elevations were still evident at week 104, the study was extended to 117 weeks for further observation. The final reticulocyte counts, however, revealed no definite pattern with respect to dosage. Platelet counts were normal in all animals. Peripheral blood smears showed more normoblasts in the high-dose animals as well as an occasional increase in erythrocyte basophilia. These effects were not observed in any other test group. Organ weight, gross pathology, and histopathology failed to reveal any compound-related changes. Electron microscopic evaluation of liver and kidney of dosed animals showed normal cellular constituents. There was no increase in the endoplasmic reticulum in liver cells of treated animals. The NOEL in this study was 1580 mg/kg diet (equal to 72 mg/kg bw per day) based on decreased haemoglobin and/or

haematocrit at various time points throughout the study and decreased RBC counts at 112 weeks in high-dose dogs of both sexes (Eastman Chemical Products, 1968).

#### Special studies on potentiation and inhibition of carcinogenesis

In a number of studies on potentiation and inhibition of carcinogenesis, TBHQ has been shown to enhance or suppress the carcinogenic or related toxic effects of other chemicals in the two-stage carcinogenesis model in rats.

The effect of TBHQ on intragastric N-dimethylnitrosamine formation was investigated in rats which were given TBHQ along with sodium nitrite and dimethylamine. TBHQ was found to suppress liver cell necrosis at a dose of 225 mg/kg but had no protective effect at lower doses (Astill and Mulligan 1977). A protective effect was also observed in a study in which a reduction in the number and area of preneoplastic GST placental form positive foci in the liver of rats resulted from a single i.p. dose of diethylnitrosamine and partial hepatectomy in combination with 1 % TBHQ in the diet for 6 weeks (compared to rats that received diethylnitrosamine alone), whereas TBHQ at a level of 0.25 % was not effective (Ito *et al.*, 1988; Hasegawa *et al.*, 1992).

Several studies have been undertaken to investigate modifying activities of TBHQ or TBHQ-containing antioxidant mixtures on BBN-induced urinary bladder carcinogenesis in rats. Feeding rats a diet containing 2 % TBHQ for 36 weeks following initiation by the bladder carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) significantly increased the incidence of papillary or nodular hyperplasia, but did not significantly affect the incidences of carcinomas and papillomas. However, this result indicated a weak promoting activity of TBHQ in BBN-initiated urinary bladder carcinogenesis (Tamano *et al.*, 1987). In a similar study also using BBN as an initiator, the incidence of bladder carcinomas and/or papillomas was essentially unaffected in rats which received 0.7 % of dietary TBHQ for 16 weeks. However, a combined treatment of 0.7 % TBHQ, 0.7 % BHA and 0.3 % BHT had an additive effect on the incidence of bladder tumours, significantly ( $p < 0.01$ ) increasing the incidences of papillomas alone and papillomas and carcinomas combined compared to individual treatment with each antioxidant as well as compared to BBN-initiated controls. This indicated synergistic or additive actions of antioxidants on bladder cancer induction (Ono *et al.*, 1992). Feeding rats a diet containing TBHQ, BHA or BHT alone (0.8 % each) or in pairs (0.4 % each) for 32 weeks following initiation by BBN, resulted in a slightly but significant higher

incidence of preneoplastic papillary or nodular hyperplasia in the group treated with TBHQ plus BHA after BBN than in controls receiving BBN only. In this study, BHA, BHT and TBHQ all exerted enhancing effects in BBN-induced urinary bladder carcinogenesis in rats, but no synergism regarding this promotion occurred (Hagiwara *et al.*, 1989). In a review article on promoting activity of antioxidants in a two-stage bladder carcinogenesis model, it was summarized that TBHQ exerted weak promoting activities in BBN-initiated male rats while those of BHA and BHT were strong. It was concluded that promoting effects of the antioxidants could not be related to urinary components or the potency of their antioxidant action (Ito and Fukushima, 1989).

In a study using 7,12-dimethylbenz[a]anthracene (DMBA) as initiator in rats, feeding 0.8 % dietary TBHQ for 51 weeks reduced the incidences of total mammary tumours. The authors suggested that this could also be due to a treatment-associated decrease in body weight gain (Hirose *et al.*, 1988).

In a multi-initiator rat multi-organ carcinogenesis model a variety of tumours were induced using a mixture of five nitrosamines and 1,2-dimethylhydrazine. TBHQ at a dietary level of 1 %, significantly elevated the incidences of oesophageal papillary or nodular hyperplasias and papillomas, as well as forestomach papillomas, but significantly decreased the multiplicity of colon adenocarcinomas (Hirose *et al.*, 1993).

In a study in which TBHQ was part of an antioxidant mixture, it was shown that incidences of liver hyperplastic nodules and hepatocellular carcinomas of rats were reduced when antioxidants were administered in combination with hepatocarcinogens and/or nitroso compounds, while for the urinary bladder, the combination of antioxidants with nitroso compounds enhanced cancer development, and the addition of hepatocarcinogens further increased tumourigenesis (Fukushima *et al.*, 1991).

*In vitro*, TBHQ was shown to be a more powerful inhibitor of Benzo(a)pyrene (BaP) metabolism and DNA-adduct formation than BHA or BHT by using a hepatic microsome preparation from 3-methylcholanthrene-induced mice containing BaP and antioxidants at a molar ratio of 1:10 and calf thymus DNA (Colovai *et al.*, 1993).

#### Studies on genotoxicity

The genotoxic potential of TBHQ has been investigated *in vitro* and *in vivo* in numerous studies comprising different genetic end-points (WHO, 1998).

No induction of gene mutations has been observed in bacterial reversion assays (Ames tests using eight *S.typhimurium* strains) with and without rat or hamster liver S9 mix, in *S.cerevisiae* D7 (reversion and gene conversion), and in forward mutation assays at the HPRT locus in V79 and CHO cells, with and without S9 mix. A positive result was reported in a forward mutation assay with mouse lymphoma cells at the TK locus, only in the presence of rat liver S9.

Several studies have shown that TBHQ induces structural chromosomal aberrations *in vitro* in the absence of S9 (V79 and CHO cells) and/or in the presence of S9 (CHO and CHL cells).

The mechanism of genotoxicity of TBHQ in V79 cells was investigated by Dobo and Eastmond (1994). In this cell system, TBHQ induced both CREST-negative micronuclei (indicative of chromosome breakage) and CREST-positive micronuclei (indicative of chromosome loss). The formation of CREST-negative micronuclei was inhibited by catalase and increased by hypoxanthine/xanthine-oxidase. Formation of both CREST-positive and CREST-negative micronuclei was inhibited by glutathione. On this basis the authors suggested that reactive oxygen species contribute to the clastogenic activity of TBHQ, while chromosome loss results from binding of quinone or semiquinone metabolites to proteins critical for microtubule assembly and spindle formation. The induction of oxidative DNA damage (8-OH-dG) *in vitro* was confirmed in other studies.

The results of *in vivo* studies measuring chromosomal aberrations or micronuclei in mouse bone marrow are contradictory. TBHQ was reported to induce sister chromatid exchanges in mouse bone marrow after i.p. administration (Mukerjee *et al.*, 1989). Out of these *in vivo* studies, only the NTP micronucleus test in mouse bone marrow, yielding negative results, can be considered as conclusive (NTP, 1995). Another mouse micronucleus assay (Société Kemin Europa, 1982), considered positive by JECFA, was not available for re-evaluation.

Equivocal results, with increases in post implantation losses with no dose dependency, were obtained in a dominant lethal assay with rats.

The genotoxicity assays on TBHQ are summarized in Tables 2 and 3 in the Annex.

### Developmental toxicity

The results of studies on reproductive toxicity on rats (Fassett *et al.*, 1965; Terhaar and Krasavage, 1968a; Krasavage and Terhaar, 1970; NTP, 1995) indicated an adverse effect of TBHQ on pup survival and/or pup body weight at dietary levels of 0.5 % or higher. The effect on pup body weight was late-occurring in terms of the lactation period and the NOEL was 0.25 % in the diet, equivalent to 125 mg / kg bw per day. In the NTP 13-week study, the effect of TBHQ on reproductive parameters in rats included significantly lower mean spermatid counts, spermatid heads per testis, and spermatid heads per gram of testis only in males exposed to 5000 ppm. The oestrous cycles of females exposed to 2500 or 5000 ppm were significantly longer than that of the controls. The number of females with oestrous cycles that were of unclear duration or were longer than 12 days was increased in the 10.000 ppm group. In mice, decreased testis weights of males and increased lengths of oestrous cycles were observed only at high doses (10.000 or 40.000 ppm, respectively) (NTP, 1995). However, in three- and single-generation studies on rats, levels of up to 0.5 % TBHQ in the diet did not affect oestrous cycles (Terhaar and Krasavage, 1968; Krasavage and Terhaar, 1970). When rats were fed diets containing TBHQ up to 0.5 % from days 6 to 16 of gestation, no teratogenic effects have been observed (Krasavage, 1977).

### Immunotoxicity

In a study on immunotoxicity (conducted under NTP contract), only minor effects have been observed:

TBHQ at doses between 25 and 150 mg/kg administered for 14 days to B6C3F1 mice by gavage produced only slight alterations in two innate immune activities. There was a slight increase in the amount of the 3rd component of complement, a slight increase in FC mediated adherence and phagocytosis by peritoneal adherent cells, and a slight increase in natural killer cell activity. These increases in innate immune response may be a physiological response to TBHQ. All other immune parameters assessed were unaffected. Host resistance to one bacterial challenge (*Streptococcus pneumoniae*) and the B16F10 pulmonary tumour challenge were not altered by TBHQ exposure. In the basic toxicology studies, liver and spleen weight increased slightly, reticulocyte number increased, total number of the polymorphonuclear leukocytes decreased, and blood glucose increased slightly in mice treated with TBHQ (NTP Study Number: IMM87036).

## Discussion

From the data found in the literature and the information provided by the petitioner, it is concluded that under normal conditions of storing and cooking the main degradation product expected to be detected is 2-*tertiary*-butyl-p-benzoquinone (TBBQ). This finding is supported by experiments heating TBHQ at frying temperatures (180°C or above). Heating was carried out with TBHQ either in pure form or dissolved in vegetable oil or in fatty foods, at different concentrations. The formation of TBBQ is likely to be due to the antioxidant action of TBHQ and does not seem to be a matter of concern because this compound would probably be reduced back to TBHQ to a certain extent when ingested. Additionally, there is a study of Terhaar and Krasavage (1968b) demonstrating that only negligible effects were observed, when rats were fed diets containing TBHQ in unheated and heated oil up to 250 mg TBHQ / kg diet for 6 month.

The toxicity of TBHQ has been extensively studied, including toxicokinetic aspects, acute, short-term and long-term toxicity, carcinogenicity and reproductive toxicity. In special studies, potential teratogenicity, genotoxicity, and immunotoxicity as well as effects on certain organs (lung, forestomach, liver, kidney and urinary bladder) have been investigated.

The most prominent effects observed at high doses in subchronic feeding studies were forestomach hyperplasia in rats and mice and haemosiderin pigmentation and atrophy of the red pulp of the spleen in rats. In carcinogenicity studies, an increase in the incidence of thyroid follicular cell hyperplasia in female mice and an increased incidence of transitional cell hyperplasia and suppurative inflammation of the kidneys in male rats and of haemosiderin pigmentation of the spleen in female rats were observed. In dogs, some haematological parameters were affected in a long-term feeding study at the highest dose tested.

The biological significance of haemosiderin pigmentation in the spleen (observed in male and female rats in a 13-week study and at the 3-month interim evaluation in the long-term study as well as in female rats at the end of this study) was considered minimal by the authors of the NTP study because other changes corroborating an anemia were not observed.

The effects observed in female mice have been commented on by JECFA (WHO, 1998, p. 40): "In female mice, TBHQ induced an increase in the incidence of thyroid follicular cell hyperplasia, affecting all dosed groups. A non-significant increase in follicular cell adenomas was reported at high dose but the incidence was within the range of historical control values. No follicular cell carcinomas were observed. (...) Since the Committee was aware that hydroquinone (the unsubstituted parent compound) induced thyrotoxicity in mice, but not rats, it considered that the follicular cell hyperplasia observed with TBHQ in this study might be a toxicologically significant effect. Consequently, it concluded that a NOEL could not be established for this study and that the low dose of 150 mg/kg bw per day represented a LOEL." However, despite the conclusion that there is no NOEL in mice, JECFA concluded finally "that the most sensitive species was the dog."

As the induction of thyroid follicular cell hyperplasia in female mice without NOEL in the NTP study suggests a hormone-mediated effect, additional information on the levels of thyroid hormones ( $T_3/T_4$ ) and thyroid stimulating hormone (TSH) *in vivo* might be useful for an interpretation of the results. On the other hand, the incidences of follicular hyperplasia in mice were not clearly dose-related (24%, 37%, 48%, and 44% in control, 1250, 2500, and 5000 ppm TBHQ groups, respectively). The incidence of hyperplasia doubled from the control to the intermediate (2500 ppm) dose group, but did not increase further. Additionally, the severity of hyperplasia in exposed groups was similar to that in the controls. Furthermore, though there are no historical control data given, control rates in the preceding and following NTP studies were reported by the petitioner to be 29% and 54%, respectively, indicating high variability of control incidences. In addition, there was a late onset of hyperplasia (no hyperplasia was detected at 15 month). Accordingly, the authors of the NTP report considered the results were not treatment related. Finally, there are differences between rodents and humans with respect to thyroid physiology and sensitivity to potential disturbances of the hypothalamus-pituitary-thyroid axis by non-genotoxic substances from which it can be concluded that humans are less sensitive to the development of epithelial follicular thyroid tumours than rodents (IARC, 2001; Alison *et al.*, 1994; Capen, 1999; van Raaij, 2001). Taking all these aspects into account, the Panel considers the increased incidences of thyroid follicular cell hyperplasia observed in female mice not relevant for humans. Therefore, the Panel considers that the mid dose of 2500 mg/kg, equal to 290 and 300 mg/kg bw in male and

female mice, respectively, represent the NOAEL (based on a decreased body weight of males and females and higher levels of reticulocytes in males at the high dose).

The results of studies on reproductive toxicity on rats indicated an adverse effect of TBHQ on pup survival and pup body weight at dietary levels of 0.5 % or higher. The NOEL was 0.25 % in the diet, equivalent to 125 mg / kg bw per day. Additionally, there are conflicting results with respect to oestrous cycles of female rats in the NTP 13 week study and the studies of Terhaar and Krasavage (1968) and Krasavage and Terhaar (1970). In the NTP long-term study, oestrous cycles were not investigated. However, there is some indication that the conflicting results are due to the use of different rat strains. In a study of Tropp and Markus (2001) it was demonstrated that the cycle of Fischer rats was disrupted during food deprivation whereas Sprague-Dawley rats continued to cycle normally. As Fischer rats were used in the NTP study (and as there was a trend toward decreased weight gain), whereas Sprague-Dawley rats were used in studies of Krasavage and Terhaar, the results of these studies are in agreement with the findings of Tropp and Markus (2001). In a teratogenicity study with rats, no teratogenic effects have been observed with diets containing TBHQ up to 0.5 %.

Long-term studies on mice, rats, and dogs including one NTP study on mice and rats revealed no evidence that TBHQ was carcinogenic in these species. However, using additional weight-matched control groups, significant differences in the incidence of preputial and clitoral gland tumours were observed when weight matched unexposed rats were compared to the high-dose animals (NTP, 1997b). These differences resulted from decreased tumour incidences observed in unexposed weight matched controls compared to *ad libitum* fed controls. These differences in neoplasm incidences were considered by the authors an uncertain finding for several reasons. According to the authors, there was some indication that incidences of clitoral and preputial gland neoplasms in the weight-matched control groups may have been unusually low and that the differences in the incidence of preputial and clitoral gland tumours in the weight-matched control groups and exposed groups are therefore an incidental finding.

In the NTP study on rats and mice, decreases of tumour incidences have also been observed at some tumour sites. The authors suggested that decreased incidences of mammary gland neoplasms found in exposed male and female rats and of pituitary gland neoplasms in

exposed male rats may be related to body weight decreases (NTP, 1995). This was supported by Seilkop (1995), who found positive relationships between body weight and pituitary gland neoplasms in male and female rats and mammary gland neoplasms in female rats.

TBHQ is structurally related to butylated hydroxyanisole (BHA) which has been shown to induce hyperplasia as well as tumours of the squamous epithelium of the forestomach of rodents. In contrast, TBHQ produced forestomach hyperplasia in rats and mice, but no carcinogenic effects have been observed in long-term studies in rats, mice and dogs.

Redox cycling of TBHQ with the corresponding quinone, *tertiary*-butyl-p-benzoquinone (TBBQ), catalyzed by prostaglandin H synthase and lipoxygenase *in vivo* and *in vitro* was accompanied by the production of reactive oxygen species, including superoxide anion, hydrogen peroxide, and hydroxyl radical, and this was shown with several microsome preparations from rat liver and forestomach.

The genotoxic potential of TBHQ has been investigated *in vitro* and *in vivo* in numerous studies comprising different genetic end-points. In studies with mammalian cells *in vitro*, TBHQ consistently induced structural chromosomal aberrations, chromosome loss and oxidative DNA damage. The results of *in vivo* studies addressing the same end-points are contradictory. However, out of the *in vivo* studies, only a micronucleus test in mouse bone marrow, yielding negative results, can be considered as conclusive. Another mouse micronucleus test, considered positive by JECFA, was not available for re-evaluation. Equivocal results were obtained in a dominant lethal assay with rats.

Mechanistic studies suggest that the clastogenicity of TBHQ is secondary to the production of reactive oxygen species, while chromosome loss is secondary to the interference of quinone metabolites with the spindle apparatus.

Taking this into account, and in the light of the negative results provided by long-term carcinogenicity studies with TBHQ in mice, rats, and dogs, further genotoxicity studies are considered unnecessary.

In the 117-week study with dogs, the NOAEL is 72 mg/kg bw per day (based on haematological changes at the higher dose of 220 mg/kg bw). In the long-term feeding studies with rats, the NOAEL is 110 mg/kg bw per day (based on effects in kidneys and spleens at the

higher dose of 225 mg/kg bw), and in the long-term feeding study with mice, the NOAEL is 290 mg/kg bw per day (based on decreased body weight and increased levels of reticulocytes at the higher dose of 600 mg/kg bw). Since the NOAEL of 72 mg/kg bw as well as the LOAEL of 220 mg/kg bw of the dog study are lower than the NOAELs and LOAELs of the long-term studies with rats and mice, the dog is regarded as the most sensitive species.

### **Conclusion and recommendations**

TBHQ has already been evaluated by the former Scientific Committee on Food (SCF) which concluded in 1987 that there was still a need for additional data. The SCF requested an adequate carcinogenicity study and an *in vivo* mutagenicity study of germ cell effects. At that time, the Committee was of the opinion that TBHQ was not acceptable for use as an antioxidant to food and did not establish an ADI. Since then additional data became available.

Based on the data reviewed the Panel concluded that TBHQ is not carcinogenic and that further genotoxicity studies are unnecessary. The Panel considered the dog as the most sensitive species and allocated an ADI of 0-0.7 mg/kg bw based on a NOAEL of 72 mg/kg bw per day in dogs to which a 100-fold safety factor was applied.

Considering that not all fat intake in high fat eaters will come from refined oils, conservative intake estimates suggest that exposure in adults and children who are high fat eaters would not exceed the ADI. However, if TBHQ were allowed to be used in infant formulae and follow on formulae at the maximum amount requested, exposure in infants and children would exceed the ADI.

The formation of 2-*tertiary*-butyl-p-benzoquinone (TBBQ) is likely to be due to the antioxidant action of TBHQ and this compound would probably be reduced back to TBHQ to a certain extent when ingested. The formation of 2-*tertiary*-butyl-p-benzoquinone (TBBQ) from the use of TBHQ in food is not considered to be a matter of concern.

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**Annex 1.**

**Table 2: Genotoxicity Studies (*in vitro*)**

Test System	Test Object	Result	References	Comments
Ames test	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538	negative	Societé Kemin Europa (1982a) (not available for reevaluation), Mueller and Lockhart (1983), Hageman <i>et al.</i> (1988), Zeiger <i>et al.</i> (1992), Matsuoka <i>et al.</i> (1990)	
Reverse mutation in yeast	<i>Saccharomyces cerevisiae</i> D7	negative	Rogers <i>et al.</i> (1992)	
TK <sup>+/−</sup> assay	Mouse lymphoma cells, line L5178Y	positive (+S9 only)	Litton Bionetics (1982a)	
HPRT assay	CHO cells	negative	Beilman and Barber (1985)	
HPRT assay	V79 cells	negative	Rogers <i>et al.</i> (1992)	Both in the absence and presence of rat or hamster hepatocytes.
Chromosomal aberration	V79 cells	positive (- S9 only)	Societé Kemin Europa (1982c)	Not available for re-evaluation.
Chromosomal aberration	CHO cells	positive	Phillips <i>et al.</i> (1989)	TBHQ was tested only without S9 mix. Although the number of aberrations and the cytotoxicity of TBHQ was shown to be dependent on the cell density on the plate, a clastogenic effect of TBHQ was shown even in the standard protocol.
Chromosomal aberration	CHO cells	positive	NTP (1995)	In the study report, it is mentioned that the high dose was limited by toxicity, however, data on toxicity (e.g. mitotic index or % survival) were not included. Although at high doses, no more than 25 cells per dose were scored, it was pointed out in the study report that only a few cells were scored per dose level, because large numbers of multiple aberrations were observed in almost 100 % of treated cells. This is in agreement with OECD guideline 473. Therefore, it seems not clear, that the studies were inadequate as it is assumed in footnote 6 of Table 2 of the JECFA monograph (WHO, 1998).
Chromosomal aberration	CHL cells	positive (+S9 only)	Matsuoka <i>et al.</i> (1990)	

**Table 2 (continued):**

Test System	Test Object	Result	References	Comments
Micronucleus	CHL cells	positive (+S9)	Suzuki <i>et al.</i> (1991)	Validity cannot be evaluated (abstract only)
Micronucleus	V79 cells (-S9 only)	positive	Dobo and Eastmond (1994)	Increased frequencies of CREST-positive micronuclei (indicating chromosome loss) and CREST-negative micronuclei (indicating chromosome breakage) were observed following exposure of a prostaglandin H synthase-containing V79 cell line. In contrast to hydroquinone, for which the formation of micronuclei was dependent upon prior supplementation of V79 cells with arachidonic acid, TBHQ induced micronuclei both in the presence and absence of arachidonic acid supplementation indicating that the genotoxic effects of TBHQ were not dependent upon prostaglandin H synthase-mediated activation.
Mitotic gene conversion	Yeast	negative	Rogers <i>et al.</i> (1992)	
SCE	CHO cells	positive (+S9 only)	NTP (1995)	Weak positive. TBHQ treatment of CHO cells in the presence of S9 mix resulted in a statistically significant ( $p < 0.01$ ) slight increase of sister chromatid exchanges per cell (14.4 SCEs/cell compared to 8.3 of the control).
SCE	V79 cells	negative	Rogers <i>et al.</i> (1992)	Inconclusive. The negative result of the SCE test on V79 cells is inconclusive since it is not clear whether TBHQ is tested up to the toxicity limit (50% reduction in cloning efficiency is not achieved, and the toxicity of TBHQ to V79 cells was determined only in the absence of hepatocytes).
Oxidative DNA damage (8-oxodG)	dG	positive	Schilderman <i>et al.</i> (1993)	
Oxidative DNA damage (8-oxodG)	Human lymphocytes	positive	Schilderman <i>et al.</i> (1995)	
Oxidative DNA damage (8-oxodG)	Calf thymus DNA	positive	Nagai <i>et al.</i> (1996)	

**Table 2 (continued):**

Test System	Test Object	Result	References	Comments
Oxidative DNA damage (8-OHdG)	Rat hepatocytes	positive	Li et al. (2002)	The authors suggested that endogenous cellular copper may be capable of bioactivating TBHQ resulting in oxidative DNA-damage in cultured cells. The capacity of copper, a nuclei- and DNA-associated transition metal, to mediate the oxidation of TBHQ resulting in DNA damage has been investigated <i>in vitro</i> with isolated DNA and cultured rat hepatocytes. Incubation of plasmid DNA with TBHQ in the presence of Cu(II) resulted in DNA strand breaks, which could be prevented by catalase or bathocuproinedisulfonic acid (BSC). Incubation of rat hepatocytes with TBHQ in phosphate buffered saline led to increased formation of 8-hydroxyguanosine (8-OHdG) in nuclear DNA. The TBHQ-induced formation of 8-OHdG was reduced in the presence of a cell permeable Cu(I)-specific chelator, suggesting that a Cu(II)/Cu(I) redox mechanism may also be involved in the induction of oxidative DNA damage by TBHQ in hepatocytes. The study demonstrates that the activation of TBHQ by Cu(II) resulted in the formation of TBBQ, semiquinone anion radical and reactive oxygen species (ROS), and that the ROS formed may participate in oxidative DNA damage observed both in isolated DNA and intact cells.
DNA strand breaks <i>in vitro</i>	φX-174 DNA	positive	Li et al. (2002)	See above.
DNA strand breaks <i>in vitro</i>	φX-174 DNA	positive	Li and Trush (1994)	

**Table 3: Genotoxicity Studies (*in vivo*)**

Test System	Test Object	Result	References	Comments
Chromosomal aberration	Mouse bone marrow	negative	Litton Bionetics (1985)	The negative result is <b>inconclusive</b> , since the bone marrow was collected only at a single sampling time 24 hours after a single i.p. administration, and the top dose (200 mg/kg i.p.) was not justified by toxicity (the animals showed no clinically toxic signs and the mitotic index was not reduced). This does not meet the criteria of current EU and OECD guidelines and recommendations. According to the OECD guideline 475, samples should be taken at two separate times following treatment. For rodents, the first sampling interval is 1.5 normal cell cycle length (the latter being normally 12-18 hr) following treatment. Since the time required for uptake and metabolism of the test substance as well as its effect on cell cycle kinetics can affect the optimum for chromosomal aberration detection, a later sample collection 24 hr after the first sample time is recommended (OECD, 1998).
Chromosomal aberration	Mouse bone marrow	positive	Giri <i>et al.</i> (1984)	In footnote 9 of Table 2 of the JECFA monograph (WHO, 1998), it is mentioned that the repeated dosing protocol was inappropriate for this assay. Although a single application should be preferred, multiple applications may also be in agreement with current EU and OECD guidelines, but this should be scientifically justified. On the other hand, the study is <b>inconclusive</b> , since the type of aberrations scored is not reported in detail and the proportion of cells with chromosomal aberrations found in the control group (up to 4.67 % of total cells) is somewhat unusual. Furthermore, it is remarkable that repeated application of 2 mg/kg bw in the diet for 30 days induced nearly the same effect as a single i.p. application of 200 mg/kg bw.
Micronucleus	Mouse bone marrow	negative	Litton Bionetics (1982b)	The negative result is <b>inconclusive</b> , since it is based on an inadequate test protocol. TBHQ was administered twice within 24 hours. The bone marrow was sampled six hours after last treatment. This protocol does not meet the criteria of current EU and OECD guidelines and recommendations. If 2 or more daily treatments are used, samples should be collected once between 18 and 24 hours following the final treatment (OECD, 1998).
Micronucleus	Mouse bone marrow	positive	Société Kemin Europa (1982b)	Not available for re-evaluation. In footnote 10 of Table 2 of the JECFA monograph (WHO, 1998), it is mentioned that the test result was positive at 24-hour harvest only but not at 48- or 72-hour harvests. However, this does not argue for an inconclusive report. According to OECD guideline 474, samples of bone marrow should be taken at least twice (between 24 and 48 h) after a single application, because the incidence of micronucleated cells may depend on sampling time (OECD, 1998; Hayashi <i>et al.</i> 1994). Therefore, a positive result at 24-hour harvest with a negative result at 48- or 72-hour harvest may be conclusive. The strength of the effect should be evaluated.
Micronucleus	Mouse bone marrow	negative	NTP (1995)	Although there are some minor objections (only 5 male mice per group were used instead of 5 male and 5 female; the multiple treatment schedule was not justified; toxicity was determined only by survival of animals and the ratio PCE/NCE was not reported), the study was essentially in agreement with current guidelines and recommendations. Therefore, the negative result appears to be <b>conclusive</b> .

Table 3 (continued):

Test System	Test Object	Result as evaluated by JECFA	References	Comments
Dominant lethal	Rat	negative	Krasavage and Faber (1983)	<b>Equivocal.</b> Male rats were fed diets containing TBHQ for 83 days. The TBHQ treated males were mated with untreated females for two consecutive weeks immediately following treatment. Although this treatment schedule is not commonly used, it may be acceptable. Post implantation losses were statistically significant ( $p \leq 0.05$ ) increased in the 285 mg/kg group after the first mating (13.9 % of total implantations compared to 6.1 for the control group), and were also increased ( $p \leq 0.05$ ) in the 145 mg/kg group after the second mating (11.9 compared to 6.1 for the control group). No dose dependency could be observed for these effects. However, the assay is not clearly negative.
<i>In vivo</i> SCE	Mouse bone marrow	positive	Mukerjee <i>et al.</i> (1989)	It is not clear whether the SCEs have been observed at doses (mg TBHQ / kg body weight) or concentrations (mg TBHQ / kg corn oil). In the former case the LOEL is 2 mg TBHQ / kg bw, in the latter case 2 mg TBHQ / kg corn oil equivalent to 0.018 mg TBHQ / kg bw.
DNA strand breaks <i>in vivo / in vitro</i>	Rat forestomach	negative	Morimoto <i>et al.</i> (1991)	

