SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of butylated hydroxyanisole – BHA (E 320) 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 (ANS) delivers a scientific opinion re-evaluating the safety of butylated hydroxyanisole (BHA) (E 320). BHA is a synthetic antioxidant authorised as a food additive in the EU that was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) several times, the latest in 1989 and the EU Scientific Committee for Food (SCF) in 1989. Both committees established an ADI of 0.5 mg/kg bw/day, with that of the SCF being classified as temporary. Both ADIs were based on proliferative changes in the rat forestomach. 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 an EFSA public call for data. The Panel concluded that BHA does not raise concern with respect to genotoxicity. A large number of long-term toxicity and carcinogenicity studies with BHA have been performed, demonstrating proliferative changes in the forestomach with $BMDL_{10}$ values in the rat of 115 and 83 mg/kg bw/day. The Panel concluded that the present database does give reason to revise the ADI. The Panel considered that forestomach hyperplasia in rodents may no longer be relevant for human risk assessment. Based on a NOAEL of 100 mg/kg bw/day for growth retardation, increased mortality and behavioural effects in rat pups at higher dose levels, and using an uncertainty factor of 100 the Panel established an ADI of 1.0 mg/kg bw/day. This NOAEL also covers the $BMDL_{10}$ values for forestomach hyperplasia observed in the rat. The Panel also concluded that at the current levels of use refined intake estimates are generally below the ADI of 1.0 mg/kg bw/day.

KEY WORDS

BHA, butylated hydroxyanisole, 3-tertiary-butyl-4-hydroxyanisole, (1,1-dimethylethyl)-4-methoxyphenol, E 320, CAS 25013-16-5, food antioxidant

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SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) of the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the re-evaluation of butylated hydroxyanisole (BHA) (E 320) as a food additive.

BHA (E 320) is a synthetic antioxidant authorised as a food additive in the EU that was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) several times, the latest in 1989 and the EU Scientific Committee for Food (SCF) in 1989. Both committees established an ADI of 0.5 mg/kg bw/day with the ADI of the SCF being a temporary ADI. Both ADIs were based on proliferative changes in the rat forestomach.

BHA is a mixture of two isomers with full chemical names 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole. Specifications have been defined in the EU legislation in Directive 2008/128/EC and by JECFA. The purity is specified to be not less than 98.5% of $C_{11}H_{16}O_2$ and not less than 85% of the 3-tertiary-butyl-4-hydroxyanisole isomer.

In general, BHA is rapidly absorbed from the gastrointestinal (GI) tract, metabolised and excreted in the form of metabolites mainly in urine and/or faeces. The major metabolites of BHA are the glucuronides, sulphates and free phenols, including *tert*-butylhydroquinone (TBHQ). The proportions of the different metabolites vary in different species and also for different dose levels. The acute toxicity of BHA is low, with an LD_{50} in mouse and rat > 2000 mg/kg bw/day.

In general, the majority of the genotoxicity studies indicate a lack of potential for BHA to induce point mutations or to interact with or damage DNA. BHA and its metabolite TBHQ have been reported to induce chromosomal aberrations *in vitro* in the presence of metabolic activation. The Panel recognised that the clastogenic activity exerted *in vitro* by BHA and TBHQ is likely to be secondary to the formation of reactive oxygen species via pro-oxidant chemistry, and that such a mechanism of genotoxicity is considered thresholded.

A large number of long-term toxicity and carcinogenicity studies have been performed with BHA, demonstrating proliferative changes in the forestomach. The studies have included species with a forestomach (rats, mice, hamsters) and without (guinea-pigs, dogs). The Panel noted that gastric epithelial hyperplasias, papillomas and carcinomas were only seen in species with a forestomach.

The Panel concluded that the long term studies in rats reveal the lower confidence limit of the benchmark dose ($BMDL_{10}$) values for forestomach hyperplasia of respectively 115 mg/kg bw/day and 83 mg/kg bw/day. Forestomach hyperplasia was the critical effect on which SCF and JECFA based their acceptable daily intake (ADI).

The Panel noted that a new reproductive and developmental toxicity study had been published since the latest SCF and JECFA evaluations. The Panel noted that the study was not performed according to OECD guidelines and that several of the endpoints studied are not part of regular OECD guidelines for testing reproductive and developmental toxicity. The Panel also noted that the decreases reported in the parameters that were stated to be affected were generally less than 10% without a clear dose response, and that the ranges reported for these parameters overlap due to large standard deviations. Thus the Panel concluded that the effects reported in this study that were statistically significant (at $p<0.05$) were not biologically relevant.

The Panel also noted that other studies gave a no-observed-adverse-effect level (NOAEL) for reproductive and developmental toxicity of 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day) in rats, and a NOAEL for reproductive and teratogenic parameters of 400 mg/kg bw/day in rabbits. The NOAEL in rats defined in the study of Vorhees et al. in 1981 was based on pups showing growth retardation, increased mortality and behavioural effects at higher dose levels.

The Panel noted that forestomach hyperplasia in rats was the critical effect on which SCF and JECFA based their ADI. The Panel considered that humans do not have a forestomach and that forestomach hyperplasia in rodents may no longer be considered relevant for human risk assessment.

Overall, the Panel concluded that the present database does give reason to revise the ADI of 0.5 mg/kg bw/day.

The Panel noted the potential endocrine effect of BHA which has been investigated in a number of studies. The Panel also noted that studies on reproductive and developmental toxicity in rats gave a NOAEL of 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day). This NOAEL was based on pups showing growth retardation, increased mortality and behavioural effects at higher dose levels. Based on a NOAEL of 100 mg/kg bw/day and using an uncertainty factor of 100 the Panel established an ADI of 1.0 mg/kg bw/day. Since the NOAEL of 100 mg/kg bw/day is in the range of the $BMDL_{10}$ values for forestomach hyperplasia in rats of respectively 115 mg/kg bw/day and 83 mg/kg bw/day the Panel concluded that this NOAEL also covers the $BMDL_{10}$ values for forestomach hyperplasia observed in the rat. This NOAEL was also below the dose of 500 mg/kg bw/day causing oesophageal basal cell proliferation in monkeys.

Exposure estimates to BHA at Tier 2 for children and the adult population at both the average and high level exposures are unlikely to exceed the ADI of 1.0 mg/kg bw/day.

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

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The Regulation (EC) No 1333/2008 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 the 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 the Regulation (EU) No 257/2010[4]. 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[5] of 2001. The report "Food additives in Europe 2000[6]" 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 a 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] OJL 80, 26.03.2010, p19
[5] COM(2001) 542 final.
[6] 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 butylated hydroxyanisole (BHA) (E 320) when used as a food additive.

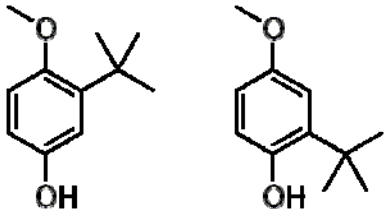
BHA (E 320) is an antioxidant authorised as a food additive in the EU and most recently evaluated in 1989 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the EU Scientific Committee for Food (SCF).

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[7].

2. Technical data

2.1. Identity of the substance

BHA (E 320) is a synthetic antioxidant with the molecular formula $C_{11}H_{16}O_2$. It has a molecular weight of 180.25 g/mol, a CAS Registry Number 25013-16-5 and EINECS number 246-563-8. BHA is a mixture of two isomers with full chemical names 2-*tert*-butyl-4-hydroxyanisole or 3-(1,1-dimethylethyl)-4-methoxyphenol (structure A in Figure 1) and 3-*tert*-butyl-4-hydroxyanisole or 2-(1,1-dimethylethyl)-4-methoxyphenol (structure B in Figure 1).



(A) (B)

Figure 1: Structural formula of the BHA isomers; 2-*tert*-butyl-4-hydroxyanisole (A) and 3-*tert*-butyl-4-hydroxyanisole (B).

The most commonly used synonyms for BHA are butylated hydroxyanisole, butyl hydroxyanisole, *tert*-butyl-4-hydroxyanisole and *tert*-butyl-4-methoxyphenol.

BHA is a white or slightly yellow crystalline or waxy solid with a faint characteristic odour. BHA is insoluble in water, freely soluble in ethanol, other alcohols, propylene glycol, petroleum ether, and fats and oils. It has a boiling point of 268 °C, a melting point of 48-63 °C, and an octanol/water partition coefficient (log P_{ow}) of 3.5. The density is 1.0587 g/cm³ at 20 °C (ChemIDplusAdvanced, 2010; Commission Directive 2008/84/EC[8]; TemaNord, 2002).

[7] "Call for scientific data on food additives permitted in the EU and belonging to the functional classes of preservatives and antioxidants Submission of data on preservatives and antioxidants" (published: 23 November 2009). Available from: http://www.efsa.europa.eu/en/dataclosed/call/ans091123a.htm

[8] Commission Directive 2008/84/EC of 27 August 2008 laying down specific purity criteria on food additives other than colours and sweeteners. OJ L 253, 20.9.2008.

2.2. Specifications

Specifications for BHA have been defined in the Commission Directive 2008/84/EC on purity criteria and by JECFA (2006) (Table 1).

Table 1: Specifications for BHA according to Commission Directive 2008/84/EC and to JECFA (2006).

	Commission Directive 2008/84/EC	JECFA (2006)
Assay	Not less than 98.5% of $C_{11}H_{16}O_2$ and not less than 85% of 3-tert-butyl-4-hydroxyanisole	Not less than 98.5% of $C_{11}H_{16}O_2$ and not less than 85% of 3-tert-butyl-4-hydroxyanisole
Identification		
Solubility	Insoluble in water; freely soluble in ethanol	Insoluble in water; freely soluble in ethanol and propane-1,2-diol
Melting range	48-63 °C	-
Spectrophotometry	Specific absorption $E^{1\%}_{1cm}$ (290 nm): not less than 190 and not more than 210 Specific absorption $E^{1\%}_{1cm}$ (228 nm): not less than 326 and not more than 345	-
Gas chromatographic method	-	Two different methods described both using flame-ionization detector
Purity		
Sulphated ash	$\leq$ 0.05%	$\leq$ 0.05%
Phenolic impurities	$\leq$ 0.5%	$\leq$ 0.5%
Lead	$\leq$ 5 mg/kg	$\leq$ 2 mg/kg
Arsenic	$\leq$ 3 mg/kg	-
Mercury	$\leq$ 1 mg/kg	-

The Panel noted that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$ 5 mg/kg, and that in the EC specifications arsenic and mercury are allowed at levels up to 3 mg/kg and 1 mg/kg, respectively .

2.3. Manufacturing process

BHA can be prepared from 4-methoxyphenol and tert-butyl alcohol at 150 °C using a silica or aluminium oxide catalyst.

Methylation of hydroquinone yields an intermediate that gives a mixture of 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole upon treatment with *tert*-butyl alcohol and phosphoric acid. Butylation of hydroquinone and subsequent methylation with dimethyl sulphate and sodium hydroxide can also be used to produce a mixture of the two BHA isomers (HSDB, 2010).

2.4. Methods of analysis in food

A number of methods for analysis of BHA in food are described in published literature of which methods based on High Performance Liquid Chromatography (HPLC) appear to be most generally employed (Hashizume et al., 1988; Riber et al., 2000; Zivanovic et al., 2005). A spectrofluorometric method for determination of BHA in foodstuffs is also described (Cruces-Blanco et al., 1999).

2.5. Reaction and fate in food

BHA has been shown to degrade over time when heated during deep fat frying (Warner et al., 1986). The major decomposition product was *tert*-butylhydroquinone (TBHQ) which could be oxidized to *tert*-butylbenzoquinone (TBBQ). Photodegradation of BHA, when irradiated by visible light, was shown to occur by a mechanism involving production of reactive oxygen species (Criado et al., 2007).

2.6. Case of need and proposed uses

Maximum permitted levels

Maximun permitted levels (MPLs) of BHA have been defined in Council Directive No 95/2/EC[9] on food additives and are described in Table 2.

BHA is a synthetic antioxidant authorised in the EU (Directive No 95/2/EC) alone or in combination with other antioxidants such as the gallates, TBHQ and butylated hydroxytoluene (BHT).

Table 2: Maximum permitted levels (MPLs) of BHA in foodstuffs according to Council Directive No 95/2/EC.

Foodstuffs	Maximum permitted level (mg/kg)
Fats and oils for the professional manufacture of heat-treated foodstuffs Frying oil and frying fat, excluding olive pomace oil Lard; fish oil; beef, poultry and sheep fat	200 mg/kg [(a)]
Cake mixes Cereal-based snack foods Milk powder for vending machines Dehydrated soups and broths Sauces Dehydrated meat Processed nuts Pre-cooked cereals	200 mg/kg (gallates, TBHQ and BHA, individually or in combination) Expressed on fat
Seasonings and condiments	200 mg/kg (gallates, TBHQ and BHA, individually or in combination)
Dehydrated potatoes	25 mg/kg (gallates, TBHQ and BHA, individually or in combination)
Chewing gum Food supplements as defined in Directive, 2002/46/EC	400 mg/kg (gallates, TBHQ and BHA, individually or in combination)
Essential oils	1000 mg/kg (gallates, TBHQ and BHA, individually or in combination)
Flavourings other than essential oils	200 mg/kg (TBHQ and BHA, individually or in combination)

(a): When combinations of the antioxidants gallates, TBHQ, BHA and BHT are used, the individual levels must be reduced proportionally.

BHA is permitted alone, or in combination with the gallates and TBHQ, in dehydrated potatoes at a maximum level of 25 mg/kg; in chewing gum at a maximum level of 400 mg/kg; in essential oils at a

[9] European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. OJ L 61, 18.3.1995, p. 1.

maximum level of 1000 mg/kg; and in flavourings other than essentials oils, at a maximum level of 200 mg/kg (Table 2). BHA is not authorised for use in beverages.

Reported actual (maximum) use levels

Only few data have been found or obtained from industry upon a public call for data, on the actual (maximum) use levels of BHA in foods. The few data provided indicated that BHA is either not used or found at levels below the limit of detection (10 mg/kg food). In view of the small amount of data provided these data were considered as too limited to be used for exposure estimation (see below).

The National Food Administration in Denmark has reported a project on monitoring and control of food additives in which the BHA levels were analysed in 122 samples of emulsified and non-emulsified sauces (dressings, ketchup etc.) and fruit and vegetable preparations (chutney, tomato paste etc.). None of these samples contained detectable amounts of BHA (Jensen, 2006).

In a survey of the French population, investigating dietary exposure to 13 selected food colours, preservatives, antioxidants, stabilizers, emulsifiers and sweeteners, 6 out of 22 analysed soups and broths (including dehydrated soups) were found to contain BHA at an average concentration of 1.8 mg/kg foodstuff (Bemrah et al., 2008).

BHA may also be used as antioxidant for cosmetics (Commission Decision 2006/257/EC[10]) and in pharmaceuticals (e.g. suppositories, ointments, coated tablets). Use levels for this range of products were not available to the Panel.

2.7. Information on existing authorisations and evaluations

BHA has previously been evaluated by the SCF in 1978, 1983 and 1989. Following their latest evaluation (1989), SCF allocated a temporary ADI of 0-0.5 mg/kg bw/day. The SCF report stated the following:

"*The Committee concluded that the production of rodent forestomach tumours by BHA was not a manifestation of genotoxicity, and that in addition to being an effect with a threshold, the preceding hyperplasia may not be of relevance for man. Further reassurance was to be found in the results of studies in species without a forestomach. In setting an ADI the Committee took into account that:*

(a) the NEL in a 90-day study for production of hyperplasia in the rat forestomach (62.5 mg/kg bw). Given the evidence of lack of effect in more relevant species, the Committee considered that a safety factor of 100 was adequate;

(b) the NEL of 250 mg/kg bw previously determined in a long-term chronic toxicity feeding study – on the basis of which JECFA had established a temporary ADI of 0-0.5 mg/kg bw (using a safety factor of 500).

The Committee established a temporary ADI of 0-0.5 mg/kg bw."

BHA has previously been evaluated by JECFA in 1974, 1976, 1982, 1986, 1987 and 1989. Following the most recent evaluation (JECFA, 1989), an ADI of 0-0.5 mg/kg bw/day was allocated. JECFA summarised that:

"*Studies have been conducted that provide additional information on the proliferative changes observed in the forestomach fed BHA. No new studies on the effect of BHA on the stomach and esophagus of species that do not have a forestomach, e.g., pigs or monkeys, were conducted. The data*

[10] Commission Decision 2006/257/EC amending Decision 96/335/EC establishing the inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97, 5.4.2006, p.1

show that continuous exposure of the rat forestomach to 2% BHA in the diet for 6-12 months is necessary to produce squamous cell carcinoma. The data also show that the induction of mild hyperplasia can occur at levels of 0.125% of BHA in the diet but not at a level of 0.1% BHA".

It was also noted that further studies in animals without a forestomach were not required (JECFA, 1989) and concluded that: "*The human relevance of the rat studies, while inherently questionable because the target tissue in the rat has no human counterpart, cannot readily be ignored. Based on the dose dependence and reversibility of the lesions produced in the rat, discussed previously in the 1986 monograph and since confirmed by more recent studies, it was concluded that an ADI can be established"* and that *"a NEL of 0.1% BHA in the diet, equivalent to 50 mg/kg bw was established"*. This was based on data showing that 0.125% BHA in the diet induced mild hyperplasia in the rat forestomach which was not observed at 0.1% BHA in the diet. The ADI seems to be based on a No-Observed-Adverse-Effect Level (NOAEL) of 0.1% in the diet, equivalent to 50.0 mg/kg bw/day from a chronic toxicity study using an uncertainty factor of 100.

In a later assessment (TemaNord, 2002) two 90-day studies were evaluated (Clayson et al., 1986; Altmann et al., 1986). These studies were important for the establishment of the NOAEL which was based on induction of hyperplasia in the rat forestomach. These studies are probably also the ones used in the JECFA (1989) and SCF (1989) evaluation. On the basis of these studies TemaNord stated:

"*That no such effects were seen in other species without a forestomach such as the guinea pig and the dog. In the pig and monkey there were indications that proliferative responses may take place in the oesophagus at dose levels considerably higher than in the rat*."

The International Agency for Research on Cancer (IARC) classified BHA as possibly carcinogenic to humans (category 2B), based on inadequate evidence in humans and sufficient evidence in experimental animals (IARC, 1986, 1987). In the evaluation from 2003, the mechanism by which BHA induces tumours in experimental animals was discussed and it was considered that the mechanism was not relevant to humans (IARC, 2003).

In a recent report from the US National Toxicology Program Report on Carcinogens (NTP, 2005), BHA is anticipated to be a carcinogen.

The Panel also noted that BHA is mentioned in a "Study on enhancing the endocrine disruptor priority list with a focus on low production volume chemicals" reported to the European Commission DG Environment as a substance to be considered with high concern in view of the results of studies on reproduction toxicity and endocrine disrupting properties and regarding its use in food and feed (DHI, 2007).

Furthermore in a Commission working document on the implementation of the "Community Strategy for Endocrine Disrupters" - a range of substances suspected of interfering with the hormone systems of humans and wildlife were evaluated, including BHA (SEC, 2007). This prioritisation work started in the year 2000. This work was completed at the end of 2006. In total 575 substances were investigated as to their endocrine disrupting (ED) effects. In terms of prioritisation, it was found that, out of this number, 320 substances showed evidence or potential evidence for ED effects, including BHA.

TBHQ, which is a metabolite of BHA, is an authorised antioxidant in the EU, alone or in combination with other antioxidants, such as gallates, BHA and BHT (Directive No 95/2/EC). For TBHQ an ADI of 0.7 mg/kg bw/day has been allocated by JECFA (1999) and later by EFSA (2004).

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 goes from the

conservative estimates that form the first Tier of screening, to progressively more realistic estimates that form the Second and Third Tiers (Annex A). In the tiered approach, Tier 1 is based on theoretical food consumption data and 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 actual usage patterns (Tier 3).

2.8.1. Crude estimates (Budget Method)

The dietary exposure to BHA from the MPLs 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).

The use of BHA in beverages is not authorised (Directive 95/2/EC). The maximum permitted level in solid foods is 1000 mg/kg for essential oils. The second highest MPL for solid foods is 400 mg/kg for chewing gum. As this is a food category which is usually not swallowed, the Panel decided to use for Tier 1 calculations a maximum permitted level in solid foods of 200 mg/kg.

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

The overall theoretical maximum daily exposure to BHA for adults would therefore be:

(200 x 0.025 x 0.25) = 1.25 mg/kg bw/day.

For children, the level of BHA considered in solid food was 200 mg/kg. This assumes that a typical 3 year-old child weighing 15 kg consumes 94 g of solid foods containing BHA.

The overall theoretical maximum daily exposure to BHA for children would therefore be:

(200 x 0.025 x 0.25) = 1.25 mg/kg bw/day.

2.8.2. Refined estimates

Refined exposure estimates have been performed for Tier 2 using national consumption data and MPLs, presented in Table 2. Data on actual use levels were made available to the Panel indicating that either BHA is not used or that the levels are below an LOD of 10 mg/kg, However, the available information on actual use levels were limited to very few foods or from very low sample numbers. Therefore, Tier 3 exposure estimates could not be calculated.

The Panel noted that its Tier 2 estimates could be considered as being conservative as it is assumed that all foods contain BHA added at the maximum permitted use levels.

For adults, the Panel calculated the exposure based on the UK consumption survey, because detailed individual food consumption data (UK NDNS, 2000-2001) were available from the UNESDA report (Tennant D, 2006).

Exposure estimates for children (1-14 years old) have been performed by the Panel based on detailed individual food consumption data from 11 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), for Tier 2. As the UK is not part of the EXPOCHI consortium, estimates for UK children (1.5 - 4.5 years old) 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).

Tier 2 exposure estimates are based on the following assumptions:

- fats and oils for the professional manufacture of heat-treated foodstuffs and frying oils and frying fat are mainly part of the food categories fine bakery wares, and snacks and snack products, which are assumed to have a fat content of 30%,

- cake mixes, cereal-based snack foods, and processed nuts are mainly part of the food category fine bakery wares and snacks, which are assumed to have a fat content of 30%,

- dehydrates soups and broths are part of the food category soups, which are assumed to have a fat content of 10%,

- sauces, seasonings and condiments are part of the food category sauces, seasonings, pickles, relishes, chutney, piccalilli, which are assumed to have a fat content of 30%,

- pre-cooked cereals are part of the food category extruded, puffed and/or fruit-flavoured breakfast cereals, which are assumed to have a fat content of 20%,

- dehydrated potatoes are part of the food category processed potato products, and

- all these foods contain BHA at the maximum permitted level as listed in table 2.

All other food categories (milk powder for vending machines, dehydrated meat, essential oils, flavourings other than essential oils) were considered as negligible in respect of the amounts consumed.

Table 3 summarises the anticipated exposure of children and adults to BHA.

In the case of BHA, when considering MPLs, estimates reported for the UK adult population give a mean dietary exposure of 0.1 mg/kg bw/day and 0.14 mg/kg bw/day for high level consumers. The main contributors to the total anticipated mean exposure to BHA (>10%) were soups (14%), sauces and seasonings (13%), fine bakery wares (33%), and breakfast cereals (20%).

The mean dietary exposure of European children (aged 1-14 years and weighing an average of 16-54 kg) ranged from 0.1 to 0.3 mg/kg bw/day, and from 0.2 to 0.7 mg/kg bw/day at the 95th percentile. At Tier 2, the main contributors to the total anticipated mean exposure to BHA (>10% in all countries, these contributions differed per country), were fine bakery wares (18-69%), snacks (10-19%), processed potato products (14-71%), and breakfast cereals (15-23%).

Table 3: Summary of anticipated exposure to BHA 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 1. Budget method	1.25	1.25
Tier 2. Maximum Permitted Level		
• Mean exposure	0.1	0.1 – 0.3
• Exposure 95th* or 97.5th percentile**	0.14	0.2 – 0.7

* 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, 2006).

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

The food consumption databases used for the exposure estimates in Table 2 do not provide data on chewing gum consumption. In a study on teenagers consumption of chewing gum was found to be in the range of 1.3 g/day on average and 2.5 g/day at the higher quartile (Leclercq et al, 2000 a, b). Data from Ireland found a chewing gum consumption of 0.07 g/day for the average adult population and of 9.5 g/day at the 95th percentile. However, it has to be noted that not all BHA from chewing gum is extracted, but ranges from no extraction at all to 1% extraction (Catherine et al., 1983). Assuming a 10% extraction of BHA from chewing gum and a consumption of chewing gum of 9.5 g/day, total exposure to BHA would increase by 0.006 mg/kg bw and day for adults and by 0.025 mg/kg bw and day for children, which would not represent a substantial increase.

3. Biological and toxicological data

BHA has been evaluated previously by JECFA and the SCF the latest in 1989. It was also evaluated by TemaNord (2002). The present opinion reports briefly the major studies evaluated in these opinions and describes any additional data in more detail.

3.1. Absorption, distribution, metabolism and excretion

Absorption, distribution, metabolism and excretion (ADME) of BHA have been studied in rats, rabbits, dogs, monkeys and humans as evaluated by JECFA (JECFA, 1982, 1986, 1987, 1989) and SCF (1989). Overall, these studies show that BHA is rapidly absorbed from the gastrointestinal (GI) tract, metabolised and excreted. Excretion is mainly via urine and faeces.

The major identified metabolites of BHA were glucuronides, sulphates and free phenols, with TBHQ, produced by *O*-demethylation, being the most relevant (JECFA 1976, 1982, 1987). TBHQ is an authorised antioxidant in the EU, alone or in combination with other antioxidants, such as gallates, BHA and BHT (Directive No 95/2/EC). For TBHQ an ADI of 0.7 mg/kg bw/day has been allocated by JECFA (1999) and later by EFSA (2004).

The most relevant ADME studies of BHA from JECFA (JECFA, 1982, 1986, 1987, 1989) and SCF (1989) evaluations and additional studies reported since then are as follows.

3.1.1. Rats

The tissue-distribution and kinetics of orally administered labelled [methyl-^{14}C]3-BHA were investigated in 39 adult male Sprague-Dawley rats. [Methyl-^{14}C]3-BHA was administered by gavage as a single dose (1.5 mmol/kg bw, equivalent to 270 mg/kg bw). Urine, faeces, blood and samples

from all major organs were collected at 0.5, 1, 3, 6, 12, 16, 17, 18, 24, 48, 72, 168 and 240 hours after dosing. The total radiolabel in most tissues increased exponentially with time, reaching a maximum between 10-24 hours after dosing, followed by an exponential decrease. By 48 hours, the radioactivity was almost totally eliminated from the rats with 41% of the methoxy-labelled radioactivity found in the urine and 53% in faeces (Ansari and Hendrix, 1985).

The elimination of BHA (isomers of 2-*tert*-butyl-4-hydroxyanisole (2-BHA) and 3-*tert*-butyl-4-hydroxyanisole (3-BHA) labelled at either the methoxy or *tert*-butyl moiety) was studied in 2 groups of 3 male F344 rats. BHA given by gavage as a single dose of 1 g/kg bw was found to be excreted via urine, faeces or respiration at a level of 87-96% within the first 48 hours after administration. Animals were dosed with one of the four compounds: 2- and 3-BHA labelled at either the methoxy or *tert*-butyl moiety. The two isomers of BHA, 3-BHA and 2-BHA, with the tert-butyl label, were found in the urine at levels of 63.7% and 69.0% of the dose of the corresponding isomers, respectively. In faeces, 28.8% and 18.1% of the dose of the corresponding isomers were found for 3-BHA and 2-BHA, with the tert-butyl label, respectively. 3-BHA and 2-BHA, labelled in the methoxy group, were found in the urine at levels of 49.8% and 46.5% of the dose of the corresponding isomer, respectively, while 28.3% and 29.6% of the dose of the corresponding isomer were found in faeces for 3-BHA and 2-BHA, labelled in the methoxy group. In exspired air 8.3% and 13.7% of the dose of 2-BHA and 3-BHA, labelled in the methoxy group, were found (Hirose et al., 1987b).

The elimination pattern of BHA was investigated in four groups of three male F344 rats (6 weeks old, weighing 120-130 g), each given by gavage a single dose (1 g/kg bw) of one of the following labelled preparations: [*tert*-butyl-^{14}C]3-BHA, [butyl-^{14}C]2-BHA, [methyl-^{14}C]3-BHA or [methyl-^{14}C]-2-BHA). The preparations were obtained by diluting the radio-labelled molecules with unlabelled 3-BHA and 2-BHA. By using thin-layer chromatography (TLC), the urinary and faecal metabolites were identified in samples of 2-day pooled urine and faeces, and further characterized and identified by enzymatic hydrolysis. Confirmation of the standards was performed by proton magnetic resonance spectroscopy and electron impact mass spectrometry. The radioactivity was almost completely eliminated and the total ^{14}C recoveries were about 92% in 3 male rats. The total ^{14}C recoveries from [methyl-^{14}C]3-BHA and [methyl-^{14}C]-2-BHA were lower than those from [*tert*-butyl-^{14}C]3-BHA and [butyl-^{14}C]2-BHA, being 81.3 and 73%, respectively. Most of the metabolites of 3-BHA and 2-BHA were found as conjugates (53% of 3-BHA and 66% of 2-BHA). The major metabolite identified in the urine of rats given 3-BHA was 3-BHA-glucuronide. A smaller amount of tert-butylhydroquinone-sulphate was detected in the faeces together with unchanged 3-BHA and 3-BHA-glucuronide. The major metabolites identified in the urine of rats given 2-BHA were the sulphate conjugates of 2-BHA, 4-tert-butyl-5-methoxy-1, 2-benzoquinone and the glucuronide of 2-BHA. Unchanged 2-BHA was found in the faeces (Hirose et al., 1988).

The metabolism of 2- and 3-BHA was investigated in rats, focusing on the forestomach and binding to tissue macromolecules (Hirose et al., 1987a). Male Fischer 344 rats (2 groups of 3 rats) were given by gavage a single dose of 1 g/kg bw [*tert*-butyl-^{14}C]3-BHA or [methyl-^{14}C]3-BHA. BHA metabolites were analysed by comparing TLC of forestomach, glandular stomach and stomach content samples, taken 6 hours after administration. No significant amount of metabolites was found in the forestomach or glandular stomach epithelium. In the binding experiment, rats were given by gavage a single dose of 1 g/kg bw of [*tert*-butyl-^{14}C]3-BHA, [butyl-^{14}C]2-BHA or [methyl-^{14}C]3-BHA with or without pre-treatment with 1% unlabelled 3-BHA in the diet for 6 days. Based on the data, it was suggested by the authors that BHA acts on forestomach epithelium without metabolic activation, and furthermore that the action was not related to its binding to DNA or RNA.

Two additional rat studies were identified in the literature review:

Morimoto et al. (1992) investigated covalent binding of BHA to forestomach protein in male F344 rats. [^{14}C] 3-BHA was administered orally (0.01, 0.1, 1 and 2%) and the radioactivity after 6 h was found to be higher in the forestomach compared to the glandular stomach, liver, kidney and plasma. The dose of 0.01% 3-BHA corresponded to about 2.25 mg/kg bw. Covalent binding to forestomach

proteins was low, at 0.1% 3-BHA, but was higher at concentrations of 1 and 2% 3-BHA. Binding to forestomach protein was up to 54-fold higher after oral 3-BHA compared to the intravenous route.

The distribution of free and conjugated BHA (glucuronide and sulfate) in liver, serum and foetuses was investigated in pregnant Wistar SPF rats (4 groups of 7 rats), using HPLC analysis with electrochemical detection (Yamada et al., 1993). BHA was administered orally at 0, 200, 400 and 800 mg/kg bw/day and was found in all foetuses from treated rats, but at lower levels compared to levels in liver and serum. The ratio of free and conjugated BHA was approximately constant in liver, serum and foetuses in all dose groups.

3.1.2. Mice

The elimination and distribution of BHA was investigated in mice by Hashizume et al. (1992) using an unspecified number of animals. BHA and its conjugated metabolites (glucuronide and sulphate) were determined in organs, blood and excreta by HPLC analysis. Male mice (Slc:ddy) were given single oral doses of 50 and 500 mg BHA/kg bw. Samples were collected up to 48 hours after dosing. Half an hour after treatment BHA was present at high concentrations in the liver, kidney and blood of the 500 mg/kg bw group, but was not detected in any samples 8 hours later. The sulphate metabolite exceeded the glucuronide metabolite in the liver after 0.5 hour and this was reversed after 1-3 hours. At the same dose level 52% of BHA was found in the urine 8 hours after dosing and about 76% within 48 hours (72.3±7.6% as glucuronide; 3.0±2.4% as sulphate and 0.3±0.1% as unchanged BHA), while 2.3% of BHA was recovered from faeces. At the 50 and 500 mg/kg bw dose levels, about 15 and 25% of BHA, respectively, could be recovered from the stomach after 8 hours (Hashizume et al., 1992).

An additional study in mice was published since the latest SCF and JECFA evaluation:

Ahmed et al. (1991), investigated the placental transport and localization in fetal and maternal tissues of [methyl-^{14}C]2-BHA and [methyl-^{14}C]3-BHA, and showed differences between 3-BHA and 2-BHA. Pregnant mice were given BHA isomers i.v. at a dose level of 50 μCi/100 g at day 11 and day 18 of gestation and distribution was investigated by whole-body autoradiography. Peak levels of radioactivity occurred in various tissues at 1-4 hours after administration of both isomers. The tissue distribution and affinity was shown to be different for the two isomers including the magnitude and extent of placental transport. 3-BHA levels in maternal liver and brown fat tissue were much higher than the radioactivity concentration in the corresponding tissues of mothers treated with 2-BHA. Fetal concentration was higher in animals treated with 2-BHA than in those treated with 3-BHA. Thus, a different placental transport pattern was shown for radiolabelled 2-BHA and 3-BHA, with 2-BHA being more accumulated in fetal tissue and 3-BHA being more accumulated in maternal tissue. Both isomers were shown to cross the placental membrane.

3.1.3. Dogs

The absorption and excretion of [methyl-^{14}C]3-BHA was investigated in 9 male beagle dogs (5 months of age). Groups of 3 dogs were fed a diet containing 0, 0.03 or 3% BHA for 7 days followed by a single injection of radiolabelled BHA. Radioactivity was determined in whole body, blood, urine, faeces and several tissues including different parts of the stomach. Within 48 hours 50-80% of the labelled BHA was detected in the urine and 15-30% in faeces. Seven days after administration the levels of radiolabelled BHA were measured in organs and showed a small amount in the stomach (0.16-0.19% of dose/g), liver (0.3-1.7%) and other tissues (0.02%). It was suggested by the authors, that the dose-independence of distribution and excretion of radiolabelled BHA could be attributed to the lack of a forestomach in the beagle dog (Takizawa et al., 1985).

3.1.4. Humans

The elimination and distribution of BHA was investigated in eight healthy male non-smoking volunteers who ingested 0.5 mg BHA/kg bw/day for 10 consecutive days (Verhagen et al., 1989b). Standard biochemical plasma parameters, kinetic parameters and urinary excretion of metabolites

(total, conjugated and unconjugated BHA) were determined. Blood samples were taken on days 6 and 0 before and on days 4 and 7 after the first BHA administration for the assessment of standard clinical plasma parameters including L-aspartate aminotransferase, L-alanine- aminotransferase, L-gamma-glutamyltranspeptidase, creatine phosphokinase, lactate dehydrogenase, total protein, albumin, urea, creatinine, Na^+, and Cl^-. Antipyrine (500 mg p.o.) and paracetamol (500 mg p.o.) were administered before and during BHA administration as test substances to measure phase-I and phase-11 biotransformation capacity. Saliva samples and urine were subsequently collected for the assessment of kinetic parameters (e.g. saliva elimination half-life, saliva clearance, apparent volume of distribution) and urinary excretion of metabolites. Kinetic plasma parameters of BHA itself were determined in plasma samples obtained via a catheter in an arm vein after oral BHA intake on days 0 and 7. Levels of antipyrine, paracetamol, BHA and metabolites in plasma, saliva or urine were quantified by standard or newly developed reversed-phase high-performance liquid chromatography methods. Urinary excretion of Na^+, K^+, and Cl^-, as well as osmolality of urine were measured on three days before and six days during BHA administration. No significant differences in kinetic parameters or urinary metabolite profiles were detected. Significantly higher amounts of unconjugated BHA and TBHQ were detected in the urine on days 3 and 7 compared to the first day of BHA administration. The study authors concluded that this may result either from an induction or inhibition of BHA-specific phase-I and -II metabolizing enzymes in man, or from a bioaccumulation of BHA and/or its metabolites in the body.

In conclusion, based upon the available studies from earlier evaluations and new studies identified through the literature search, the Panel concluded that BHA is rapidly absorbed from the GI tract, metabolised and excreted in the form of metabolites mainly in urine and/or faeces. The major metabolites of BHA are the glucuronides, sulphates and free phenols including TBHQ. The proportions of the different metabolites vary in different species and also for different dosage levels.

3.2. Toxicological data

3.2.1. Acute oral toxicity

In the twentieth report from JECFA (1976), two acute oral toxicity studies were reviewed. These studies included a rat study from which an LD_{50} of 2200-5000 mg/kg bw was reported (Bunnell et al., 1955; Lehman et al, 1951) and a study in mice giving an LD_{50} of > 2000 mg/kg bw (Bunnell et al., 1955; Lehman et al, 1951). No information on acute oral toxicity was given in the latest evaluation from SCF (1989).

In the study reported by Corte and Sgaragli (1984) the acute oral LD_{50} of BHA was 1500-1700 mg/kg bw in mice and 2900–3000 mg/kg bw in rats.

The Panel concluded that the acute oral toxicity of BHA is low.

3.2.2. Short-term and subchronic toxicity

A large number of short-term and subchronic toxicity studies have been performed in rats, mice, hamsters, pigs, dogs and monkeys investigating the sub-acute and subchronic toxicity of BHA. These studies are referenced in the JECFA evaluations (JECFA, 1982, 1986, 1987, 1989) and in the SCF evaluation (SCF, 1989).

The most important studies evaluated in the latest JECFA (1989) and SCF (1989) evaluations and new studies not included in these previous evaluation are summarized below.

3.2.2.1. Rats

Studies evaluated already by SCF and JECFA

In the study by Clayson et al. (1986), 5 groups of 5 male F344 rats were fed 0, 0.1, 0.25, 0.5 or 2% BHA *ad libitum* in the diet (estimated to be equivalent to a dose of approximately 0, 50, 125, 250 or 1000 mg/kg bw/day) for between 9 and 13 weeks. BHA at 2% in the diet (approximately 1000 mg/kg bw/day) induced forestomach epithelial cell regeneration and necrosis that were more prevalent in the prefundic part of the forestomach compared to the mid region. Histological data were only given for the animals administered 2% BHA in the feed for 27 days or 13 weeks. The incorporation of i.v. injected radiolabelled thymidine showed that the effect of BHA on the forestomach was at a threshold at 0.25% (approximately 125 mg/kg bw/day) after both 9 days and 13 weeks of feeding. The effects reversed towards normal after return to basal diet.

In a subchronic 90-day feeding study, 4 groups of 10 Wistar rats of each sex received 0, 0.125, 0.5 or 2% BHA in crystalline form in the diet (estimated to be equivalent to a dose of approximately 0, 62.5, 250 or 1000 mg/kg bw/day). Marked hyperkeratosis and hyperplasia with epithelial dysplasia in some basal areas of the forestomach were seen in the group fed the highest concentration of BHA. This was less pronounced at the 0.5% level, and in the 0.125% group only mild lesions were seen. In a second experiment, 0, 0.025, 0.125 or 2% BHA (estimated to be equivalent to a dose of approximately 0, 12.5, 62.5 or 1000 mg/kg bw/day), dissolved in arachis oil, was administered in the diet to groups of 20 rats of each sex. Pronounced hyperplasia was seen in the forestomach in the high dose group but none of the lower dose levels showed any effect (Altmann et al., 1986). Hirose et al. (1987), gave 2% powdered BHA (equivalent to a dose of approximately 1000 mg/kg bw/day) in the diet to a group of 5 male F344 rats for 4 weeks. A number of effects were observed when compared to the control group, including a significant reduction in body weight gain and a significant increase in relative liver weight. Severe hyperplasia was observed in the prefundic region of the stomach, near the oesophageal orifice.

New studies (Two new rat studies have been identified that were not included in the previous SCF or JECFA evaluation.)

Verhagen et al., (1990), fed a diet containing 0, 0.25, 0.50, 0.75, 1.0 or 2.0% BHA (estimated to be equivalent to approximately 0, 125, 250, 375, 500 or 1000 mg/kg bw/day) *ad libitum* to groups of ten 5-week-old male Wistar rats for 2 weeks; another group of rats served as a pair-fed control (PFC) group for the 2% BHA-fed animals. Subsequently, rats were injected i.p. with the thymidine-analogue 5-bromo-2'-deoxyuridine (BrdU) which was incorporated into the DNA of cells during DNA synthesis. In the forestomach, glandular stomach, small intestine and colon/rectum the mean tissue labelling index (LI) was significantly increased in rats fed 2% BHA compared to both *ad libitum* and PFC. The LI was significantly higher in the oesophagus of rats fed 2% BHA in comparison with their PFC group, but not with the *ad libitum* controls. The authors indicated that in rat forestomach, an apparent NOAEL for *ad libitum* fed rats was 0.5% BHA (LI) and at 0.75% BHA (potential doubling time). The authors also concluded that the oesophagus, glandular stomach, small intestine and large bowel, in addition to the forestomach, are possible target tissues in rats for the proliferation-enhancing effects of BHA. At the time of termination of the experiment, plasma BHA concentrations were dose-dependently increased. The induction of cell proliferation in the forestomach was investigated in four groups of five male F344 rats given diets containing 0, 0.5, 1 or 2% BHA (calculated by the authors to be equivalent to a doses of 0, 350, 710, 1400 mg/kg bw/day) for 4 weeks. Twenty four hours before termination of the experiment, the rats were implanted with osmotic minipumps delivering BrdU. Cell proliferation in the forestomach was assessed by immunohistochemistry for BrdU incorporated into DNA. The cell number/mm section length and fraction of replicating cells (LI) were determined in 3 domains of the forestomach (saccus caecus, mid region, prefundic region). A dose-dependent increase in number and size of hyperplastic lesions, together with a significant increase of LI was observed in the forestomach (Cantoreggi et al., 1993). BHA increased the rate of cell division in all treated groups. Overall, studies performed in rats showed induction of mild hyperplastic lesions starting at 0.125% BHA in the diet rising in severity up to 2% BHA in the diet, as evaluated from the two 90-day studies in rats (Clayson et al., 1986; Altmann et al., 1986). The two new studies identified were of shorter duration and were in line with the results from the 90 day studies.

3.2.2.2. Hamsters

The potential of 2-BHA, 3-BHA and crude BHA to induce forestomach hyperplasia and neoplastic lesions was investigated in male Syrian golden hamsters. Diets containing 1% 2-BHA, 1% 3-BHA or 1% crude BHA (estimated to be equivalent to 1200 mg/kg bw/day) were given to groups of 26-32 hamsters (7 weeks old). Three hamsters from each group were sacrificed on days 1 and 3 and in weeks 1, 2, 3, 4 and 16 for histological and auto-radiographic examinations. The remaining animals were sacrificed at week 16. From week 4 onwards 2-BHA induced severe hyperplasia which reached a maximum level at week 16, when papillomatous lesions were seen. In contrast from week 1 onwards 3-BHA and crude BHA induced hyperplasia which reached a maximum level at week 4 and then decreased. Papillomatous lesions reached a maximum level in week 16. The authors concluded that 2-BHA, 3-BHA and crude BHA induced hyperplasia and papillomatous lesions; some of them were reversible for 3-BHA and crude BHA. The authors concluded that the tumourigenic action of crude BHA on hamster forestomach is largely due to 3-BHA (Hirose et al., 1986b).

Overall, at a 1% dose level (equivalent to 1200 mg/kg bw/day), 2-BHA, 3-BHA and crude BHA were shown to induce hyperplasia and papillomatous lesions in the forestomach of hamsters.

3.2.2.3. Pigs

Groups of 9-13 pigs were fed pellets containing 0, 0.5, 1.9 or 3.7% BHA (indicated by the authors to be equivalent to a dose of 0, 50, 200, or 400 mg BHA/kg bw/day, respectively) for the first 110 days of pregnancy (Würtzen and Olsen, 1986). A significantly lower weight gain was observed in the group of dams given the highest dose. Dose-related increases in the absolute and relative organ weights of the liver and thyroid were found. Proliferative and parakeratotic proliferative changes of the stratified epithelium of the stomach were found in both control and treated pigs. In addition, proliferative and parakeratotic changes of the oesophageal epithelium were observed in a few pigs in the two highest dose groups. No papillomas or other histopathological changes were reported in the glandular part of the stomach. Linear yellow-brown, rough epithelium was seen in the entire length of the oesophagus in a few pigs in the middle and high dose groups.

3.2.2.4. Monkeys

Groups of 8 female Cynomologus monkeys were given 0, 125 or 500 mg BHA/kg bw/day by gavage in corn oil, 5 days per week for 4 weeks, after which the dose was halved and continued for a total of 85 days. No dose-related changes were seen in blood clinical parameters or abnormal observations. Also, there were no proliferative effects seen in the forestomach. There were no treatment-related histopathological changes reported; the only observation was a statistically significant elevation in the mitotic index (1.9 times) in the basal cell layer of the squamous epithelium of the distal oesophagus in the highest dose group. Elevated relative liver weight was observed at the end of the study for both dose groups ($2.19 \pm 0.11\%$ in the control versus $2.64 \pm 0.26\%$ ($p < 0.05$) at 125 mg/kg bw/day and 2.89 ± 0.39 ($p < 0.05$) at 500 mg/kg bw/day) (Iverson et al., 1986).

Overall, the Panel derived a NOAEL of 125 mg/kg bw/day from the Clayson et al. rat study (1986) based on hyperplasia in the forestomach and a Lowest-Observed-Adverse-Effect Level (LOAEL) of 62.5 mg/kg bw/day from the Altmann et al. (1986) rat study also based on hyperplasia in the forestomach. The Panel noted that the study of Clayson et al. (1986) was based on only 5 rats/group, whereas in the first study of Altmann et al. (1986) 10 males and 10 females per dose groups were used and that a second study, using 20 males and 20 females per dose level indicated the dose level of 62.5 mg/kg bw/day to be a NOAEL.

3.2.3. Genotoxicity

The genotoxicity of BHA has been studied *in vitro* and *in vivo* and was evaluated by JECFA that stated that:

"the results of several tests on the genotoxicity of BHA, involving both bacterial and mammalian cells, gave additional support to previous results in several in vitro and in vivo systems which did not prove BHA to be mutagenic" (JECFA, 1987).

SCF concluded in 1989 that:

"*Nearly all the available mutagenicity data were negative and BHA has not been demonstrated to be a genotoxic compound*".

In addition, TemaNord (2002) reported that:

"*BHA was not genotoxic in the Ames test and several other in vitro genotoxicity tests. Furthermore, it was reported that in vivo investigations showed no cytogenetic effects in the bone marrow of rats dosed with BHA at 15-1500 mg/kg bw. This was confirmed in a host-mediated assay using S. typhimurium G-46 and TA 1530 strains in mice at the same dose levels showing no mutagenic effects*".

The genotoxicity of BHA was also evaluated in a publication from The Ministers of The Environment and of Health in Canada (Environment Canada, 2010). In this report, existing data on BHA were reviewed. Table 4 gives an overview of the studies evaluated.

Table 4: Overview of the results from *in vitro* and *in vivo* genotoxicity studies of BHA. The table has been modified from the report by The Ministers of The Environment and of Health in Canada (2010).

Genotoxicity and related endpoints (*in vitro*):	**Reverse mutation** ***Negative*:** *Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 and TA 1538 with or without metabolic activation (Williams et al., 1990a) *Salmonella typhimurium* TA 97, TA 98, TA 100 and TA 102 with or without metabolic activation (Matsuoka et al., 1990) *Salmonella typhimurium* TA 97, TA 100, TA 102 and TA 104 with or without metabolic activation (Hageman et al., 1988) *Saccharomyces cerevisiae* strain D7 with and without metabolic activation (Rogers et al., 1992)
	Gene mutation ***Positive*:** *Staphylococcus aureus* without metabolic activation (Degre and Saheb, 1982) ***Negative*:** Adult rat liver (ARL) epithelial cell mutagenesis assay with hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus without metabolic activation (Williams et al., 1990a). Hepatocyte- mediated assay with V79 Chinese hamster lung (CHL) cells (HGPRT locus) with or without metabolic activation (Rogers et al., 1985).
	Chromosomal aberrations ***Positive*:** Chinese hamster ovary (CHO) cells with metabolic activation (Phillips et al., 1989). CHO cells with metabolic activation (Murli H, 1992). CHL cells with metabolic activation (Matsuoka et al., 1990). ***Negative*:** CHO cells without metabolic activation (Phillips et al., 1989). CHO cells without metabolic activation (Murli H, 1992).

	CHL cells without metabolic activation (Matsuoka et al., 1990).
	Sister chromatid exchange ***Negative*:** CHO cells with and without metabolic activation (Williams et al., 1990a). V79 CHL cells with and without metabolic activation (Rogers et al., 1985).
	Unscheduled DNA synthesis ***Negative*:** Rat hepatocyte primary culture (HPC)/DNA repair test (Williams et al., 1990a).
Genotoxicity and related endpoints (*in vivo*):	**Unscheduled DNA synthesis and replicative DNA synthesis** ***Positive (replicative DNA synthesis)*:** B6C3F1 mice (group of five mice, no information on gender), induction of S-phase in forestomach epithelium cells, single dose by gastric gavage with 300 mg/kg bw, 6, 8, 10 and 12 hours assessment, consistent effect after 10 hours (Benford et al., 1994). ***Negative (unscheduled DNA synthesis)*:** B6C3F1 mice (group of five mice, no information on gender), forestomach epithelium, single dose by gastric gavage with 300 mg/kg bw, 2 and 4 hours assessment (Benford et al., 1994).
	DNA adduct formation ***Negative*:** F344 rats (group of six male rats), forestomach, single dose and 5 day repeated oral by gavage, 1000 mg/kg bw (Saito et al., 1989). F344 rats (group of six male rats), forestomach epithelium, 5 day repeated intragastric dose, 1000 mg/kg bw (Ito et al., 1991).
	DNA strand breaks and repair ***Positive*:** ddY mice (group of five male mice), Comet Assay performed after 3 and 24 hours on glandular stomach and colon, single oral dose of 2000 mg/kg bw (Sasaki et al., 2002). *Negative:* F344 rat (male rat but no information on number), forestomach epithelium, single dose by gastric tube with 1% equivalent to 220 mg/kg bw (Morimoto et al., 1991).
	Sex-linked recessive lethal ***Negative*:** No spontaneous frequency of recessive sex linked lethal in *Drosophila melanogaster* (Myiagi and Goodheart, 1976). ***Positive*:** Doubling of the spontaneous frequency of recessive sex linked lethal in *Drosophila melanogaster* was observed (Prasad and Kamra, 1974).

Table 4 reveals that a large number of studies have been performed investigating the genotoxic potential of BHA. No reverse mutation was observed when tested in a battery of *Salmonella typhimurium* strains. Positive results were obtained in the *in vitro* chromosomal aberration studies using metabolic activation and these tests were negative without metabolic activation. No effects were seen in the sister chromatid exchange **(**SCE) assay and in the unscheduled DNA assay *in vitro*. A number of *in vivo* studies investigating possible DNA adduct formation and unscheduled DNA synthesis were all negative. A single study showed an effect of BHA on DNA replication in forestomach epithelial cells. In general, studies investigating DNA strand breaks and repair were negative using hepatocytes and in the forestomach epithelium of rats. In contrast, a study in mice showed positive effects in tissue from glandular stomach and colon, using the Comet assay. The Panel noted that the positive effect for the Comet assay reported by Sasaki et al., (2002) and in the

replicative DNA synthesis (Benford et al. 1994) may be due to the induction of cell proliferation and may not reflect genotoxicity. In the sex-linked recessive lethal test using *Drosophila melanogaster*, equivocal results were obtained.

In general, the majority of the genotoxicity studies indicate a lack of potential for BHA to induce point mutations or to interact with or damage DNA. BHA and its metabolite TBHQ have been reported to induce chromosomal aberrations *in vitro* in the presence of metabolic activation. The Panel recognised that the clastogenic activity exerted *in vitro* by BHA and TBHQ is likely to be secondary to the formation of reactive oxygen species via pro-oxidant chemistry (see also Dobo and Eastmond 1994, cited below), and that such a mechanism of genotoxicity is considered thresholded.

3.2.3.1. Genotoxicity of metabolites

A number of the reported studies in Table 4 also investigated possible genotoxicity of the metabolites of BHA and these are described below:

In the study by Matsuoka et al. (1990), TBHQ, tert-butylquinone (TBQ), BHA dimer (diBHA), 3-*tert*-butyl-4,5-dihydroxyanisole (BHA-OH), 3-*tert*-butylanisol-4,5-quinone (BHA-o-O) and *tert*-butylquinone oxide (TBQO) were investigated for possible genotoxicity. In the reverse mutation assay using *Salmonella typhimurium* TA 97, TA 98, TA 100 and TA 102 strains, with or without metabolic activation, TBHQ, TBQ and diBHA were not mutagenic with and without metabolic activation. BHA-OH, BHA-o-O and BQO were not tested in the reverse mutation assay. In the same study, all metabolites were screened for the capacity to induce chromosomal aberrations. The metabolite diBHA did not induce significant increases in the frequency of chromosomal aberrations with or without metabolic activation. Chromosomal aberrations were induced by TBHQ and TBQ with metabolic activation, while BHA-OH, BHA-o-O and TBQO induced chromosomal aberrations without metabolic activation.

In another study using *Salmonella typhimurium* TA 97, TA 100, TA 102 and TA 104 strains with or without metabolic activation, TBHQ was shown not to be mutagenic (Hageman et al., 1988). This was also the result when TBHQ was tested in *Saccharomyces cerevisiae* strain D7 with and without metabolic activation (Rogers et al., 1992). In the same study using V79 CHL cells, TBHQ was shown to have inconsistent and weak activity in the frequency of SCE and in the mutation frequency of the HGPRT gene locus with or without metabolic activation, using hepatocytes.

Formation of DNA-adducts by the BHA metabolites TBQ and 5-methoxy-3-*tert*-butyl-1,2-benzoquinone (3-TBOQ) was investigated in groups of six male F344 rats using an enzymatic ^{32}P-postlabelling assay. No DNA adducts were observed in the forestomach after a single oral dose by gavage or repeated administration for five days at dose levels of 125 mg/kg bw TBBQ and 250 mg/kg bw (3-TBOQ) (Saito et al., 1989).

The metabolites TBHQ and TBQ were tested in male F344 rats (no information on the number of rats could be retrieved from the reference) for their ability to induce DNA damage in the forestomach epithelium using an alkaline elution assay. Rats were given single oral doses by gavage of 0.001% TBHQ (equivalent to 0.22 mg/kg bw), 0.001% TBQ (equivalent to 0.22 mg/kg bw) and 0.00001% TBQ (equivalent to 0.0022 mg/kg bw). No detectable DNA damage was observed in the group given 0.001% TBHQ. In the TBQ dosed groups, strong DNA damage activity was seen at the dose level of 0.001% TBQ and weak DNA damage activity at 0.00001% TBQ (Morimoto et al., 1991).

Further studies investigating the possible genotoxicity of BHA metabolites were identified by the literature search and can be summarised as follows:

In the study by Kalus et al. (1990), BHA and its reaction products, TBQ and 3-TBOQ were tested using *Salmonella typhimurium* TA 98 and TA 100 strains**.** No evidence of mutagenicity was observed, with or without metabolic activation.

Two microsomal metabolites of BHA, TBHQ and TBQ, were examined for mutagenic activity using *Salmonella typhimurium* TA 98 and TA 100 strains. No evidence of mutagenicity was observed, with or without metabolic activation (Kalus et al., 1994).

In another study by Schilderman et al., (1995a), the effect of BHA, TBHQ and TBQ on the formation of 7-hydro-8-oxo-deoxyguanosine (8-oxodG) and cell proliferation was investigated in human lymphocytes. Analysis showed that BHA and TBHQ undergo biotransformation and that TBQ could be reduced to TBHQ. It was also shown that TBHQ or TBQ bind to macromolecules. In a study on cell proliferation of phytohemagglutinin-stimulated lymphocytes a dose-dependent increase was seen with BHA, TBHQ and TBQ. Further, it was shown that BHA did not induce oxidative DNA damage in lymphocytes, but TBQ and particularly TBHQ did. Finally, it was shown that prostaglandin H synthase is involved in the mechanism of toxicity of BHA.

In another study by Schilderman et al. (1995b), the involvement of prostaglandin H synthase in BHA induced toxicity was investigated further. Four groups of 18 male Wistar rats were maintained on a diet containing 0 and 1.5% BHA and drinking water containing 0 and 0.2% acetylsalicylic acid (ASA). Groups of 6 rats within each treatment group were designated to be killed at day 3, 7 and 14. Oxidative DNA damage, as measured by increased levels of 8-oxodG, was observed in the epithelial cells of the glandular stomach. Hyperplasia and inflammation was observed in the forestomach and glandular stomach. It was shown that prostaglandin H synthase is involved in the enhancement of cellular proliferation.

The observed effects of BHA in the chromosomal aberration studies using metabolic activation indicate a possible genotoxic potential of BHA metabolites (Matsuoka et al., 1990; Murli H, 1992; Phillips et al., 1989). The BHA metabolites TBHQ and TBQ induced chromosomal aberrations with metabolic activation while BHA-OH, BHA-o-O and TBQO induced chromosomal aberrations without metabolic activation (Matsuoka et al., 1990). Furthermore, TBQ induced DNA damage in forestomach epithelium of rats (Morimoto et al., 1991). It is speculated that the carcinogenicity of BHA seen in certain mouse and rat strains may in part be explained by oxidative damage of DNA caused by the metabolites TBHQ and TBQ.

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.

3.2.4. Chronic toxicity and carcinogenicity

The most relevant and important studies in the latest JECFA and SCF evaluations (1989) and studies reported since these previous evaluations, are summarized below:

3.2.4.1. Rats

In a long-term study by Ito et al. (1982) groups of 50-52 male and female F344 rats (6 weeks of age) were maintained on diets containing 0, 0.5 or 2% BHA (equivalent to dose levels of approximately 0, 250, 1000 mg/kg bw/day) for a total of 104 weeks. After this period, animals were fed a basal diet until week 112. The animals were sacrificed and gross and histopathological examinations were

performed. Tumours were found in both sexes fed with the diet containing BHA. The histological investigation showed marked proliferation of basal cells, and in the papillomas keratin-forming elements were seen. In the investigation of squamous cell carcinomas, two different types of cells were seen; well-differentiated (more keratinisation) and poorly-differentiated (many mitotic figures and atypism). The data on the incidence of changes in the forestomach as measured by hyperplasia and neoplasia (papillomas and squamous cell carcinomas), showed a dose-related response (Table 5). Table 6 presents the results from a Benchmark Dose (BMD) analysis of the data from Ito et al. (1982) on the incidence of hyperplasia in the forestomach of male and female rats (combined) induced by BHA.

Table 5: Incidences of hyperplasia and neoplasia (papillomas and squamous cell carcinomas) in the forestomach of F344 rats (Ito et al., 1982).

	0% BHA	**0.5% BHA**	**2% BHA**
Approximate dose levels mg/kg bw/day	0	250	1000
Hyperplasia (male)	0/50	13/50**	52/52**
Hyperplasia (female)	0/51	10/51*	50/51**
Papillomas (male)	0/50	1/50	52/52**
Papillomas (female)	0/51	1/51	49/51**
Carcinomas (male	0/50	0/50	18/52**
Carcinomas (female)	0/51	1/51	15/51**

* $p < 0.01$; ** $p < 0.001$

Table 6: Results of a BMD analysis of the data from Ito et al. (1982) on the incidence of hyperplasia in the forestomach of male and female rats (combined) induced by BHA using Benchmark Dose Software (BMDS) version 2.1.2., the default settings of extra risk, a Benchmark Response (BMR) of 10%, and a 95% confidence limit and no constraints.

Model	No of parameters	Log likelihood	p value	accepted	BMD_{10} mg/kg bw/day	$BMDL_{10}$ mg/kg bw/day
null	1	-206.424				
full	3	-59.8174				
gamma	2	-59.8174	1.000	yes	181.439	146.645
logistic	2	-62.8053	0.0923	yes	187.471	157.163
probit	2	-62.5991	0.1088	yes	184.659	152.897
multistage	2	-59.8174	1.000	yes	166.508	114.857
Weibull	2	-59.8174	0.7351	yes	162.491	126.947

Based on the BMD analysis, using the data reported by Ito et al. (1982) on hyperplasia in the forestomach of male and female rats (combined) the Panel derived a lower confidence limit of the benchmark dose ($BMDL_{10}$) of 115 mg/kg bw/day.

In another long-term study (104 weeks) by Ito et al., (1986a), groups of 50 male F344 rats (6 weeks of age) were maintained on diets containing 0, 0.125, 0.25, 0.5, 1 or 2% BHA. The authors of the study report corresponding average dose levels of BHA of 0, 55, 110, 230, 428, 1323 mg/kg bw/day. A thorough histopathological examination was performed, with specific emphasis on the oesophagus and stomach, where epithelial lesions were classified into three categories: hyperplasia, papilloma and squamous cell carcinoma. There was no effect on the survival rate, the average food intake was comparable and a dose-related weight gain was observed. No metastases were reported. The incidences of proliferative and neoplastic lesions in the forestomach increased in a dose-related manner (Table 7). Table 8 presents the result from a BMD analysis of these data from Ito et al. (1986a) on the incidence of hyperplasia in the forestomach induced by BHA.

Table 7: Incidences of hyperplasia and neoplasia (papillomas and squamous cell carcinomas) in the forestomach of male rats exposed to BHA (Ito et al., 1986a).

	0% BHA	0.125% BHA	0.25% BHA	0.5% BHA	1% BHA	2% BHA
Average dose levels mg/kg bw/day	0	55	110	230	428	1323
Hyperplasia	0/50	1/50	7/50*	16/50**	44/50**	50/50**
Papillomas	0/50	0/50	0/50	0/50	10/50*	50/50**
Carcinomas	0/50	0/50	0/50	0/50	0/50	11/50**

* $p < 0.01$; ** $p < 0.001$

Table 8: Results of a BMD analysis of the data from Ito et al. (1986a) on the incidence of hyperplasia in the forestomach of male rats induced by BHA using BMDS version 2.1.2. software, the default settings of extra risk, a BMR of 10%, and a 95% confidence limit and no constraints.

Model	No of parameters	Log likelihood	p value	accepted	BMD_{10} mg/kg bw/day	$BMDL_{10}$ mg/kg bw/day
null	1	-201.065				
full	6	-74.8399				
gamma	2	-76.9644	0.3733	yes	109.577	86.3975
logistic	2	-77.0503	0.352	yes	136.646	114.482
probit	2	-76.5376	0.4939	yes	128.085	106.922
multistage	2	-76.2476	0.5892	yes	107.705	82.6926
Weibull	2	-76.1037	0.6397	yes	112.251	87.1522

Based on the BMD analysis using the data reported by Ito et al. (1986a) on hyperplasia in the forestomach of male rats the Panel derived a $BMDL_{10}$ of 83 mg/kg bw/day.

In another study Ito et al., (1986b) investigated proliferative and neoplastic lesions in 3 groups of male F344 rats to which a pellet diet containing 0, 1 or 2% (equivalent to a dose of approximately 0, 500, or 1000 mg/kg bw/day) BHA was given for 104 weeks. The number of animals surviving after 104 weeks was 23 (0% BHA), 25 (1% BHA) and 26 (2% BHA). In both the 1 and 2 % dose group, incidence of hyperplasia and papilloma was significant, while in the 2% group there was also a significant incidence of carcinoma. The Panel noted that the dose levels tested in this study were all effect levels and were higher than the $BMDL_{10}$ values derived from the two other long term studies.

In a study in male F344 rats (Masui et al., 1986a) 10 animals received a pellet diet containing 2% BHA (equivalent to a dose of approximately 1000 mg/kg bw/day) for 24 weeks and were then sacrificed. Another 20 rats were administered the same 2% BHA diet for 24 weeks and were then given feed without BHA for 72 weeks prior to sacrifice. A third group of 20 animals were given a basal diet without BHA for 96 weeks and served as controls. In the rats given 2% BHA for 24 weeks, the forestomach showed epithelial thickening compared to controls. After an exposure-free period of 72 weeks the effect was almost completely reversed. Rats exposed to BHA for 24 weeks developed forestomach hyperplasia and papillomas, including proliferation with inflammatory reactions. At week 96, after 72 weeks without treatment the hyperplasias disappeared. Although reduced, basal cell hyperplasia persisted in all animals and papillomas were also still observed in 17% of the rats. No proliferative changes were observed in control animals.

In a study investigating the gastric and oesophageal carcinogenesis of BHA, a group of 30 male Sprague-Dawley rats was given 1% BHA (equivalent to a dose of approximately 500 mg/kg bw/day) in the diet for 3 months (Newberne et al., 1986). Another group of 30 male Sprague-Dawley rats was given BHA dissolved in corn oil, by gavage five times weekly. The administered mg/kg dose was equivalent to the dose calculated from the food consumption in the dietary study. Forestomach lesions were classified as hyperplasia, papillomas and carcinomas based on [^{3}H]-thymidine LI as a parameter. The data showed that the LI was 11 and 15 times higher than in the control animals for the diet and gavage group, respectively. Furthermore, in the diet group 66% of the animals had hyperplasia, 26% papillomas and 6% carcinomas. The incidence of lesions in the gavage group indicated more severe effects, with 5% showing hyperplasia, 28% papillomas and 66% carcinomas.

The effect of length of feeding with BHA on the incidence of forestomach lesions in rats, including tumours, was investigated over 24-months (Nera et al., 1988). Groups of 44-54 male Fischer 344 rats were fed 2% BHA (equivalent to a dose of approximately 1000 mg/kg bw/day) for 0, 3, 6, 12, and 24 months followed by an exposure-free period on basal diet until the completion of the 24-month experimental period. At termination, it was shown that cellular proliferation in the epithelium lining the greater and the lesser curvature of the forestomach was dependent on the continuous presence of 2% BHA for at least 12 months. Papillomas were found in both the greater and the lesser curvature of the forestomach in all rats dosed with 2% BHA for 24 months. There were no differences in the incidence of squamous cell carcinomas in rats fed 2% BHA for 12 months followed by basal diet 12 months compared to rats receiving 2% BHA for 24 months.

Abraham et al. (1986), investigated the induction of forestomach tumours in partially hepatectomized rats. A total of 40 rats was equally divided into four groups of 10 each and treated as follows: Group I rats served as controls; Group II rats received a dietary level of 2% BHA (equivalent to a dose of approximately 1000 mg/kg bw/day) for three months (BHA control group). Histopathological investigations performed on forestomach tissue from the BHA control group (Group II) showed induction of mild hyperplasia when compared to a control group (group I) given normal diet for three months. No papillomas or carcinomas were observed in the 2% BHA control group.

The following five rat studies were not included in the latest evaluations by JECFA or SCF:

In a 110-week chronic feeding study, a group of 27 male F344 rats was given diets containing BHA at a concentration of 12 000 mg/kg (equivalent to a dose of approximately 600 mg/kg bw/day). Normal growth and histopathological examinations were performed including investigations of gastric lesions in the squamous and glandular stomach. In the squamous portion of the stomach BHA induced a moderate level of hyperplasia and a moderate incidence of squamous cell papillomas. Incidences of neoplasms in other organs were comparable to the control group (Williams et al., 1990b).

A carcinogenicity study of BHA included groups of 30-50 F344 rats of each sex treated with 0% or 2% BHA (equivalent to a dose of approximately 0 or 1000 mg/kg bw/day) in the diet for 104 weeks prior to sacrifice (Ito et al., 1991). Histopathological examinations of the forestomach found the incidences of forestomach papillomas and squamous cell carcinomas to be significantly increased in both male and female rats treated with 2% BHA compared to the control.

The reversibility of BHA-induced rat forestomach hyperplasia was investigated in a group of ten F344 male rats treated with 2% BHA in the diet for 24 weeks, followed by 24 weeks of basal diet. A control group was treated with basal diet alone during the experiment. Moderate hyperplasia was observed in all animals after 24 weeks of 2% BHA treatment which decreased to 40% of the animals 24 weeks after cessation of treatment (Ito et al., 1993).

In a carcinogenicity study, groups of 30 or 31 male F344 rats were treated with 0 or 0.4% BHA (equivalent to a dose of approximately 0 or 200 mg/kg bw/day, respectively) for up to 104 weeks prior to sacrifice. Histopathological examination of the forestomach and the glandular stomach was performed investigating the incidence of hyperplasia, papilloma, adenoma and carcinoma. No effect of

BHA was seen at a dose level of 200 mg/kg bw/day BHA (Hirose et al., 1997). The Panel noted that this NOAEL was higher than the $BMDL_{10}$ values derived from the two long term rat studies reported by Ito (Ito et al 1982; Ito et al. 1986a).

A study of BHA-induced forestomach carcinogenicity compared effects in groups of thirty 6-week-old male rats of F344, SHR, Lewis and Sprague-Dawley strains over 104 weeks. Animals were administered basal diet or a diet containing 2% (equivalent to a dose of approximately 1000 mg/kg bw/day) BHA. Forestomach squamous cell papillomas and hyperplasia were found in all rats of each strain however, incidences of squamous cell carcinoma differed considerably between rat strains: F344 rats, 26.7% (8/30); SHR rats, 76.7% (23/30); SD rats, 36.7% (11/30) and Lewis rats, 6.7% (2/30). Inflammation was most pronounced in the SHR rat strain. The authors concluded "that major strain differences exist regarding BHA rat forestomach carcinogenesis and that sensitivity to cytotoxicity might be an important parameter" (Tamano et al., 1998).

Overall, studies performed in rats with BHA at dose levels of 0.125%-2% showed significant incidences of forestomach hyperplasia at dietary concentration of 0.125% or higher, significant incidences of papillomas at dietary concentration of 1% or higher and significant incidences of squamous cell carcinomas at dietary concentration of 2%. Reversibility of the hyperplastic lesions was shown to be dependent on dose and duration. In a strain comparison study the SHR rat strain was shown to be most sensitive, with the highest incidence of squamous cell carcinoma. $BMDL_{10}$ values for induction of forestomach hyperplasia of 115 and 83 mg/kg bw/day can be derived from data from the study of Ito et al. (1982) and Ito et al. (1986a), respectively.

3.2.4.2. Hamsters

In a study by Ito et al. (1986b), the carcinogenic activity of BHA, as measured by proliferation and neoplastic lesions, was investigated in male Syrian golden hamsters. A pelleted diet containing 1% or 2% BHA (equivalent to a dose of approximately 1200 and 2400 mg/kg bw/day) was given for 104 weeks. The surviving number of animals after 96 weeks was 12 (0% BHA), 13 (1% BHA) and 4 (2% BHA). In the 1% dose group, there was a significant incidence of hyperplasia and papilloma while for the 2% dose group, the incidence of papilloma was also significant and a squamous cell carcinoma was found in one hamster. In a study by Hirose et al., (1986a), groups of 15 male Syrian golden hamsters were given a powdered diet containing 1% (equivalent to a dose of approximately 1200 mg/kg bw/day) BHA for 20 weeks. The LI in the forestomach epithelium was investigated by injection with radioactive thymidine just prior to sacrifice. When compared to control, the LI in the forestomach epithelium was significantly increased. All animals in the treated group displayed severe hyperplasia and more than half of the group had papillomas.

One hamster study was identified which was not previously evaluated by JECFA or SCF.

Hirose et al. (1990) investigated the reversibility of BHA-induced hamster forestomach hyperplasia in groups of 10-15 male Syrian golden hamsters treated with 2% BHA (equivalent to a dose of approximately 2400 mg/kg bw/day), for 12, 24 or 48 weeks followed by basal diet until week 72. Other animals were treated with 2% BHA continuously for 72 weeks. The results showed that the BHA-induced hyperplasia reversed after cessation of treatment, but that squamous cell dysplasia and basal cell dysplasia, observed in some animals treated with BHA for 24 weeks or more, persisted and tended to increase with time on BHA. The authors concluded that "the results suggest that basal cell dysplasia, including regions of squamous cell dysplasia, may be of particular importance as a precursor pre-neoplastic lesion" (Hirose et al., 1990).

Overall, studies performed in hamsters showed proliferative changes in the forestomach including induction of papillomas at a dose level of 1% BHA. A study by Hirose et al. (1990) was identified in the literature search in which it was shown that squamous cell dysplasia was not reverted at a dose level of 2% BHA.

3.2.4.3. Mice

In a study by Ito et al., (1986b), the carcinogenic activity of BHA, as measured by proliferative and neoplastic lesions, was investigated in male B6C3F mice. A pelleted diet containing 0, 0.5% or 1% BHA (equivalent to a dose of approximately 0, 750, 1500 mg/kg bw/day) was given for 104 weeks. The surviving number of animals after 104 weeks was 16 (0% BHA), 21 (1% BHA) and 22 (2% BHA). Incidence of hyperplasia was significantly increased in the 0.5% and 1% dose groups but no papillomas or squamous cell carcinomas were found. The Panel noted that there was no information on the number of animals used in the study.

In a study by Masui et al., (1986b), 3 groups of 150 male $B6C3F_1$ mice were given dietary doses of 0, 0.5% and 1% BHA (equivalent to a dose of approximately 0, 750, 1500 mg/kg bw/day) for up to 104 weeks (Masui et al., 1986b). The incidence of hyperplasia and papilloma were significantly increased at both dose levels. One carcinoma was found in the 0.5% dose group and two in the 1% dose group. The incidences of proliferation and neoplastic lesions in the forestomach are summarized in Table 9 Table 10 presents the result from a BMD analysis of these data from Masui (1986b) on the incidence of hyperplasia in the forestomach induced by BHA.

Table 9: Incidences of hyperplasia and neoplasia (papillomas and squamous cell carcinomas) in the forestomach of B6C3F1 mice (Masui et al., 1986b).

	0% BHA	**0.5% BHA**	**1% BHA**
Approximate dose level in mg/kg bw/day	0	750	1500
Hyperplasia	0/39	10/37**	35/43**
Papillomas	0/39	5/37*	5/43*
Carcinomas	0/39	1/37	2/43

* $p < 0.05$; ** $p < 0.001$

Table 10: Results of a BMD analysis of the data from Masui et al. (1986b) on the incidence of hyperplasia in the forestomach of male mice induced by BHA using BMDS version 2.1.2. software, the default settings of extra risk, a BMR of 10%, and a 95% confidence limit, and no constraints.

Model	**No of parameters**	**Log likelihood**	**p value**	**accepted**	**BMD_{10} mg/kg bw/day**	**$BMDL_{10}$ mg/kg bw/day**
null	1	-78.9151				
full	3	-42.2494				
gamma	2	-42.2494	1.0000	yes	520.329	320.838
logistic	2	-43.1566	0.1780	yes	527.033	394.022
probit	2	-42.7278	0.3280	yes	510.174	373.173
multistage	1	-42.2494	1.0000	yes	518.116	244.793
Weibull	2	-42.2494	1.0000	yes	476.608	285.396

Based on the BMD analysis using the data reported by Masui et al. (1986b) on hyperplasia in the forestomach of male mice the Panel derived a $BMDL_{10}$ of 245 mg/kg bw/day.

Overall, studies performed in mice showed proliferative changes in the forestomach including induction of papillomas and carcinomas with a $BMDL_{10}$ of 245 mg/kg bw/day. No new studies were identified.

3.2.4.4. Guinea pigs

Guinea pigs fed a diet containing up to 1% BHA (equivalent to a dose of approximately 400 mg/kg bw/day) for 20 months, did not show any gross changes in the stomach as stated in the review by Ito (1987).

3.2.4.5. Dogs

Groups of 29 male and 30 female Beagle dogs were randomly assigned to three treatment groups fed diets containing 0, 1.0 or 1.3% BHA (equivalent to doses of approximately 0, 250, 325 mg/kg bw/day) for 180 days. In the highest dose group, food consumption and body weight gain were reduced. The liver weight was increased in animals receiving BHA at both doses, as was proliferation of smooth endoplasmic reticulum. There were no proliferative or hyperplastic lesions (Ikeda et al., 1986).

Groups of 3-4 male or female Beagle dogs were fed diets containing 0.25, 0.5 or 1.0% BHA (equivalent to a dose of approximately 0, 62.5, 125, 250 mg/kg bw/day) for six months. Dose-related retardation of growth was reported. Liver weights were increased but no histopathological changes were seen. Investigation of the mitotic index in the squamous epithelium of the distal oesophagus showed no changes. The data showed no histopathological effects after 6 months of exposure to BHA in the diet in the stomach, oesophagus, duodenum or liver of beagle dogs (Tobe et al., 1986).

Overall, studies performed in dogs did not show any proliferative changes in the stomach at dose levels up to 325 mg/kg bw/day, the highest dose tested. No new studies were identified.

In conclusion, a large number of long-term toxicity and carcinogenicity studies have been performed, investigating the induction of proliferative changes in the forestomach by BHA and the reversibility of the lesions. The studies have included species with and without a forestomach i.e. rats, mice, hamsters, guinea pigs and dogs. The Panel noted that gastric epithelial hyperplasias, papillomas and carcinomas were only seen in species with a forestomach.

3.2.5. Reproductive and developmental toxicity

A number of studies investigating reproduction and developmental toxicity of BHA have been performed in rats, mice, rabbits, monkeys and pigs and were evaluated by JECFA and the SCF in 1989. Following that evaluation JECFA requested that a multi-generation reproduction study with BHA should be submitted when available.

The conclusions of the SCF (1989) stated that "*in a number of (1 generation) reproduction studies with rats, mice and monkeys, at dose levels of 50 mg/kg bw or higher no effects were observed except on the behaviour of mice. Teratogenicity studies were all negative*". The TemaNord report (2002) reached the same conclusion as the SCF.

Studies evaluated in the latest JECFA and SCF evaluations (1989) and one additional reproduction and developmental study in rats (Jeong et al., 2005) are summarised below.

3.2.5.1. Rats

BHA was administered in the diet to Sprague-Dawley rats at dose levels of 0, 0.125, 0.25 and 0.5% BHA before mating, during gestation and through to 90 days of postnatal age (Vorhees et al., 1981). The authors indicate that the dose levels for the 0.5 % group amounted to approximately 420 mg/kg bw/day prior to breeding, to 410 mg/kg bw/day during gestation and to 800 mg/kg bw/day during lactation. For the 0.25 % group these values amounted to 220, 210 mg/kg bw/day and 440 mg/kg bw/day, and for the 0.125% group the values were 110, 100 and 220 mg/kg bw/day. A battery of standardized behavioural tests was applied to offspring between 3 and 90 days of age. There was no effect of BHA on reproductive parameters and there were no changes in maternal weight. However, the offspring showed impaired growth during the last week of pre-weaning development and there was increased (pre)weaning mortality in the 0.5% group and marginally in the 0.25% group up to postnatal

day 30, amounting to respectively 13.5 % ($p<0.01$) and 8.3% ($p<0.06$) of the offspring dying based on the number of live progeny on day 30 after birth. Delayed startle response was found in the 0.25 and 0.5% groups, in the behavioural investigations. The Panel concluded that a NOAEL for reproductive and developmental toxicity was 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day).

One additional study has been published since the latest JECFA and SCF evaluations:

A study was performed in Sprague-Dawley rats on reproduction and development in order to characterise the potential endocrine-disrupting effects of BHA, using rats exposed through the F_0 and F_1 generations (Jeong et al., 2005). BHA, dissolved in corn oil, was administered by gavage at dose levels of 0, 10, 100 or 500 mg/kg bw/day to groups of 12 rats of each sex. F_0 rats were dosed through the pre-gestation, gestation and lactation period while the offspring (F_1) were dosed until 13 weeks of age. Twelve offspring (F_1) representing each sex, litter and treatment group (1–2 pups/sex/litter) were given BHA from postnatal day 21 (PND 21) until 13 weeks old and another 12 offspring (F_1) representing sex, litter and treatment group were sacrificed for the evaluation of anogenital distance, necropsy findings and organ weights on PND 21.

The Panel noted that the study was not performed according to OECD guidelines (OECD TG 416) and that several of the endpoints studied are not part of regular OECD guidelines for testing reproductive and developmental toxicity. The Panel noted that when compared to standard guidelines for multi-generation reproduction studies, this study contains a number of discrepancies i.e. the F_1 generation was terminated after 13 weeks (normally production of a F_2 generation), pre-mating period was 2 weeks (normally 10 weeks) and the investigations of sperm morphology was performed on dried preparations from 10 sperm cells per animal (normally wet and from at least 200 sperm cells per animal).

The Panel also noted that the decreases reported in the parameters that were stated to be affected were generally less than 10% without a clear dose response, and that the ranges reported for these parameters overlap due to large standard deviations. Thus the Panel concluded that the effects reported in this study that were statistically significant (at $p < 0.05$) were not biologically relevant.

Taking all together the Panel concluded that the effects reported in this new study cannot be used to derive a point of departure for the risk assessment on BHA.

3.2.5.2. Mice

Diets containing 0 and 0.5% BHA (equivalent to approximately 0 and 750 mg/kg bw/day) were given to mated pairs of Swiss Webster. The litters obtained from the mated pairs were culled to 8 pups at birth and weaned at 21 days of age. The young were then continued on a diet that was similar to that of their mothers. When the pups were 6 weeks old, behavioural testing was initiated. A number of behavioural changes were observed including decreased orientation reflex, decreased sleeping, decreased grooming of self, slow learning and increased exploration (Stokes and Schudder, 1974).

3.2.5.3. Rabbits

In a study by Hansen and Meyer (1978), doses of 0, 50, 200 or 400 mg/kg bw/day of BHA were given orally by gavage to groups of pregnant New Zealand white rabbits from day 7 to day 18 of pregnancy. There were no observed differences between the treated groups and controls in the parameters measured (body weight, incidence of soft tissue or skeletal abnormalities, number of foetuses born dead or alive, number of corpora lutea and implantations, general reproductive parameters). The Panel concluded that a NOAEL for developmental toxicity of 400 mg/kg bw/day can be derived from this study.

3.2.5.4. Pigs

A diet containing the equivalent of 0, 50, 200, or 400 mg/kg bw/day of BHA was given to groups of 10 young adult pigs (Hansen et al., 1982). The pigs were maintained on the diet for three weeks and then artificially inseminated. Feeding of the diet was continued throughout the study from mating (artificial insemination) to day 110 of the gestation period when the foetuses were removed. No effect was seen on food consumption. A significant effect was observed on the body weight of dams in the dose group given 400 mg/kg bw/day compared to the control group. There was a dose-related increase in absolute and relative liver and thyroid weight and absolute and relative liver and thyroid weight were statistically significantly increased relative to the control at all dose levels tested. There was no effect of BHA on the reproductive and developmental parameters. The Panel concluded that a NOAEL for developmental toxicity of 400 mg/kg bw/day can be derived from this study and that the NOAEL for maternal toxicity is 200 mg/kg bw/day.

3.2.5.5. Monkeys

A group of six adult female rhesus monkeys were maintained on a diet containing a mixture of BHT and BHA. The daily intake corresponded to 50 mg BHT/kg bw/day and 50 mg BHA/kg bw/day. Another group of six adult female rhesus monkeys were used as controls. The monkeys were fed the diet for one year prior to breeding and then for an additional year, including a 165-day gestation period. Haematology studies including haemoglobin, hematocrit, total and differential white blood cell (WBC) counts, cholesterol, Na+, K+, total protein, serum glutamic-pyruvic transaminase (GPT) and serum glutamic-oxaloacetic transaminase (GOT), were carried out at monthly intervals. Body weights were recorded at monthly intervals. Records of menstrual cycles were maintained through the test period. After a year of exposure, the females were mated with rhesus males that had received "control diet". During pregnancy complete blood counts were done on days 40, 80, 120 and 160 of gestation and on days 30 and 60 postpartum. The gestation of test animals was free of complications and normal offspring were delivered. A total of five offspring were born of the experimental monkeys and six of the control monkeys. Haematological evaluations were made on infants of the test and control monkeys at days 1, 5, 15, 30 and 60, and observations of the infants were continued through two years of age. Two experimental and two control infants, 3 months of age, were removed from their mothers for a month of home cage observations. No clinical abnormalities were observed in parent or offspring during the period of study. Adult females continued to have normal offspring. Offspring born during the exposure period remained healthy, with the exception of one infant that died from unrelated causes. Home cage observations at the third month of life did not reveal any behavioural abnormalities (Allen, 1976). The Panel concluded that a NOAEL from this study would be 100 mg/kg bw/day of BHA and BHT combined.

In conclusion, reproduction and developmental studies have been performed in rats, rabbits, pigs and monkeys. The Panel concluded that the effects reported in a new reproduction and development study using rats exposed by gavage through F_0 and F_1 generations (Jeong et al., 2005) cannot be used to derive a point of departure for the risk assessment on BHA. In an earlier reproduction study performed in rats using dietary administration of BHA (Vorhees, 1981), a NOAEL of 100 mg/kg bw/day was derived. This NOAEL was based upon pups showing growth retardation, increased mortality and behavioural effects.

The Panel noted that there is no two generation reproduction toxicity study.

3.2.6. Hypersensitivity, allergenicity, intolerance

Two patients with chronic idiopathic urticaria were subjected to double-blind, placebo-controlled, oral challenges with a series of food additives. During testing, BHT and BHA were identified as causative agents. Avoidance of foods containing BHT and BHA resulted in long-term reduction in severity and frequency of urticarial episodes (Goodman et al., 1990). The authors concluded that BHT and BHA were capable of aggravating symptoms in certain patients with chronic urticaria.

In a double-blind placebo controlled challenge study by Young (1997), a total of 132 human subjects were submitted to additive challenge going through a double sequence of a low dose (1 mg BHA and BHT) and a high dose (50 mg BHA and BHT). The additives were ingested by capsules. No relationship could be established with ingestion of the food additives and the symptoms reported.

A couple of review papers (Anderson, 1996; Kitts et al., 1997) describe BHA to be involved in allergic reactions to foods, but the physiological mechanism is unclear and individual with food allergies appear to be most sensitive. The symptoms are described to be urticaria and possible hyperactivity. Nothing conclusive can be derived from these papers.

In addition, TemaNord (2002) stated that a few cases of contact dermatitis from BHA are known, but allergy or intolerance to dietary BHA has not been described.

The Panel considered that no conclusion on allergy or intolerance to dietary BHA could be established based on the available information.

No other relevant studies on hypersensitivity and intolerance of orally administered BHA have been found in the literature.

3.2.7. Other studies

3.2.7.1. Epidemiological studies

The association between dietary intake of BHA and stomach cancer risk was investigated in the Netherlands Cohort Study (NLCS) that started in 1986 among 120,852 men and women aged 55 to 69 years. A semi-quantitative food frequency questionnaire was used to assess food consumption. Information on BHA content of cooking fats, oils, mayonnaise and other creamy salad dressings and dried soups was obtained by chemical analysis, a Dutch database of food additives (ALBA) and the Dutch Compendium of Foods and Diet Products. After 6.3 years of follow-up, complete data on BHA intake of 192 incident stomach cancer cases and 2035 subcohort members were available for case-cohort analysis. Mean intake of BHA among subcohort members was 105 mg/day. For consumption of mayonnaise and other creamy salad dressings with BHA no association with stomach cancer risk was observed. A statistically non-significant decrease in stomach cancer risk was observed with increasing BHA intake [rate ratio (RR) highest/lowest intake of BHA = 0.57 (95% confidence interval (CI): 0.25-1.30]. In this study, no significant association with stomach cancer risk was found for usual intake of low levels of BHA (Botterweck et al., 2000).

3.2.7.2. Special studies on gene expression

Nair et al. (2006) studied the pharmacogenomics and the regulation of gene expression profiles elicited by BHA in small intestine and liver in mice. Identification of BHA-modulated nuclear factor-E2-related factor 2 (Nrf2)-dependent genes was also performed. C57BL/6J (+/+; wild type) and C57BL/6J/Nrf2 (-/-; knockout) young female mice in groups of four were administered a single dose by gavage of 200 mg BHA/kg bw or vehicle (50% PG 400 solution). Animals were sacrificed 3 hours after BHA and vehicle treatment and the liver and small intestines were collected. Gene expression profiles were analyzed using microarray analyses of total RNA from liver and small intestine tissue. A number of BHA regulated genes were identified that were either induced or suppressed more than two fold by BHA treatment compared with vehicle in C57BL/6J/Nrf2(-/-; knockout) and C57BL/6J Nrf2 (+/+; wild type) mouse genotypes. In the small intestine and liver, 1,490 and 493 genes respectively were identified as Nrf2-dependent and up-regulated, and 1,090 and 824 genes respectively as Nrf2-dependent and down-regulated. In this study, BHA-regulated and Nrf2-dependent genes were identified as new molecular targets involved in the molecular mechanism of BHA induced pharmacogenomics.

The molecular basis underlying the biological action of BHA was investigated by Hu et al. (2006). The *in vivo* pharmacokinetics, the induction of mitogen-activated protein kinase (MAPK) signalling

and the induction of Phase II/III drug metabolizing enzymes/transporter gene expression was studied in mice. Swiss Webster mice were dosed by gavage with 200 mg/kg bw of BHA suspended in corn oil or by i.v. injection with 10 mg/kg bw of BHA in 50% ethanol. Four mice were randomly assigned to each treatment group. The mice were sacrificed at specific time points 0-12 hour post–treatment, and plasma and liver samples were obtained. The oral bioavailability of BHA was relatively poor, about 43%, with a peak plasma concentration of 2 µg/ml (10 µM) after 1 hour. In mouse liver, BHA induced the expression of phase II genes (including NQO-1, HO-1, gamma-GCS, GST-pi and UGT 1A6), as well as some of the phase III transporter genes (MRP1 and Slcolb2). Furthermore in relation to early signalling events, BHA activated distinct mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK), as well as p38 enzymes. Based on these results, the authors suggested that the MAPK pathways may play an important role in early signalling events leading to the regulation of gene expression including phase II drug metabolizing and some phase III drug transporter genes. In this study, the molecular basis underlying the biological action of BHA was shown by *in vivo* pharmacokinetics, *in vivo* activation of MAPK signalling proteins and *in vivo* induction of Phase II/III drug metabolizing enzymes/transporters in the mouse livers. It should be noted that no information on the sex of the animals or detailed placebo treatment could be retrieved.

3.2.7.3. Special studies on cytotoxicity

The acute cytotoxicity of BHA to different types of human cells of dermal origin (cultured human dermal fibroblasts, keratinocytes, melanocytes and melanoma tumour cells) was determined *in vitro* using the neutral red assay (Babich and Borenfreund, 1990). Cytotoxicity was observed in all cell types but the effect was more pronounced over time in the assay using keratinocytes. The authors speculate that as these cells are the only ones that can maintain appreciable xenobiotic metabolizing ability in culture, the enhanced toxicity of BHA could reflect conversion to metabolites more toxic than the parent molecule.

Okubo et al. (2004) investigated the possible cytotoxicity and molecular mechanisms of cell death induced by BHA in human monocytic leukaemia U937 cells. BHA was shown to induce cytotoxicity and caused caspase activation, nuclear condensation and fragmentation, structural damage in mitochondria, decrease in mitochondrial trans-membrane potential, and inter-nucleosomal DNA cleavage at 0.75 mM. The authors speculate that cell death in human monocytic leukaemia U937 cells by BHA could be linked to apoptosis pathways.

The cytotoxicity of BHA was investigated in non-tumoural mammalian cells, using Vero, an established line of monkey kidney cells which is a known screening assay for investigating chemical toxicity *in vitro*. Cell growth inhibition was assessed by colorimetric assays, whereas cellular alterations after BHA exposure were evaluated using conventional light and fluorescence microscopy. The results obtained showed that low doses of BHA exerted a significant cytotoxic effect, associated with loss of mitochondrial function. This was accompanied by morphological alterations in critical subcellular targets such as lysosomes, mitochondria and actin cytoskeleton as the concentration of BHA was increased. An irreversible loss of cell proliferative capacity, preceding apoptosis induction was also observed and it was therefore concluded by the authors that the dose-dependent activity of BHA on Vero cells appears to be cytotoxic as well as cytostatic (Labrador et al., 2007).

In a study by Nakagawa et al. (1994), rat hepatocytes were used in the investigation of cytotoxic effects of BHA and the metabolites TBHQ and BHA-OH. Cell death was observed in a time-dependent manner. In the same study the effect of BHA and the metabolites on mitochondrial respiration was investigated in isolated hepatic mitochondria. It was shown that the cellular respiratory system is an important target for BHA and its metabolites as significant inhibition was observed for BHA, TBHQ and BHA-OH, with BHA-OH being most cytotoxic.

In a study by Saito et al. (2003), the human promyelocytic leukaemia cell line (HL-60) and the human squamous cell carcinoma cell line (HSC-2) were used in the investigation of cytotoxicity and

apoptosis induced by BHA and BHT. BHA alone and in combination with BHT was shown to be cytotoxic, more pronounced in the combination. This was also observed in the investigations of inter-nucleosomal DNA fragmentation, in the MnSOD mRNA expression analysis and in the activation of caspases 3, 8 and 9. In this study, BHA was shown to be a more potent inducer of cytotoxicity and apoptosis in combination with BHT. The authors stated that "*The great cytotoxicityy and apoptosis induction by BHA/BHT may be due to reactive intermediates derived from the interaction between BHA phenoxyl radical and BHT or BHT phenoxyl radical*".

The reactive oxygen species (ROS) scavenger activity of BHA was investigated by Festjens et al. (2006) in cell systems using L929 cells, inhibition of mitochondrial complex activity using spectrophotometric analysis and inhibition of lipoxygenase (LOX) activity. It was shown that BHA has ROS scavenging effects and the ability to inhibit complex I mitochondrial and LOX activity leading to a possible necrotic cell death.

In conclusion, a number of studies using different cell types have shown BHA to be cytotoxic and the inhibition of mitochondrial activity and induction of ROS activity to be central elements.

3.2.7.4. Special studies on endocrine disrupting effect

BHA was investigated for oestrogenic and androgenic activity using the immature rat uterotrophic assay and the Hershberger assay (Kang et al., 2005). In the immature rat uterotrophic assay, BHA at doses of 50, 100, 250, 500 mg/kg bw/day was administered subcutaneously to 20-day-old immature female Crj:CD (SD) rats for three consecutive days where 17beta-estradiol was the positive control. Anti-oestrogenic activity was investigated for BHA doses of 50 and 500 mg/kg bw/day followed by 17beta-estradiol administration (2 µg/kg). In all the BHA exposed groups, the absolute and relative uterine weights were significantly decreased. 17 Beta-estradiol-stimulated weights of uterine and vagina were also decreased by BHA (500 mg/kg), while uterine epithelial cell height was not affected. In a Hershberger assay, BHA was administered daily for 10 days at dose levels of 50, 100, 250, 500 mg/kg bw/day alone or with testosterone propionate to 51-day-old castrated male rats. No significant effect was observed. Overall, the data showed that BHA has anti-oestrogenic activity and a negligible effect on the androgenic activity in castrated male rats.

In a study by Zhu et al. (1997), the effect of BHA on liver microsomal enzyme activity on oestradiol or oestrone was investigated. BHA was administered in the diet to female CD-1 mice for 3 weeks at a dose level of 0.75% (equivalent to a dose of approximately 1125 mg/kg bw/day). BHA was shown to increase liver microsomal glucuronidation of oestradiol, oestrone, 4-aminophenol, and 4-nitrophenol by 103, 187, 162, and 92%, respectively. When a single i.p. injection of 100 or 300 ng of oestradiol or oestrone was given to ovariectomised CD-1 mice, measurable levels of oestradiol and oestrone were observed in the serum and uterus after 30 min. In comparison, a decrease by 30-60% was observed in mice prior to the injection of oestrogen, when mice were given a diet of 0.75% BHA diet for 18 days. When ovariectomised CD-1 mice were given a 0.75% BHA-supplemented diet for 18 days, a number of effects were seen in relation to the uterotrophic action. An inhibition of the uterotrophic effect of oestradiol or oestrone was observed together with an inhibition of oestradiol- or oestrone-stimulated [^{3}H]-thymidine incorporation into uterine DNA. Based on the obtained results, it was stated by the authors that an increased activity of liver microsomal enzymes that catalyze glucuronidation and oxidation of oestradiol and oestrone was observed when 0.75% BHA was supplemented in the diet to female CD-1 mice for 2-3 weeks. Furthermore, an enhanced *in vivo* metabolism of oestrogens was observed together with an inhibition of the uterotrophic action. In the oestrogen receptor-dependent proliferation of MCF-7 cells, BHA was shown to have the capacity to compete with 17-beta-estradiol. BHA induced a decrease in gene expression of ER alpha and an increase in that of progesterone receptor in a time-dependent manner. These effects were similar to that of 17-beta-estradiol, although much higher concentrations were required for BHA than for 17- beta-estradiol (Okubo and Kano, 2003).

The interaction of BHA and the human androgen receptor was investigated in a human prostate cancer cell line (recombinant human PC-3 prostate carcinoma cell line) (Schrader and Cooke, 2000). The effect of BHA was measured as the inhibition of the transcriptional activity of dehydrotestosterone. It was shown that BHA could have a possible anti-androgen effect, as BHA acted as an androgen antagonist in the assay. The possible estrogenic effect of BHA was investigated in an assay (named E-SCREEN by the authors) using the MCF-7 cell line transfected with an oestrogen-regulated luciferase construct (Soto et al., 1995). In the assay, BHA was shown to have oestrogen-like cell proliferation effects.

3.2.7.5. Special studies on induction of liver enzymes

The induction of Phase I and II drug-metabolizing enzymes, proteins and enzyme activity measurements by BHA was investigated in male Sprague-Dawley rats. Three rats were given diets containing 0.75% BHA (equivalent to a dose of approximately 375 mg/kg bw/day) and 6 rats were given normal diet for 3 days. Overall, induction of messenger ribonucleic acid (mRNA) correlated well with induction of protein levels and enzyme activity measurements. However, for BHA treated rats increased levels of hepatic mRNA encoding for the uridine 5′-diphosphate (UDP)-glucuronyl transferase isoenzyme UGT1*06 was seen, whereas little effect was noted on the expression levels of mRNA for cytochrome P450 enzymes (Buetler et al., 1995).

The induction of specific isozymes of cytochrome P450 was studied in groups of five male mice (ddY). Co-administration of BHA (0.2% equivalent to a dose of approximately 300 mg/kg bw/day) and flavonoid (0.1% flavone or 0.1% flavanone equivalent to a dose of approximately 120 mg/kg bw/day) in the diet for two weeks was shown to elevate CYP1A isozymes (Sun and Fukuhara, 1997).

The oxidative cytochrome P-450-mediated biotransformation of BHA was studied in four groups of six male Wistar rats fed a diet containing 0% and 2% BHA (equivalent to a dose of approximately 0 and 1000 mg/kg bw/day) for 2 weeks. Other groups, fed with 0% BHA and one 2% BHA-fed group of rats were injected i.p. daily with the cytochrome P-450 inducer phenobarbital (PB; 60 mg/kg) in saline. Additionally two groups were injected i.p. with saline only. Based on the results of TBHQ monitoring and LI in the forestomach, it was shown that the oxidative cytochrome P450-mediated demethylation of BHA into TBHQ does not correlate with the oral dose of BHA. Further, it was speculated by the authors that "oxidative cytochrome P-450-mediated biotransformation of BHA does not contribute to the tumorigenicity of BHA in rat forestomach" (Verhagen et al., 1989a).

The effects of dietary BHA on the enzyme activities of glutathione (GSH) S-transferase and catechol O-methyltransferase (COMT) were examined in thirty male Syrian golden hamsters and female ICR/Ha mice given diets containing 0, 0.25, 0.5, 1 or 2% BHA (equivalent to a dose of approximately 0, 300, 600, 1200, 2400 mg/kg bw/day). The effects were examined in the forestomach, small intestinal mucosa, and liver. Activity of GSH S-transferase in the forestomach of hamsters showed a dose-dependent increase which was not apparent in the liver or in the small intestinal mucosa. Similar activity was found in the COMT assay. In mice, an increased GSH S-transferase activity was observed in the liver and intestinal mucosa; there was also a dose-related increase in COMT activity in the forestomach and small intestinal mucosa. The data indicate that the detoxifying enzyme system is different in hamsters and mice in relation to formation of forestomach tumours caused by BHA (Lam, 1988).

The stimulation of superoxide formation by BHA in liver microsomes was investigated, especially in relation to the metabolite TBHQ in a study by Kahl et al. (1989). This metabolite was shown to auto-oxidize to TBQ. TBQ was shown to exceed TBHQ in its capacity for superoxide production in microsomes.

In conclusion, a number of studies have investigated the induction of liver enzymes showing that detoxifying enzyme systems are different in hamster and mice and the involvement of oxidative

cytochrome P-450-mediated biotransformation of BHA is not fully disclosed. It was also shown that microsomal superoxide formation was more pronounced for TBQ than TBHQ.

4. Discussion

The present opinion deals with the re-evaluation of the safety of BHA when used as a food additive.

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 an EFSA public call for data. BHA (E 320) is a synthetic antioxidant authorised as a food additive in the EU that was previously evaluated by JECFA several times, the latest in 1989 (JECFA, 1989) and the SCF in 1989 (SCF, 1989). Both committees established an ADI of 0.5 mg/kg bw/day, with that of the SCF being classified as temporary. The ADI defined by both the SCF and JECFA was based on proliferative changes in the rat forestomach.

BHA is a mixture of two isomers with full chemical names 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole. Specifications have been defined in the EU in Directive 2008/128/EC and by JECFA (JECFA, 2004). The purity is specified to be not less than 98.5% of $C_{11}H_{16}O_2$ and not less than 85% of the 3-tertiary-butyl-4-hydroxyanisole isomer.

In general, BHA is rapidly absorbed from the GI tract, metabolised and excreted in the form of metabolites mainly in urine and/or faeces. The major metabolites of BHA are the glucuronides, sulphates and free phenols including TBHQ. The proportions of the different metabolites vary in different species and also for different dose levels. The acute toxicity of BHA is low with an LD_{50} in mouse and rat > 2000 mg/kg bw/day.

In general, the majority of the genotoxicity studies indicate a lack of potential for BHA to induce point mutations or to interact with or damage DNA. BHA and its metabolite TBHQ have been reported to induce chromosomal aberrations *in vitro* in the presence of metabolic activation. The Panel recognised that the clastogenic activity exerted *in vitro* by BHA and TBHQ is likely to be secondary to the formation of reactive oxygen species via pro-oxidant chemistry (see also Dobo and Eastmond 1994), and that such a mechanism of genotoxicity is considered thresholded.

A large number of long-term toxicity and carcinogenicity studies have been performed with BHA, demonstrating proliferative changes in the forestomach. The studies have included species with and without a forestomach i.e. rats, mice, hamsters, guinea pigs and dogs. The Panel noted that gastric epithelial hyperplasias, papillomas and carcinomas were only seen in species with a forestomach.

A number of publications discuss the relevance of the observed incidences of rat forestomach squamous cell carcinomas in relation to man and discuss the mode of action. Overall, BHA does not seem to operate through a direct DNA-reactive mechanism and the tumour formation appears to involve tumour promotion. The mechanism underlying BHA induced cytotoxicity is not fully understood (Iverson, 1999; Parke and Lewis, 1992; Verhagen et al., 1991; Whysner, 1993; Whysner et al., 1994; Whysner and Williams, 1996). This was also discussed in two other references in which it was concluded that BHA is not DNA-reactive and the epigenetic mechanism of tumour formation appears to involve tumour promotion (Williams and Whysner, 1995, 1996).

The Panel concluded that the long term studies in rats (Ito et al., 1982, Ito et al., 1986a) and mice (Masui et al., 1986b) yield $BMDL_{10}$ values for forestomach hyperplasia of respectively 115 mg/kg bw/day (Ito et al., 1982), 83 mg/kg bw/day (Ito et al., 1986a) in rats and 245 mg/kg bw/day in mice (Masui et al. 1986b). Forestomach hyperplasia was the critical effect on which the SCF and JECFA based their ADI.

The Panel considered that humans do not have a forestomach and that forestomach hyperplasia in rodents may no longer be considered relevant for human risk assessment. The Panel noted that a new

reproductive and developmental toxicity study (Jeong et al., 2005) has been published since the previous SCF and JECFA evaluations. The Panel noted that the study was not performed according to OECD guidelines and that several of the endpoints studied are not part of regular OECD guidelines for testing reproductive and developmental toxicity. The Panel also noted that the decreases reported in the parameters that were stated to be affected were generally less than 10% without a clear dose response, and that the ranges reported for these parameters overlap due to large standard deviations. Thus the Panel concluded that the effects reported in this study that were statistically significant (at $p<0.05$) were not biologically relevant.

The Panel also noted that other studies gave a NOAEL for reproductive and developmental toxicity of 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day) in rats (Vorhees et al., 1981), and a NOAEL for reproductive and teratogenic parameters of 400 mg/kg bw/day in rabbits (Hansen and Meyer, 1978). The NOAEL in rats defined in the study of Vorhees et al. (1981) was based on pups showing growth retardation, increased mortality and behavioural effects at higher dose levels.

The Panel also noted that the potential endocrine effect of BHA has been investigated in a number of studies, but these were either performed at high dose levels (900 mg/kg bw/day) or *in vitro* (Kang et al., 2005; Zhu et al. 1997; Okubo and Kano 2003; Schrader and Cooke 2000; Soto et al., 1995).

Overall, the Panel concluded that the present database does give reason to revise the ADI of 0.5 mg/kg bw/day.

The Panel noted the potential endocrine effect of BHA which has been investigated in a number of studies. The Panel also noted that studies on reproductive and developmental toxicity in rats gave a NOAEL of 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day). This NOAEL was based on pups showing growth retardation, increased mortality and behavioural effects at higher dose levels. Based on a NOAEL of 100 mg/kg bw/day and using an uncertainty factor of 100 the Panel established an ADI of 1.0 mg/kg bw/day. Since the NOAEL of 100 mg/kg bw/day is in the range of the $BMDL_{10}$ values for forestomach hyperplasia in rats of respectively 115 mg/kg bw/day and 83 mg/kg bw/day the Panel concluded that this NOAEL also covers the $BMDL_{10}$ values for forestomach hyperplasia observed in the rat. This NOAEL was also below the dose of 500 mg/kg bw/day causing oesophageal basal cell proliferation in monkeys.

The dietary exposure to BHA at Tier 2 was estimated for adults to be 0.1 mg/kg bw/day on average and 0.14 mg/kg bw/ day at the 95th percentile and for children in the range of 0.1-0.3 mg/kg bw/day on average and 0.2-0.7 mg/kg bw/day at the 95th/97.5th percentile based on several assumptions made on the proportion of fat in different food categories and the maximum permitted levels of BHA added to the foods listed in Table 2. As no actual use levels were available for Tier 3 calculations and the assumptions made were rather conservative, the Tier 2 exposure estimates in total are very conservative and it is likely that actual exposure is considerably lower.

Exposure estimates to BHA at Tier 2 for children and the adult population at both the average and high level exposures are unlikely to exceed the ADI of 1.0 mg/kg bw/day.

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

CONCLUSIONS

The Panel concluded that the available database does give reason to revise the ADI of 0.5 mg/kg bw/day.

The Panel considered that forestomach hyperplasia in rodents may no longer be considered relevant for human risk assessment.

Based on a NOAEL of 100 mg/kg bw/day for growth retardation, increased mortality and behavioural effects in rat pups at higher dose levels, and using an uncertainty factor of 100 the Panel established an ADI of 1.0 mg/kg bw/day.

Exposure estimates to BHA at Tier 2 for children and the adult population at both the average and high level exposures are unlikely to exceed the ADI of 1.0 mg/kg bw/day.

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

DOCUMENTATION PROVIDED TO EFSA

MARS CHOCOLATE UK. Reply to EFSA "Call for scientific data on food additives permitted in the EU and belonging to the functional classes of preservatives and antioxidants" (published: 23 November 2009) on 23April 2010

Chemical Safety Group, Food Safety Authority of Ireland. Reply to EFSA "Call for scientific data on food additives permitted in the EU and belonging to the functional classes of preservatives and antioxidants" (published: 23 November 2009) on 11 May2010

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GLOSSARY AND ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	Absorption, distribution, metabolism and excretion
ALBA	Dutch database of food additives
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
ARL	Adult rat liver
ASA	Acetylsalicylic acid
BHA	Butylated hydroxyanisole
2-BHA	2-*tert*-butyl-4-hydroxyanisole
3-BHA	3-*tert*-butyl-4-hydroxyanisole
diBHA	BHA dimer; 2,2'-dihydroxy-3,3'-di-tert-butyl-5,5'-dimethoxy-1,1'-biphenyl
BHA-OH	3-*tert*-butyl-4,5-dihydroxyanisole
BHA-o-O	3-*tert*-butylanisol-4,5-quinone
BHT	Butylated hydroxytoluene
BMD	Benchmark Dose
BMDL	Lower confidence limit of the benchmark dose
BMDS	Benchmark Dose Software
BMR	Benchmark Response
BrdU	5-bromo-2'-deoxyuridine
CAS	Chemical Abstracts Service
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CI	Confidence interval
COMT	Catechol O-methyltransferase
ED	Endocrine disrupting
EFSA	European Food Safety Authority
EC	European Commission
ERK	Extracellular signal-regulated protein kinase
EU	European Union
EXPOCHI	Refers to EFSA Article 36 2008 call for Proposals Focused on Children and Food Consumption
GI	Gastrointestinal Tract
HL-60	Human promyelocytic leukaemia cell line
GOT	Glutamic-oxaloacetic transaminase
HPC	Hepatocyte primary culture
i.p.	Intraperitoneal

GPT	Glutamic-pyruvic transaminase
GSH	Glutathione
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HPLC	High Performance Liquid Chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
IARC	International Agency for Research on Cancer
JNK	c-Jun N-terminal kinase
LD_{50}	Median Lethal dose
LI	Labelling index
LOAEL	Lowest-Observed-Adverse-Effect Level
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
MPL	Maximum permitted levels
NEL	No-effect -level
NLCS	Netherlands Cohort Study
NOAEL	No-Observed-Adverse-Effect Level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PFC	Pair-fed controls
PND	Postnatal day
ROS	Reactive Oxygen Species
RR	Rate ratio
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
SCF	Scientific Committee on Food
TBHQ	*tert*-butylhydroquinone
TBQ	*tert*-butylquinone
TBQO	*tert*-butylquinone oxide
TLC	Thin Layer Chromatography
UDP	Uridine 5′-diphosphate
WBC	White Blood Cell