SCIENTIFIC OPINION

on the re-evaluation of caramel colours (E 150 a,b,c,d) as food additives[1]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The ANS Panel provides a scientific opinion re-evaluating the safety of the caramel colours (E150a (Class I), E150b (Class II), E150c (Class III), E150d (Class IV)) used as food additives. The caramel colours are a complex mixture of compounds produced by heating carbohydrates under controlled heat and chemical processing conditions; they are divided into four classes according to the manufacturing reactants used. The caramel colours were previously evaluated by the SCF and by JECFA, which concluded that a numerical ADI was not necessary for Class I, but established ADIs for the other classes of caramels, ranging from160-200 mg/kg bw/day. Given the consistency in the toxicological database, the Panel establishes a group ADI of 300 mg/kg bw/day for the caramel colours, by applying an uncertainty factor of 100 to a NOAEL of 30 g/kg bw/day (highest dose tested) identified in 13-week rat studies with Class IV and a similar NOAEL identified in a rat reproductive toxicity study, also with Class IV. Comparable NOAELS for Classes II, III and IV were reported in the SCF and JECFA evaluations. Within this group ADI, the Panel establishes an individual ADI of 100 mg/kg bw/day for Class III due to new information regarding the immunotoxicity of THI. The Panel concludes that the anticipated dietary exposure of child and adult populations may exceed the ADIs for Classes I, III and IV caramels, but exposure estimates to Class II were below the ADI. Exposure estimates for the caramel constituents THI, 4-MEI and SO_2 are not of concern, but the Panel welcomes additional studies to clarify remaining uncertainties regarding effects of THI on the immune system. The Panel notes that other constituents of caramel colours including 5-HMF and furan may be present at levels that may be of concern, and considers that the specifications should include maximum levels for these constituents.

KEY WORDS

Plain Caramel, Caustic Caramel, caramel colour I, E 150a, Caustic Sulphite Caramel, caramel colour II, E 150b, Ammonia Caramel, caramel colour III, E 150c, Sulphite Ammonia Caramel, caramel colour IV, E 150d, CAS Registry Number 8028-89-5, food colouring substance.

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2 Panel members: F. Aguilar, B. Dusemund, P. Galtier, J. Gilbert, D.M. Gott, S. Grilli, R. Gürtler, J. König, C. Lambré, J-C. Larsen, J-C. Leblanc, A. Mortensen, D. Parent-Massin, I. Pratt, I.M.C.M. Rietjens, I. Stankovic, P. Tobback, T. Verguieva, R.A. Woutersen. Correspondence: ans@efsa.europa.eu

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SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion re-evaluating the safety of caramel colours (E 150a,b,c,d) when used as food colouring substances.

Caramel colours are colouring substances authorised as food additives in the EU, and are classified according to the reactants used in their manufacture as follows: Class I Plain Caramel or Caustic Caramel (E 150a); Class II Caustic Sulphite Caramel (E 150b); Class III Ammonia Caramel (E 150c) and Class IV Sulphite Ammonia Caramel (E 150d).

The four classes of caramel colours have been previously evaluated by the EU Scientific Committee for Food (SCF), by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the Nordic Council of Ministers (TemaNord). Both JECFA and the SCF concluded that a numerical Acceptable Daily Intake (ADI) was not necessary for Class I Plain Caramel, considering that it contains no added ammonia or sulphite and that it is likely to be produced in normal cooking processes. For Class II Caustic Sulphite Caramel, JECFA established an ADI of 0-160 mg/kg bw/day, while the SCF included Class II Caustic Sulphite Caramel within the ADI of 200 mg/kg bw/day that it had already established for Class IV Sulphite Ammonia Caramel, based on information indicating that its chemical composition was similar to and intermediate between that of Class I Plain Caramel and Class IV Sulphite Ammonia Caramel. For Class III Ammonia Caramel, the SCF allocated an ADI of 200 mg/kg bw/day with the proviso that the content of the constituent 2-acetyl-4-tetrahydroxy-butylimidazole (THI) should not exceed 10 mg/kg colour on a colour intensity basis. JECFA has also allocated an ADI of 0-200 mg/kg bw/day to this colour, together with a specification for a maximum level for THI of 25 mg/kg caramel colour. For Class IV Sulphite Ammonia Caramel, both the SCF and JECFA have established an ADI of 200 mg/kg bw/day.

Specifications for the four classes of caramel colours have been defined in Commission Directive 2008/128/EC[4] and by JECFA (2006). The different classes of caramel colours are variously defined (in addition to their method of production) by the degree of binding to Diethylamino Ethyl (DEAE) cellulose and to phosphoryl cellulose (for Classes I Plain Caramel and Class III Ammonia Caramel), by the absorbance ratio (Classes II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel) and by their colour intensity. The solids content for the different classes range from 62-77% (Class I Plain Caramel), 65-72% (Class II Caustic Sulphite Caramel), 53-83% (Class III Ammonia Caramel) or 40-75% (Class IV Sulphite Ammonia Caramel). The maximum level of the constituent 4-methylimidazole (4-MEI), found in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel only, is restricted to $\leq$ 250 mg/kg caramel on a colour intensity basis under Commission Directive 2008/128/EC, while the constituent THI, found in Class III Ammonia Caramel only, is restricted to $\leq$ 10 mg/kg caramel on a colour intensity basis.

The Panel noted that the caramel colours are poorly characterised, and it is not clear whether the controls on manufacturing processes are sufficient to minimise batch-to-batch variability, particularly with respect to levels of individual Low Molecular Weight (LMW) constituents. The wide range of starting materials and reactants that may be used for the production of caramel colours may result in a variety of end products, with different physical, chemical and toxicological properties. The Panel noted that concerns about e.g. chemical composition, purity and similarity of various caramel colours have also been raised in the past by the SCF. The Panel also noted that a number of the identified or theoretical LMW constituents of caramel colours are genotoxic under certain experimental conditions and in some cases have carcinogenic potential, e.g. furan and 5-hydroxymethyl-2-furfural (5-HMF),

[4] Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. Official Journal of the European Communities, L 6, 10.1.2009, p.6.

which may be relevant to the toxicological profile of the caramel colours. The Panel considered that the toxicological studies carried out on specific caramel colours would have involved exposure to these compounds, and therefore the anticipated toxicological effect should have been detected in these studies, as exemplified by the toxicological profiles of Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel due to the presence of the imidazoles THI and 4-MEI

According to information from industry, slight variation of manufacturing process parameters (starting material, temperature and time) allows the production of a large range of different qualities of product within each caramel category and also results in differences in chemical composition and physical properties. This is further evidenced by variation in THI and/or 4-MEI concentrations in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, respectively. The Panel noted that there was limited information about the relationship between processing parameters for the caramel colours and the formation and nature of heat-derived constituents of these colours.

The Panel noted that data on the toxicokinetics of the caramel colours are very limited, but indicate little uptake of the high molecular weight fraction of the colours from the gastrointestinal tract, with the bulk of the material being excreted in the faeces. Specific data showed that the small fraction of Class IV Sulphite Ammonia Caramel that is absorbed has been shown to be distributed to lymphoreticular tissue, and eventually excreted in the urine. The Panel considered that individual constituents of the LMW fraction of caramel colours (e.g. MW less than 500 g/mol) are likely to be absorbed, although little information is available to confirm this assumption. The caramel colours are of low toxicity both in short-term tests and in chronic toxicity/carcinogenicity studies. The available short-term studies on Class I Plain Caramel, Class II Caustic Sulphite Caramel, Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, employing generally high dose levels in drinking water, show some dose-related effects, including reduced body weight gain associated with reduced food and fluid consumption, pigmentation of mesenteric lymph nodes, enlargement of the caecum, reduced urinary output associated with increases in specific gravity of the urine, diarrhoea and increases in caecal and kidney weights, unaccompanied by any histopathological change. Additionally, animal studies on Class III Ammonia Caramel have shown evidence of lymphocyte depression and other evidence of immunotoxicity, which are considered to be due to the presence of THI, a potent immunosuppressant, in this caramel.

The Panel concluded that the effects on body-weight were in part due to the reduced water intake caused by poor palatability of the drinking water rather than toxic effects of the caramel colours per se, and that the other effects seen were secondary both to the reduced fluid intake and the intake of large quantities of osmotically-active caramel material. The Panel considered that these effects are not of toxicological significance in establishing the safety of the caramel colours.

Caramel colours have been extensively tested for genotoxic potential in a variety of assays *in vitro* and *in vivo*. The results in *in vitro* systems were generally negative, with a few marginally positive findings, and no positive findings have been reported in *in vivo* assays. Overall the Panel concluded that there were no concerns regarding the genotoxic potential of caramel colours.

The findings in the long-term toxicity studies carried out with Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel were similar to, and did not reveal any pattern of toxicity not already seen in, the 90-day oral toxicity studies carried out with these caramel colours. No evidence of carcinogenicity was seen in 2-year studies in rats on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel. In a parallel study in mice, there was similarly no evidence for a carcinogenic potential of Class IV Sulphite Ammonia Caramel. The Panel noted that no long-term toxicity or carcinogenicity data were available for Class I Plain Caramel and Class II Caustic Sulphite Caramel. The Panel considered however that given the fact that Class I Plain Caramel is likely to be produced in normal cooking processes, also considering the long-term toxicity and carcinogenicity data available on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, and the rather similar toxicological profile of all the caramel colours, there are no concerns regarding the long-term

toxicity or carcinogenicity of Class I Plain Caramel or Class II Caustic Sulphite Caramel, nor regarding the carcinogenicity of Class III Ammonia Caramel or Class IV Sulphite Ammonia Caramel.

In relation to the reproductive and developmental toxicity of the caramel colours, the Panel noted that no data were available for Class I Plain Caramel and Class II Caustic Sulphite Caramel. However given the data available on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel and the toxicological similarities between all four classes of caramel colours, the Panel concluded that there are no concerns regarding reproductive and or developmental toxicity of Class I Plain Caramel or Class II Caustic Sulphite Caramel. There were no indications that either Class III Ammonia Caramel or Class IV Sulphite Ammonia Caramel can induce reproductive and or developmental toxicity in mice, rats, or rabbits following gavage administration at levels of up to 1600 mg/kg bw/day. In a reproductive toxicity study with Class IV Sulphite Ammonia Caramel in rats, at the top dose of 25% in the diet, equivalent to approximately 28 g/kg bw/day, pups showed a higher incidence of alopecia compared with pups in the control group and a generalized poor condition during the last 7 days of suckling. The number of implantation sites, litter size and of live pups at days 0, 4, and 21 of lactation in the 20%-dose group were significantly lower than control values, the Panel noted however that there was no dose-related trend, since in the 25% dose group these parameters were not statistically different from controls. The Panel considered that a No-Observed-Adverse-Effect-Level (NOAEL) of 25-30 g/kg bw/day for female rats could be identified in this study.

The Panel noted that no multigeneration study is available on any of the four classes of caramel colours.

Due to a lack of data, no definite conclusion can be drawn with respect to intolerance and allergenicity to the four classes of caramel colours under evaluation. The Panel noted, however that no cases of intolerance and allergenicity intolerance and allergenicity have been reported in published literature.

In relation to the potential haematotoxicity/immunotoxicity of caramel colours, effects have been identified in animal studies with Class III Ammonia Caramel, but not with the other classes of caramel colours. The Panel noted that lymphocytopenic and immunomodulatory effects have been seen in a number of studies with Class III Ammonia Caramel, and that the overall conclusion to be drawn from these studies is that THI, a constituent in Class III Ammonia Caramel, together with deficiency of pyridoxine (vitamin B_6), as a dietary influence, was primarily responsible for these effects. The Panel also noted that these effects were (apparently) transient in nature, disappearing in the later stages of longer-term studies with Class III Ammonia Caramel. The Panel noted that in the pivotal 90-day study of MacKenzie et al. on Class III Ammonia Caramel, in which rats were dosed with up to 20 g/kg bw/day caramel colour, containing either 15 mg THI/kg caramel or 295 mg THI/kg caramel, on a solids basis, no dose-related lymphocytopenia occurred in the animals fed caramel containing approximately 15 mg THI/kg. The latter level is higher than the current maximum level for THI laid down in the specifications for Class III Ammonia Caramel. Class III Ammonia Caramel containing 295 mg THI/kg, at a dose level of 20 g/kg bw/day, induced a statistically significant decrease in lymphocyte counts in both sexes at 2 weeks and only in male rats at 6 weeks. All lymphocyte values in these groups were normal at the termination of the study. The Panel noted however that the results of a short-term oral study carried out by Thuvander and Oskarssen indicated that Class III Ammonia Caramel that meets the limit of less than 25 mg THI/kg established in the JECFA specifications may, nevertheless, interfere with the lymphoid system in mice with an adequate vitamin B_6 status.

The 90-day oral toxicity study on Class III Ammonia Caramel in rats carried out by Mackenzie et al. included an evaluation of the toxicity of THI after a 4-week dosing period. In rats maintained on a normal, pyridoxine-replete diet, the short-term NOAEL of THI for the reduction of total lymphocytes was determined to be 120 µg/kg bw/day in female rats, and 380 µg/kg bw/day in male rats. Houben et al. reported intakes of THI alone or Class III Ammonia Caramel containing THI that, in combination with manipulation of dietary pyridoxine levels, resulted in lymphocytopenia and could therefore be regarded as Low-Observed-Adverse-Effect-Levels (LOAELs) for this effect. These ranged from 57.2

µg THI/kg bw/day (provided by a level of 0.4% Class III Ammonia Caramel in drinking water, in rats maintained on a low-pyridoxine diet, 2-3 mg/kg diet) to levels of 200 µg THI/kg bw/day or higher. Sinkeldam and co-workers reported decreases in lymphocyte counts in rats receiving 0.1% Caramel Colour (III) in drinking water, equivalent to 20 µg THI/ kg bw/day, for 1 week and maintained on a low-pyridoxine diet (2-3 mg/kg diet). The Panel considered that these findings in pyridoxine-deficient rats may be of limited relevance for human health risk assessment. Overall the Panel concluded from these results that a NOAEL for the lymphocytopenic effects of THI in pyridoxine-replete rats lies in the range of 120-400 µg/kg bw/day as indicated by the studies of MacKenzie et al, Sinkeldam et al and Houben et al.

The Panel noted that, in contrast to the findings in rats and mice, in the available short-term human studies consumption of Class III Ammonia Caramel had no effects on total or specified white blood cell counts at dose levels of up to 200 mg/kg bw/day (with THI levels almost 20 times above those permitted according to current specifications) albeit following short term exposure periods. The study of Houben et al. included subjects with (mild) pyridoxine deficiency, and the authors, in comparing the results obtained in this study with data in rats maintained on normal and pyridoxine-reduced diets, suggested that, with regard to oral intake of THI, humans are less sensitive to Class III Ammonia Caramel-induced lymphocytophenia than are rats. The Panel agreed with this interpretation, based on the available data.

The Panel noted that another imidazole constituent of Class III Ammonia Caramel, 4-MEI, which is also found in Class IV Sulphite Ammonia Caramel, is considered to be responsible for the convulsions observed after administration of high doses of this caramel to a range of species. The Panel considered that the acute toxicity of 4-MEI is not of toxicological concern since the maximum level of 4-MEI is restricted to $\leq$ 250 mg/kg in these caramel colours under Commission Directive 2008/128/EC. The Panel noted that 4-MEI has been demonstrated to have a carcinogenic potential in mice in a recent National Toxicology Programme (NTP) study. The Panel considered, however, that the carcinogenic effect of 4-MEI seen in mice in this study was thresholded, based on the lack of genotoxicity of 4-MEI, also noting that alveolar/bronchiolar neoplasms occur spontaneously at high incidence in B6C3F1 mice. The Panel concluded therefore that the intermediate dose of 625 mg 4-MEI/kg diet, equivalent to 80 mg 4-MEI/kg bw/day could be considered to be a NOAEL in this study.

The Panel, in evaluating the overall toxicological database on the four classes of caramel colours, considered that while the potential constituents 4-MEI (present in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel) and THI (found in Class III Ammonia Caramel only) must be taken into account in the safety evaluation of these caramel colours as food additives, as discussed above, all four caramel colours are otherwise similar in their toxicological effects. The Panel also noted that the effects produced by the caramel colours can be anticipated to be additive in nature.

The Panel considered that, in spite of the absence of full chemical characterisation of the four classes of caramel colours, given the consistency in the toxicological database, the caramel colours can be considered as a single group in terms of assessing their safety. The Panel considered therefore that a group ADI can be established for the caramel colours. Given, however, concerns regarding the immunotoxicity of THI, present in Class III Ammonia Caramel, the Panel decided to define an individual ADI for this caramel within the overall group ADI based on the currently available database. While the Panel noted the toxicological data gaps for caramel colours, including the generally sparse database on reproductive toxicity for caramel colours as a whole and the absence of long-term toxicity and carcinogenicity studies on Class I Plain Caramel and Class II Sulphite Caramel, the Panel considered that missing data for these classes of caramel colours can be accounted for by data from another class.

The Panel considered that several studies carried out in rats with the different caramel colours are relevant for establishment of a group ADI for the caramel colours. These include the three studies used previously by JECFA and SCF to define the respective ADIs for the individual Classes:

- a 90-day study with Class II Caustic Sulphite Caramel, providing a NOAEL of 16 g/kg bw/day, the highest dose tested,
- a 90-day study with Class III Ammonia Caramel, providing a NOAEL of 20 g/kg bw/day, the highest dose tested,
- a 2-year oral toxicity study in rats with Class IV Sulphite Ammonia Caramel, providing a NOAEL of 10 g/kg bw/day, the highest dose tested.

The Panel considered that several additional toxicological studies should be taken into account when establishing the group ADI, and these include:

- a 13-week toxicity study in rats with Class IV Sulphite Ammonia Caramel in drinking water , providing a NOAEL of 30 g/kg bw/day, the highest dose level tested,
- another 13-week drinking water study in rats with Class IV Sulphite Ammonia Caramel providing a NOAEL of 30 g/kg bw/day, the highest dose level tested,
- a 90-day study in Beagle dogs with Class IV Sulphite Ammonia Caramel providing a NOAEL of 6.25 g/kg bw/day, the highest dose tested,
- a 96-week drinking water study in mice with Class III Ammonia Caramel, providing a NOAEL of 8.4 g/kg bw/day, the highest dose tested,
- a 2-year dietary study in rats with Class III Ammonia Caramel providing a NOAEL of 3 g/kg bw/day, the highest dose tested,
- a 104-week study in rats with Class III Ammonia Caramel, providing a NOAEL of 2 g/kg bw/day, the highest dose tested,
- a reproductive toxicity study in rats with Class IV Sulphite Ammonia Caramel providing in the dams a NOAEL of 25-30 g/kg bw/day.

Given that:

- the NOAELs in all these studies were the highest dose levels tested,
- the effects of the caramel colours in 90-day studies were generally similar to those reported in the long-term studies,
- available reproduction and developmental toxicity studies, although limited, do not reveal any effects of concern,
- the studies reveal no effects on the reproductive organs,
- the effect of most concern, i.e. lymphocytopenia, can, as also stated by JECFA, best be evaluated from short-term studies, and
- the long-term studies support the conclusion that the caramel colours are not carcinogenic,

the Panel decided to use the highest NOAEL of 30 g/kg bw/day reported in several of these studies, still the highest dose level tested, as the basis to derive a group ADI for the caramel colours. The Panel

noted that whilst there were arguments for increasing the default uncertainty factor of 100, to compensate for limitations in the toxicological databases on reproductive toxicity, equally compelling arguments could be advanced for deriving a chemical-specific adjustment factor below the default uncertainty factor. The Panel therefore applied an uncertainty factor of 100 to the NOAEL of 30 g/kg bw/day to derive a group ADI of 300 mg/kg bw/day. Overall, based on the available database, the Panel considered that this would provide a sufficient margin of safety.

In relation to Class III Ammonia Caramel, the Panel considered the available data on the immunotoxicity of THI, a constituent of this caramel class only. The Panel noted that no dose-related effects on haematological parameters were reported in the 90-day study of MacKenzie, using a Class III Ammonia Caramel containing a THI level of 15 mg/kg, while with a Class III Ammonia Caramel containing a much higher level of THI of 295 mg/kg, only transient effects on lymphocytes were seen. The study of MacKenzie provided a NOAEL of 20 g/kg bw/day for Class III Ammonia Caramel, the highest dose tested. The Panel also noted, however, the results of the study of Thuvander and Oskarssen, indicating that Class III Ammonia Caramel that meets the limit of less than 25 mg THI/kg established in the JECFA specifications may, nevertheless, interfere with the lymphoid system in mice with an adequate vitamin B_6 status. While the Panel considered that this study should not be used as a pivotal study for the purposes of risk assessment without further substantiation, given a number of studies in rats showing no effect on haematological parameters over longer periods and at higher dose levels than those used in the study of Thuvander and Oskarssen, the Panel considered that it should be taken into account in establishing an ADI for Class III Ammonia Caramel. The Panel applied an additional uncertainty factor of 2 together with the default uncertainty factor of 100 to the NOAEL of 20 g/kg bw/day identified from the MacKenzie study. The Panel therefore establishes, within the group ADI for all caramel colours and based on the currently available database, an ADI of 100 mg/kg bw/day for Class III Ammonia Caramel.

The Panel noted that this means that within the group ADI of 300 mg/kg bw/day established for the four caramel colours, only 100 mg/kg bw/day of this 300 mg/kg bw/day can be made up by Class III Ammonia Caramel.

The exposure assessment 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. As caramel colours Class I, II, III and IV are authorised *quantum satis* in almost all categories, the refined exposure estimates have been performed only for Tier 3 using the maximum reported use levels or when no usages were reported to EFSA, values defined by decision rules for *quantum satis* usages were used.

Exposure estimates for children (1-14 years old) have been done by the Panel for 11 European countries (Belgium, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Denmark, Cyprus, Greece) based on detailed individual food consumption data provided by the EXPOCHI consortium. As the UK is not part of the EXPOCHI consortium, estimates for UK children (aged 1.5- 4.5 years) were made by the Panel with the use of detailed individual food consumption data available from the Union of European Beverage Associations (UNESDA) report. For the adult population, the Panel has selected the UK population as representative of the EU consumers for estimates of exposure.

The mean dietary exposure of European children including UK pre-school children ranged from 76.9 to 427.2 mg/kg bw/day for Class I Plain Caramel, from 8.7 to 34.6 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 21.7 to 302.4 mg/kg bw/day for Class III Ammonia Caramel, and from 23.2 to 506.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel. At the 95th or 97.5th percentile, estimates ranged from 179.6 to 882.2 mg/kg bw/day for Class I Plain Caramel, from 18.5 to 117.3 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 107.9 to 757.3 mg/kg bw/day for Class III Ammonia Caramel, and from 129.7 to 1480.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors (>10% in all or several countries) to the total anticipated exposure of children were for Class I Plain Caramel: non alcoholic flavoured drinks (12% to 55%), fine bakery wares (15% to 32%), desserts including flavoured milk products (11% to 48%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 56%), soups (11% to 32%) and malt bread (16% to 49%). For Class II Caustic Sulphite Caramel the main contributors were fine bakery wares (12% to 53%), desserts including flavoured milk products (11% to 41%), edibles ices (11% to 22%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 45%), soups (18% to 54%) and malt bread (19% to 55%). For Class III Ammonia Caramel the main contributors were fine bakery wares (13% to 45%), desserts including flavoured milk products (12% to 44%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 79%), and vinegar (12% to 45%), while in one country non alcoholic flavoured drink, malt bread confectionery, and sausages, pates and terrines contributed 29%, 15%, 13% and 10%, respectively. For Class IV Sulphite Ammonia Caramel the main contributors were non alcoholic flavoured drinks (13% to 51%), confectionery (20% to 81%), fine bakery wares (10% to 29%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (10% to 24%), and malt bread (10% to 34%).

The anticipated dietary exposure reported for the UK adult population gives a mean of 136.6 mg/kg bw/day and a 97.5th percentile of 429.3 mg/kg bw/day for Class I Plain Caramel; a mean of 21.7 mg/kg bw/day and a 97.5th percentile of 109.5 mg/kg bw/day for Class II Caustic Sulphite Caramel; a mean of 60.8 mg/kg bw/day and a 97.5th percentile of 295.0 mg/kg bw/day for Class III Ammonia Caramel; and a mean of 89.4 mg/kg bw/day and 97.5th percentile of 368.9 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors to the total anticipated exposure of adults were for Class I Plain Caramel non alcoholic flavoured drinks (30%), beer and cidre bouché (27%), soups (16%), and sauces, seasonings (e.g. curry powder, tandoori) and pickles (10%). For Class II Caustic Sulphite Caramel the main contributors were beer and cidre bouché (50%) and soups (20%). For Class III Ammonia Caramel the main contributors were beer and cidre bouché (48%) and sauces, seasonings (e.g. curry powder, tandoori) and pickles (22%). For Class IV Sulphite Ammonia Caramel the main contributors were confectionery (65%) and non alcoholic flavoured drinks (23%).

The Panel also evaluated combined anticipated dietary exposure to all four classes of caramel colours, taking into account the highest maximum reported level for all caramel classes, described in Table 8, from each food category. When considering this scenario, as presented in Table 10, anticipated mean combined dietary exposure reported for European children, including UK pre-school children, ranged from 83.5 to 698.3 mg/kg bw/day. At the 95th/97.5th percentile, estimates ranged from 224.8 to 1672.3 mg/kg bw/day. For the UK adult population this scenario gave a range of exposures of 194.8 and 474.3 mg/kg bw/day for the mean and the 97.5th percentile, respectively.

The main contributors to the total combined anticipated exposure to caramel colours for children were non alcoholic flavoured drinks (11% to 28%), confectionery (19% to 58%), fine bakery wares (15% to 29%), desserts including flavoured milk products (10% to 31%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (14% to 44%), and malt bread (16% to 46%). Soups were estimated to contribute from 25% to 28% in two countries and vinegar was estimated to contribute 20% in one country. For the adult population the main contributors (>10%) were confectionery (30%), non alcoholic flavoured drinks (21%), beer, cidre bouché (19%) and soups (11%).

The Panel noted that the anticipated dietary exposure of the adult population at the 97.5th percentile to Class I Plain Caramel exceeds the group ADI of 300 mg/kg bw/day proposed for the caramel colours. Similarly, the anticipated dietary exposure of the adult population at the 97.5th percentile to Class IV Ammonia Caramel exceeds this group ADI. For children, the upper end of both the mean intake ranges and also the 95th/97.5th percentile intakes for Class I Plain Caramel exceed the group ADI of 300 mg/kg bw/day. Similarly, for children, the upper end of both the mean intake ranges and also the

95th/97.5th percentile intakes for IV Sulphite Ammonia Caramel exceed the group ADI of 300 mg/kg bw/day.

The anticipated dietary exposure to Class II Sulphite Caramel for both adults and children was below the group ADI of 300 mg/kg bw/day.

For Class III Ammonia Caramel the upper end of the mean intake range for children exceeds the individual ADI of 100 mg/kg bw/day established for this colour within the group ADI, while the 97.5th percentile anticipated dietary exposures of both the child and adult populations are above this ADI of 100 mg/kg bw/day.

The Panel noted that anticipated combined dietary exposures of both adults and children to all caramel colours exceed the group ADI of 300 mg/kg bw/day at the 95th/97.5th percentile, while the ADI is also exceeded by the combined mean intake for children. In the case of children, this exceedance applies to the upper end of the exposure range only.

Reflecting the concerns regarding the immunotoxicity seen in a number of studies with either Class III Ammonia Caramel or with THI alone, found as a constituent in Class III Ammonia Caramel, and the carcinogenicity of 4-MEI, found as a constituent in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, the Panel has estimated exposure to THI and 4-MEI as a result of consumption of these caramel colours in the diet. Additionally the Panel has also estimated exposure to sulphur dioxide, present in Class II and Class IV caramel colours as a result of the production method. The Panel concludes overall that the exposure estimates for THI, 4-MEI or sulphur dioxide are not of concern, but notes remaining uncertainties regarding the effects of THI on the immune system. The Panel would welcome additional studies to clarify these effects.

The Panel notes that variations in the manufacturing processes of the caramel colours may result in a wide variability in the nature and levels of the various constituents, including constituents of toxicological concern such as 5-HMF and furan. Given this likely variability, the Panel considers that in order to further guarantee the safety of caramel colours with respect to their minor constituents, such as THI, 4-MEI, 5-HMF and furan, it would be prudent to reduce their levels as much as technologically feasible. The Panel considers therefore that the specifications for the caramel colours should be updated and extended to also include maximum levels for constituents of possible concern not yet included in the specifications, such as for example 5-HMF and furan.

The Panel additionally concludes that there is limited information about the relationship between processing parameters for the caramel colours and the formation and nature of heat-derived constituents, which is also relevant for the control of manufacturing processes. Future research work is recommended in this respect.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to the Framework Directive 89/107/EEC[5] on food additives, the Scientific Committee for Food (SCF) should be consulted before the adoption of provisions likely to affect public health, such as the drawing up of lists of additives and the conditions for their use. Accordingly, all food additives, prior to their authorization, have been evaluated for their safety by the SCF or by its successor the European Food Safety Authority (EFSA).

Directive 89/107/EEC as well as Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives[6] which applied as from 20 January 2010, require that food additives must be kept under continuous observation and must be re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. In addition Regulation (EC) No 1333/2008 requires that all food additives which were permitted before 20 January 2009 shall be subject to a new risk assessment carried out by EFSA.

In accordance with Regulation (EC) No 1333/2008, the Commission should, after consultation with EFSA, set up by 20 January 2010 an evaluation programme for EFSA to re-evaluate the safety of the permitted food additives. That programme will define the needs and the order of priorities according to which the approved food additives are to be examined.

Food colours were among the first additives to be evaluated, therefore many of the evaluations are old. For some of these colours new studies have become available and the results of these studies should be included in the evaluation. Therefore, food colours should be evaluated with priority. The order of priorities for the re-evaluation of the remaining permitted food additives will be set in the Regulation for the re-evaluation program.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to start a systematic re-evaluation of all authorised food additives and to issue scientific opinions on these additives, taking into account that colours as a group should be given the highest priority for re-evaluation for the reasons outlined above.

[5] OJ L 40, 11.2.1989, p. 27
[6] OJ L 354, 31.12.2008, p. 16.

ASSESSMENT

1. INTRODUCTION

The present opinion deals with the re-evaluation of the safety of caramel colours (E 150a,b,c,d) when used as food colouring substances.

Caramel colours are colouring substances authorised as food additives in the EU. The caramel colours are divided into four classes, Class I, Class II Class III, and Class IV, according to the reactants used in their manufacture. The four classes of caramel colours have been previously evaluated by the EU Scientific Committee for Food (SCF) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Nordic Council of Ministers have reviewed the caramel colours in a report which takes into account the literature published on these colouring substances up to the year 2000 (TemaNord, 2002).

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

2. TECHNICAL DATA

2.1. Identity of the substances

The caramel colours are a complex mixture of compounds produced by heating carbohydrates under controlled heat and chemical processing conditions (JECFA, 2006). They are dark brown to black liquids or solids having an odour of burnt sugar and a pleasant, somewhat bitter taste (Kamuf et al, 2003; JECFA, 2006). They are miscible with water and contain colloidal aggregates that account for most of their colouring properties and characteristic behaviour towards acids, electrolytes and tannins (Kamuf et al., 2003; JECFA, 2006).

The caramel colours are covered by the general CAS Registry number of 8028-89-5 and EINECS number 232-435-9. They have no specific chemical name, structural or chemical formula. The caramel colours are grouped into four classes according to the reactants used in their manufacture as described in section 2.3: Manufacturing Process, and synonyms used for the four classes of caramel colours are as follows:

- Class I Plain Caramel (E 150a): caramel colour I, caustic caramel and spirit caramel.
- Class II Caustic Sulphite Caramel (E 150b): caramel colour II and caustic sulphite process.
- Class III Ammonia Caramel (E 150c): caramel colour III, ammonia process caramel, closed-pan ammonia process caramel, open-pan ammonia process caramel, bakers' caramel, confectioners' caramel, and beer caramel.
- Class IV Sulphite Ammonia Caramel (E 150d): caramel colour IV, Sulphite Ammonia process caramel, sulphite ammonia process, acid-proof caramel, beverage caramel, and soft-drink caramel.

Each caramel colour carries an isoelectric charge which is different for each class (Licht et al., 1992a).

Dross and Baltes (1989), Licht et al. (1992a) and others describe caramel colours as complex mixtures with a composition approximating that shown below:

Mass Range (g/mol)	Composition
0-1000	56-60%
1000-10 000	ca. 20%
>10 000	20-30%

In the analysis of the Low Molecular Weight (LMW) fraction (constituents with MW below 1000 g/mol) of Caramel Class IV, five of the major constituents have been identified as disaccharides, glucose, 1,6-anhydroglucose, laevulinic acid and 5-hydroxymethyl-2-furfural (5-HMF) (Licht et al.,1992c). 5-HMF is used as a marker substance of the LMW fractions (Licht et al., 1992 b-c). Licht and co-workers found 5-HMF in all 157 samples of Caramel Class I, II, III and IV analysed. For Class I, levels of 5-HMF ranged between 700 and 27 300 mg/kg on an as is basis (9 samples analysed); for Class II, levels were between 3300 and 33 700 mg 5-HMF/kg (18 samples analysed); for Class III, between 10 and 3900 mg 5-HMF/kg reported; Class IV, between 4900 and 21 400 mg 5-HMF/kg reported (90 samples analysed) (Licht et al., 1992b-c; EUTECA, 2011a,b). Another study indicated that caramel products may contain 110-9500 mg 5-HMF/kg (Murcovic and Pichler, 2006).

The Panel noted that extensive research has been carried out into the chemistry of the browning process of complex foods (e.g. the Maillard reaction (O'Brien et al, 1998)), and also into the chemistry of caramelisation of sugars such as sucrose (Kitts et al., 2006), with identification of LMW substances such as acrylamide, 5-HMF, 2-furfyl methyl ketone and 5-methyl-2-furfyl methyl ketone, which have either also been detected in the caramel colours or are theoretical LMW constituents of these mixtures. LMW constituents of caramel colours produced with ammonia include *N*-heterocyclic compounds such as pyridines, pyrazines, pyrroles and imidazoles (Patey et al., 1987; Myers and Howell, 1992) although no quantitative data are available (EUTECA, 2011b). The occurrence of furanoid compounds has also been reported (Meyers and Howell, 1992), with furan being specifically identified in some caramel colours e.g. Class IV Sulphite Ammonia Caramel (Bononi and Tateo, 2007). Industry has reported that levels of furan up to 0.18 mg/kg caramel colour have been detected (Class I (1 sample): 0.15 mg/kg; Class II (1 sample): 0.05 mg/kg; Class III (1 sample): 0.18 mg/kg; Class IV (28 samples): average: 0.02 mg/kg, maximum: 0.06 mg/kg) (EUTECA, 2010b; EUTECA, 2011).

Industry has reported that acrylamide was not detectable in 16 commercial samples of Caramel Class IV at a limit of detection of 1 ug/kg caramel colour (EUTECA, 2011a,b). Caramel Classes I, II and III have not been investigated in this study, the formation of acrylamide not being expected in caramel classes I or II due to the lack of a nitrogen source in their manufacturing process (EUTECA, 2011a). In a single sample of Caramel Class III analysed, acrylamide was not detectable at a limit of detection of 100 µg/kg caramel colour (EUTECA, 2011b).

Furthermore industry presented recent analytical data of caramel colours manufactured in the years between 2008 and 2010 on levels of 2-acetyl-4-tetrahydroxy-butylimidazole (THI) (Class III (45 samples): mean: 7.7 mg/kg, range: 2.4–10 mg/kg) and 4-methylimidazole (4-MEI) (Class III (103 samples): mean: 41.6 mg/kg, range: 5–140 mg/kg; Class IV single strength (35 samples): mean: 102.4 mg/kg, range: 48–183 mg/kg; Class IV double strength (152 samples): mean: 88.3mg/kg, range: 22.7–147 mg/kg). The data on the occurrence of heat-derived constituents in caramel colours which have been made available by industry are compiled in Table 1.

Table 1: Occurrence of heat-derived constituents in Caramel Classes I, II, III and IV according to data provided by industry (EUTECA, 2011)

	Class I	Class II	Class III	Class IV
5-HMF	700 – 27 300 mg/kg (n = 9)	3300 – 33 700 mg/kg (n = 18)	10 – 3900 mg/kg (n = 40)	4900– 21 400 mg/kg (n = 90)
Furan	151 µg/kg (n = 1)	52 µg/kg (n= 1)	177 µg/kg (n = 1)	Maximum: 59 µg/kg (n = 28)
THI	No data	No data	2.4 – 10 mg/kg (n = 45)	No data
4-MEI	No data	No data	5 - 140 mg/kg (n = 103)	Single strength: 48 -183 mg/kg (n = 35) Double strength: 23-147 mg/kg (n = 152)
Acrylamide	No data	No data	Not detected (limit of detection = 100 µg/kg) (n = 1)	Not detected (limit of detection = 1 µg/kg) (n = 16)
Otherfuran-, pyridines-, pyrazines- and imidazole-derivatives	No data	No data	No data	No data

The Panel noted that the complexity of caramel colours means that they are rather poorly characterised, and it is not clear whether manufacturing processes are sufficiently controlled to guarantee limited batch-to-batch variability of both LMW and higher molecular weight constituents. The wide range of starting materials and reactants that may be used for the production of caramel colours may result in a variation of end products, with different physical and chemical properties. Concerns about e.g. chemical composition, purity and similarity of various caramel colours have also been raised in the past by the SCF although overall the SCF noted that "*although the caramelisation reaction is a complex one, and many different compounds are formed, especially in the presence of ammonia, within a given class there is a broad similarity in qualitative chemical composition*" (SCF, 1989).

According to information from industry, slight changes in time and temperatures of the reaction, or in starting materials, can produce changes in the chemical composition and physical properties of the final product (EUTECA, 2010b; EUTECA, 2011a). Furthermore industry indicated that variation of manufacturing process parameters allows the production of a large range of different qualities of products within each caramel class. Concerning Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel there is also a wide range of products made by caramel manufacturers, their differences in chemical composition including some variation in THI and/or 4-MEI concentrations, respectively (EUTECA, 2011a).

The Panel noted that there was limited information about the detailed relationship between processing parameters for the caramel colours and the formation and nature of heat-derived constituents. The Panel also noted that a number of the identified or theoretical constituents of caramel colours, e.g. furan, acrylamide and 5-HMF, are genotoxic under certain experimental conditions and in some cases have carcinogenic potential, which may be relevant to the toxicological profile of the caramel colours. The Panel considered however that the toxicological studies carried out on the caramel colours would

have involved exposure to these compounds, and therefore the anticipated toxicological effect due to the LMW fraction constituents should have been detected in these studies.

2.2. Specifications

Specifications for the four classes of caramel colours have been defined in Commission Directive 2008/128/EC[7] and by JECFA (2006) (Tables 2-5). The different classes of caramel colours are variously defined (in addition to their method of production) by the degree of binding to Diethylamino Ethyl (DEAE) cellulose in an acidic solution (0.025 *N* HCl) (Licht et al., 1992a; JECFA, 2006; Directive 2008/128/EC). Under these conditions Class II Sulphite Caramel and Class IV Sulphite Ammonia Caramel are negatively charged and bind to the positively charged DEAE cellulose, while Class III Ammonia Caramel, being positively charged, and Class I Plain Caramel, carrying no net charge, do not bind to DEAE cellulose (Licht et al., 1992a).

Classes I Plain Caramel and Class III Ammonia Caramel are differentiated from each other by binding to negatively charged phosphoryl cellulose, the positively charged Class III Ammonia Caramel showing binding, while Class I Plain Caramel shows little or no binding (Licht et al., 1992a). The absorbance ratio (A280/560) is used to further distinguish between Classes II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel, while the absorbance ratio of the coloured constituents of the various caramel colours (other than Class I Plain Caramel) bound either to DEAE cellulose or to phosphoryl cellulose provides further differentiation between the classes (Licht et al., 1992a). The solids content for the different classes range from 62-77% (Class I Plain Caramel), 65-72% (Class II Caustic Sulphite Caramel), 53-83% (Class III Ammonia Caramel) or 40-75% (Class IV) (JECFA, 2006).

The caramel colours are also defined in terms of their colour intensity, colour intensity being expressed as the absorbance of a 0.1% (w/v) solution of caramel colour solids in water in a 1 cm cell at 610 nm (Licht et al., 1992a; JECFA, 2006; Directive 2008/128/EC). The colour intensities of the various classes are specified as follows: Class I Plain Caramel: 0.01-0.12; Class II Caustic Sulphite Caramel 0.06-0.10; Class III Ammonia Caramel: 0.08-0.36; and Class IV Sulphite Ammonia Caramel: 0.10-0.60 (JECFA, 2006). The Panel noted that the specifications for colour intensity laid down in Directive 2008/128/EC are identical to those of JECFA (2006), except for those for Class II Sulphite Caramel, for which Directive 2008/128/EC specifies a range of 0.05 – 0.13. The latter range is also proposed for Class II Sulphite Caramel by Licht et al. (1992a).

Table 2: Specifications for Class I Plain Caramel according to Commission Directive 2008/128/EC and JECFA (2006)

Purity	**Commission Directive 2008/128/EC**	**JECFA (2006) ([2])**
Colour bound by DEAE cellulose	≤ 50 %	≤ 50 %
Colour bound by phosphoryl cellulose	≤ 50 %	≤ 50 %
Solid content	-	62 – 77 %
Colour intensity	0.01 - 0.12[(1)]	0.01 – 0.12
Total nitrogen	≤ 0.1 %	≤ 0.1 %
Total sulphur	≤ 0.2 %	≤ 0.3 %
Arsenic	≤ 1 mg/kg	≤ 1 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb) [(3)]	≤ 25 mg/kg	-

7 Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. Official Journal of the European Communities, L 6, 10.1.2009, p.6.

([1]) Colour intensity is defined as the absorbance of a 0.1 % (w/v) solution of caramel colour solids in water in a 1 cm cell at 610 nm.
([2]) Arsenic and lead metals limits are expressed on the basis of the product as is; other limits and ranges are, unless otherwise stated, expressed on a solids basis.
([3]) The specifications for total heavy metals (as Pb), of < 25 mg/kg will be deleted from the specifications for caramel colours in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008 and repealing Directives: 2008/128/EC (purity criteria concerning colours), 2008/84/EC (purity criteria on food additives other than colours and sweeteners) as amended and 2008/60/EC (purity criteria concerning sweeteners), currently under discussion.

Table 3: Specifications for Class II Caustic Sulphite Caramel according to Commission Directive 2008/128/EC and JECFA (2006)

Purity	**Commission Directive 2008/128/EC**	**JECFA (2006) [(3)]**
Colour bound by DEAE cellulose	> 50%	> 50%
Solid content	-	65 – 72 %
Colour intensity	0.05 – 0.13[(1)]	0.06 – 0.10
Total nitrogen	≤ 0.3 % [(2)]	≤ 0.2 %
Sulphur dioxide	≤ 0.2 % [(2)]	≤ 0.2 %
Total sulphur	0.3 – 3.5 %[(2)]	1.3 – 2.5 %
Sulphur bound by DEAE cellulose	> 40 %	-
Absorbance ratio of colour bound by DEAE cellulose	19 – 34	-
Absorbance ratio (A 280/560)	> 50	> 50
Arsenic	≤ 1 mg/kg	≤ 1 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb) [(4)]	≤ 25 mg/kg	-

([1]) Colour intensity is defined as the absorbance of a 0.1 % (w/v) solution of caramel colour solids in water in a 1 cm cell at 610 nm.
([2]) Expressed on equivalent colour basis, i.e. expressed in terms of a product having a colour intensity of 0.1 absorbance units.
([3]) Arsenic and lead metals limits are expressed on the basis of the product as is; other limits and ranges are, unless otherwise stated, expressed on a solids basis.
([4]) The specifications for total heavy metals (as Pb), of < 25 mg/kg will be deleted from the specifications for caramel colours in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008 and repealing Directives: 2008/128/EC (purity criteria concerning colours), 2008/84/EC (purity criteria on food additives other than colours and sweeteners) as amended and 2008/60/EC (purity criteria concerning sweeteners), currently under discussion.

Table 4: Specifications for Class III Ammonia Caramel according to Commission Directive 2008/128/EC and JECFA (2006)

Purity	**Commission Directive 2008/128/EC**	**JECFA (2006) [(3)]**
Colour bound by DEAE cellulose	≤ 50 %	≤ 50 %
Colour bound by phosphoryl cellulose	> 50 %	> 50 %
Solid content	-	53 – 83 %
Colour intensity	0.08 – 0.36[(1)]	0.08 – 0.36
Ammoniacal nitrogen	≤ 0.3 % [(2)]	≤ 0.4 % ([2])
4-Methylimidazole (4-MEI)	≤ 250 mg/kg [(2)]	≤ 300 mg/kg (≤ 200 mg/kg [(2)])
2-Acetyl-4-tetrahydroxy-butylimidazole (THI)	≤ 10 mg/kg [(2)]	≤ 40 mg/kg (≤ 25 mg/kg [(2)])
Total sulphur	≤ 0.2 % [(2)]	≤ 0.3 % [(2)]
Total nitrogen	0.7 – 3.3 % [(2)]	1.3 – 6.8 % [(2)]
Absorbance ratio of colour bound by phosphoryl cellulose	13 - 35	-
Arsenic	≤ 1 mg/kg	≤ 1 mg/kg

Lead	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb) [4]	≤ 25 mg/kg	-

([1]) Colour intensity is defined as the absorbance of a 0.1 % (w/v) solution of caramel colour solids in water in a 1 cm cell at 610 nm.

([2]) Expressed on an equivalent colour basis, i.e. expressed in terms of a product having a colour intensity of 0.1 absorbance units.

([3]) Arsenic and lead metals limits are expressed on the basis of the product as is; other limits and ranges are, unless otherwise stated, expressed on a solids basis

([4]) The specifications for total heavy metals (as Pb), of < 25 mg/kg will be deleted from the specifications for caramel colours in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008 and repealing Directives: 2008/128/EC (purity criteria concerning colours), 2008/84/EC (purity criteria on food additives other than colours and sweeteners) as amended and 2008/60/EC (purity criteria concerning sweeteners), currently under discussion.

Table 5: Specifications for Class IV Sulphite Ammonia Caramel according to Commission Directive 2008/128/EC and JECFA (2006)

Purity	**Commission Directive 2008/128/EC**	**JECFA (2006) [4]**
Colour bound by DEAE cellulose	> 50 %	> 50 %
Solid content	-	40 - 75 %
Colour intensity	0.10 - 0.60[1]	0.10 - 0.60
Ammoniacal nitrogen	≤ 0.6 % [2]	≤ 2.8 %
Sulphur dioxide	≤ 0.2 % [2]	≤ 0.5 %
4-methylimidazole (4-MEI)	≤ 250 mg/kg [2]	≤ 1000 mg/kg (≤ 250 mg/kg [2])
Total nitrogen	0.3 - 1.7 % [2]	0.5 - 7.5 %
Total sulphur	0.8 - 2.5 % [2]	1.4 - 10.0 %
Nitrogen/sulphur ratio of alcohol precipitate	0.7 - 2.7	-
Absorbance ratio of alcohol precipitate	8 - 14 [3]	-
Absorbance ratio ($A_{280/560}$)	≤ 50	≤ 50
Arsenic	≤ 1 mg/kg	≤ 1 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb) [5]	≤ 25 mg/kg	-

([1]) Colour intensity is defined as the absorbance of a 0.1 % (w/v) solution of caramel colour solids in water in a 1 cm cell at 610 nm.

([2]) Expressed on an equivalent colour basis, i.e. expressed in terms of a product having a colour intensity of 0.1 absorbance units.

([3]) Absorbance ratio of alcohol precipitate is defined as the absorbance of the precipitate at 280 nm divided by the absorbance at 560 nm (1 cm cell).

([4]) Arsenic and lead metals limits are expressed on the basis of the product as is; other limits and ranges are, unless otherwise stated, expressed on a solids basis.

([5]) The specifications for total heavy metals (as Pb), of < 25 mg/kg will be deleted from the specifications for caramel colours in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008 and repealing Directives: 2008/128/EC (purity criteria concerning colours), 2008/84/EC (purity criteria on food additives other than colours and sweeteners) as amended and 2008/60/EC (purity criteria concerning sweeteners), currently under discussion.

The constituents 4-MEI, found in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, and THI, found only in Class III Ammonia Caramel, are of particular relevance in relation to the toxicological profile of these classes of caramel colours. 4-MEI is limited to a maximum level of 250 mg/kg[8] caramel colour, expressed on an equivalent colour basis (as defined in the footnotes to

[8] The limit for 4-MEI in Class III and Class IV caramels is proposed to be 200 mg/kg in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008 and repealing Directives: 2008/128/EC (purity criteria concerning colours), 2008/84/EC (purity criteria on food additives other than colours and sweeteners) as amended and 2008/60/EC (purity criteria concerning sweeteners), currently under discussion.

Tables 4 and 5), under Commission Directive 2008/128/EC and to a maximum level of 200 mg/kg caramel colour according to the JECFA specifications. Analysis of food grade caramel colours at the time of the initial evaluations by JECFA (JECFA, 1972, 1977) indicated that commercial Class III Ammonia Caramel of undefined origin contained 50-500 mg 4-MEI/kg (Heyns, 1971), while other examinations have shown ranges of 100-700 mg 4-MEI /kg (Battelle Memorial Institute, 1971). 4-MEI is formed by interaction of ammonia with reducing sugars (Morgan and Edwards, 1986), and it has been shown that the yields of imidazole compounds increased linearly with the increment of molar ratio of ammonia to glucose (Komoto, 1962).

The residual levels for THI are < 10 mg/kg Class III Ammonia Caramel under Commission Directive 2008/128/EC and < 25 mg/kg according to the JECFA specifications. The absence of THI in Caramel Classes I and II is due to the fact that there is no nitrogen source present that would allow its formation (EUTECA, 2010a). The presence of THI in Class IV Sulphite Ammonia Caramel has not been demonstrated by current analytical methodologies, and it is suggested by the industry that the presence of sulphite/sulphur dioxide may interfere with or inhibits the formation of THI (EUTECA, 2010a).

2.3. Manufacturing process

All four caramel classes are prepared by the heat treatment (caramelisation) of carbohydrates. According to the European Technical Committee on Caramels (EUTECA) website, the reaction is controlled with respect to temperature and pressure, but no information was provided about the actual temperature or pressure used. In all cases the carbohydrate raw materials are commercially available food grade nutritive sweeteners consisting of glucose, fructose, invert sugar and/or polymers thereof (e.g. glucose syrups, sucrose or invert sugars, and dextrose). To promote caramelisation and hence produce greater colour intensity, food-grade acids, alkalis or salts may be used in amounts consistent with Good Manufacturing Practices (GMP) (Kamuf et al, 2003; Emerton, 2008).

The acids used are food-grade sulphuric or citric acids, although phosphoric or carbonic acids may also be used; the alkalis used are ammonium, sodium, potassium or calcium hydroxides or mixtures thereof; the salts used are carbonate, hydrogen carbonates, sulphates, ammonium, sodium, potassium or calcium phosphate (EUTECA, 2007a).

According to JECFA (JECFA, 2006) and EUTECA (2007a) the four classes into which the caramel colours are divided depend on the reagent used in their manufacture:

Class I Plain Caramel. No reagent (no sulphite and/or ammonium compounds).

Class II Caustic Sulphite Caramel. Reagent: sulphite compounds (no ammonium compounds).

Class III Ammonia Caramel. Reagent: ammonium compounds (no sulphite compounds).

Class IV Sulphite Ammonia Caramel. Reagent: sulphite and ammonium compounds.

The ammonium compounds used are one or any of the following: ammonium hydroxide, ammonium carbonate and ammonium hydrogen carbonate, ammonium phosphate, ammonium sulphate, ammonium sulphite and ammonium hydrogen sulphite. Where sulphite compounds are used, they are one or any of the following: sulphurous acid, potassium, sodium and ammonium sulphites and hydrogen sulphites (JECFA, 2006).

Food-grade polyglycerol esters of fatty acids may be used as processing aids (antifoam) in amounts not greater than those required to produce the intended effect. Caramel colour is Polymerase Chain Reaction (PCR) negative, because any trace of protein in corn syrup raw material is denatured under the high temperatures used in caramel manufacturing (Kamuf et al., 2003).

The shelf life for a caramel colour under ambient storage conditions will normally be listed as either one or two years depending on the class. The caramelisation reaction continues at a slow rate during

ambient temperature storage, with both colour and viscosity increasing with time. For example, a class IV double-strength liquid stored at ambient temperature increases in colour intensity from 0.235 to 0.282 over 33 months (Kamuf et al., 2003).

2.4. Methods of analysis in food

Licht et al. (1992b, 1992c) have characterised caramel colour classes, looking for consistency within a class, regardless of manufacturer and intended end-use. Specifications for caramel colours were proposed to ensure comparability within classes (Licht et al., 1992a). Licht et al. (1992a) indicated that methods used for the characterisation of the classes include: ultrafiltration, cellulose chromatography, High Performance Liquid Chromatography (HPLC), size-exclusion HPLC, chromatographic profiling using refractive index and ultraviolet detection, HPLC combined with carbohydrate analysis and Gas Chromatography/Mass Spectrometry (GC/MS). Patey et al. (1985) used Gas Chromatography (GC) and GC/MS to analyse trimethylsilyl derivatives of LMW fractions from caramel colours which revealed as many as 147 individual compounds, with considerable variability in patterns and levels. The technique allowed identification of the manufacturer of UK-produced Class III Ammonia Caramel but not of class type. UK-produced Class I Plain Caramel and Class IV Sulphite Ammonia Caramel and Canadian and Japanese-produced Class II Caustic Sulphite Caramel each had a distinctive fingerprint unlike UK-produced Class III Ammonia Caramel.

Each caramel molecule carries a net electrical (colloidal) charge formed during processing. Class I Plain Caramel carries a slightly negative charge, Class II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel are strongly negative and Class III Ammonia Caramel is strongly positive. These electrical properties can be used in caramel analysis particularly using Capillary Electrophoresis (CE). Royle and Radcliffe (1999) used CE and ultrafiltration to distinguish caramel colours, and later extended this work to distinguish malts from caramel colours (Royle et al., 2002).

The complex nature and limited knowledge of chemical composition of caramel colours makes their identification in food products a significant analytical challenge. However, Ciolino (1998) used a combination of marker compounds and HPLC-UV profiling to detect caramel in adulterated acerloa juice. Sádecká and Tóthová (2010) have used synchronous fluorescence spectroscopy to measure caramel content in brandy and mixed wine spirits at levels above 4 mg/l. There are no recent publications attempting the analysis of caramel colours in more complex foods, although modern instrumental methods and the use of chemometrics for fingerprinting do hold some promise.

2.5. Reaction and fate in food

The complexity of caramel colours makes it almost impossible to study the reaction and fate of individual caramel colours in foods. This can only be addressed by analysing for each of the individual constituents, which to date has not been pursued. However, in general the caramel colours are all heat- and light-stable, although acid stability varies considerably, the negatively-charged caramel colours generally being more stable under acid conditions (Emerton, 2008).

2.6. Case of need and proposed uses

Caramel colours are permitted *quantum satis*[9] in all foodstuffs except those listed in Annexes II and III of Directive 94/36/EC[10] on colours for use in foodstuffs, in which the use of colours is prohibited or

[9] Article 2 (7) of Directive 94/36/EC states: *'In the Annexes to this Directive 'quantum satis' means that no maximum level is specified. However, coloring matters shall be used according to good manufacturing practice at a level not higher than is necessary to achieve the intended purpose and provided that they do not mislead the customer'.*

[10] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. Official Journal of the European Communities, L 237, 10.9.94.

restricted to certain food colours. The main use of the caramel colours is to impart a brown colour and associated characteristic caramel flavour to a range of foodstuffs including, in particular, non-alcoholic (cola-type) drinks, beers and ciders, meat/fish analogues and soups and gravies (Tennant, 2007). The relative proportions of the four classes of caramel colours used in Europe are as follows: 1 %: Class I Plain Caramel, 2 %: Class II Caustic Sulphite Caramel, 25-27 %: Class III Ammonia Caramel and 70-72 %: Class IV Sulphite Ammonia Caramel (EUTECA, 2007b). Use of Class IV Sulphite Ammonia Caramel is mostly associated with cola-flavoured drinks.

2.6.1. Actual levels of use of caramel colours Class I, II, III and IV

The current use levels of the caramel colours was made available to the Panel for several food categories for finished products by EUTECA (Tennant, 2007,) and by the Confederation of the Food and Drink Industries of the EU (CIAA) (CIAA, 2009). These data are presented in Tables 6 and 7.

EUTECA carried out a survey of usage as part of a wider survey by the Natural Food Colours Association (NATCOL) in 2006-2007 (Tennant, 2007). All manufacturers of caramel colours who are members of EUTECA were asked at the time of the survey to provide information about the formulation of the products in combination with the amounts of the formulation used in each food. No usage data were made available as a result of this survey for Class I Plain Caramel (E150a) or Class II Caustic Sulphite Caramel (E150b). However new information on usage data was made available to the Panel from EUTECA, based on a study implemented on February 2010 by the SFFCC (Syndicat des Fabricants Français de Caramels Colorants) (EUTECA 2010c). The Panel noted that in comparison to the data provided in the Tennant report (2007) based on a food additive usage survey, the new data provided by EUTECA in 2010 contain levels of use for Class I Plain Caramel or Class II Caustic Sulphite Caramel, as well as some new levels of use for Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel in foodstuffs that had not been mentioned in the previous report.

2.6.1.1. Beverages

Class I Plain Caramel:

For non-alcoholic flavoured drinks, the CIAA reported typical use levels ranging from 13 to 1500 mg/l and an extreme use level of 2000 mg/l (CIAA, 2009). EUTECA (2010c) provided use levels ranging from 500-10 000 mg/l with a typical use level of 4000 mg/l.

For beer, the CIAA (2009) provided values ranging from 0 to 6000 mg/l with a typical use level of 3000 mg/l. EUTECA (2010c) provided values for beer and cidre bouché ranging from 2000-10 000 mg/l with a typical use level of 6000 mg/l for beer and 2000 mg/l for cidre bouché.

For whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit (other than Tsikoudia and Tsipouro and eau de vie de marc marque nation), the CIAA (2009) provided typical values ranging from 0 to 2000 mg/l with a extreme use level of 15000 mg/l. EUTECA (2010c) provided a typical use level of 2000 mg/l.

For aromatised wines-based drinks (except bitter soda), americano, liqueur wines and quality liqueur wines produced in specific regions, and spirit drinks, EUTECA (2010c) provided a typical use level of 5000 mg/l.

For aromatised wines, aromatised wines-based drinks and aromatised wine-products cocktails, EUTECA (2010c) provided a typical use level of 10 000 mg/l. For fruit wine, EUTECA (2010c) provided a typical use level of 6000 mg/l.

For liquid food supplements/dietary integrators, EUTECA (2010c) provided a typical value of 15 000 mg/l.

Class II Caustic Sulphite Caramel:

For non-alcoholic flavoured drinks, the CIAA reported typical use levels ranging from 0 to 30 mg/l (CIAA, 2009).

For whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit (other than Tsikoudia and Tsipouro and eau de vie de marc marque nation), EUTECA (2010c) provided a typical use level of 2000 mg/l. For cidre bouché, EUTECA (2010c) provided a typical use level of 3000 mg/l.

For aromatised wines-based drinks (except bitter soda), americano, liqueur wines and quality liqueur wines produced in specific regions, spirit drinks, and aromatised wines, aromatised wines-based drinks and aromatised wine-products cocktails, EUTECA (2010c) provided a typical use level of 5000 mg/l. For fruit wine, EUTECA (2010c) provided a typical use level of 3000 mg/l.

For liquid food supplements/dietary integrators, the CIAA (2009) provided a typical value of 620 mg/l. EUTECA (2010c) provided a typical use level of 15 000 mg/l.

Class III Ammonia Caramel:

For non-alcoholic flavoured drinks, the CIAA reported typical use levels ranging from 55 to 100 mg/l and extreme use levels ranging from 55 to 500 mg/l (CIAA, 2009). Tennant (2007) and EUTECA (2010c) reported usage data range from 340 to 1360 mg/l. Tennant (2007) reported a typical use level of 1000 mg/l, whilst EUTECA (2010c) indicated typical use levels 55-100 mg/l for "carbonate" category.

For spirituous beverages, aromatized wines and fruit wines, Tennant (2007) and EUTECA (2010c) provided values ranging from 136 to 680 mg/l with a typical use level of 600 mg/l. For fruit wines category, CIAA (2009) also provided extreme values ranging from 30 to 60 mg/l with a typical use level of 40 mg/l.

For beer, the CIAA (2009) provided typical values ranging from 0 to 7500 mg/l with extreme use levels ranging from 0 to 8000 mg/l. Tennant (2007) and EUTECA (2010c) provided data for the same food category. The use levels range from 340 to 2040 mg/l with a typical value of 2000 mg/l. The same data were reported by Tennant (2007) and EUTECA (2010c) for cidre bouché.

Class IV Sulphite Ammonia Caramel:

For non-alcoholic flavoured drinks, the CIAA reported typical use levels ranging from 0 to 1600 mg/l and extreme values ranging from 0 to 2800 mg/l. Tennant (2007) and EUTECA (2010c) reported usage data range from 3000 to 4000 mg/l. Tennant (2007) reported a typical use level of 1600 mg/l,

whilst EUTECA (2010c) indicated a typical use level of 5000 mg/l for "carbonate" category. UNESDA reported an actual maximum use level of 1600 mg/l (UNESDA, 2006).

For cidre bouché, EUTECA (2010c) provided a typical use level of 500 mg/l.For spirituous beverages, Tennant (2007) and EUTECA (2010c) provided values ranging from 4000 to 5000 mg/l with a typical use level of 5000 mg/l.

For fruit wines, CIAA (2009) provided extreme values ranging from 30 to 560 mg/l with typical use levels ranging from 70 to 330 mg/l. EUTECA (2010c) provided a typical use level of 500 mg/l.

For Americano and aromatised wines, Tennant (2007) and EUTECA (2010c) provided a typical use level of 1000 mg/l and 5000 mg/l, respectively. For whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit (other than Tsikoudia and Tsipouro and eau de vie de marc marque nation), Tennant (2007) and EUTECA (2010c) provided values ranging from 4000 to 5000 mg/l with a typical use level of 5000 mg/l.

For liquid food supplements and dietary integrators, the CIAA (2009) reported a range of typical data ranging from 25 to 615 mg/l.

Table 6: Data reviewed on use levels of caramel in beverages

	Beverages	**Data provided by**	**Reported typical use levels (range lowest-highest or maximum) (mg/l)**	**Reported maximum use levels (mg/l)**
Class I Plain Caramel	Non-alcoholic flavoured drinks	CIAA (2009)	13- 1500	2000
		EUTECA, 2010c	4000	10 000
	Beer, cidre bouché	CIAA (2009)	3000	6000
		EUTECA, 2010c	6000 Cidre: 2000	10 000
	Whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit	CIAA (2009)	0 -2000	15 000
		EUTECA, 2010c	2000	
	Aromatised wines-based drinks (except bitter soda)	EUTECA, 2010c	5000	
	Americano	EUTECA, 2010c	5000	
	Liqueur wines and quality liqueur wines produced in specific regions	EUTECA, 2010c	5000	
	Spirit drinks, (including products less than 15% alcohol by volume)[1]	EUTECA, 2010c	5000	
	Aromatized wines, aromatised wine-based drinks and aromatized wine-products cocktails as mentioned in Regulation 1601/91[1]	EUTECA, 2010c	10 000	
	Fruit wine (still or sparkling)	EUTECA, 2010c	6000	

	Beverages	Data provided by	Reported typical use levels (range lowest-highest or maximum) (mg/l)	Reported maximum use levels (mg/l)
	Liquid food supplements/dietary integrators	EUTECA, 2010c	15 000	
Class II Caustic Sulphite Caramel	Non-alcoholic flavoured drinks	CIAA (2009)	0- 30	
	Cidre bouché	EUTECA, 2010c	3000	
	Whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit	EUTECA, 2010c	2000	
	Aromatised wines-based drinks (except bitter soda)	EUTECA, 2010c	5000	
	Americano	EUTECA, 2010c	5000	
	Liqueur wines and quality liqueur wines produced in specific regions	EUTECA, 2010c	5000	
	Spirit drinks, (including products less than 15% alcohol by volume)[1]		5000	
	Aromatized wines, aromatised wine-based drinks and aromatized wine-products cocktails as mentioned in Regulation 1601/91[1]	EUTECA, 2010c	5000	
	Fruit wine (still or sparkling)	EUTECA, 2010c	3000	
	Liquid food supplements/dietary integrators	CIAA (2009)	620	
		EUTECA, 2010c	15 000	
Class III Ammonia Caramel	Non-alcoholic flavoured drinks	CIAA (2009)	55- 100	500
		Tennant (2007) EUTECA, 2010c	1000 55-100[3]	1360
	Spirit drinks, aromatized wines and fruit wines	Tennant (2007); EUTECA, 2010c	600	680
		CIAA (2009)[2]	40	60
	Beer	CIAA (2009)	0-7500	8000
		Tennant (2007); EUTECA, 2010c	2000	2040
	Cidre bouché	Tennant (2007); EUTECA, 2010c	2000	2040
Class IV Sulphite Ammonia Caramel	Non-alcoholic flavoured drinks	UNESDA (2006)		1600
		CIAA (2009)	0-1600	2800
		Tennant (2007) EUTECA, 2010c	1600 5000[3]	4000
	Spirit drinks (including products less than 15% alcohol by volume)[1]	Tennant (2007); EUTECA, 2010c	5000	5000
	Cidre bouché	EUTECA, 2010c	500	

	Beverages	Data provided by	Reported typical use levels (range lowest-highest or maximum) (mg/l)	Reported maximum use levels (mg/l)
	Fruit wines (still or sparkling)	CIAA (2009)	70-330	560
		EUTECA, 2010c	500	
	Aromatised wines wine-based drinks (except bitter soda)	Tennant (2007); EUTECA, 2010c	5000	5000
	Americano	Tennant (2007); EUTECA, 2010c	1000	1000
	Whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit	Tennant (2007); EUTECA, 2010c	5000	5000
	Liquid food supplements/dietary integrators	CIAA (2009)	25-615	

[1] Except those mentioned in Annex II and III of Directive 94/36/EC
[2] Only for fruit wines
3 In the "Carbonates" category

2.6.1.2. Foodstuffs

Class I Plain Caramel

For confectionery products, the Panel was provided with data from the CIAA which reported typical use levels ranging from 25 to 9800 mg/kg and extreme use levels from 2 to 3800 mg/kg (CIAA, 2009). EUTECA (2010c) provided use levels ranging from 500-10 000 mg/kg with a typical use level of 2500 mg/kg.

For decorations and coatings, the CIAA (2009) reported a typical use level of 4 mg/kg and extreme values of 40 mg/kg.

For fine bakery wares, the CIAA (2009) reported extreme values ranging from 25 to 4900 mg/kg with typical values ranging from 25 to 800 mg/kg. EUTECA (2010c) provided use levels ranging from 1000-15 000 mg/kg with a typical use level of 3000 mg/kg.

For edible ices, the Panel was provided with data from the CIAA which reported extreme use levels ranging from 1.3 to 1990 mg/kg, with typical values ranging from 1.3 to 900 mg/kg (CIAA, 2009). EUTECA (2010c) provided a typical use level of 5000 mg/kg.

For desserts, including flavoured milk products, the CIAA (2009) reported typical and extreme use levels of 2774 mg/kg. EUTECA (2010c) provided use levels ranging from 1000-15 000 mg/kg with a typical use level of 5000 mg/kg.

Use level data have been reported for sauces and seasonings by the CIAA (2009) with a range of typical use levels from 0.5 to 13000 mg/kg and extreme use levels ranging from 0.5 to 21 000 mg/kg.

EUTECA (2010c) provided use levels ranging from 1000-100 000 mg/kg with a typical use level of 5000 mg/kg.

For snacks (dry, savoury potato, cereal or starch-based snack products: extruded or expanded savoury snack products) as well as for other savoury snack products and savoury peanuts, nuts or hazelnuts, typical and extreme use levels have been reported by the CIAA (2009) and range from 200 to 1600 mg/kg. EUTECA (2010c) provided a typical use level of 3000 mg/kg.

For dietetic formulae for weight control intended to replace total daily food intake or an individual meal, the CIAA (2009) reported a typical use level of 1800 and maximum usage of 2500 mg/kg.

Data for soups have been provided by CIAA (2009) which reported typical levels ranging from 50 to 300 mg/kg and maximum levels of 50 to 1332 mg/kg. EUTECA (2010c) provided use levels ranging from 2000-50 000 mg/kg with a typical use level of 10 000 mg/kg.

For meat and fish analogues based on vegetable proteins, the CIAA (2009) provided a typical use level of 16 000 mg/kg. EUTECA (2010c) provided use levels ranging from 1000-40 000 mg/kg with a typical use level of 10 000 mg/kg.

For the category extruded, puffed and/or fruit-flavoured breakfast cereals, CIAA (2009) provided a typical use level of 5610 mg/kg and a maximum value of 10 010 mg/kg.

For jams, jellies and marmalades, the Panel was provided with data from the CIAA which reported a typical use level of 1000 mg/kg and a maximum value of 4000 mg/kg (CIAA, 2009).

For malt bread, EUTECA (2010c) provided use levels ranging from 1000-30 000 mg/kg with a typical use level of 1000 mg/kg.

For sausages, pâtés and terrines, EUTECA (2010c) provided use levels ranging from 1000-8000 mg/kg with a typical use level of 2000 mg/kg.

For vinegar and smoked fish, EUTECA (2010c) provided a typical use level of 6000 and 10 000 mg/kg, respectively.

For complete formulae for weight control, EUTECA (2010c) provided a typical use level of 7000 mg/kg.

Class II Sulphite Caramel

For confectionery products, the Panel was provided with data from the CIAA which reported no use in such products (CIAA, 2009).

For fine bakery wares, the CIAA (2009) reported extreme values ranging from 14 to 3000 mg/kg with typical values ranging from 14 to 1500 mg/kg. EUTECA (2010c) provided a typical use level of 1000 mg/kg.

For edible ices, the Panel was provided with data from the CIAA which reported extreme use levels ranging from 120 to 7620 mg/kg, with typical values ranging from 2840 to 4000 mg/kg (CIAA, 2009). EUTECA (2010c) provided a typical use level of 3000 mg/kg.

For flavoured processed cheese, the CIAA (2009) reported no use in such products.

Use level data have been reported for sauces and seasonings by the CIAA (2009) with a range of typical use levels from 0 to 2000 mg/kg. EUTECA (2010c) provided a typical use level of 6000 mg/kg.

For sausages, pâtés and terrines, EUTECA (2010c) provided a typical use level of 1000 mg/kg. For malt bread, vinegar and smoked fish, EUTECA (2010c) provided a typical use level of 3000, 5000 and 10 000 mg/kg, respectively.

For desserts, EUTECA (2010c) provided a typical use level of 1000 mg/kg.

For snacks (dry, savoury potato, cereal or starch-based snack products: extruded or expanded savoury snack products) as well as for other savoury snack products and savoury peanuts, nuts or hazelnuts, EUTECA (2010c) provided a typical use level of 1000 mg/kg.

For complete formulae for weight control, meat and fish, and soups EUTECA (2010c) provided a typical use level of 5000, 5000 and 10 000 mg/kg, respectively.

Class III Ammonia Caramel

For confectionery products, the Panel was provided with data from the CIAA which reported use levels ranging from 0 to 8000 mg/kg with typical values ranging from 0 to 5000 mg/kg (CIAA, 2009). Tennant (2007) and EUTECA (2010c) reported use levels ranging from 136 to 680 mg/kg with a typical value of 600 mg/kg.

For candied fruit, vegetables, mustarda di frutta, Tennant (2007) and EUTECA (2010c) provided use level data ranging from 340 to 2040 mg/kg with a typical level of 2000 mg/kg.

For decorations and coatings, Tennant (2007) and EUTECA (2010c) provided use level data ranging from 2040 to 3400 mg/kg with a typical level of 3000 mg/kg.

For fine bakery wares, the CIAA (2009) reported extreme values ranging from 40 to 11500 mg/kg with typical values ranging from 70 to 11500 mg/kg. Tennant (2007) and EUTECA (2010c) reported values ranging from 340 to 1360 mg/kg and a typical use level of 1000 mg/kg.

For edible ices, Tennant (2007) and EUTECA (2010c) reported values ranging from 340 to 1360 mg/kg and a typical use level of 1000 mg/kg.

For flavoured processed cheese, the CIAA (2009) reported no use in such products.

For desserts, including flavoured milk products, the CIAA (2009) reported typical use levels of 5120 mg/kg, while Tennant (2007) and EUTECA (2010c) reported values ranging from 340 to 1360 mg/kg and a typical use level of 1000 mg/kg.

Use level data have been reported for sauces and seasonings by the CIAA (2009) with a range of extreme use levels from 0 to 25000 mg/kg and typical use levels ranging from 600 to 3200 mg/kg. Tennant (2007) and EUTECA (2010c) provided data ranging from 10 000 to 100 000 mg/kg, high use levels relating to concentrated products.

For snacks (dry, savoury potato, cereal or starch-based snack products: extruded or expanded savoury snack products) as well as for other savoury snack products and savoury peanuts, nuts or hazelnuts, data have been reported by Tennant (2007) and EUTECA (2010c) and range from 340 to 2040 mg/kg with a typical value of 2000 mg/kg.

For complete formulae and nutritional supplements for use under medical supervision, the CIAA (2009) reported a typical use level of 9500 mg/kg.

Data for soups have been provided by CIAA (2009) which reported extreme levels ranging from 30 to 3000 mg/kg and typical levels of 100 to 2000 mg/kg. EUTECA (2010c) provided a typical use level of 2000 mg/kg.

For noodles, CIAA (2009) reported a typical use level of 510 mg/kg.

For meat and fish analogues based on vegetable proteins, Tennant (2007) and EUTECA (2010c) reported use levels of 10 000 mg/kg. The CIAA (2009) reported extreme use levels ranging from 100 to 5000 mg/kg and typical use levels ranging from 200 to 5000 mg/kg

For the category extruded, puffed and/or fruit-flavoured breakfast cereals and malt bread, Tennant (2007) and EUTECA (2010c) provided usage data ranging from 340 to 2040 mg/kg with a typical use level of 2000 mg/kg.

For malt bread, Tennant (2007) and EUTECA (2010c) provided use levels ranging from 340-2040 mg/kg with a typical use level of 2000 mg/kg.

For sausages, pâtés and terrines, EUTECA (2010c) provided use levels ranging from 1000-5000 mg/kg with a typical use level of 1000 mg/kg.

For vinegar and mustard, EUTECA (2010c) provided a typical use level of 2000 mg/kg.

Class IV Sulphite Ammonia Caramel

For confectionery products, the Panel was provided with data from the CIAA which reported extreme use levels ranging from 0 to 8000 mg/kg with typical values ranging from 0 to 5000 mg/kg (CIAA, 2009). Tennant (2007) and EUTECA (2010c) reported use levels ranging from 1000 to 300 000 mg/kg (high use levels relating to concentrated products) with a typical value of 1000 mg/kg.

For fine bakery wares, the CIAA (2009) reported extreme values ranging from 30 to 1700 mg/kg with typical values ranging of 250 mg/kg. Tennant (2007) and EUTECA (2010c) reported extreme values ranging from 3000 to 5000 mg/kg and a typical use level of 5000 mg/kg.

For edible ices, the CIAA (2009) reported extreme values ranging from 100 to 5200 mg/kg and typical use levels ranging from 400 to 3500 mg/kg. EUTECA (2010c) provided a typical use level of 3000 mg/kg.

For flavoured processed cheese, the CIAA (2009) reported no use in such products.

Use level data have been reported for sauces and seasonings by the CIAA (2009) with a range of extreme use levels from 0 to 10 000 mg/kg and typical use levels ranging from 0 to 2000 mg/kg. Tennant (2007) and EUTECA (2010c) provided data ranging from 20 000 to 100 000 mg/kg (high use levels relates to concentrated products) with typical values of 20 000 mg/kg.

For meat and fish analogues based on vegetable proteins, Tennant (2007) and EUTECA (2010c) reported a use level of 1000 mg/kg. The CIAA (2009) provided extreme use levels ranging from 0 to 2000 mg/kg and typical use levels ranging from 280 to 2000 mg/kg

Data for soups have been provided by Tennant (2007) and EUTECA (2010c) which reported a typical level of 4000 mg/kg.

For malt bread, Tennant (2007) and EUTECA (2010c) reported use level data of 5000 mg/kg.

For vinegar, the typical use level reported by Tennant (2007) and EUTECA (2010c) was 2000 mg/kg.

For sausages, pâtés and terrines, EUTECA (2010c) provided use levels ranging from 1000-5000 mg/kg with a typical use level of 1000 mg/kg.

For desserts and snacks (dry, savoury potato, cereal or starch-based snack products: extruded or expanded savoury snack products) as well as for other savoury snack products and savoury peanuts, nuts or hazelnuts, EUTECA (2010c) provided a typical use level of 1000 mg/kg.

Table 7. Data reviewed on use levels of caramel in foodstuff.

	Foodstuff	Data provided by	Reported typical use levels (range lowest-highest or maximum) (mg/kg)	Reported maximum use levels (mg/kg)
Class I Plain Caramel	Confectionery product	CIAA (2009)	25-9800	9800
		EUTECA, 2010c	2500	10 000
	Decorations and coatings	CIAA (2009)	4	40
	Fine bakery wares	CIAA (2009)	25-800	4900
		EUTECA, 2010c	3000	15 000
	Edible ices	CIAA (2009)	1.3-900	1990
		EUTECA, 2010c	5000	
	Dessert (including flavoured milk products)	CIAA (2009)	2774	2774
		EUTECA, 2010c	5000	15 000
	Sauces and seasonings	CIAA (2009)	0.5-13 000	21 000
		EUTECA, 2010c	5000	100 000
	Snacks, savoury peanuts, nuts and hazelnuts	CIAA (2009)	200-1600	1600
		EUTECA, 2010c	3000	
	Soups	CIAA (2009)	50-300	1332
		EUTECA, 2010c	10 000	50 000
	Meat and fish analogues based on vegetable proteins	CIAA (2009)	16 000	
		EUTECA, 2010c	10 000	40 000
	Extruded, puffed and/or fruit flavoured breakfast cereals	CIAA (2009)	5610	10 010
	Jam, jellies and marmelades	CIAA (2009)	1000	4000
	Malt bread	EUTECA, 2010c	10 000	30 000
	Vinegar	EUTECA, 2010c	6000	
	Sausages, pâtés and terrines	EUTECA, 2010c	2000	8000
	Dietetic formulae for weight control	CIAA (2009)	1800	2500
		EUTECA, 2010c	7000	
	Smoked fish	EUTECA, 2010c	10 000	
Class II Caustic Sulphite	Fine bakery wares	CIAA (2009)	14-1500	3000
		EUTECA, 2010c	1000	
	Edible ices	CIAA (2009)	2840-4000	7620
		EUTECA, 2010c	3000	
	Sauces and seasonings	CIAA (2009)	0-2000	
		EUTECA, 2010c	6000	
	Malt bread	EUTECA, 2010c	3000	
	Vinegar	EUTECA, 2010c	5000	
	Sausages, pâtés and terrines	EUTECA, 2010c	1000	

	Foodstuff	Data provided by	Reported typical use levels (range lowest-highest or maximum) (mg/kg)	Reported maximum use levels (mg/kg)
Caramel	Dessert	EUTECA, 2010c	1000	
	Smoked fish	EUTECA, 2010c	10 000	
	Snacks, savoury peanuts, nuts and hazelnuts	EUTECA, 2010c	1000	
	Complete formulae for weight control	EUTECA, 2010c	5000	
	Soups	EUTECA, 2010c	10 000	
	Meat and fish analogues based on vegetable proteins	EUTECA, 2010c	5000	
Class III Ammonia Caramel	Confectionery product	CIAA (2009)	0-5000	8000
		Tennant (2007); EUTECA, 2010c	600	680
	Candied fruit, vegetables, mustarda di fruta	Tennant (2007); EUTECA, 2010c	2000	2040
	Decorations and coatings	Tennant (2007); EUTECA, 2010c	3000	3400
	Fine bakery wares	CIAA (2009)	70-11 500	11500
		Tennant (2007); EUTECA, 2010c	1000	1360
	Edible ices	Tennant (2007); EUTECA, 2010c	1000	1360
	Desserts (including flavoured milk products)	CIAA (2009)	5120	
		Tennant (2007); EUTECA, 2010c	1000	1360
	Sauces and seasonings	CIAA (2009)	600-3200	25 000
		Tennant (2007); EUTECA, 2010c		100 000*
	Snacks, savoury peanuts, nuts and hazelnuts	Tennant (2007); EUTECA, 2010c	2000	2040
	Complete formulae and nutritional supplements	CIAA (2009)	9500	
	Soups	CIAA (2009)	100-2000	3000
		EUTECA, 2010c	2000	
	Noodles	CIAA (2009)	510	
	Meat and fish analogues based on vegetable proteins	Tennant (2007); EUTECA, 2010c	10 000	10 000
		CIAA (2009)	200-5000	5000
	Extruded, puffed and/or fruit –flavoured breakfast cereals and malt bread	Tennant (2007); EUTECA, 2010c	2000	2040
	Malt bread	Tennant (2007); EUTECA, 2010c	2000	2040
	Vinegar	EUTECA, 2010c	2000 (balsamic: 100 g/l)	
	Sausages, pâtés and terrines	EUTECA, 2010c	1000	5000
	Mustard	EUTECA, 2010c	2000	

	Foodstuff	Data provided by	Reported typical use levels (range lowest-highest or maximum) (mg/kg)	Reported maximum use levels (mg/kg)
Class IV Sulphite Ammonia Caramel	Confectionery products	CIAA (2009)	0-5000	8000
		Tennant (2007); EUTECA, 2010c	1000	300 000*
	Fine bakery wares	CIAA (2009)	250	1700
		Tennant (2007); EUTECA, 2010c	5000	5000
	Edible ices	CIAA (2009)	400-3500	5200
		EUTECA, 2010c	3000	
	Sauces and seasonings	CIAA (2009)	0-2000	10 000
		Tennant (2007); EUTECA, 2010c	20 000	20 000
	Soups	Tennant (2007); EUTECA, 2010c	4000	4000
	Meat and fish analogues based on vegetable proteins	Tennant (2007); EUTECA, 2010c	1000	1000
		CIAA (2009)	280-2000	2000
	Malt bread	Tennant (2007); EUTECA, 2010c	5000	5000
	Vinegar	Tennant (2007); EUTECA, 2010c	2000 (balsamic: 100 g/l)	2000
	Sausages, pâtés and terrines	EUTECA, 2010c	1000	5000
	Desserts	EUTECA, 2010c	1000	
	Snacks, savoury peanuts, nuts and hazelnuts	EUTECA, 2010c	1000	

* High use levels related to concentrated products

In order to refine the exposure assessment for children and adults to food colours, the Panel has defined some rules to identify maximum reported use levels based on maximum actual usage or maximum analytical data. The rules followed in order to deal with *quantum satis* authorisation, with use levels data or observed analytical data, for all regulated colours re-evaluated by the Panel, are given in Annex A. Table 8 summarises the maximum reported use levels for caramel colours Class I, II, III and IV in beverages and foodstuffs used for the refined exposure assessment. They have been defined by applying the rules reported in Annex A to the data available to EFSA.

Table 8: Maximum Permitted Levels of use for caramel colours Class I, II, III and IV in beverages and foodstuffs according to European Parliament and Council Directive 94/36/EC and maximum reported use levels of caramel colours Class I, II, III and IV in beverages and foodstuffs used for the refined exposure assessment (Annex A)

Beverages	Maximum Permitted Level (mg/l)	Maximum Reported Use Level (mg/l)			
		Class I	Class II	Class III	Class IV
Non-alcoholic flavoured drinks	*quantum satis*	10 000[1]	30[1]	1360[1]	5000[1]

Beverages	Maximum Permitted Level (mg/l)	Maximum Reported Use Level (mg/l)			
		Class I	Class II	Class III	Class IV
Bitter soda, bitter vino as mentioned in Reg (EEC) 1601/91	*quantum satis*	100[2]	100[2]	100[2]	100[2]
Cider and perry	*quantum satis*	6000[1]	3000[1]	680[1]	600[1**]
cidre bouché	*quantum satis*	2000[1]	3000[1]	2040[1]	500[1]
Americano	*quantum satis*	5000[1]	5000[1]	100[2]	1000[1]
Aperitif wines, spirit drinks including products with less than 15 % alcohol by volume	*quantum satis*	30[2]	30[2]	30[2]	30[2]
Aromatised wine, aromatised wine-based drinks and aromatised wine product cocktails as mentioned in Reg (EEC) 1601/91, except those mentioned in Annex II or IIIs	*quantum satis*	10000[1]	5000[1]	680[1]	200[2]
Aromatised wine-based drinks (except bitter soda) and aromatised wines as mentioned in Reg (EEC) 1601/91	*quantum satis*	5000[1]	5000[1]	680[1]	5000[1]
Spirit drinks, (including products less than 15 % alcohol by volume) except those mentioned in Annex II or III	*quantum satis*	5000[1]	5000[1]	680[1]	5000[1]
Beer	*quantum satis*	10 000[1]	3000[2]	8000[1]	500[2]
Whisky, whiskey, grain spirit (other than Korn or Kornbrand or eau de vie de seigle marque nationale luxembougeoise), wine spirit, rum brandy, weinbrand, grape marc, grape marc spirit (other than Tsikoudia and Tsipouro and eau de vie de marc marque nation	*quantum satis*	15 000[1]	2000[1]	200[2]	5000[1]
Fruit wines (still or sparkling)	*quantum satis*	6000[1]	3000[1]	680[1]	560[1]
Liqueur wines and quality liqueur wines produced in specified regions	*quantum satis*	5000[1]	5000[1]	200[2]	200[2]
Liqueurs, including fortified beverages with less than 15 % alcohol by volume	*quantum satis*	200[2]	200[2]	200[2]	200[2]
Liquid food supplements/dietary integrators	*quantum satis*	15 000[1]	15 000[1]	100[2]	620[1**]
Bigarreaux cherries in syrup and in cocktails	*quantum satis*	150[2]	150[2]	150[2]	150[2]

Foodstuffs	Maximum Permitted Level (mg/kg)	Maximum reported use level (mg/kg)			
		Class I	Class II	Class III	Class IV
Breakfast sausages with a minimum cereal content of 6 %	*quantum satis*	100[2]	100[2]	100[2]	100[2]

Foodstuffs	Maximum Permitted Level (mg/kg)	Maximum reported use level (mg/kg)			
		Class I	Class II	Class III	Class IV
Burger meat with a minimum vegetable and/or cereal content of 4 %	*quantum satis*	100[2]	100[2]	100[2]	100[2]
Candied fruits and vegetables, Mostarda di frutta	*quantum satis*	200[2]	200[2]	2050[1**]	200[2]
cocktail cherries and candies cherries	*quantum satis*	200[2]	200[2]	200[2]	200[2]
Complete formulae and nutritional supplements for use under medical supervision	*quantum satis*	50[2]	50[2]	9500[1]	1000[2]
Confectionery	*quantum satis*	10 000[1]	-*	8000[1]	300 000[1]
Decoration of chocolates	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Decorations and coatings	*quantum satis*	40[1]	500[2]	3400[1]	500[2]
Desserts including flavoured milk products	*quantum satis*	15 000[1]	1000[1]	5120[1]	1000[1]
Dietetic formulae for weight control intended to replace total daily food intake or an individual meal	*quantum satis*	7000[1]	5000[1]	50[2]	50[2]
Edible casings	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Edible cheese rind	*quantum satis*	100[2]	100[2]	100[2]	100[2]
Edible ices	*quantum satis*	5000[1]	8000[1**]	1360[1]	5200[1]
External coating of confectionery	*quantum satis*	500[2]	500[2]	500[2]	500[2]
External coating of sugar confectionery for the decoration of cake and pastries	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Extruded, puffed and/or fruit-flavoured breakfast cereals	*quantum satis*	10 010[1]	500[2]	2040[1]	500[2]
Fine bakery wares	*quantum satis*	15 000[1]	3000[1]	11500[1]	5000[1]
fish analogues based on vegetable proteins	*quantum satis*	40 000[1]	5000[1]	10 000[1]	2000[1]
Fish paste and crustacean paste	*quantum satis*	100[2]	100[2]	100[2]	100[2]
Fish roe	*quantum satis*	300[2]	300[2]	300[2]	300[2]
Flavoured processed cheese	*quantum satis*	100[2]	-*	-*	-*
Jam, jellies and marmalades and other similar fruit preparations including low calorie products	*quantum satis*	4000[1]	100[2]	100[2]	100[2]
Kippers	*quantum satis*	20[2]	20[2]	20[2]	20[2]
Malt bread	*quantum satis*	30 000[1]	3000[1]	2040[1]	5000[1]
Meat analogues based on vegetable proteins	*quantum satis*	40 000[1]	5000[1]	10 000[1]	2000[1]
Mustard	*quantum satis*	300[2]	300[2]	2000[1]	300[2]
Other savoury snack products and savoury peanuts, nuts or hazelnuts	*quantum satis*	3000[1]	1000[1]	2040[1]	1000[1]
Pre-cooked crustaceans	*quantum satis*	250[2]	250[2]	250[2]	250[2]
Preserves of red fruits	*quantum satis*	200[2]	200[2]	200[2]	200[2]

Foodstuffs	Maximum Permitted Level (mg/kg)	Maximum reported use level (mg/kg)			
		Class I	Class II	Class III	Class IV
Salmon substitutes	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Sauces, seasonings (for example curry powder, tandoori), pickles, relishes, chutney and piccalilli	*quantum satis*	100 000[1]	6000[1]	100 000[1]	20 000[1]
Saucisses de Strasbourg	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Sausages, pâtés and terrines	*quantum satis*	8000[1]	1000[1]	5000[1]	5000[1]
Smoked fish	*quantum satis*	10 000[1]	10 000[1]	100[2]	100[2]
Snacks: dry, savoury potato, cereal or starch-based snack products: extruded or expanded savoury snack products	*quantum satis*	3000[1]	1000[1]	2040[1]	1000[1]
Solid food supplements/dietary integrators	*quantum satis*	300[2]	300[2]	300[2]	300[2]
Soups	*quantum satis*	50 000[1]	10 000[1]	3000[1]	4000[1]
Surimi	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Vegetables in vinegar, brine or oil (excluding olives)	*quantum satis*	500[2]	500[2]	500[2]	500[2]
Vinegar	*quantum satis*	6000[1]	5000[1]	100 000[1]	100 000[1]

1 Maximum reported use level or maximum level determined by analysis.
2 *quantum satis* rules data.
* No usage reported
**Rounded value

2.7. Information on existing authorisations and evaluations

The caramel colours have previously been evaluated by the SCF and by JECFA. They have additionally been reviewed by TemaNord (TemaNord, 2002).

Evaluations of Class I Plain Caramel

The JECFA evaluation of caramel colours in 1969 concluded that caramel colour (Class I Plain Caramel) could be considered as a natural constituent of the diet, and that a toxicological discrimination between caramel produced by cooking or heating sugars (burnt sugar caramel) and caramel colours commercially produced by processes not using ammonia or ammonium salts was unwarranted (JECFA, 1970). JECFA allocated an ADI "not limited" to Class I Plain Caramel at their fifteenth meeting (JECFA, 1972a), and reconfirmed this ADI in 1985 (JECFA, 1986). In an opinion published in 1975, SCF concurred with the JECFA position on Class I Plain Caramel, and following further discussions in 1983 and 1987, the SCF saw no reason to change its previous opinion on Class I Plain Caramel. A numerical ADI was thus found not necessary (SCF, 1975, 1984 and 1989).

Evaluations of Class II Caustic Sulphite Caramel

The SCF in 1987 concluded that Class II Caustic Sulphite Caramel was temporarily acceptable pending provision of further information on the composition and homogeneity of caramel colours falling within this class (SCF, 1989). Following provision of additional information indicating that the composition of Caustic Sulphite Caramel is intermediate between that of Class I Plain Caramel and that of Class IV Sulphite Ammonia Caramel, SCF considered, at its 76th meeting in 1990, that the existing toxicity data on Class II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel

provided adequate documentation for the safety in use for both these classes of caramel and it therefore included Class II Caustic Sulphite Caramel within the ADI of 200 mg/kg bw/day it had already established for Class IV Sulphite Ammonia Caramel.

In 1985, JECFA concluded that Class II Caustic Sulphite Caramel was sufficiently different from other classes of caramel colours to warrant a separate evaluation but that there were insufficient data to do so. No ADI was established (JECFA, 1986). At its 55th meeting, the Committee re-evaluated the data and established an ADI of 160 mg/kg bw/day for Class II Caustic Sulphite Caramel, based on a No-Observed-Adverse-Effect-Level (NOAEL) of 16 g/kg bw/day identified in a 90-day study in rats (JECFA, 2001).

Evaluations of Class III Ammonia Caramel

The SCF in 1975 established an ADI of 100 mg/kg bw/day for Class III Ammonia Caramel SCF, 1975). In 1989 the ADI was revised and the SCF allocated a value of 200 mg/kg bw/day, possibly based on the 90-day rat study by MacKenzie (1985b; subsequently published in the open literature as MacKenzie et al., 1992b). The ADI was made temporary pending a review of further studies and especially of the attempts to reduce the content of THI in Class III Ammonia Caramel (SCF, 1989). Finally, at the latest SCF evaluation of 1997, on the basis of the data supplied to the SCF, the Committee decided to allocate a full ADI of 200 mg/kg bw/day to Class III Ammonia Caramel with the provision that the THI content should not exceed 10 mg/kg colour (SCF, 1997).

JECFA in 1972 first established a temporary ADI of 100 mg/kg bw/day for caramel colours prepared by processes using ammonia or ammonium salts (Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel) (JECFA, 1972b). In 1977 JECFA noted that the specifications for Class III Ammonia Caramel were ambiguous since they also covered caramel colours manufactured by the Class IV Sulphite Ammonia Caramel process. Separate specifications were prepared for Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel. It was at that time determined that the principal toxic effect of Class III Ammonia Caramel was depression of circulating lymphocytes and leucocytes, an effect for which a clear no-effect level could not be determined, based on the available studies. Accordingly, the temporary ADI was revoked (JECFA, 1977, 1978). JECFA at its 29th meeting (JECFA, 1986, 1987) carried out a detailed evaluation of all available data on Class III Ammonia Caramel and concluded that it could also allocate an ADI of 200 mg/kg bw/day for this caramel colour. JECFA concluded that the effect of most concern, i.e. lymphocytopenia, could best be evaluated from short-term studies, and based its ADI on the no-effect level of 20 g/kg bw/day in a 90-day study in rats using Class III Ammonia Caramel which contained approximately 15 mg THI/kg on a solids basis.

Evaluations of Class IV Sulphite Ammonia Caramel

In 1975 the SCF established a temporary group ADI of 100 mg/kg bw/day for Class IV Sulphite Ammonia Caramel and Class III Ammonia Caramel, because of the inadequacy of the available chronic studies. In 1983 the SCF noted that the genotoxicity data on Class IV Sulphite Ammonia Caramel did not indicate mutagenic potential and that subchronic studies did not show haematological effects (as were observed in the studies with Class III Ammonia Caramel). The available subchronic studies, including a one generation study in rats, indicated a NOAEL for Class IV Sulphite Ammonia Caramel of 10% in the diet. The SCF concluded that continued use of Class IV Sulphite Ammonia Caramel was justified until the results of long-term studies and adequate specifications were prepared (SCF, 1984). In 1987, the SCF concluded that the available long-term studies using Class IV Sulphite Ammonia Caramel on rats and mice showed no significant effects and considered that Class IV Sulphite Ammonia Caramel was toxicologically acceptable. An ADI of 200 mg/kg bw/day was established (SCF, 1989), based on a NOAEL for Class IV Sulphite Ammonia Caramel of 10% in the diet, however, it is not clear which uncertainty factor was used to reach this ADI.

JECFA in 1972 first established a temporary ADI of 100 mg/kg bw/day for caramel colours prepared by processes using ammonia or ammonium salts (Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel) (JECFA, 1972). Separate specifications were subsequently prepared for Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, and at the 21st meeting of JECFA (JECFA, 1978) the temporary ADI of 100 mg/kg bw/day was retained only for Class IV Sulphite Ammonia Caramel. The temporary ADI for Class III Ammonia Caramel was revoked at that time.

JECFA had drawn attention to the need for adequate specifications for Class IV Sulphite Ammonia Caramel and for a long-term study of carcinogenicity (JECFA, 1980). The temporary ADI of 100 mg/kg bw/day was extended pending the results of long-term toxicity studies. At its 29th meeting (JECFA, 1986) the JECFA based its evaluation on the no-effect level of 10 g/kg bw/day in the then provided long-term/carcinogenicity study in the rat (MacKenzie, 1985c, now published as MacKenzie et al. 1992c), to which (in view of the ancillary human data in which no adverse effects other than laxation were observed) a uncertainty factor of 50 was applied. This resulted in the allocation of an ADI of 200 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

Caramel colour is listed as generally recognized as safe (GRAS) at defined use levels as a general-purpose food additive in CFR 21, Section 182.1235 (CFR, 2006).

2.8. Exposure

2.8.1. Exposure assessment

The Panel agreed to follow the principles of the stepwise approach, which were used in the report of the Scientific Cooperation (SCOOP) Task 4.2 (EC, 1998), to estimate the intake of food additives. For each successive Tier, this involves a further refinement of intakes assessment. The approach goes from the conservative estimates that form the first Tier (Tier 1) of screening, to progressively more realistic estimates that form the Second (Tier 2) and Third (Tier 3) steps.

2.8.1.1. Crude estimates (Budget Method)

In the case of caramel colours Class I, II, III and IV, as these colours are permitted at *quantum satis* in all foodstuffs, the Panel could not estimate the dietary exposure using the Budget Method (Tier 1).

2.8.1.2. Refined estimates

As caramel colours Class I, II, III and IV are authorised *quantum satis* in almost all categories, the refined exposure estimates have been performed only for Tier 3 using the maximum reported use levels or when no maximum usages were reported to EFSA, maximum values defined by decision rules for *quantum satis* usages were used (Table 8).

Exposure estimates for children (1-14 years old) have been done by the Panel for 11 European countries (Belgium, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Denmark, Cyprus, Greece) based on detailed individual food consumption data provided by the EXPOCHI consortium (Huybrechts et al., 2010). As the UK is not part of the EXPOCHI consortium, estimates for UK children (aged 1.5- 4.5 years 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, 2008).

Since the UK population is considered to be one of the highest consumers of soft drinks in Europe and also because estimates are provided on more refined adult food consumption data rather than those available to the Panel (e.g. EFSA concise food consumption database, which gives access to aggregate

food categories consumed by 15 European countries), the Panel decided to select the UK population as representative of the EU consumers for estimates of dietary exposure to all 4 classes of caramel colours. Estimates of exposure to each of the classes of caramel colours, caramels Class I, II, III and IV, for the UK adult population (>18 years old) have been made by the Panel with the use of the detailed individual food consumption data (UK NDNS, 2000-2001) available from the UNESDA report (Tennant, 2008) .

Table 9 summarises the anticipated exposure of children and adults to each of the classes of caramel colours, caramels Class I, II, III and IV

When considering the maximum reported use levels from Table 8, the mean dietary exposure of European children (aged 1-10 years and weighing 15-29 kg, except for Cypriot children where the reported body weight was 54 kg for 11-14 years old), including UK pre-school children, ranged from 76.9 to 427.2 mg/kg bw/day for Class I Plain Caramel, from 8.7 to 34.6 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 21.7 to 302.4 mg/kg bw/day for Class III Ammonia Caramel, and from 23.2 to 506.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel. At the 95th or 97.5th percentile, estimates ranged from 179.6 to 882.2 mg/kg bw/day for Class I Plain Caramel, from 18.5 to 117.3 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 107.9 to 757.3 mg/kg bw/day for Class III Ammonia Caramel, and from 129.7 to 1480.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors (>10% in all or several countries) to the total anticipated exposure of children were for Class I Plain Caramel: non alcoholic flavoured drinks (12% to 55%), fine bakery wares (15% to 32%), desserts including flavoured milk products (11% to 48%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 56%), soups (11% to 32%) and malt bread (16% to 49%). For Class II Caustic Sulphite Caramel the main contributors were fine bakery wares (12% to 53%), desserts including flavoured milk products (11% to 41%), edibles ices (11% to 22%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 45%), soups (18% to 54%) and malt bread (19% to 55%). For Class III Ammonia Caramel the main contributors were fine bakery wares (13% to 45%), desserts including flavoured milk products (12% to 44%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 79%), and vinegar (12% to 45%), while in one country non alcoholic flavoured drink, malt bread confectionery, and sausages, pates and terrines contributed 29%, 15%, 13% and 10%, respectively. For Class IV Sulphite Ammonia Caramel the main contributors were non alcoholic flavoured drinks (13% to 51%), confectionery (20% to 81%), fine bakery wares (10% to 29%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (10% to 24%), and malt bread (10% to 34%).

When considering the maximum reported use levels from Table 8, anticipated dietary exposure reported for the UK adult population give a mean of 136.6 mg/kg bw/day and a 97.5th percentile of 429.3 mg/kg bw/day for Class I Plain Caramel; a mean of 21.7 mg/kg bw/day and a 97.5th percentile of 109.5 mg/kg bw/day for Class II Caustic Sulphite Caramel; a mean of 60.8 mg/kg bw/day and a 97.5th percentile of 295.0 mg/kg bw/day for Class III Ammonia Caramel; and a mean of 89.4 mg/kg bw/day and 97.5th percentile of 368.9 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors (>10% in all or several countries) to the total anticipated exposure of adults were for Class I Plain Caramel non alcoholic flavoured drinks (30%), beer and cidre bouché (27%), soups (16%), and sauces, seasonings (e.g. curry powder, tandoori) and pickles (10%). For Class II Caustic Sulphite Caramel the main contributors were beer and cidre bouché (50%) and soups (20%). For Class III Ammonia Caramel the main contributors were beer and cidre bouché (48%) and sauces, seasonings (e.g. curry powder, tandoori) and pickles (22%). For Class IV Sulphite Ammonia Caramel the main contributors were confectionery (65%) and non alcoholic flavoured drinks (23%).

Table 9: Summary of anticipated exposure to caramel colours Class I, II, III and IV using the tiered approach (EC, 2001) in children and adult populations

Tier 3. Maximum reported use levels	**Adult** (>18 years old) [2]	**Children** (1-10 years old, 15-29 kg body weight)[1]

	Mean Exposure (mg/kg bw/day)	**Exposure 97.5th-percentile** (mg/kg bw/day)	**Mean exposure** (mg/kg bw/day)	**Exposure 95th(3) or 97.5th(2) percentile** (mg/kg bw/day)
Class I	136.6	429.3	76.9 -427.2	179.6-882.2
Class II	21.7	109.5	8.7-34.6	18.5-117.3
Class III	60.8	295	21.7-302.4	107.9-757.3
Class IV	89.4	368.9	23.2-506.2	129.7-1480.2

[1] Except for Cypriot children where the reported body weight was 54 kg for 11-14 years old.
[2] For the UK population, estimates are based on the UNESDA report which gives the 97.5th percentile intake (Tennant, 2008).
[3] For EU children, estimates are based on the EXPOCHI report, which gives the 95th percentile intake.

2.8.1.3. Combined exposure

The Panel also evaluated combined anticipated dietary exposure to all four classes of caramel colours, taking into account the highest maximum reported level for all caramel classes, described in Table 8, from each food category. When considering this scenario, as presented in Table 10, anticipated mean combined dietary exposure reported for European children, including UK pre-school children, ranged from 83.5 to 698.3 mg/kg bw/day. At the 95th/97.5th percentile, estimates ranged from 224.8 to 1672.3 mg/kg bw/day. For the UK adult population this scenario gave a range of exposures of 194.8 and 474.3 mg/kg bw/day for the mean and the 97.5th percentile, respectively.

The main contributors (>10% in several countries) to the total combined anticipated exposure to caramel colours for children were non alcoholic flavoured drinks (11% to 28%), confectionery (19% to 58%), fine bakery wares (15% to 29%), desserts including flavoured milk products (10% to 31%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (14% to 44%), and malt bread (16% to 46%). Soups were estimated to contribute from 25% to 28% in two countries and vinegar was estimated to contribute 20% in one country.

For the adult population the main contributors (>10%) were confectionery (30%), non alcoholic flavoured drinks (21%), beer and cidre bouché (19%), and soups (11%).

Table 10: Summary of anticipated combined exposure to all caramel colours in children and adult populations

Tier 3. Maximum reported use levels	**Adult** (>18 years old) [2]		**Children** (1-10 years old, 15-29 kg body weight)[1]	
	Mean Exposure (mg/kg bw/day)	**Exposure 97.5th percentile** (mg/kg bw/day)	**Mean exposure** (mg/kg bw/day)	**Exposure 95th(3) or 97.5th(2) percentile** (mg/kg bw/day)
All caramel colours	194.8	474.3	83.5 - 698.3	224.8 - 1672.3

[1] Except for Cypriot children where the reported body weight was 54 kg for 11-14 years old.
[2] For the UK population, estimates are based on the UNESDA report which gives the 97.5th percentile intake (Tennant, 2008).
[3] For EU children, estimates are based on the EXPOCHI report, which gives the 95th percentile intake.

2.8.1.4. Dietary exposure to 4-MEI, present in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel; THI, present in Class III Ammonia Caramel and sulphur dioxide (SO_2), present in Class II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel.

Maximum Levels (ML) for the constituents 4-MEI (< 250 mg/kg caramel colour) found in Class III Ammonia caramel and Class IV Sulphite Ammonia Caramel; THI (< 10 mg/kg caramel colour) found in Class III Ammonia caramel and sulphur dioxide (<0.2% caramel colour) found in Class II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel have been specified under Commission Directive 2008/128/EC. These constituents are of particular relevance in relation to the toxicological profile of these classes of caramel colours.

Maximum Reported Levels (MRL) have been provided by the Industry (EUTECA, 2011) for 4-MEI, being 140 mg/kg for Class III Ammonia Caramel and 183 mg/kg for Class IV Sulphite Ammonia Caramel. The MRL for THI (EUTECA, 2011) are the same as the maximum levels specified in Commission Directive 2008/128/EC (10 mg/kg) for Class III Ammonia Caramel. Using the maximum levels defined for these constituents in the specifications for caramel colours and the MRL from EUTECA (2011), the Panel derived anticipated exposure to 4-MEI, THI and sulphur dioxide arising from the anticipated exposure from the diet to caramel colours for children and adults, as estimated by the Panel and presented in Table 9.

Table 11 summarises the anticipated exposure of children and adults to 4-MEI, THI and sulphur dioxide.

Table 11: Summary of anticipated exposure to 4-MEI, THI and sulphur dioxide in child and adult populations from uses of caramel colours Class II, Class III and Class IV.

Constituents	**MLs or MRLs[4]**	**Adult** (>18 years old) [2]		**Children** (1-10 years old, 15-29[1] kg body weight) [3]	
		Mean Exposure (mg/kg bw/day)	**Exposure 97.5th percentile** (mg/kg bw/day)	**Mean exposure (range lowest-highest)** (mg/kg bw/day)	**Exposure 95th or 97.5th percentile[2] (range lowest-highest)** (mg/kg bw/day)
Sulphur dioxide	0.2%*				
Class II		0.043	0.219	0.017-0.069	0.037-0.235
Class IV		0.179	0.738	0.046-1.012	0.259-2.960
4-MEI					
Tier 2 : MLs (EC)	0.025%*				
Class III		0.015	0.074	0.005-0.076	0.027-0.189
Class IV		0.022	0.092	0.006-0.127	0.032-0.370
Tier 3: MRLs (EUTECA, 2011a,b)					
Class III	0.014%**	0.009	0.041	0.003-0.042	0.015-0.106
Class IV	0.0183%**	0.016	0.068	0.004-0.093	0.024-0.271
THI	0.001%*				
Class III		0.0006	0.003	0.0002-0.003	0.0011 - 0.0076

[1] Except for Cypriot children where the reported body weight was 54 kg for 11-14 years old.
[2] For the UK population, estimates are based on the UNESDA report which gives the 97.5th percentile intake (Tennant, 2008).
[3] For EU children, estimates are based on the EXPOCHI report, which gives the 95th percentile intake.
[4] ML: Maximum Levels from the Commission Directive 2008/128/EC; MRL: Maximum Reported Levels from the industry (EUTECA, 2011a,b)
*ML from the Commission Directive 2008/128/EC
**MRL from the industry (EUTECA, 2011a,b)

Based on the MLs defined for these constituents in the specifications for caramel colours and on the MRLs from industry (EUTECA, 2011a,b), anticipated dietary exposure calculated for the UK adult population gives the lowest means and highest 97.5th percentiles of 0.04 and 0.74 mg/kg bw/day for sulphur dioxide; of 0.009 and 0.092 mg/kg bw/day for 4-MEI; and of 0.0006 and 0.003 mg/kg bw/day for THI.

In children, anticipated dietary exposure gives the lowest means and highest 95th/97.5th percentiles of 0.017 and 2.96 mg/kg bw/day for sulphur dioxide; of 0.003 and 0.370 mg/kg bw/day for 4-MEI; and of 0.0002 and 0.008 mg/kg bw/day for THI.

3. BIOLOGICAL AND TOXICOLOGICAL DATA

In considering the biological and toxicological data available on the four classes of caramel colours, the Panel noted, as described in more detail in Section 2.1, that the wide range of starting materials and reactants used for the production of caramel colours may result in variability in chemical composition and purity, and that information on the composition of the materials tested is generally lacking, other than on the broad class of caramel that was tested together with information on some key constituents such as THI and 4-MEI. As already outlined in section 2.2, the Panel noted that LMW constituents of caramel colours produced with ammonia (Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel) include N-heterocyclic compounds such as pyridines, pyrazines, pyrroles and imidazoles. The occurrence of furanoid compounds in caramel colours is also reported, with furan being specifically identified in some caramel colours e.g. Class IV Sulphite Ammonia Caramel. The presence of acrylamide has also been reported.

The Panel also noted that a number of the identified or theoretical constituents of caramel colours, e.g. furan, acrylamide and 5-HMF, have genotoxic and in some cases carcinogenic potential, which may be relevant to the toxicological profile of the caramel colours. The Panel considered that the toxicological studies carried out on the caramel colours will have involved exposure to these compounds, in addition to the constituents THI and 4-MEI, and any anticipated toxicological effect due to the LMW fraction constituents of caramel colours should have been detected in these studies. A brief overview of the toxicological profiles of THI, 4-MEI, furan and 5-HMF is however provided in Sections 3.2.9 and 3.2.10 of this opinion. Acrylamide has not been included in this overview since the data provided by industry, as reported in Section 2.1, indicate that acrylamide was not detectable (limit of detection 1 µg/kg) in Class IV Sulphite Ammonia Caramel and Class III Ammonia Caramel (limit of detection 100 µg/kg), and its presence in Classes I and II caramels is not anticipated (EUTECA, 2011a,b).

3.1. Absorption, distribution, metabolism and excretion

In a study on the toxicokinetic behaviour of Class III Ammonia Caramel reported by JECFA (JECFA, 1987), groups of rats (2-4) received 10 or 20% caramel solutions (volume not defined) for 100 days. Examination of the faecal contents revealed that about one-third of the colour-giving constituents (not defined) appeared to be absorbed. According to the authors, no conclusions could be drawn regarding the absorption of colourless constituents (Haldi and Wynn, 1951). The Panel considered that few conclusions regarding the toxicokinetics of the caramel colours could be drawn from this very limited study.

JECFA also described a more comprehensive study on the toxicokinetics of Class IV Sulphite Ammonia Caramel in male F344 rats (Selim et al., 1992). In this study Caramel Colour IV (Class IV Sulphite Ammonia Caramel) prepared from uniformly-labelled [U-^{14}C]-glucose was ultrafiltered in order to isolate the High Molecular weight Colour Fraction (HMCF). The colour fraction that was non-permeable to a 10 000-Da porosity membrane contained 84% of the colour, 22% of the solids and 24% of the radioactivity of the ^{14}C-Caramel Colour IV. The Panel noted that no further characterisation of the HCMF was carried out, and hence no information is available on its composition. The absorption, distribution and excretion of ^{14}C-HMCF was evaluated after administration of single or multiple oral doses of the material at a dose level of 2.5 g/kg bw. Following

administration of a single oral dose of ^{14}C-labelled material, groups of 4 rats were killed at intervals of 4, 8, 12, 24, and 96 hours after dosing, and radioactivity was measured in the following tissues/organs; blood, brain, heart, lungs, liver, kidneys, spleen, thymus, mesenteric and cervical lymph nodes, gastrointestinal tract (contents and tissues), and carcass. Most of the radioactivity was located in the gastrointestinal tract, and only low levels of radioactivity were found in blood and tissues. The specific radioactivity in the thymus, mesenteric lymph nodes, spleen, kidneys, and liver exceeded that in blood but, with the exception of the mesenteric lymph nodes, the radioactivity was cleared rapidly over the 96-hour study period. With the exception of the gastrointestinal tract, the highest tissue levels attained, in the liver and kidneys, never exceeded 0.02 % and 0.01 % respectively of the administered dose.

Rats on the multiple oral dosage regimen were given unlabelled HMCF in their drinking water for 13 days before the administration of a bolus dose of ^{14}C-HMCF at a dose level of 2.5 g/kg bw on day 14. On both dosage regimes, the predominant route of excretion was in the faeces. Only 1-2 % of the administered radioactivity was excreted in the urine and only a negligible amount was found in the expired air. More than 99% of the administered radioactivity was excreted in the faeces within 96 hours. The principal tissues in which radioactivity were found were the mesenteric lymph nodes, liver, kidney and tissues of the gastro-intestinal tract. No major differences were observed in the absorption, distribution or excretion patterns between the single and multiple oral dose regimens. The authors concluded that after administration of large doses of the coloured constituents of Class IV Sulphite Ammonia Caramel, only a small fraction was absorbed, distributed in lymphoreticular tissue, and eventually excreted in the urine; uptake by the mesenteric lymph nodes appeared to account for the pigmentation observed in this tissue (Selim et al., 1992).

The Panel noted that, except for the study on HCMF of Class IV Ammonium Sulphite Caramel described above, data on the toxicokinetics of the caramel colours are lacking. The Panel considered that individual constituents of the low molecular weight fraction of caramel colours (e.g. MW less than 500) are likely to be absorbed, although little information is available to confirm this.

3.2. Toxicological data

3.2.1. Acute oral toxicity

No data are available on the acute oral toxicity of Class I Plain Caramel, Class II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel. As reported by JECFA (JECFA, 1987), the oral LD_{50} of Class III Ammonia Caramel in rats was reported to be higher than 17 500 mg/kg bw (Chacharonis, 1960, 1963). In mice and rabbits Class III Ammonia Caramel administered at single doses of up to 10 000 and 15 000 mg/kg bw respectively did not result in treatment-related effects (Sharratt, 1971). In earlier JECFA evaluations (JECFA, 1972, 1977), it was reported that convulsions were observed after administration of Class III Ammonia Caramel (conceivably at higher doses that those used by Sharrat, 1971) to a range of species and by different routes. These convulsions were however considered to be attributable to the presence of 4-MEI in the Class III Ammonia Caramel product. Additional information on the toxicological profile of 4-MEI is provided in Section 3.2.9.

3.2.2. Short-term and subchronic toxicity

3.2.2.1. Class I Plain Caramel

One short-term study on Class I Plain Caramel is described by JECFA and TemaNord. Class I Plain Caramel was administered to groups of 20 weanling female Wistar rats at levels of 0, 15, or 30% in the diet (highest dose equivalent to 30 g/kg bw/day) for 8 weeks, followed by a 4-week recovery period. Dose-related diarrhoea was observed in the treated animals and food efficiency was decreased (dose not specified), but the growth rate was normal. Haematological indices, in particular leucocyte counts, were normal throughout the study. The relative caecal weights were increased after eight weeks, but

returned to normal by the end of the 4-week recovery period. Discolouration of the mesenteric lymph nodes was observed in animals of both treatment groups after eight weeks, but the discolouration diminished during the recovery period. No other gross or microscopic pathological changes were reported (Sinkeldam and van der Heyden, 1976a).

In an additional study published subsequent to the JECFA and TemaNord reports, Caramel A (described by the authors as being in accordance with the definition, properties, identity test results and purity test results stipulated for Class I Plain Caramel) was orally administered at dosage levels of 0.5, 1, and 2 ml/kg bw/day to 5 Crj:CD (SD) IGS strain rats of each sex for 28 consecutive days (Harigae et al., 2000a). The control group received water alone. No deaths and no clinical signs of toxicity were noted in either sex of any group, and no treatment-related effects were reported on body weight, food consumption, urinalysis, ophthalmology, blood chemical analysis, organ weights or on histopathological findings. Females in all caramel colour A groups showed significant decreases in Activated Partial Thromboplastin Time (APTT) in comparison with the control group, and females receiving 0.5 ml/kg bw/day or 2 ml/kg bw/day showed significant increases in the lymphocyte fraction of total white blood cells and significant decreases in the neutrophil fraction. However all values were within the historical control range, and the authors concluded that these findings occurred by chance, and were not due to caramel colour A. Occasional histopathological findings including vitreous inclusions in proximal renal tubular epithelial cells in rats receiving Caramel A were also concluded by the authors to have occurred by chance, and were not due to caramel colour A. The non-toxic dose of caramel colour A was estimated by the authors to be at least 2 ml/kg bw/day (equivalent to 2.66 g/kg bw/day, sp. gr. 1.33) (Harigae, 2000a). The Panel agreed with this conclusion.

3.2.2.2. Class II Caustic Sulphite Caramel

A 90-day oral toxicity study has been carried out with Class II Caustic Sulphite Caramel in rats. Five groups of 20 male and 20 female weanling Fischer F-344 rats were given Class II Caustic Sulphite Caramel in drinking water at dose levels of 0, 4, 8, 12, or 16 g/kg bw/day for 90 days. Body weights and food and water intakes were recorded weekly. The principal observations were a dose-related decrease in body weight and reduced food consumption and fluid intake, mainly affecting the groups that received doses of 12 and 16 g/kg bw/day of Class II Caustic Sulphite Caramel, and slight, dose-related increases in absolute and relative kidney weights and full and empty caecum weights, with no evidence of significant histopathological changes in any tissue. Dose-related staining of the gastrointestinal tract and mesenteric lymph nodes was observed, with deposits of yellow pigment observed microscopically in the caecal mucosa and mesenteric lymph nodes (only the highest dose groups were examined microscopically) (MacKenzie, 1985a; MacKenzie et al., 1992a).

JECFA, in evaluating this study, concluded in agreement with the authors of the study that the decreased body-weight gain and increased kidney weight observed in the rats were the consequence of the reduced consumption of food and fluids, probably resulting from the reduced palatability of the drinking-water due to the presence of the caramel (JECFA, 1987). JECFA considered that the pigmentation of mesenteric lymph nodes and enlargement of the caecum were not of toxicological significance and concluded that the NOAEL was the highest dose tested, 16 g/kg bw/day. The Panel agreed with this NOAEL.

3.2.2.3. Class III Ammonia Caramel

The constituents THI and 4-MEI, found in Class III Ammonia Caramel, are of particular relevance in relation to the toxicological profile of this class of caramel.

In previous evaluations of Class III Ammonia Caramel including those of JECFA and SCF, a substantial number of studies on the short-term toxicity of Class III Ammonia Caramel have been described. One of the most notable effects in these studies was a reduction in circulating lymphocytes (lymphocytopenia), measured in haematological investigations. The overall conclusion that was drawn

from these studies at the time is that THI, a constituent in Class III Ammonia Caramel, and pyridoxine (vitamin B_6), a general constituent of the diet, play a major role in Class III Ammonia Caramel-related lymphocyte depression. Regarding the effects on white blood cells, JECFA in its evaluations commented that depression of lymphocyte counts has been shown to be due largely, if not solely, to THI. This conclusion was probably based on the studies of Sinkeldam and co-workers (Sinkeldam et al., 1982b, 1988) and Kroplien (1984, 1985), among others. Due to the large number of studies on Class III Ammonia Caramel, including mechanistic studies on the leucocytopenia observed in studies with this caramel colour, summaries of these studies are provided in Annex B, with overviews of key studies being provided in the body of the opinion. Similarly, summaries of specific studies on THI are provided in Annex D.

In these studies, as the normal proliferative activity of all lymphoid organs was not affected, it was concluded that THI did not induce lymphocytopenia by reduced production of lymphocytes (Houben et al., 1992a; Houben and Penninks, 1994). There were however indications of a diminished migration of mature cells from the thymus to the periphery (Sinkeldam et al., 1988; Houben et al., 1992a; Houben and Penninks, 1994). In addition, splenic cells of rats did not show the expected response upon mitogenic stimulation (Houben et al., 1992a; Houben and Penninks, 1994). More recently it has been shown, in a study in which mice were given THI in drinking water at a level of 50 mg/l for 3 days, that the diminished migration of lymphocytes from lymphoid tissues such as the thymus and spleen induced by THI was related to an increase in sphingosine 1-phosphate (S1P) in these tissues (Schwab et al., 2005). Further discussion of the immunotoxicity of both Class III Ammonia Caramel and THI is provided in sections 3.2.7 and 3.2.9 of this opinion.

The pivotal study used by both the SCF and JECFA to derive an ADI for Class III Ammonia Caramel was the 90-day oral toxicity study in rats carried out by MacKenzie et al. (1985b, 1992b). In this study, groups of weanling F344 rats (20 animals/sex/group) were given 2 different samples of Class III Ammonia Caramel in the drinking water at dose levels of 0, 10, 15, or 20 g/kg bw/day (sample A; containing approximately 15 mg THI/kg caramel on a solids basis;) and 20 g/kg bw/day (sample B; containing 295 mg THI/kg caramel on a solids basis) for 90 days. The diet contained >10 mg pyridoxine/kg. Although the findings were not always consistent, the animals treated with 15 and 20 g/kg bw/day of both sample A and B generally had decreased body weights and body weight gains compared with controls. All treated groups had significantly decreased food and drinking water intake compared with controls. The authors concluded that the effects on body weight gain were in part due to the reduced water intake caused by poor palatability of the drinking water rather than toxic effects of the caramel colour per se. The authors also suggested that a reduction in food intake is an invariable response to either voluntary or forced restriction of water intake by the rat (MacKenzie et al., 1992b).

Both sample A and sample B Class III Ammonia Caramel induced treatment-related increases in the absolute and relative weights of the caecum (full and empty) in both sexes at all dose levels. Absolute and relative kidney weights were also increased in a dose-related manner and were considered by the authors to be a consequence of reduced water intake. No histopathological changes in the kidneys of any of the test groups were observed. A dose-dependent decrease in the absolute weight of the thymus was observed in sample A males and sample B males and females. This effect was however only statistically significant at a dose of 20 g/kg bw/day. Neither sample had an effect on the relative thymus weight, and the Panel concluded overall that the effect on absolute thymus weight was not of toxicological relevance. Other differences in absolute and relative organ weights appeared to be a consequence of the dose-related reduction in body weight.

Sample B Class III Ammonia Caramel (containing 295 mg THI/kg) at a dose level of 20 g/kg bw/day induced a statistically significant decrease in lymphocyte counts in both sexes at 2 weeks and only in male rats at 6 weeks. All lymphocyte values in these groups were normal at the termination of the study. No statistically significant decreases in lymphocyte counts occurred in male or female groups fed batch A Class III Ammonia Caramel (containing approximately 15 mg THI/kg) at any of the dose levels, with the exception of male rats receiving 10 g/kg bw/day (but not at 15 or 20 g/kg bw/day) at 2 weeks only. There were no consistent changes in clinical chemistry values, except for a slight but

statistically significant increase in blood urea nitrogen values in rats of both sexes receiving sample B Class III Ammonia Caramel at 6 and 13 weeks, and in male rats receiving 20 g/kg bw/day of sample A Class III Ammonia Caramel at 13 weeks. Urinalysis revealed decreased urinary volume and increased specific gravity in animals receiving either sample A or sample B Class III Ammonia Caramel at 6 weeks. At termination at 13 weeks, these differences were only significant in males receiving 20 g/kg bw/day of either sample A or sample B Class III Ammonia Caramel. The only treatment-related microscopic changes noted were minimal to moderate accumulation of pigment in the tissues of the intestinal tract and mesenteric lymph nodes without other histopathological evidence of tissue damage.

To determine whether the poor palatability of the drinking water due to the presence of high levels of Class III Ammonia Caramel was the mechanism underlying the decreased body-weight gains frequently noted in toxicity studies with Class III Ammonia Caramel, a paired-feeding study was conducted (Sinkeldam, 1979). In this study a group of 10 male Wistar rats received drinking water containing 12 % Class III Ammonia Caramel (equivalent to 6 g/kg bw/day) whereas controls were permitted food and water intake quantitatively equivalent to that consumed by the exposed group. As similar decreases in body weight gain were noted in both exposed and control animals, it was concluded that growth depression after exposure to Class III Ammonia Caramel is the result of decreased fluid and food intake. Poor palatability of the drinking water containing the colour was the probable cause of these changes (Sinkeldam, 1979), and it has also been suggested that a reduction in food intake is an invariable response to either voluntary or forced restriction of water intake by the rat (MacKenzie et al., 1992b). The Panel agreed with this interpretation.

In a study in female Balb/c mice, the animals received a diet containing 0, 2 or 10% (equivalent to approximately 0, 3.3, or 16.7 g/kg bw/day) of a commercial Class III Ammonia Caramel preparation in the drinking water for 9 weeks. The sample of Class III Ammonia Caramel used contained < 25 mg THI/kg; the diet contained relatively high vitamin B_6 content (not specified). Although a treatment-related lymphocytopenia was not evident, flow cytometric analysis demonstrated reductions in the $CD4^+$ and $CD8^+$ lymphocyte subpopulations in exposed mice. In addition, the proliferative response of spleen cells to B and T cell mitogens was significantly reduced in the mice exposed to 2% Ammonia Caramel. No changes were observed in natural killer cell activity or in the humoral antibody response to a viral antigen (Thuvander and Oskarsson, 1994).

Overall, although some statistically-significant effects were seen in animals receiving Class III Ammonia Caramel compared with controls in the study of MacKenzie et al, they were not considered by the authors to be toxicologically relevant, and consequently the NOAEL for Class III Ammonia Caramel was considered to be 20 g/kg bw/day (MacKenzie, 1985b; MacKenzie et al., 1992b). JECFA concurred with this NOAEL and consequently allocated an ADI of 200 mg/kg bw/day to the colour (JECFA, 1987). The Panel also agreed that the NOAEL in this study was 20 g/kg bw/day.

3.2.2.4. Class IV Sulphite Ammonia Caramel

JECFA describes a number of short-term and subchronic studies with Class IV Sulphite Ammonia Caramel. Summaries of the more important studies for this opinion are provided in this section, other studies are provided as summaries in Annex C, as background information. The constituent 4-MEI is of particular relevance in relation to the toxicological profile of this class of caramel.

Female Wistar rats (20/group) received stock diet to which 0, 10, or 20% Class IV Sulphite Ammonia Caramel containing 202 mg 4-MEI/kg was added. From week 2-6 the levels of Class IV Sulphite Ammonia Caramel in the diet were increased to 15 and 25% and from week 7-16, the levels were increased to 25 and 30%. A 4-week recovery period followed. No effects were found on food consumption, growth, leucocyte count or relative thymus weight. The relative weights of the caecum, both filled and empty were increased after 4 weeks of feeding Class IV Sulphite Ammonia Caramel, however after the recovery period of 4 weeks the increases had disappeared. Gross examination at autopsy after 16 weeks of feeding Class IV Sulphite Ammonia Caramel revealed a dose-related, brown-greenish coloration of the mesenteric lymph nodes in all test animals at the highest dose level.

Microscopically, the lymph nodes of the test rats showed pigment accumulation. After the recovery period of 4 weeks the colour change and pigmentation were less, but still visible (Sinkeldam and van der Heyden, 1975).

Wistar rats (10 animals/sex/group) were fed diets containing 0, 1.25, 2.5, 5, 10, and 15% of low colour intensity Class IV Sulphite Ammonia Caramel or 0, 0.5, 1, 2, 4, and 6% high colour intensity Class IV Sulphite Ammonia Caramel for 10 weeks. In this study and also those of Procter et al., 1976, BIBRA, 1977, and in the genotoxicity studies on Class IV Sulphite Ammonia Caramel reported by Brusick and co-workers, as reported below, the descriptor low intensity caramel referred to a 'single strength' Class IV caramel (typically colour intensity up to 0.18) and high intensity caramel refers to a 'double strength' Class IV caramel (typically, >0.2 colour intensity). Class IV Sulphite Ammonia Caramel caused loose stools at the highest dose levels, although body weight gains were not affected. Leucocyte counts were not affected. Slight indications of caecal enlargement were observed. Minimal amounts of pigment were observed in the mesenteric lymph nodes of rats fed 2.5% and higher of the low colour intensity sample, and 1% and higher of the high colour intensity sample (Sinkeldam and van der Heyden, 1976b).

Sprague-Dawley rats (15 animals/sex/group) were fed diets containing 1.25, 2.5, 5, 10, and 15% of low colour intensity Class IV Sulphite Ammonia Caramel or 0.5, 1, 2, 4, and 6% high colour intensity Class IV Sulphite Ammonia Caramel for 10 weeks. In the animals fed 15% low colour intensity Class IV Sulphite Ammonia Caramel, the faeces became soft within 2 weeks. The water content of the faeces from the highest dose groups (both high and low intensity) was higher than that of the controls. Body weight gains were slightly decreased in male, but not female, rats fed high and low intensity caramel colour. No effects were found on total white cell or lymphocyte counts or pigmentation of the mesenteric lymph nodes. Caecal weights were generally increased in all test groups fed Class IV Sulphite Ammonia Caramel (Procter et al., 1976).

Weanling Wistar rats (10-60 animals/sex/group) were fed diets containing 0, 0.5, 1.0, 2.0, 4.0, or 16.0% of high or low colour intensity Class IV Sulphite Ammonia Caramel for 10 weeks, followed by a recovery period with basal diet (7 or 28 days). Decreased body weight gains were noted in animals of both sexes fed 16% high colour intensity Class IV Sulphite Ammonia Caramel, but not in the groups fed low colour intensity caramel. Food intake was not consistently altered. There were no consistent or dose-related differences in lymphocyte counts or leucocyte counts. Liver weights were significantly increased in the group fed 16% Class IV Sulphite Ammonia Caramel (high colour intensity). Increased relative kidney weights were observed in the groups fed 2, 4, and 16% Class IV Sulphite Ammonia Caramel (high colour intensity) and 16% Class IV Sulphite Ammonia Caramel (low colour intensity), although no histological changes were observed. Increased caecal weights were seen only at the 16% feeding level for both the low intensity and the high intensity Class IV Sulphite Ammonia Caramel. At necropsy pigmentation of the lymph nodes was seen at the 16% feeding level of both low intensity and high intensity caramel colour. Microscopically, pigmentation was observed in the mesenteric lymph nodes in the males and females in the groups fed 16% Class IV Sulphite Ammonia Caramel (high colour intensity). Relative weights of the liver and kidneys and caecal weights returned to normal during the recovery period (BIBRA, 1977).

Weanling F344 rats (groups of 30 males and 30 females) were given Class IV Sulphite Ammonia Caramel in drinking water at concentrations which provided intakes of 0, 15, 20, 25, or 30 g/kg bw/day for 13 weeks (Heidt and Rao, 1980). All rats given Class IV Sulphite Ammonia Caramel produced dark-coloured, soft or sticky, liquid and/or odorous faeces which stained and caused alopecia of the perianal area, most noticeably at the higher dose levels. Dose-related significant decreases in food intake, water consumption (after correction for caramel content), and body weight gains were observed in both males and females at all dose levels of caramel and were attributed to the poor palatability of the drinking solutions. All treatment groups of both sexes had significantly reduced blood urea nitrogen and alkaline phosphatase levels at both 45 and 90 days. Total serum protein values of both sexes in the treatment groups were lower than controls at 90, but not at 45, days. The authors suggested that these effects may be due to reduced food intake and growth retardation. All treated rats

had reduced urine volume and increased urine specific gravity, protein, ketones, and acidity, which were associated with decreased water consumption. Treatment-related changes were seen in absolute and relative weights of brain, liver, heart, male gonads, thymus, spleen and kidney weights were observed, although no histopathological changes were seen in these organs that correlated with the organ weight changes. Both absolute and relative thymus weights were significantly decreased in all male treatment groups and in females receiving 25 or 30 g/kg bw/day. Absolute spleen weights were significantly decreased in males receiving 25 or 30 g/kg bw/day and in females receiving 20, 25 and 30 g/kg bw/day. Both absolute and relative kidney weights were significantly increased in all treated rats, both males and females. Full and empty caecum weights were also increased in all treatment groups, with macroscopically visible caecal enlargement. Dark staining of the mucosa of the caecum and colon was noted in all treatment groups. The only treatment-related histopathological changes reported were an accumulation of yellowish-tan pigment in macrophages of the mesenteric lymph nodes, together with a thickening of the caecal mucosa and the tunical muscularis of the caecum. The authors considered that these changes in the caecum and mesenteric lymph nodes were nonspecific consequences of administration of large quantities of caramel, as also concluded by JECFA in their 1970 evaluation, and that the observed decreases in organ weights were largely related to the changes in body weight seen in the study (Heidt and Rao, 1980) . The Panel concurred with this interpretation and noted that the effects reported in the study have been seen in the majority of other studies in the rat conducted with high dose levels of the caramel colours (see also section 3.2.4.3). The Panel concluded therefore that 30 g/kg bw/day, the highest dose tested, could be considered a NOAEL in this study.

Class IV Sulphite Ammonia Caramel was evaluated in a 13-week drinking water study in F344 rats, as a preliminary study for chronic toxicity and carcinogenicity studies (MacKenzie, 1985b; MacKenzie et al., 1992c). Class IV Sulphite Ammonia Caramel was mixed with demineralised water and the solutions given to the animals ad libitum in the drinking water. Groups of 30 male and 30 female rats were given Class IV Sulphite Ammonia Caramel for 13 weeks at levels of 0, 15, 20, 25 or 30 g/kg bw/day, based on body weight data and drinking water consumption data. Treated rats had dose-related lower water consumption than controls. This was attributed by the authors to poor palatability of the drinking water, and was generally associated with decreased food consumption and body weights, as previously described for the parallel study on Class III Ammonia Caramel (section 3.2.2.3). Rats given Class IV Sulphite Ammonia Caramel often had soft or liquid malodorous faeces. Blood biochemical changes in the rat (i.e. reduced blood urea nitrogen, alkaline phosphatase and total serum protein) appeared to be related to dietary influences and were not considered by the authors to be toxicologically significant. There were no treatment-related alterations in haematological variables (MacKenzie, 1985b, MacKenzie et al., 1992c). The authors noted that "*it is well known that when the water intake of rodents is limited, food intake will be proportionally reduced and there will be a reduced rate of body weight gain. Reduced water intake also affects renal function, reducing urinary output and increasing specific gravity. A compensatory hypertrophy of the kidney, without pathological changes, also occurs*" (MacKenzie et al., 1992c). The authors considered therefore that the NOAEL in this study was 30 g/kg bw/day. The Panel agreed with this conclusion.

As reported by JECFA, adult beagle dogs (groups of 3 males and 3 females) received 0, 6, 12.5, or 25% Class IV Sulphite Ammonia Caramel in their diet (estimated by the Panel to be equivalent to 0, 1.5, 3.1 or 6.25 g/kg bw/day for a 10 kg dog eating 250 g of chow per day) 5 days per week for 90 days. No significant adverse effects on growth, behaviour, food consumption, mortality, liver function, kidney function, haematology, urinalysis, gross pathology, or histopathology were noted (Kay and Calandra, 1962c). Based on this report, the Panel considered that the NOAEL in this study was 6.25 g/kg bw/day, the highest dose tested.

3.2.3. Genotoxicity

Caramel colours have been extensively tested for genotoxic potential in a variety of assays *in vitro,* reflecting the fact that browning and caramelising products formed in food have been suggested or demonstrated to contain potentially genotoxic chemicals. Overall the results in a range of *in vitro*

systems were generally negative, with a few marginally positive findings (e.g. Kawachi et al., 1980; Ishidate and Yoshikawa, 1980; Jensen et al., 1983; Ishidate et al., 1984; Yu et al., 1984; Aeschbacher, 1986). The caramel colours investigated in these publications were generally commercial caramel colours whose nature and classification was not further specified by the authors (JECFA, 1987). On the basis of these marginally inconclusive results, the International Technical Caramel Association (ITCA) in the period 1980–1990 carried out a comprehensive testing programme with samples of well-defined caramel colours to determine the genotoxic potential of the different caramel classes in a battery of genotoxicity tests *in vitro* and *in vivo* (ITCA, 1990). The results of these studies are summarised below for the different caramel classes.

3.2.3.1. Class I Plain Caramel

As described by JECFA, two samples of Class I Plain Caramel with different colour intensities were tested in a bacterial mutagenicity (Ames) assay using *Salmonella typhimurium (S. typhimurium)* strains TA98, TA100, TA1535, TA1537, and TA1538. Class I Plain Caramel was neither mutagenic nor cytotoxic, either with or without activation by rat liver S-9 fraction, at up to 20 µl per plate (Richold and Jones, 1980a; 1980b, unpublished study not available to the Panel).

The genotoxicity of Class I Plain Caramel was also investigated in a battery of three valid *in vitro* short-term tests and one *in vivo* (Adams et al., 1992). In this study a total of 15 caramel colours representative of all four caramel classes were examined for genotoxic activity using the *S. typhimurium* plate incorporation assay (Ames test). Five bacterial strains, TA1535, TA1537, TA1538, TA98 and TA100 were used in all the plate incorporation tests, some of the caramel colours being tested both with and without a pre-incubation stage. The results of the bacterial mutation assay with Class I Plain Caramel showed no evidence of genotoxic activity. The Panel noted that it is not clear whether the result reported for Class I Plain Caramel in the publication of Adams et al. (1992) was also that reported in the earlier unpublished reports of Richold and Jones (1980a, 1980b).

In the mammalian cell mouse lymphoma L5178Y assay Class I Plain Caramel produced a statistically significant increase in mutation frequencies, but only at dose levels of 6.5 mg/ml and above in the absence of S-9 metabolic activation system. A cytogenetic assay *in vitro*, using Chinese Hamster Ovarian (CHO) cells, showed evidence of chromosomal damage in the presence of Class I Plain Caramel but only in the absence of S-9 and only at the highest dose level of 10 mg/ml. A negative result for Class I Plain Caramel was obtained in the mouse micronucleus assay *in vivo*, using a single oral dose of 5000 mg/kg bw Class I Plain Caramel with examination of bone marrow from groups of 5 male and 5 female mice at 24, 48 or 72 hours after dosing (Adams et al., 1992).

In a more recent study, the genotoxicity of Caramel Colour A (described by the authors as being in accordance with the definition, properties, identity test results and purity test results stipulated for Class I Plain Caramel) was investigated *in vitro* in a bacterial reverse mutation test and a chromosome aberration test, and a mouse micronucleus test *in vivo* (Harigae, 2000b). Negative results were reported at 5000 µg Caramel Colour A/plate without and with metabolic activation in the reverse mutation test, carried out with five bacterial strains, *S. typhimurium* TA100, TA98, TA1535 and TA1537, and *Escherichia coli* WP2*uvrA*, using the pre-incubation method. The chromosome aberration test was carried out using cultured mammalian fibroblasts derived from Chinese hamster lung tissue. In a cell growth inhibition test performed in the same culture system prior to the chromosomal aberration test the IC_{50} of Caramel Colour A was 3309 µg/ml without S9, but the IC_{50} was not reached up to 5000 µg/ml in the presence of S9. In the chromosomal aberration test, no effects were noted at concentrations up to and including 5000 µg/ml with metabolic activation and up to and including 3500 µg/ml without activation. However, in the absence of metabolic activation Caramel Colour A at 4000 µg/ml, a concentration inducing overt cytotoxicity, caused chromosomal aberrations, although the aberration potential was considered by the authors to be weak and without a marked dose-dependency.

The mouse micronucleus test was performed in CD-1 mice, dosed orally by gavage at levels of 500, 1000 or 2000 mg/kg bw and killed 24 or 48 hours after administration of the second of two doses,

given 24 hours apart. Negative results for micronuclei induction were obtained at 2000 mg/kg bw, the highest dose level tested (Harigae, 2000b). The Panel noted that there was no change in the NCE/PCE ratio in animals receiving Caramel Colour A compared with controls.

3.2.3.2. Class II Caustic Sulphite Caramel

As reported by JECFA, Class II Caustic Sulphite Caramel at 2.5-20 µl/plate was neither mutagenic nor bacteriotoxic in the Ames test using *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538, with or without metabolic activation by rat liver S-9 fraction (Richold and Jones, 1980c; Richold et al., 1984a, unpublished studies not available to the Panel). No mutagenic activity of Class II Caustic Sulphite Caramel in *S. typhimurium* at exposure levels of up to 20 mg/plate and no *in vitro* evidence for clastogenic effects in CHO cells at exposure levels of up to 5 mg/ml were reported in the study of Allen et al., which examined these two endpoints for all four classes of caramel colours (Allen et al., 1992). The Panel noted that it is not clear whether the result reported for Class II Caustic Sulphite Caramel in the publication of Allen et al. (1992) was also that reported in the earlier unpublished reports of Richold and Jones (1980c) and Richold et al. (1984a).

Class II Caustic Sulphite Caramel was tested for potential mutagenic activity based on induction of DNA repair (unscheduled DNA synthesis) in cultured human epithelial (HeLa 53) cells. Caustic Sulphite Caramel was incorporated in the culture medium at concentrations of 25-51 200 µg/ml and the test was performed on two occasions both in the presence and absence of rat liver S-9 mix. In both tests, in the absence of the S-9 mix a small (not further specified) but statistically significant increase in the number of silver grains over nuclei was observed at a concentration of 25 600 µg/ml; no significant increases were seen at higher concentrations in this test or at any concentration in the repeat test (Allen and Proudlock, 1984).

Class II Caustic Sulphite Caramel was not clastogenic to cultured CHO cells at concentrations of 500, 2500, or 5000 µg/ml either in the presence or absence of rat liver S-9 mix (Allen et al., 1984).

No additional information has been found on the genotoxicity of Class II Caustic Sulphite Caramel.

3.2.3.3. Class III Ammonia Caramel

As reported by JECFA, Class III Ammonia Caramel was evaluated in a bacterial mutagenicity (Ames) test using *S. typhimurium* (strains TA98, TA100, TA1535, TA1537, and TA1538) with or without metabolic activation, and also in a yeast mutagenicity assay using *Saccharomyces cerevisiae (S. cerevisiae)* (strain D4), again with or without metabolic activation. The dose range employed was 1-50 mg/plate for TA100 and 1-20 mg/plate for all other testers. Class III Ammonia Caramel was non-mutagenic under these conditions (Jagannath and Brusick, 1978a, unpublished study not available to the Panel). Negative results were also obtained in a further series of bacterial mutagenicity studies using a range of caramel concentrations and *S. typhimurium* strains, with or without metabolic activation (e.g. Jagannath and Brusick, 1978b, Kawana et al., 1980; Richold and Jones, 1980d; Ashoor and Monte, 1983; Richold et al., 1984b; Allen et al., 1992).

In an Ames test using *S. typhimurium*, a series of samples taken at various stages in the manufacture of Class III Ammonia Caramel were found to be non-mutagenic in strains TA98 and TA1535 with and without metabolic activation. However, exposure of strain TA100 to samples taken late in the production process, increased the number of revertants in the absence of metabolic activation (Jensen et al., 1983).

As reported by JECFA, the same batch of Class III Ammonia Caramel as that tested by Richold et al. (1984b) in an Ames test study with negative results was investigated for its ability to induce unscheduled DNA synthesis in cultured human epithelial (HeLa 53) cells with and without metabolic activation. The test was performed on two occasions at Class III Ammonia Caramel concentrations of

0.025-51.2 mg /ml in the culture medium. In both tests, Class III Ammonia Caramel caused a statistically significant increase in nuclear grains at concentrations of 6.4 and 12.8 mg/ml only in the absence of metabolic activation (Allen and Proudlock, 1984, unpublished study not available to the Panel).

Five samples of caramel colour (one being Class III Ammonia Caramel) gave positive results in a chromosome aberration test in a cultured Chinese hamster lung fibroblast cell line, with or without metabolic activation (aberrations in 20% of metaphase cells). No further details were provided in the publication (Ishidate and Yoshikawa, 1980). In contrast, Class III Ammonia Caramel was not clastogenic to cultured CHO cells (no further details) at concentrations of 0.5, 2.5, or 5 mg/ml either in the presence or absence of metabolic activation (Allen et al., 1984, unpublished study not available to the Panel). In another *in vitro* cytogenetic assay using cultured CHO cells, Class III Ammonia Caramel was found to induce a significant increase in chromosome aberrations at concentrations of 3 mg/ml and above in the absence of metabolic activation. In the presence of metabolic activation of the colour no cytogenetic activity was observed at concentrations up to 5 mg/ml (Galloway and Brusick, 1981a, 1981b, unpublished study not available to the Panel).

In an *in vivo* mouse micronucleus test, Class III Ammonia Caramel was not clastogenic when administered to animals (5 per sex) by gavage at doses of 0, 1050, or 3500 mg/kg bw (2 doses 24 hours apart), with sampling of the bone marrow 6 hour after the last dose (Cimino and Brusick, 1981; Brusick et al., 1992). The Panel noted that the bone marrow sampling regime in this study was not in line with that recommended in OECD TG 474.

No additional information has been found on the genotoxicity of Class III Ammonia Caramel.

3.2.3.4. Class IV Sulphite Ammonia Caramel

A number of studies concerning the genotoxicity of Class IV Sulphite Ammonia Caramel are described by JECFA (JECFA, 1987).

Class IV Sulphite Ammonia Caramel was evaluated for mutagenicity using the Ames *S typhimurium* bacterial mutagenicity test and the *S. cervisiae* mutagenicity test. The samples tested were blends of 3 samples of Class IV Sulphite Ammonia Caramel (low colour intensity) and 3 samples of Class IV Sulphite Ammonia Caramel (high colour intensity). The *Salmonella* strains used were TA98, TA100, TA1535, TA1537, and TA1538. Tests were conducted with or without metabolic activation (metabolic activation system was a liver microsomal enzyme preparation from Aroclor-induced rats). Tests were conducted over a range of concentrations from 1-50 mg/plate. No signs of genotoxic activity were observed with any of the samples of Sulphite Ammonia Caramel tested using either the *S. typhimurium* or *S. cervisiae* test organisms (Jagannath and Brusick, 1978a). Similar negative results had also been obtained for Class IV Sulphite Ammonia Caramel in an earlier study by one of the authors (Brusick, 1974), and in a subsequent study by Ashoor and Monte (Ashoor and Monte, 1983).

No mutagenic activity of Class IV Sulphite Ammonia Caramel (low and high colour intensity) in *S. typhimurium* and no *in vitro* evidence for clastogenic effects in CHO cells were reported in the study of Allen et al. (Allen et al., 1992), which examined all four classes of caramel colours. Similar results were reported by Brusick and co-workers (Galloway and Brusick, 1981a, 1981b; Brusick et al., 1992).

No additional information has been found on the genotoxicity of Class IV Sulphite Ammonia Caramel.

Overall the Panel concluded that there were no concerns regarding the genotoxic potential of caramel colours.

3.2.4. Chronic toxicity and carcinogenicity

3.2.4.1. Class I Plain Caramel and Class II Caustic Sulphite Caramel

No studies on the chronic toxicity and/or carcinogenicity of Class I Plain Caramel or Class II Caustic Sulphite Caramel were found in any of the previous evaluations, and no additional information has been found on the chronic toxicity and/or carcinogenicity of these classes of caramel colours.

3.2.4.2. Class III Ammonia Caramel

In the JECFA evaluation three chronic studies with Class III Ammonia Caramel are described in detail (JECFA, 1987).

Three groups of B6C3F1 mice (50 animals/sex/group) were given drinking water containing 0, 1.25, or 5% Class III Ammonia Caramel (equivalent to approximately 0, 2.1, or 8.4 g/kg bw/day) for 96 weeks followed by 8 weeks of drinking water without caramel colour (the Class III Ammonia Caramel contained less than 25 mg THI/kg; pyridoxine content of the diet not specified). No consistent differences compared with controls were noted with respect to growth or water intake. Although the cumulative mortality of males in the 5% group was higher than controls in weeks 100-104, no clear pathological differences in any organs and no treatment-related abnormalities in urinalyses were noted. In males of both treatment groups statistically significant elevations of the total leucocyte counts were observed, but were considered by the authors to be within the range encountered for B6C3F1 mice. No treatment-related gross pathology was noted during or at the end of the experiment and no significant differences in tumour incidence were found compared to controls (Hagiwara et al., 1983).

Four groups of Wistar rats (48 animals/sex/group) were fed diets containing 0, 1, 3, or 6% Class III Ammonia Caramel (equivalent to 0, 0.5, 1.5, and 3 g/kg bw/day) for 2 years (Evans et al., 1976). The material used in the study was described as a straight ammonia-catalysed "half open-half closed" caramel, and while general specifications for the material tested were provided, no information on the THI or 4-MEI content of the sample was provided, nor were dietary pyridoxine levels given. A significant decrease in body weight was observed in males receiving caramel in the diet compared with controls at all dose levels, of the order of 11% lower in the 1% group, 17% in the 3% group and 18% in the 6% group. Body weight gain was also reduced at all dose levels in males compared with controls (statistical analysis not presented in the paper). Food intake in males was reduced in males at a number of time intervals for which data were reported, although these reductions were not statistically significant. Body weights and hence body weight gains were also reduced in females at all dose levels compared with controls, particularly towards the end of the study, although decreases were not consistently dose-related and were never statistically significant (< 10% in any treated group). Water consumption was reported not to be affected (data not presented in the publication). Weights of major organs were reduced in both sexes in parallel with the reduced body weights, however these were not statistically significantly different from controls when adjusted for body weight. In particular, absolute spleen weights were reported to be reduced in a dose-related manner, being statistically significantly different from controls in all male treated animals and in females receiving 3% or 6% Class III Ammonia Caramel in the diet. Data for absolute spleen weights were not provided in the publication, which only provided data for organ weights relative to body weight. Reductions in spleen weight were not however statistically significant when adjusted for body weight. The only statistically significant changes in relative organ weights were reported for relative caecal (full) weights, which were increased in males receiving 3% or 6% caramel in the diet and in females receiving 1% or 6% caramel; for relative brain weight, increased in males receiving 3% or 6% caramel; and for relative thyroid and kidney weight, increased in females receiving 3% or 6% caramel.

In this study, a significant reduction in leucocyte counts compared with controls in both males and females receiving 6% Class III Ammonia Caramel in the diet was associated with a lymphocytopenia in the early part of the study. This effect was sustained until week 80 and 52 in males and females respectively. Lymphocytopenia was reported at week 13 in both males and females receiving 3%

caramel in the diet, and in males at this dose level at week 52. Neoplastic findings found in control and treated rats were typical of rats of this age and strain and none were considered by the authors to be treatment-related. There was no evidence of a carcinogenic effect. The authors concluded that a no-observed-effect level could not be identified for the caramel used in this particular study, on the basis of the reduction of absolute spleen weights observed in males at all dose levels of caramel and in females receiving 3% and 6% caramel, which they considered to be linked to the lymphocytopenia seen in the animals at earlier stages in the study (Evans et al., 1976). The authors attributed the other main finding in the study, the reduction in body weight gain in animals receiving caramel, to be due to a dilution effect of the test material when given in the diet (i.e. reduction in the amount of diet available to the animals due to the high levels of caramel present), with the effect in the treated males being exacerbated by a reduction in food intake.

The Panel concluded that the lymphocytopenia seen in the animals, together with the splenic effects, could be attributed to the presence of THI in the material tested, by analogy with other studies showing this effect (e.g. Mackenzie 1985b; Mackenzie et al. 1992b), and that since information was not provided on the level of THI in the material tested, these endpoints could not be used as the basis for identifying a NOAEL in the study. The Panel noted that other findings in the study were typical of those reported generally for the caramel colours (reduced body weight gain and increased caecal weight), considered by Mackenzie and others not to be toxicologically important (Mackenzie 1985b), and on this basis the Panel considered that a NOAEL of 3 g/kg bw/day (the highest dose tested) could be identified.

Three groups of F344 rats (50 animals/sex/group) received Class III Ammonia Caramel in drinking water at levels of 0, 1, or 4% (equivalent to 0, 0.5, or 2 g/kg bw/day) for 104 weeks. The Class III Ammonia Caramel used contained less than 25 mg THI/kg; the basal diet contained 11-12 mg pyridoxine/kg. After the exposure period animals received drinking water without caramel for 9 weeks. No treatment-related differences in growth or survival rates were noted. In the pituitary gland of males of the 4% group a significantly higher incidence of tumours was observed. This finding was however attributed by the authors to the variability of spontaneous tumour incidence in F344 rats. No dose-related effects were found regarding incidence and/or induction time of tumours in various other organs and tissues (Maekawa et al., 1983). The only parameters monitored in this study other than histopathological investigations of neoplastic change were clinical signs, mortality and body weight changes. The authors did not report a NOAEL for the study, however on the basis of the absence of any treatment-related effect in the study the Panel concluded that 2 g/kg bw/day (the highest dose tested) could be considered a NOAEL.

No additional information has been found on the chronic toxicity or carcinogenicity of Class III Ammonia Caramel.

3.2.4.3. Class IV Sulphite Ammonia Caramel

The 1987 JECFA evaluation describes a number of chronic toxicity/carcinogenicity studies with Class IV Sulphite Ammonia Caramel.

Six samples of Class IV Sulphite Ammonia Caramel (3 samples of low colour intensity and 3 samples of high colour intensity) were tested in a long-term toxicity study in rats (Sinkeldam et al., 1975; Sinkeldam et al., 1976). The Panel noted that the report of this study indicates that the material tested was “ammonia caramel”. The Panel concluded that this was Class IV Sulphite Ammonia Caramel, as described by JECFA, based on the fact that information was provided on levels of 4-MEI only, not on 4-MEI and THI, as would have been the case for Class III Ammonia Caramel. Each test group consisted of 40 male or 40 female weanling Wistar rats, except the control group which had double this number of animals. Animals were selected from the first litter of parents fed diets containing the various caramel colours from weaning age (see section 3.2.5, reproduction studies, Til and Spanjers, 1973). The dose levels tested were 5, 10, or 15% (nominal) Class IV Sulphite Ammonia Caramel (low colour intensity) and 2, 4, or 6% (nominal) Class IV Sulphite Ammonia Caramel (high colour

intensity). The caramel samples contained, respectively, 202, 400 or 600 mg 4-MEI/kg (5, 10, or 15% low intensity caramel) or 350, 639 or 852 mg 4-MEI/kg (2, 4 or 6% high intensity caramel). The study design was such that the highest daily intake of 4-MEI was provided by a dose level of 10% (nominal) low colour intensity Class IV Sulphite Ammonia Caramel in the diet, containing 600 mg 4-MEI/kg, equivalent to approximately 3 mg 4-MEI/kg bw/day. Parameters investigated were body weight, food intake, mortality, blood biochemistry, haematology, liver and kidney function tests, urine composition, organ weights, macroscopic and microscopic pathology. Haematological examinations were carried out at weeks 13, 26, 52, 79 and 98, while blood biochemical parameters and urinalysis were investigated at 13, 26, 52 and 98 weeks. For interim investigations, 10 males and 10 females per treatment group were killed at 52 weeks.

No treatment-related effects on behaviour, growth, food intake, mortality, liver and kidney function tests, urine composition, or organ weights were observed. No clinical changes were observed, except for slightly decreased haemoglobin and haematocrit values at weeks 78 and 98 in males fed high colour intensity Class IV Sulphite Ammonia Caramel. Leucocyte counts were decreased in females fed 10 and 15% of one sample of low colour intensity Class IV Sulphite Ammonia Caramel at weeks 13 and 52, but these changes were not consistent and decreases in lymphocyte counts were not observed in the differential count. In males, no such effect was noted and in a subsequent experiment in which the same sample of Class IV Sulphite Ammonia Caramel was fed to female rats at levels as high as 15 and 30%, no indications of decreased leucocyte counts were observed after 4, 8, 12, or 16 weeks. At autopsy, an increased incidence of greenish discoloured mesenteric lymph nodes was observed in most groups fed high levels of Class IV Sulphite Ammonia Caramel. Microscopically, an increased pigment-phagocytosis in the mesenteric lymph nodes in all test groups (except the group fed 2% high colour intensity Class IV Sulphite Ammonia Caramel) was observed. Enlarged caecae were observed in a number of animals at 52 weeks, this change was not evident at the terminal kill. No evidence of any other adverse structural or cellular alteration was found. Gross and microscopic examination of the other organs did not reveal any pathological changes attributable to the ingestion of Class IV Sulphite Ammonia Caramel. After about 14 months, mortality attributed to intercurrent disease was observed in both control and treated groups. Approximately three-quarters of the animals died or were killed before the experiment was terminated at week 104. An increase in the incidence of neoplastic lesions in the different groups was not found (Sinkeldam et al., 1976). The Panel considered that the haematological findings in this study could not be attributed to treatment, and that the other findings in the study (pigmentation of lymph nodes and caecal enlargement) were non-adverse effects of administration of large amounts of caramel, reported in most long-term studies with the caramel colours. The Panel concluded therefore that the highest dose tested, of 15% low intensity caramel, estimated to be equivalent to 7.5 g/kg bw/day, was a NOAEL. This caramel contained a level of 202 mg 4-MEI/kg caramel, representing a daily intake of 1.5 mg 4-MEI/kg bw/day, which could be taken as a NOAEL for 4-MEI. However, since no treatment-related effects were identified in the dose group receiving 10% (nominal) low colour intensity Class IV Sulphite Ammonia Caramel in the diet, containing 600 mg 4-MEI/kg, the Panel considered that an intake (and hence a NOAEL) of 3 mg/kg bw/day could be identified for 4-MEI in this study.

Five groups of F344 rats (each group consisting of 50 male and 50 female weanling rats) were given drinking water containing Class IV Sulphite Ammonia Caramel at 2.5, 5.0, or 10 g/kg bw/day for 24 months (MacKenzie, 1985c; MacKenzie et al., 1992c). In addition, groups of F344 rats (25 male and 25 female weanling rats/group) were given drinking water containing Class IV Sulphite Ammonia Caramel at 2.5, 5.0, 7.5 or 10 g/kg bw/day for 1 year (chronic toxicity phase). Class IV Sulphite Ammonia Caramel did not affect survival in either the chronic toxicity or carcinogenicity phases of this study and, other than dark-stained and soft faeces, there were no treatment-related ante-mortem observations. Body weights were reduced for both males and females at the 5 and 10 g/kg bw/day dose levels, but the effects were correlated with reduced water and food consumption at these dose levels and were considered by the authors to reflect the reduced palatability of the drinking water, as already described in the 13-week dose range finding study (section 3.2.2.4). According to the authors, clinical chemistry and haematological studies at 6 and 12 months in the chronic toxicity study did not reveal changes of toxicological concern. At 6 months, serum concentrations of blood urea nitrogen and

creatinine were reduced in male groups treated with 5.0, 7.5, and 10 g/kg bw/day and in female groups treated with 7.5 and 10 g/kg bw/day caramel colour. Similar changes were noted at 12 months. Creatinine levels were within the normal range for the F344 rat, whereas blood urea nitrogen levels were slightly outside this range. Decreased levels of serum total protein, albumin, and globulin were also noted at the 6-month sampling, particularly in male rats. These changes, which were less marked at 12 months, were not accompanied by any pathology in the liver or kidneys and were not considered by the authors to be of toxicological importance. The urinalysis studies revealed generally reduced urine volume and increased specific gravity in both sexes.

At necropsy the changes noted were characteristic of the feeding of high levels of caramel colour, and consisted primarily of brown staining of the gastrointestinal tract and mesenteric lymph nodes and caecal enlargement. There was no evidence of reactive hyperplasia to the pigment in the mesenteric lymph nodes of the gastrointestinal tract. Increased kidney weights were noted in both sexes in animals fed Class IV Sulphite Ammonia Caramel; which the authors considered to be related to the water imbalance in these animals, since no histological alterations were present that could be associated with the increased renal weight. Random variations in both benign and malignant neoplasms typical of the F344 strain and this age of animal were observed; however, there were no treatment-related differences. The authors concluded that the feeding of Class IV Sulphite Ammonia Caramel at doses up to 10 g/kg bw/day for 24 months did not induce neoplastic changes or non-neoplastic changes of toxicological importance. Although statistically significant changes were noted in some parameters, they were not considered to be toxicologically important. The highest dose fed, 10 g/kg bw/day, was considered to be the NOAEL (MacKenzie, 1985c; MacKenzie et al., 1992c). The Panel agreed with this NOAEL.

Class IV Sulphite Ammonia Caramel was administered in drinking water at dose levels of 2.5, 5.0, or 10.0 g/kg bw/day to B6C3F1 mice (groups of 50 male and 50 female mice) for 104 weeks (MacKenzie, 1985d; MacKenzie et al., 1992c). A complete necropsy was performed on all animals dying on test or sacrificed in a moribund condition, and on all survivors at termination. Except for some sporadic significant differences in mean body weights of treated male and female groups compared to controls, no consistent effects on body weight were observed. Males of the 10 g/kg bw/day dose group had lower mean food consumption than the control groups for 67 of the 104 weeks and males receiving 2.5 g/kg bw/day had lower mean food consumption for 21 of the 104 weeks. There were no consistent differences in food intake of males at the 5 g/kg bw/day dose level nor in any of the females. Decreased fluid intake was noted, particularly in males at the higher dose levels, but not in females. There were no treatment-related differences in survival rates. At necropsy, there were dose- and/or treatment-related effects on the gastrointestinal tract and mesenteric lymph nodes, including dark gastrointestinal contents, staining of the mucosa, and diffusely red and congested mesenteric lymph nodes. These changes were not considered by the authors to be toxicologically important and there were no other changes of toxicological significance. There was no evidence of treatment-related neoplastic lesions in any organs (MacKenzie, 1985d; MacKenzie et al., 1992c). The Panel agreed with the authors' conclusions and considered that the dose level of 10.0 g/kg bw/day was a NOAEL for Class IV Caramel in B6C3F1 mice.

No additional information has been found on the chronic toxicity or carcinogenicity of Class IV Sulphite Ammonia Caramel.

3.2.5. Reproductive and developmental toxicity

3.2.5.1. Class I Plain Caramel and Class II Caustic Sulphite Caramel

No studies on the reproductive and/or developmental toxicity of Class I Plain Caramel and Class II Caustic Sulphite Caramel were described in any of the previous evaluations, and no additional information has been found on the reproductive and/or developmental toxicity of these classes of caramel colours.

3.2.5.2. Class III Ammonia Caramel

JECFA describes three developmental toxicity studies on Class III Ammonia Caramel in mice, rats and rabbits (Morgareidge, 1974a). The Panel noted that the reports of these studies indicate that the material tested was described as "Caramel; Bakers & Confectioners", sample description FDA 71-82, and that bakers' caramel and confectioners' caramel are synonyms for Class III Ammonia Caramel.

Groups of pregnant CD1 mice (22-23 animals/group) received Class III Ammonia Caramel by gavage at doses of 0, 16, 74, 345, and 1600 mg/kg bw/day on days 6 to 15 of gestation (Morgareidge, 1974a). After Caesarean section on day 17 no treatment-related effects were observed regarding the number of implantation sites and resorption sites, or maternal and fetal survival. In addition dams were examined for urogenital anatomical abnormalities, fetal weight was recorded and fetuses were examined for gross external abnormalities, visceral abnormalities, and skeletal defects. The number of abnormalities did not differ from those occurring spontaneously in controls and no treatment-related effects were noted on fetal weight (Morgareidge, 1974a).

Pregnant Wistar rats (21-24 animals/group) were given Class III Ammonia Caramel by gavage at doses of 0, 16, 74, 345, and 1600 mg/kg bw/day on days 6 to 15 of gestation. After Caesarean section on day 20 no treatment-related effects were observed regarding the numbers of implantation sites and resorption sites, or maternal and fetal survival. In addition dams were examined for urogenital anatomical abnormalities, fetal weight was recorded and fetuses were examined for gross external abnormalities, visceral abnormalities, and skeletal defects. No treatment-related effects were seen in the urogenital tract of the dams, nor on fetal body weight, and fetuses displayed no gross external, visceral, or skeletal abnormalities other than those occurring spontaneously in controls (Morgareidge, 1974a).

Pregnant Dutch-belted female rabbits (11-12 animals/group) were administered caramel colour by gavage at doses of 0, 16, 74, 345, and 1600 mg/kg bw/day on days 6 to 18 of gestation. After Caesarean section on day 29 no treatment-related effects were observed on numbers of corpora lutea, implantation sites and resorption sites, or maternal and fetal survival. In addition dams were examined for urogenital anatomical abnormalities, fetal weight was recorded and fetuses were examined for gross external abnormalities, visceral abnormalities, and skeletal defects. No treatment-related effects were seen in the urogenital tract of the dams, nor on fetal body weight, and fetuses displayed no gross external, visceral, or skeletal abnormalities other than those occurring spontaneously in controls (Morgareidge, 1974a).

No additional information has been found on the reproductive and/or developmental toxicity of Class III Ammonia Caramel. The Panel noted that no multigeneration study was available on Class III Ammonia Caramel.

3.2.5.3. Class IV Sulphite Ammonia Caramel

JECFA described three developmental toxicity studies on Class IV Sulphite Ammonia Caramel in mice, rats and rabbits (JECFA, 1987).

Fifteen male and female Wistar rats were given 0 or 10% Class IV Sulphite Ammonia Caramel solution as their sole fluid source until day 100 and were then mated. Animals of the F1generation (25 males and 25 females) were weaned and again given 0 or 10% caramel solution until day 100. There were no adverse effects with regard to the number of litters born and the number of pups/litter. No influence on haematology, growth, food consumption, gross pathology, or histopathology of the F1-generation at 100 days of age was observed (Haldi and Wynn, 1951). The full report of this unpublished study was unavailable for independent evaluation by the Panel.

Six different samples of Class IV Sulphite Ammonia Caramel, three double strength and three single strength, each containing a different level of 4-MEI (between 200 and 850 mg 4-MEI/kg caramel) were tested in a reproduction study in rats (Til and Spanjers, 1973). The Panel noted that the report of this study indicates that the material tested was "ammonia caramel". The Panel concluded that this was Class IV Sulphite Ammonia Caramel, as described by JECFA, based on the fact that information was provided on levels of 4-MEI only, not on 4-MEI and THI, as would have been the case for Class III Ammonia Caramel. Twelve groups of 10 male and 20 female weanling Wistar rats were provided with diets containing 2-15% caramel. At week 12, the rats were mated in subgroups of 5 males and 10 females. After a 3-week mating period, the females were caged individually. Following parturition, the numbers of pups per litter and the weight of the litter were recorded on days 1, 10 and 20, and the young were inspected grossly for club-feet, cleft palate and hydrocephalus. After weaning, the dams were killed and numbers of implantation sites per dam were recorded. No consistent, dose-related effects on growth of the F0 animals were noted. No adverse effects were seen on female fertility, litter size, average weight and growth of the pups, or number of implantation sites or sex ratio of the young. In one group (10% SS (single strength) Class IV Sulphite Ammonia Caramel + 600 mg 4-MEI/kg) there was a slight increase in mortality at birth. No developmental effects were found (Til and Spanjers, 1973). The Panel estimated that the maximum intake of 4-MEI in this study would have been approximately 6 mg/kg bw/day, based on consumption of a diet containing 10% single strength Sulphite Ammonia Caramel with measured level of 600 mg 4-MEI/kg caramel, and assuming a body weight of 100 g for the rats at the start of the study.

Mature F344 rats (12 animals/sex/group) were given Class IV Sulphite Ammonia Caramel at concentrations of 0, 10, 15, 20, or 25% in drinking water (8-28 g/kg bw/day) for 21 days prior to mating until lactation (Tierney, 1980). In the 20- and 25% dose groups of both generations there was a higher incidence of soft stools than in the controls. In addition, dose-related depression of body-weight gain was noted in animals of the F0 and F1 generation. Mating, pregnancy, and fertility rates were considered by the authors to be comparable for all groups. Pups in the 25% dose group showed a higher incidence of alopecia compared with controls, and a generalised poor condition during the last 7 days of suckling. The authors attributed these findings to the fact that the bodies of most of the pups in this dose group were covered with faecal/test material and to a lack of grooming during this period. One pup at this dose level had an arched spine, which was again attributed to the general poor condition of the pups in this group. Alopecia was also seen in the pups in the 20% Class IV Sulphite Ammonia Caramel in the diet group, and in the F0 animals in these two dose groups, attributed by the authors to the increased grooming activity of the animals in an effort to remove test substance/faecal material. The Panel agreed with the conclusions of the authors regarding these findings. The number of implantation sites and of live pups at days 0, 4, and 21 of lactation in the 20%-dose groups were significantly lower than control values. The Panel noted however that there was no dose-related trend, since in the 25% dose group these parameters were not statistically different from controls. No significant haematological changes were observed at 45 or 90 days post-weaning. Blood urea nitrogen values were lower than controls at 45 and 90 days, as has been noted in several other studies in which animals received high levels of caramel in the diet, but other clinical chemistry values were normal. At necropsy, dose-related increases in absolute and relative weights of the liver, kidneys, and caecum (full and empty) were observed from animals in the 15% and higher dose groups. The only gross treatment-related morphological changes reported were brown/black or green colouration of the contents and mucous membrane of the lower gut and mesenteric lymph nodes (Tierney, 1980).

The study report did not identify a NOAEL. The Panel concluded that the effects observed were in line with the effects on body and organ weights observed in other subchronic and chronic studies and that for reasons already outlined above these were not considered adverse. The Panel also considered that the findings in the pups in the 25% dose group were attributable to the general condition of the dams and the body contamination with faecal/test material rather than a specific developmental or toxic response, while the decreases in implantation sites and numbers of live pups seen in the 20% group did not show a dose response relationship. Therefore the Panel concluded that the dose level of 25% Class IV Sulphite Ammonia Caramel in the diet, the highest dose level tested was a NOAEL. This dietary dose level was reported by the authors to be equivalent to an intake of 25-30 g/kg bw/day for the

female rats during the period of mating, gestation and parturition, although intakes by the males in this dose group were lower, at approximately 20-25 g/kg bw/day. The Panel considered that a NOAEL of 25-30 g/kg bw/day for female rats could be identified in this study, for the purposes of risk assessment.

In addition, JECFA described a study on developmental toxicity in three different species (Morgareidge, 1974b). The Panel noted that the material tested was described as caramel beverage, sample description FDA 71-83, which the Panel concluded was Class IV Sulphite Ammonia Caramel, as described by JECFA.

Pregnant albino CD1 mice (19-22 animals/group) were administered 0, 16, 74.3, 345, and 1600 mg/kg bw/day Class IV Sulphite Ammonia Caramel by gavage, from day 6-15 of gestation. After Caesarean section on day 17 no treatment-related effects were observed regarding the numbers implantation sites and resorption sites, or maternal and fetal survival. In addition dams were examined for urogenital anatomical abnormalities and fetuses were examined for gross external abnormalities, visceral abnormalities, and skeletal defects. The number of abnormalities did not differ from those occurring spontaneously in controls (Morgareidge, 1974b).

Pregnant Wistar rats (21-24 animals/group) were given Class IV Sulphite Ammonia Caramel by gavage at doses of 0, 16, 74, 345, and 1600 mg/kg bw/day on days 6 to 15 of gestation. After Caesarean section on day 20 no treatment-related effects were observed regarding the numbers of implantation sites and resorption sites, or maternal and fetal survival. Fetuses displayed no gross external, visceral, or skeletal abnormalities other than those occurring spontaneously in controls. Finally, the body weights of live pups were recorded but the effects were not described (Morgareidge, 1974b).

Pregnant Dutch-belted female rabbits (11-12 animals/group) were administered Class IV Sulphite Ammonia Caramel by gavage at doses of 0, 16, 74, 345, and 1600 mg/kg bw/day on days 6 to 18 of gestation. After Caesarean section on day 29 no effects were observed regarding numbers of corpora lutea, implantation sites and resorption sites, or maternal and fetal survival. Fetuses displayed no gross external, visceral, or skeletal abnormalities other than those occurring spontaneously in controls (Morgareidge, 1974b).

No additional information has been found on the reproductive and/or developmental toxicity of Class IV Sulphite Ammonia Caramel. The Panel noted that no multigeneration study was available on Class IV Sulphite Ammonia Caramel.

3.2.6. Hypersensitivity (intolerance/allergenicity)

No studies on intolerance to and/or allergenicity of any of the caramel colours were found in any of the previous evaluations, and no additional information has been found on these aspects.

3.2.7. Haematotoxicity and immunotoxicity

A large number of studies have been carried out to investigate the potential immunotoxicity of Class III Ammonia Caramel containing THI and also that of THI alone, reflecting the lymphocytopenia seen in studies with Class III Ammonia Caramel (see also section 3.2.2.3). A number of relevant studies on Class III Ammonia Caramel are described below while summaries of other relevant studies have been included in Annex B as background information, or in section 3.2.9, relating to the toxicity of 4-MEI and THI, or Annex D, relating to specific toxicity studies carried out with THI.

The effects of Class III Ammonia Caramel and THI and the influence of dietary pyridoxine on these effects were investigated in two studies in rats (Houben et al., 1992a). Weanling rats fed a diet containing 2-3 mg pyridoxine/kg and exposed to 4% Class III Ammonia Caramel or 5.72 mg THI/kg

bw in drinking water for 4 weeks showed reduced cell numbers in spleen and popliteal lymph nodes, as well as in the blood. Flow cytometric analyses demonstrated a comparable reduction in B and T lymphocytes. In blood, spleen, and popliteal lymph nodes, $CD4^+$ lymphocytes were more reduced than $CD8^+$ cells. The number of bone marrow cells was not affected. Although thymus weight and cell number were also not affected, a decreased cortex over medulla area ratio and an increase in medullary cell density was observed, largely due to an increase in $CD4^+$ thymocytes. Decreased numbers of $ED2^+$ macrophages were observed in the thymic cortex and in the spleen red pulp. All effects observed were either less pronounced or absent in a study with rats fed a diet containing 11-12 mg pyridoxine/kg in addition to Class III Ammonia Caramel. The effects of Ammonia Caramel and THI on the immune system were similar. Whereas the lymphocytopenia induced by Class III Ammonia Caramel exposure was associated with changes in vitamin B_6 status, THI did not induce obvious effects on vitamin B_6 parameters. The authors proposed that the effects of Class III Ammonia Caramel and THI on the immune system are not caused by a disturbance of vitamin B_6 metabolism, but may in fact result from a disturbance of a specific pyridoxal phosphate-dependent process (Houben et al., 1992a).

Rats were exposed to 0.4 or 4% Class III Ammonia Caramel or to 5.72 mg THI/kg bw in drinking water during and for 28 days prior to the start of immune function assays (Houben et al., 1993). Resistance to *T. spiralis* was examined in an oral infection model and clearance of *L. monocytogenes* upon an intravenous infection was studied. In addition, natural cell-mediated cytotoxicity of splenic and non-adherent peritoneal cells and the antibody response to sheep red blood cells were studied. From the results the authors concluded that exposure of rats to Class III Ammonia Caramel or THI influenced various immune function parameters. Thymus-dependent immunity was suppressed, while parameters of non-specific resistance were also affected, as shown by a decreased natural cell-mediated cytotoxicity in the spleen and an enhanced clearance of *L.monocytogenes* (Houben et al., 1993).

Female Balb/c mice (3 week-old) fed a diet with a relatively high vitamin B_6 content were exposed to 2 or 10% (equivalent to 3 or 15 g/kg bw/day) of a commercial Class III Ammonia Caramel preparation with a low THI content (less than 25 mg THI/kg) in the drinking water for 9 week. Although this treatment did not induce a lymphocytopenia in the exposed mice, flow cytometric analysis of lymphocyte subpopulations demonstrated reductions in the $CD4^+$ and $CD8^+$ cell populations. In addition, the proliferative response of spleen cells to B and T cell mitogens was significantly reduced in the mice exposed to 2% Class III Ammonia Caramel. No changes were observed in natural killer cell activity or in the humoral antibody response to a viral antigen. The authors considered that their results indicate that Class III Ammonia Caramel that meets the limit of less than 25 mg THI/kg established in the JECFA specifications may, nevertheless, interfere with the lymphoid system in mice with an adequate vitamin B_6 status (Thuvander and Oskarssen, 1994). The Panel agreed with the conclusions of the authors that this study is indicative of effects of Class III Ammonia Caramel on the immune system in mice, with a Low-Observed-Adverse-Effect-Level (LOAEL) of 3 g/kg bw/day, but considered that the study should not be used as a pivotal study for the purposes of risk assessment without further substantiation, given a number of studies in rats showing no effect on haematological parameters over longer periods and at higher dose levels than those used in the study of Thuvander and Oskarssen.

The results of similar studies on THI alone are described in Section 3.2.9 and in Annex D.

3.2.8. Human studies

No studies with Class I Plain Caramel or Class II Caustic Sulphite Caramel in human volunteers were found in any of the previous evaluations and no additional information has been found on human studies with these caramel colours.

Houben et al. (1992b; 1992c) reported on a detailed study with Class III Ammonia Caramel in elderly men selected because of the presence of marginal vitamin B_6 deficiency. In this study, groups of 8 male human volunteers (above 65 years of age) were given a dessert twice a day for 7 days containing

100 mg Class III Ammonia Caramel/kg bw with either 23 mg THI/kg (commercial sample) or 143 mg THI/kg (research sample). The intake of THI from the latter Class III Ammonia Caramel sample was estimated to be 28.6 µg/kg bw/day. A control group was given a dessert twice a day without Class III Ammonia Caramel. Two weeks prior to and during the experiment Class III Ammonia Caramel and foods rich in vitamin B_6 were excluded from the diet. Intake of Class III Ammonia Caramel by these volunteers did not induce effects on blood lymphocyte numbers or on proliferative responses to mitogenic stimulation. In addition, no effect was seen on other haematological parameters including serum immunoglobulin levels and on immunogen production *in vitro*. Houben et al. (1992b) state that spontaneous vitamin B_6 deficiency in humans is not seen frequently, but it may occur under certain conditions. For example, chronic alcohol abusers, females on oral contraceptives, pregnant women, breast-fed infants, and elderly men and women may have increased risk of vitamin B_6 deficiency. Houben et al. (1992c), in comparing the results obtained in this study with data in rats maintained on normal and pyridoxine-reduced diets, suggested that, with regard to oral intake of THI, humans are less sensitive to Caramel Colour III-induced lymphocytopenia than are rats (Houben et al., 1992c).

A pilot study was carried out in which 9 volunteers ingested 1.5 g of Class III Ammonia Caramel daily (approximately 20-25 mg/kg bw/day; no details on THI content) for 21 days. No changes in total circulating leucocytes, lymphocytes, and erythrocytes, or in haemoglobin concentrations were found that could be attributed to treatment with Class III Ammonia Caramel. Three volunteers occasionally had soft stools; no control groups were used (BIBRA, 1976).

Tolerance studies of Class IV Sulphite Ammonia Caramel were conducted in human volunteers. The subjects, 10 men and 10 women, ingested Class IV Sulphite Ammonia Caramel once daily in simulated soft drinks over 3 test periods of 21 days each separated by 7-day rest intervals. The test doses were 6 g/day during the first test period, 12 g/day during the second period, and 18 g/day (equivalent to 100, 200 or 300 mg/kg bw/day for a 60 kg person in the respective test periods). Haematological, clinical chemical and routine urinary parameters were studied at the beginning of each ingestion period, after 10 days of ingestion, and at the end of each ingestion period. Most individual values for haemoglobin, haematocrit, RBC, corrected sedimentation rate, WBC, and differentials (neutrophils, basophils, eosinophils, monocytes, and lymphocytes) were found to be within normal limits. There were a few instances of values outside the normal range, indicating mild neutropenia and mild lymphocytosis, but these were not consistent and were unrelated to the ingestion of Class IV Sulphite Ammonia Caramel. On the other hand, caramel ingestion was associated with an increased frequency of bowel movements and softening or increased liquidity of faeces (Marier et al., 1977a; 1977b).

3.2.9. Studies on 4-methylimidazole (4-MEI) and 2-acetyl-4-tetrahydroxy-butylimidazole (THI)

The imidazole constituents 4-MEI and THI, the former occurring in both Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, and the latter occurring only in Class III Ammonia Caramel, have been investigated independently in a number of studies because of their role in the toxicological profile of these two classes of caramel colours.

4-MEI

4-MEI is formed by interaction of ammonia with reducing sugars, a chemical reaction occurring during the ammonia process used in the production of both Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel. 4-MEI has been identified as a toxic by-product of fermentation in ammoniated hay forage for livestock animals (Ray et al., 1984) and has also been identified as the causal agent of convulsions in cattle and sheep fed ammonia-treated molasses (Wiggins, 1956a,b; Bartlett and Broster, 1957). Neurologic signs have been reported in calves of nursing cows fed ammoniated hay and sheep fed ammoniated hay (Weiss et al., 1986; Motoi et al., 1997) and were attributed to the presence of 4-MEI in the feed (Motoi et al., 1997).

4-MEI has been shown to cause convulsions at acutely toxic doses, in a range of species and by different routes. The LD_{50} values for 4-MEI were determined to be 370 mg/kg bw in mice and 599 mg/kg bw in chicks, and the Convulsive Doses (CD_{50}) were only slightly lower than the LD_{50} values. In a study testing the convulsant effects of a range of imidazoles, 4-MEI was found to be the most potent, producing convulsions in rabbits, mice, and chicks at single oral doses of 360 mg/kg bw (Nishie et al., 1969, 1970). The LD_{50} values of several other imidazoles were also determined and found to be at least four times higher than those for 4-MEI (Nishie et al., 1969, 1970).

Further relevant publications on the toxicology of 4-MEI are presented in Annex D, for information.

JECFA at its twenty-first meeting concluded that the toxicity of 4-MEI as a constituent in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel was no longer a cause for concern since the introduction of chemical specifications limits the concentration of 4-MEI in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel produced by the ammonia process, to a level of 200 mg 4-MEI/kg caramel (JECFA, 1978). JECFA concluded that exposure to 4-MEI as a result of use of Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel as food colours at the levels foreseen would be substantially below the levels found to induce neurological effects in a range of animal species.

More recently, the National Toxicology Programme (NTP) of the US National Institutes of Health has carried out a series of toxicity studies on 4-MEI and its structural isomer 2-MEI (NTP, 2004), and has also completed chronic toxicity and carcinogenicity studies on 4-MEI in Fischer 344 rats and in B6C3F1 mice (NTP, 2007). The NTP summaries of these studies relevant to 4-MEI are provided in Annex D. Notably, the NTP has concluded that "*Under the conditions of these 2-year feed studies, there was no evidence of carcinogenic activity of 4-methylimidazole in male F344/N rats exposed to 625, 1,250, or 2,500 mg/kg. There was equivocal evidence of carcinogenic activity of 4-methylimidazole in female F344/N rats based on increased incidences of mononuclear cell leukemia. There was clear evidence of carcinogenic activity of 4-methylimidazole in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms. Exposure to 4-methylimidazole resulted in non-neoplastic lesions in the liver of male and female rats and the lung of female mice and in clinical findings of neurotoxicity in female rats.*" (NTP, 2007).

The Panel noted that NTP concluded that 4-MEI is not genotoxic *in vitro* or *in vivo* (NTP, 2007). The Panel also noted that in rats the increased incidence of mononuclear cell leukemia was only seen at the highest dose of 2500 mg 4-MEI in the diet, equivalent to 170 mg 4-MEI/kg bw/day. In mice, the statistically significant effects noted by the NTP referred in the main to benign neoplastic lesions of the lung (adenomas), the incidences for malignant neoplastic lesions of the lung (carcinomas) only being statistically significantly increased in male mice at the top dose of 1250 mg 4-MEI/kg diet, equivalent to 170 mg 4-MEI/kg bw/day. The Panel concluded that the carcinogenic effect of 4-MEI seen in mice in this study was thresholded, and that the intermediate dose of 625 mg 4-MEI/kg diet, equivalent to 80 mg 4-MEI/kg bw/day could be considered to be a NOAEL for these effects.

The Panel noted that 4-MEI has recently been listed by the US State of California as a chemical known to cause cancer (OEHHA, 2011)

THI

The key toxicological effect of THI, as already indicated in previous sections of this opinion, is its immunosuppressive potential, mediated by its effect on components of the immune system, primarily the immunomodulatory molecule S1P (Hla, 2004, 2005; Schwab et al., 2005; Rivera et al., 2008; Yu et al., 2010). S1P is a lysophospholipid that plays an important role as a regulator of the immune and cardiovascular systems, acting through G-protein-coupled receptors on cell surfaces and regulating immune cell trafficking between lymphoid tissue and the blood and other tissues of the body (Hla, 2004, 2005; Rivera et al., 2008). Levels of S1P are normally low in most tissues, including lymphoid tissue, but are higher in blood and lymph, providing a driving force for migration of lymphocytes and

other immunocompetent cells into the peripheral circulation (Rivera et al., 2008). THI has been shown to mediate an increase in S1P in lymphoid tissue via inhibition of S1P lyase, the enzyme involved in degradation of S1P in the cell, and thereby inhibiting the egress of immunocompetent cells from the lymphoid organs (Schwab et al., 2005). This is considered to be the underlying mechanism for the lymphocytopenia seen in studies in rodents with THI alone and with Class III Ammonia Caramel containing levels of THI in levels in excess of 25 mg/kg caramel (the level established in the JECFA specifications (JECFA, 2006)).

As reported in more detail in Annex B, in studies in rats Sinkeldam and co-workers demonstrated significant reductions in peripheral blood lymphocytes and total leucocytes at a dose level of 1% Class III Ammonia Caramel in drinking water, equivalent to 1 g caramel/kg bw/day and estimated to be equivalent to 200 µg THI/ kg bw/day (Sinkeldam et al., 1988). Similar statistically significant reductions in lymphocytes were observed when the rats were given 200 µg, 500 µg or 2 mg THI/kg bw/day alone in drinking water. The authors reported decreases in lymphocyte counts in rats receiving as little as 0.1% Class III Ammonia Caramel in drinking water for 1 week and maintained on a low-pyridoxine diet (2-3 mg/kg). Class III Ammonia Caramel intake at this level in drinking water was 0.1 g kg bw/day, equivalent to 20 µg THI/ kg bw/day. Statistical analysis was not reported for this aspect of the study, but evaluation of the data provided would indicate that while lymphocytopenia was evident at this dose level, the reduction was unlikely to be statistically significant.

The 90-day oral toxicity study in rats on Class III Ammonia Caramel carried out by Mackenzie et al. also included an evaluation of the toxicity of THI after a 4-week dosing period (MacKenzie, 1985b; MacKenzie et al., 1992b). Groups of 10 rats per sex were given 0, 1, 2, 4, 16 or 32 mg THI/l in drinking water for 4 weeks (equivalent to approximately 0.1, 0.2, 0.5, 1.9 and 3.7 mg THI/kg bw/day in the treated groups), and groups of 20 rats per sex were given 0, 8 or 64 mg THI/l, equivalent to 0, 0.9, or 7.2 mg THI/kg bw/day. The rats were maintained on a normal rat chow, with adequate levels of pyridoxine. After 4 weeks 10 rats per sex of the 0, 0.9 and 7.2 mg/kg groups were given water without THI for 2 weeks (recovery phase). Decreased mean body weights in all test groups after 2 and 4 weeks were noted but ascribed to fasting for blood sampling and urinalysis. Gross and microscopic pathology in a large range of organs and tissues revealed no treatment-related effects. Treatment-related decreases in white blood cell and lymphocyte counts, and increases in neutrophil counts were observed. These changes returned to normal after 3 days recovery phase and remained normal. The NOAEL for the reduction in total lymphocytes was determined to be 4 mg THI/l in drinking water, equivalent to 380 µg/kg bw/day, in males and 1 mg THI/l in drinking water, equivalent to 120 µg/kg bw/day, in females (MacKenzie et al., 1992b).

Houben et al. (1992c) reported a range of estimated intakes of THI or Class III Ammonia Caramel that, in combination with manipulation of dietary pyridoxine levels, resulted in lymphocytopenia and could therefore be regarded as LOAELs for this effect. They ranged from an estimated intake of 1600 µg THI/ kg bw/day (provided by a level of 8% Class III Ammonia Caramel in drinking water) at a dietary pyridoxine level of 1.2 mg/kg, 800 µg THI/kg bw/day (provided by a level of 4% Class III Ammonia Caramel in drinking water) at a dietary pyridoxine levels of 8.2 mg/kg, 200 µg THI/kg bw/day in drinking water at dietary pyridoxine level of 1.8, 4.9 or 8.2 mg/kg and 20 µg THI/kg bw/day at a dietary pyridoxine level of 2-3 mg/kg. The majority of the LOAELs reported by Houben et al. (1992c) were derived from the paper of Sinkeldam et al. (1988) referred to above, although the authors also provided a LOAEL of 57.2 µg THI/ kg bw/day (provided by a level of 0.4% Class III Ammonia Caramel in drinking water) derived from their own work (Houben, 1992).

Houben et al. suggested that since rats fed a diet containing 2-3 mg pyridoxine/kg diet may have a vitamin B_6 status comparable to that of marginally vitamin B_6-deficient humans, the absence of effects indicative of immunotoxicity in humans exposed to approximately 28 µg THI/kg bw/day in Class III Ammonia Caramel may indicate that humans are less sensitive to THI- or Class III Ammonia Caramel-induced immunotoxic effects than are rats (Houben et al. 1992c; Houben and Penninks, 1994).

Comparison of the effects of Class III Ammonia Caramel in rats and mice indicate that mice are equally sensitive to the immunotoxic effects of THI as rats (Houben and Penninks, 1994; Thuvander and Oskarssen, 1994). A more recent study by Schwab et al. (2005) has shown that in mice (age not indicated) receiving 7.5 mg/kg bw/day THI (50 mg/l in drinking water) for 3 consecutive days, THI induced a marked decrease in the number of circulating lymphocytes and suppressed immunity. The mean THI plasma level was 135 ng/ml. S1P levels in lymphoid tissues of mice increased more than 100-fold after THI treatment, due to inhibition of the S1P degrading enzyme S1P lyase, an intracellular degrading enzyme which is expressed by hematopoietic cells (Schwab et al., 2005).

In a study exploring the relationship between systemic THI exposure, splenic S1P concentrations, and lymphocytopenia in rats, a single dose of THI was administered by gavage at levels of 10 and 100 mg/kg bw to male rats (n=8), and blood samples taken at intervals of up to 48 hours after dosing for determination of THI absorption and blood lymphocyte counts (Yu et al., 2010). Another 30 male rats gavage-dosed at levels of 10 and 100 mg/kg bw were sacrificed at intervals of up to 48 hours after dosing for assessment of splenic levels of S1P. The study showed that THI was rapidly absorbed and reached a plasma peak level at 1 hour after dosing. Splenic S1P increased and reached a peak level at 24 hours after dosing. THI plasma concentration was linked to splenic S1P concentration and as the splenic S1P level increased, blood lymphocyte counts decreased. According to the authors, the results of this pharmacokinetic–biomarker–pharmacodynamic model explain the discrepancies between plasma THI concentration and the pharmacological response and the relationship of THI exposure, S1P, and lymphocytopenic response (Yu et al., 2010).

Annexes B and D provide further summaries of a range of studies carried out on the immunomodulatory effects of THI, in particular its effects on lymphocytes, leading to the lymphocytopenia seen in studies on Class III Ammonia Caramel containing THI as a constituent. Based on a review of these studies, including the more recent study of Schwab et al. (2005) showing effects on S1P, an important immunoregulatory substance, THI can be considered to be a potent immunosuppressive agent. The Panel noted that additional studies on THI to determine a consistent NOAEL for the immunomodulatory effects, based on Tier I and Tier II immunotoxicity parameters, would provide useful information to further characterise the immunotoxicity of THI.

3.2.10. Overview of the toxicological profiles of furan and 5-HMF, LMW constituents of the caramel colours

Furan

In 2004, the EFSA Panel on Contaminants in the Food Chain (CONTAM) considered available data on exposure to furan as a result of its presence in a number of foods that undergo heat treatment, such as canned and jarred foods (EFSA, 2004b). The CONTAM Panel additionally reviewed the available data on the toxicity of furan, and noted that *"The toxicity database of furan in is incomplete as no data are available on reproductive and developmental toxicity. There are also no human studies. Furan is cytotoxic and the liver is the primary target organ of furan toxicity after oral application. Furan is clearly carcinogenic to rats and mice, showing a dose-dependent increase in hepatocellular adenomas and carcinomas in both sexes. In rats, also a dose-dependent increase in mononuclear leukaemia was seen in both sexes. A very high incidence of cholangiocarcinomas of the liver was present in both sexes, even at the lowest dose. Taking into account all the presently available data on the mode of action of furan, the Panel concluded that the weight of evidence indicates that furan-induced carcinogenicity is probably attributable to a genotoxic mechanism. However, chronic toxicity with secondary cell proliferation may indirectly amplify the tumour response. From the presently available data it appears that there is a relative small difference between possible human exposures and the doses in experimental animals that produce carcinogenic effects, probably by a genotoxic mechanism. However, a reliable risk assessment would need further data on both toxicity and exposure."* (EFSA, 2004b). The ANS Panel agreed with the conclusions of the CONTAM Panel.

5-hydroxymethyl-2-furfural (5-HMF)

5-HMF has been identified in a wide variety of heat-processed foods including coffee, milk, fruit juices, spirits, honey, etc, in addition to the caramel colours. Along with many other flavour- and colour-related substances, 5-HMF is formed in the Maillard reaction as well as during caramelisation. 5-HMF is thus a ubiquitous component of many foodstuffs. It is also a flavouring substance [FL-no: 13.139], and is included in the Community Register of flavouring substances used in or on foodstuffs. 5-HMF as a flavouring substance has been evaluated by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) (EFSA, 2010) and is currently under re-evaluation in the light of new data on genotoxicity, subchronic toxicity and carcinogenicity.

For 5-HMF, the Panel noted that substantial toxicological data are available, including a 3-week and 13-week subchronic studies, and chronic and carcinogenicity studies in B6C3F1 mice and F344/N rats (NTP, 2008). The carcinogenicity study in mice demonstrated that 5-HMF may induce liver tumours, but these were considered not to be relevant for human risk assessment. In contrast, no carcinogenic responses were reported in the study with rats. The critical non-neoplastic effect seen in these NTP studies was cytoplasmic alterations in renal proximal tubule epithelium in mice, observed in the 13-weeks study with mice at 188 mg/kg bw/day and above with an intermittent dose regimen of 5 days per week.

In relation to the genotoxicity of 5-HMF, taking into account additional data on metabolism, the Panel noted that the substance is negative in the conventional Ames test. Mutagenicity is observed only upon inclusion of 3'-phosphoadenosine-5´-phosphosulphate (PAPS), a sulpho-group donor and liver cytosol into the metabolic system, suggesting the formation of a sulphate-ester, 5-sulphoxymethyl-2-furfural (SMF). SMF itself was mutagenic in the absence of any metabolic activation system. In an *in vitro* assay, 5-HMF induced dose-dependent increase in DNA damage (Comet assay), but the Panel noted that this study has major drawbacks and inconsistencies and has to be considered of limited validity. In another Comet assay in HepG2 cells, able to express both cytochrome P450 (CYP) and sulphotransferase (SULT) enzymes, some indication of DNA damage were observed, but the substance did not induce clastogenic or aneugenic effects (micronucleus assay) in the same cell system. *In vivo*, a non-standard micronucleus assay in peripheral blood erythrocytes, conducted as part of a sub-chronic study in mice, provided no indication of a genotoxic potential (NTP, 2008). The Panel noted however that this study has limited validity since no bone marrow cell toxicity was observed.

4. DISCUSSION

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

Caramel colours are colouring substances authorised as food additives in the EU, and are classified according to the reactants used in their manufacture as follows: Class I Plain Caramel or Caustic Caramel (E 150a); Class II Caustic Sulphite Caramel (E 150b); Class III Ammonia Caramel (E 150c) and Class IV Sulphite Ammonia Caramel (E 150d).

The four classes of caramel colours have been previously evaluated on a number of occasions by the SCF, JECFA and TemaNord. Both JECFA and the SCF concluded that a numerical ADI was not necessary for Class I Plain Caramel, considering that it contains no added ammonia or sulphite and that it is likely to be produced in normal cooking processes (SCF, 1989). For Class II Caustic Sulphite Caramel, JECFA established an ADI of 0-160 mg/kg bw/day based on a NOAEL of 16 g/kg bw/day identified in the 90-day study in rats of MacKenzie et al. (1992a) and using an uncertainty factor of 100 (JECFA, 2001). The SCF included Class II Caustic Sulphite Caramel within the ADI of 200

mg/kg bw/day that it had already established for Class IV Sulphite Ammonia Caramel based on information indicating that its chemical composition was similar to and intermediate between that of Class I Plain Caramel and Class IV Sulphite Ammonia Caramel (SCF, 1997).

For Class III Ammonia Caramel, the SCF allocated an ADI of 200 mg/kg bw/day, apparently based on the 90-day rat study carried out on this caramel by MacKenzie (1985b), with the proviso that the content of THI should not exceed 10 mg/kg colour on a colour intensity basis (SCF, 1997). In 1987 JECFA identified a NOAEL of 20 g/kg bw/day from the same 90-day rat study, and consequently allocated an ADI of 200 mg/kg bw/day to Class III Ammonia Caramel using an uncertainty factor of 100 (JECFA, 1987). For Class IV Sulphite Ammonia Caramel, the SCF established an ADI of 200 mg/kg bw/day (SCF, 1989), based on a NOAEL for Class IV Sulphite Ammonia Caramel of 10% in the diet in an oral feeding study in rats, probably the long-term/carcinogenicity study (MacKenzie, 1985c). JECFA applied an uncertainty factor of 50 to the NOAEL of 10 g/kg bw/day in this long-term/carcinogenicity study in the rat (MacKenzie, 1985c) to derive an ADI of 200 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The Panel noted that the design of various studies in the previously evaluated dataset on the caramel colours would not be in full compliance with current regulatory protocols. However, from the study descriptions available, the Panel considered that these studies were of sufficient quality for evaluation.

Specifications for the four classes of caramel colours have been defined in Commission Directive 2008/128/EC and by JECFA (2006). The different classes of caramel colours are variously defined (in addition to their method of production) by the degree of binding to DEAE cellulose and to phosphoryl cellulose (for Classes I Plain Caramel and Class III Ammonia Caramel), by the absorbance ratio (Classes II Caustic Sulphite Caramel and Class IV Sulphite Ammonia Caramel) and by their colour intensity. The solids content for the different classes range from 62-77% (Class I Plain Caramel), 65-72% (Class II Caustic Sulphite Caramel), 53-83% (Class III Ammonia Caramel) or 40-75% (Class IV Sulphite Ammonia Caramel). The Panel noted minor differences between the EU specifications and those of JECFA but did not consider that these were generally of significance for the safety evaluation of the caramel colours.

The Panel noted that maximum levels of the imidazole 4-MEI are restricted to $\leq$ 250 mg/kg in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel under Commission Directive 2008/128/EC. The Panel is aware, however that a revised limit for 4-MEI in Class III and Class IV caramels of 200 mg/kg, to bring the limit in line with that in the JECFA specifications, is proposed in the draft Commission Regulation laying down specifications of food additives listed in Annex II and III of (EC) Regulation 1333/2008, currently under discussion. The Panel also noted that maximum levels of the imidazole THI are restricted to $\leq$ 10 mg/kg in Class III Ammonia Caramel under Commission Directive 2008/128/EC.

The Panel noted that the caramel colours are poorly characterised, and it is not clear whether the controls on manufacturing processes are sufficient to minimise batch-to-batch variability, particularly with respect to levels of individual LMW constituents. The wide range of starting materials and reactants that may be used for the production of caramel colours may result in a variation of end products, with different physical, chemical and toxicological properties. The Panel noted that concerns about e.g. chemical composition, purity and similarity of various caramel colours have also been raised in the past by the SCF. The Panel also noted that a number of the identified or theoretical LMW constituents of caramel colours, e.g. furan, acrylamide and 5-HMF, are genotoxic under certain experimental conditions and in some cases have carcinogenic potential, which may be relevant to the toxicological profile of the caramel colours. The Panel considered that the toxicological studies carried out on specific caramel colours would have involved exposure to these compounds, and therefore the anticipated toxicological effect should have been detected in these studies, as exemplified by the toxicological profiles of Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel due to the presence of the imidazoles THI and 4-MEI.

According to information from industry, slight changes in time and temperatures of the reaction, or in starting materials, can produce changes in the chemical composition and physical properties of the final product. Furthermore industry indicated that variation of manufacturing process parameters allows the production of a large range of different qualities of products within each caramel class. Concerning Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel there is also a wide range of products made by caramel manufacturers, with differences in chemical composition including some variation in THI and/or 4-MEI concentrations, respectively (EUTECA, 2011a).

The Panel noted that there was limited information about the relationship between processing parameters for the caramel colours and the formation and nature of heat-derived constituents of these colours.

The Panel noted that data on the toxicokinetics of the caramel colours are very limited. Data available concerning the toxicokinetics of Class IV Sulphite Ammonia Caramel indicate that the majority of the high molecular weight fraction of orally administered Class IV Sulphite Ammonia Caramel is directly excreted in the faeces. The small fraction that is absorbed is distributed to lymphoreticular tissue, and eventually excreted in the urine. The Panel considered that individual constituents of the LMW fraction of caramel colours (e.g. MW less than 500 g/mol) are likely to be absorbed, although little information is available to confirm this assumption.

The caramel colours are of low toxicity both in short-term tests and in chronic toxicity/carcinogenicity studies. A large number of short-term oral toxicity studies are available, particularly on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, including 90-day feeding studies in the rat. The results of these studies do not indicate any significant differences in the toxicological profile between the four classes. The only significant toxic effect, namely the lymphocytopenia produced by Class III Ammonia Caramel, particularly in animals with low pyridoxine levels, has been specifically linked with the presence of THI in this caramel. Animal studies on Class III Ammonia Caramel have shown evidence of lymphocyte depression and other evidence of immunotoxicity, as described in section 3.2.2.3 and Annex B and discussed further below.

The available short-term studies, employing generally high dose levels in drinking water, show some dose-related effects, including mild diarrhoea, reduced body weight gain associated with reduced food and fluid consumption, pigmentation of mesenteric lymph nodes, enlargement of the caecum, reduced urinary output associated with increases in specific gravity of the urine, and increases in caecal and kidney weights, unaccompanied by any histopathological change. The authors of the studies concluded that the effects on body weight gain were in part due to the reduced water intake caused by poor palatability of the drinking water containing high levels of caramel rather than toxic effects of the caramel colours *per se*, and that the other effects seen (increases in caecal and kidney weight and changes in urinary parameters), were secondary both to the reduced fluid intake and the intake of large quantities of osmotically-active caramel material. The authors also suggested that a reduction in food intake is an invariable response to either voluntary or forced restriction of water intake by the rat (MacKenzie et al., 1992b). The Panel agrees that these are likely explanations for the treatment-related effects on body weight and other parameters seen in studies with caramel colours, and considers that these effects are not of toxicological significance in establishing the safety of the caramel colours.

Caramel colours have been extensively tested for genotoxic potential in a variety of assays *in vitro*. Overall the results in a range of *in vitro* systems were generally negative, with a few marginally positive findings (e.g. Kawachi et al., 1980; Ishidate and Yoshikawa, 1980; Jensen et al., 1983; Ishidate et al., 1984; Yu et al., 1984; Aeschbacher et al., 1986). The caramel colours investigated in these publications were generally commercial caramel colours whose nature and classification was not further specified by the authors (JECFA, 1987). On the basis of these marginally inconclusive results, the ITCA in the period 1980–1990 carried out a comprehensive testing programme with samples of well-defined caramel colours to determine the genotoxic potential of the different caramel classes in a battery of genotoxicity tests *in vitro* and *in vivo*. The results of these studies largely indicated a lack of

genotoxic potential in vitro, with occasional positive responses being seen at very high exposure levels showing cytotoxicity. *In vivo* mouse micronucleus studies on Class I Plain Caramel and Class III Ammonia Caramel have not shown evidence of cytogenetic damage. Overall the Panel concluded that there were no concerns regarding the genotoxic potential of caramel colours.

No studies on the chronic toxicity and/or carcinogenicity of Class I Plain Caramel or Class II Caustic Sulphite Caramel are available. The findings in the long-term toxicity studies carried out with Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel did not reveal any pattern of toxicity not already seen in the 90-day oral toxicity studies carried out with these caramel colours. The only effects of Class III Ammonia Caramel observed in the chronic toxicity studies in rats or mice were decreased spleen weight, increased caecum weight, and (transient) lymphocytopenia. The Panel noted that decreases in splenic weight in the chronic study with Class III Ammonia Caramel in rats carried out by Evans et al. (1976) were significant in male rats at all dose levels, and were associated with reductions in lymphocyte numbers seen at earlier stages in the study. Evans *et al.* concluded that a NOAEL could not be identified in this study. The Panel noted however that the lymphocytopenia seen in the animals, together with the splenic effects, could be attributed to the presence of THI in the material tested, by analogy with other studies showing this effect (e.g. Mackenzie 1985b; Mackenzie et al., 1992b), and that since specific information was not provided on the level of THI in the material tested, this endpoint could not be used to identify a NOAEL for Class III Ammonia Caramel in this study.

Similarly, in the 2-year carcinogenicity study carried out with Class III Ammonia Caramel by Maekawa et al., 1983) the only parameters monitored, other than histopathological investigations of neoplastic change, were clinical signs, mortality and body weight changes. The authors did not report a NOAEL for the study, however on the basis of the absence of any treatment-related effect in the study the Panel concluded that 2 g/kg bw/day (the highest dose tested) could be considered a NOAEL.

In the 2-year oral toxicity study on Class IV Sulphite Ammonia Caramel in rats carried out at dose levels of up to 10 g/kg bw/day by MacKenzie (Mackenzie, 1985c; MacKenzie et al., 1992c) dark-stained and soft faeces were observed, as was caecal enlargement and increased kidney weight. In addition, body weight, food intake and water intake in male and female rats were decreased in the groups receiving 5 or 10 g Class IV Sulphite Ammonia Caramel/kg bw/day, probably reflecting a reduced palatability of the caramel containing drinking water. No pathological changes attributable to the treatment were found after examination of the organs. The highest dose tested, 10 g/kg bw/day, was considered by the authors to be the NOAEL in rats, on the basis that the effects seen were primarily related to reduced fluid intake, as discussed for the short-term toxicity studies on Class III and Class IV caramels above, and are of no toxicological significance. The Panel agreed with this NOAEL.

No evidence of carcinogenicity was seen in 2-year studies in rats on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel. In a parallel study in mice, there was similarly no evidence for a carcinogenic potential of Class IV Sulphite Ammonia Caramel. The Panel noted that no carcinogenicity data were available for Class I Plain Caramel and Class II Caustic Sulphite Caramel. The Panel considered however that given the fact that Class I Plain Caramel is likely to be produced in normal cooking processes, also considering the long-term toxicity and carcinogenicity data available on Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel and the relatively similar toxicological profile of all the caramel colours, there are no concerns regarding the carcinogenicity of Class I Plain Caramel and Class II Caustic Sulphite Caramel, nor regarding the carcinogenicity of Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel.

In relation to the reproductive and developmental toxicity of the caramel colours, the Panel noted that no data were available for Class I Plain Caramel and Class II Caustic Sulphite Caramel. There were no indications of reproductive and or developmental toxicity in mice, rats, or rabbits given Class III Ammonia Caramel by gavage at levels of up to 1600 mg/kg bw/day (Morgareidge, 1974a). In a reproduction study with Class IV Sulphite Ammonia Caramel in rats (Tierney et al., 1980), at the top dose of 25% in the diet, equivalent to approximately 28 g/kg bw/day, pups showed a higher incidence

of alopecia compared with pups in the control group and a generalized poor condition during the last 7 days of suckling. One pup at this dose level also had an arched spine, an incidence which was not statistically significant compared with controls. The number of implantation sites, litter size and of live pups at days 0, 4, and 21 of lactation in the 20%-dose group were significantly lower than control values, the Panel noted however that there was no dose-related trend, since in the 25% dose group these parameters were not statistically different from controls. The Panel considered that a NOAEL of 25-30 g/kg bw/day for female rats could be identified in this study.

The Panel noted that no multigeneration study is available on any of the four classes of caramel colours.

Due to a lack of data, no conclusion can be drawn with respect to the intolerance and allergenicity of the four caramel colours under evaluation. The Panel noted, however that no cases of intolerance and allergenicity linked to caramel exposure have been reported in published literature.

In relation to the potential haematotoxicity/immunotoxicity of caramel colours, effects have been identified in animal studies with Class III Ammonia Caramel, but not with the other classes of caramel colours. Lymphocytopenic and immunomodulatory effects have been seen in a number of studies with Class III Ammonia Caramel, and the overall conclusion of the authors of these studies was that THI, a constituent of Class III Ammonia Caramel, together with deficiency of pyridoxine, as a dietary influence, were primarily responsible for these effects. The THI-induced lymphocyte depression was largely ameliorated by pyridoxine. The Panel agreed with this interpretation and noted that where these changes were reported, the effects were (apparently) transient in nature, disappearing in the later stages of longer-term studies with Class III Ammonia Caramel. A recent study (Schwab et al., 2005) has shown that in mice receiving 7.5 mg/kg bw/day THI (50 mg/l drinking water) for 3 consecutive days, the level of S1P in plasma increased more than 100-fold. Treatment with THI also induced a marked decrease in the number of circulating lymphocytes and suppressed immunity. The migration of lymphocytes from the thymus and peripheral lymphoid organs is regulated by levels of S1P in these tissues relative to that in the peripheral circulation and inhibition of migration of immunocompetent cells occurs in response to elevated concentrations of circulatory S1P. S1P is thus an important immunoregulatory substance (Hla, 2004, 2005; Schwab et al., 2005; Rivera et al., 2008 Yu et al., 2010). THI has been shown to inhibit the S1P degrading enzyme S1P lyase, an intracellular degrading enzyme which is expressed by hematopoietic cells (Schwab et al., 2005), and these findings may provide a mechanistic explanation for the immunosuppressive effects of THI.

The Panel noted that in the pivotal 90-day study on Class III Ammonia Caramel (MacKenzie et al., 1985b, 1992b), in which rats were dosed with up to 20 g/kg bw/day caramel colour, containing either 15 mg THI/kg caramel or 295 mg THI/kg caramel, on a solids basis, no dose-related lymphocytopenia occurred in the animals fed caramel containing approximately 15 mg THI/kg. Class III Ammonia Caramel containing 295 mg THI/kg, at a dose level of 20 g/kg bw/day, induced a statistically significant decrease in lymphocyte counts in both sexes at 2 weeks and only in male rats at 6 weeks. All lymphocyte values in these groups were normal at the termination of the study.

Comparison of the effects of Class III Ammonia Caramel in rats and mice indicates that both species are equally sensitive to the immunotoxic effects of THI, although in contrast humans may be less sensitive than rodents (Houben and Penninks, 1994), as discussed below. The Panel noted that the results of a study carried out by Thuvander and Oskarssen indicate that Class III Ammonia Caramel that meets the limit of less than 25 mg THI/kg established in the JECFA specifications may, nevertheless, interfere with the lymphoid system in mice with an adequate vitamin B_6 status (Thuvander and Oskarssen, 1994). Sinkeldam and co- workers demonstrated significant reductions in lymphocytes and total leucocytes at a dose level of 1% Class III Ammonia Caramel in drinking water, estimated to be equivalent to 200 µg THI/ kg bw/day (Sinkeldam et al., 1988). Similar treatment-related, statistically significant reductions in lymphocytes were observed when the rats were given 200 µg, 500 µg or 2 mg THI/ kg bw/day alone in drinking water. The 90-day oral toxicity study in rats carried out by Mackenzie et al. included an evaluation of the toxicity of THI after a 4-week dosing

period (MacKenzie, 1985b; MacKenzie et al., 1992b). In rats maintained on a pyridoxine-replete diet, the short-term NOAEL of THI for the reduction of total lymphocytes was determined to be 120 µg/kg bw/day in female rats, and 380 µg/kg bw/day in male rats (MacKenzie et al., 1992b). Houben et al. (1992; 1992c) reported intakes of THI alone or Class III Ammonia Caramel containing THI that, in combination with manipulation of dietary pyridoxine levels, resulted in lymphocytopenia and could therefore be regarded as LOAELs for this effect. These ranged from 57.2 µg THI/kg bw/day (provided by a level of 0.4% Class III Ammonia Caramel in drinking water, in rats maintained on a low-pyridoxine diet, 2-3 mg/kg diet) to levels of 200 µg THI/kg bw/day or higher. Sinkeldam and co-workers reported decreases in lymphocyte counts in rats receiving 0.1% Class III Ammonia Caramel in drinking water, equivalent to 20 µg THI/ kg bw/day, for 1 week and maintained on a low-pyridoxine diet (2-3 mg/kg diet) (Sinkeldam et al., 1988). The Panel noted that statistical analysis was not reported for this aspect of the study, but evaluation of the data indicated that the decrease in lymphocytes was unlikely to be statistically significant at this dose level. The Panel considered that these findings in pyridoxine-deficient rats may be of limited relevance for human health risk assessment.

Overall the Panel concluded that a NOAEL for the lymphocytopenic effects of THI in pyridoxine-replete rats may lie in the range of 120-400 µg/kg bw/day as indicated by the studies of MacKenzie et al., Sinkeldam et al. and Houben et al.

The Panel noted that, in contrast to the findings in rats and mice, in the available short-term human studies consumption of Class III Ammonia Caramel had no effects on total or specified white blood cell counts at dose levels of up to 200 mg/kg bw/day (with THI levels almost 20 times above those permitted according to current specifications) albeit during short term exposure periods (BIBRA, 1976; Marier et al., 1977a; 1977b; Houben et al., 1992b; 1992c). The study of Houben et al. included subjects with (mild) pyridoxine deficiency, and the authors, in comparing the results obtained in this study with data in rats maintained on normal and pyridoxine-reduced diets, suggested that, "*with regard to oral intake of THI, humans are less sensitive to Caramel Colour III-induced lymphocytopenia than are rats*" (Houben et al., 1992c; Houben and Penninks, 1994). The Panel agreed with this interpretation, based on the available data.

The Panel considered that while theoretically THI could also be a constituent of Class IV Sulphite Ammonia Caramel, since formation of this imidazole is linked to the use of the ammonia process in the manufacture of Class III and Class IV caramels, THI has only been identified in Class III Ammonia Caramel Colour using current analytical methodologies (EUTECA, 2010a). No maximum limit for THI has therefore been established in the specifications for Class IV Sulphite Ammonia Caramel. It is suggested by the industry that the presence of sulphite/sulphur dioxide in Class IV Sulphite Ammonia Caramel may interfere with or inhibits the formation of THI (EUTECA, 2010a). Its absence in Caramel Classes I and II is due to the fact that there is no nitrogen source present that would allow its formation (EUTECA, 2010a).

The Panel noted that, in addition to the influence of the imidazole THI on the toxicological profile of Class III Ammonia Caramel, another imidazole contaminant of this class of caramel colours, 4-MEI, also found in Class IV Sulphite Ammonia Caramel, is considered to be responsible for the convulsions observed after administration of high doses of Class III Ammonia Caramel to a range of species and by different routes. The Panel considered, in line with the JECFA opinion (1978), that the acute toxicity of 4-MEI as a constituent in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel is not of toxicological concern since maximum levels of 4-MEI are legally restricted to $\leq$ 250 mg/kg in these caramel colours, at which level these acute toxic effects of 4-MEI will not be manifest. The Panel also noted however that in recent carcinogenicity studies, NTP has concluded that 4-MEI shows "*clear evidence of carcinogenic activity in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms, and also "equivocal evidence of carcinogenic activity of 4-methylimidazole in female F344/N rats based on increased incidences of mononuclear cell leukaemia*." The Panel considered, however, that the carcinogenic effect of 4-MEI seen in mice in this study was thresholded, based on the lack of genotoxicity of 4-MEI (NTP, 2007), also noting that

alveolar/bronchiolar neoplasms occur spontaneously at high incidence in B6C3F1 mice (Fox et al., 2006). The Panel concluded therefore that the intermediate dose of 625 mg 4-MEI/kg diet, equivalent to 80 mg 4-MEI/kg bw/day could be considered to be a NOAEL in this study. The Panel noted that 4-MEI has recently been listed by the US State of California as a chemical known to cause cancer (OEHHA, 2011).

The Panel, in evaluating the overall toxicological database on the four classes of caramel colours, considered that while the constituents 4-MEI (present in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel) and THI (found in Class III Ammonia Caramel only) must be taken into account in the safety evaluation of these caramel colours as food additives, as discussed above, all four caramel colours are otherwise similar in their toxicological effects. These effects include, variously, mild diarrhoea, reduced body weight gain associated with reduced food and fluid consumption, pigmentation of mesenteric lymph nodes, enlargement of the caecum, reduced urinary output associated with increases in specific gravity of the urine, and increases in caecal and kidney weights, unaccompanied by any histopathological change, although not all these changes were reported in every study with the individual caramel colours. Such effects can be expected following administration of a complex mixture of high molecular weight, insoluble materials, such as the caramel colours, and the commonality of effect can be compared to e.g. the polyol sweeteners, producing local physiological effects including osmotic diarrhoea in the gastrointestinal tract. The Panel noted that the available human volunteer studies with the caramel colours (e.g. BIBRA, 1976; Marier et al., 1977a; 1977b) indicate that repeated caramel ingestion at levels of up to 300 mg/kg bw/day can result in an increased frequency of bowel movements and softening or increased liquidity of faeces. The Panel also noted that the effects produced by the caramel colours can be anticipated to be additive in nature.

The Panel considered that, in spite of the absence of full chemical characterisation of the four classes of caramel colours, given the consistency in the toxicological database, the caramel colours can be considered as a single group in terms of assessing their safety. The Panel considered therefore that a group ADI can be established for the caramel colours. Given, however, concerns regarding the immunotoxicity of THI, present in Class III Ammonia Caramel, the Panel decided to define an individual ADI for this caramel within the overall group ADI, based on the currently available database. While the Panel noted the toxicological data gaps for caramel colours, including the generally sparse database on reproductive toxicity for caramel colours as a whole and the absence of long-term toxicity and carcinogenicity studies on Class I Plain Caramel and Class II Sulphite Caramel, the Panel considered that missing data for these classes of caramel colours can be accounted for by data from another class. The Panel noted that specific constituents of concern resulting from differences in the production processes for the four classes of caramel colours, including especially THI in Class III Ammonia Caramel and 4-MEI in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel should be dealt with separately as discussed in detail later in this Discussion section.

The Panel considered that several studies carried out in rats with the different caramel colours are relevant for establishment of a group ADI for the caramel colours. These include the three studies used previously by JECFA and SCF to define the respective ADIs for the individual classes:

- a 90-day study with Class II Caustic Sulphite Caramel (MacKenzie, 1985a; MacKenzie et al., 1992a), providing a NOAEL of 16 g/kg bw/day, the highest dose tested,
- a 90-day study with Class III Ammonia Caramel (MacKenzie, 1985b, MacKenzie et al., 1992b) providing a NOAEL of 20 g/kg bw/day, the highest dose tested,
- a 2-year oral toxicity study in rats carried out by MacKenzie (MacKenzie, 1985b, MacKenzie et al., 1992c) with Class IV Sulphite Ammonia Caramel, providing a NOAEL of 10 g/kg bw/day, the highest dose tested,

The Panel considered that several additional toxicological studies should be taken into account when establishing the group ADI, and these include:

- a 13-week toxicity study in rats with Class IV Sulphite Ammonia Caramel in drinking water (Heidt and Rao 1980), providing a NOAEL of 30 g/kg bw/day, the highest dose level tested,
- another 13-week drinking water study in rats with Class IV Sulphite Ammonia Caramel (MacKenzie, 1985b; MacKenzie et al., 1992c) providing a NOAEL of 30 g/kg bw/day, the highest dose level tested,
- a 90-day study in Beagle dogs with Class IV Sulphite Ammonia Caramel (Kay and Calandra, 1962c) providing a NOAEL of 6.25 g/kg bw/day, the highest dose tested,
- a 96-week drinking water study in mice with Class III Ammonia Caramel (Hagiwara et al., 1983), providing a NOAEL of 8.4 g/kg bw/day, the highest dose tested,
- a 2-year dietary study in rats with Class III Ammonia Caramel (Evans et al.,1976) providing a NOAEL of 3 g/kg bw/day, the highest dose tested,
- a 104-week study in rats with Class III Ammonia Caramel (Maekawa et al., 1983), providing a NOAEL of 2 g/kg bw/day, the highest dose tested,
- a reproductive toxicity study in rats with Class IV Sulphite Ammonia Caramel (Tierney, 1973) providing in the dams a NOAEL of 25-30 g/kg bw/day.

Given that:

- the NOAELs in all these studies were the highest dose levels tested,
- the effects of the caramel colours in 90-day studies were generally similar to those reported in the long-term studies,
- available reproduction and developmental toxicity studies, although limited, do not reveal any effects of concern,
- the studies reveal no effects on the reproductive organs,
- the effect of most concern, i.e. lymphocytopenia, can, as also stated by JECFA, best be evaluated from short term studies, and
- the long-term studies support the conclusion that the caramel colours are not carcinogenic,

the Panel decided to use the highest NOAEL of 30 g/kg bw/day reported in several of these studies, still the highest dose level tested, as the basis to derive a group ADI for the caramel colours. The Panel noted that whilst there were arguments for increasing the default uncertainty factor of 100, to compensate for limitations in the toxicological databases on reproductive toxicity, equally compelling arguments could be advanced for deriving a chemical-specific adjustment factor below the default uncertainty factor. The Panel therefore applied an uncertainty factor of 100 to the NOAEL of 30 g/kg bw/day to derive a group ADI of 300 mg/kg bw/day for the caramel colours. Overall, based on the available database, the Panel considered that this would provide a sufficient margin of safety.

In relation to Class III Ammonia Caramel, the Panel considered the available data on the immunotoxicity of THI, a constituent of this caramel class only. The Panel noted that no dose-related effects on haematological parameters were reported in the 90-day study of MacKenzie (1985b, 1992b),

using a Class III Ammonia Caramel containing a THI level of 15 mg/kg, while with a Class III Ammonia Caramel containing a much higher level of THI of 295 mg/kg, only transient effects on lymphocytes were seen. The MacKenzie study provided a NOAEL of 20 g/kg bw/day for Class III Ammonia Caramel, the highest dose tested. The Panel also noted, however, the results of the study of Thuvander and Oskarssen, indicating that Class III Ammonia Caramel that meets the limit of less than 25 mg THI/kg established in the JECFA specifications may, nevertheless, interfere with the lymphoid system in mice with an adequate vitamin B_6 status (Thuvander and Oskarssen, 1994). While the Panel considered that this study should not be used as a pivotal study for the purposes of risk assessment without further substantiation, given a number of studies in rats showing no effect on haematological parameters over longer periods and at higher dose levels than those used in the study of Thuvander and Oskarssen, the Panel considered that it should be taken into account in establishing an ADI for Class III Ammonia Caramel. The Panel applied an additional uncertainty factor of 2 together with the default uncertainty factor of 100 to the NOAEL of 20 g/kg bw/day identified from the MacKenzie study. The Panel therefore establishes, within the group ADI for all caramel colours and based on the currently available database, an ADI of 100 mg/kg bw/day for Class III Ammonia Caramel.

The Panel noted that this means that within the group ADI of 300 mg/kg bw/day established for the four caramel colours, only 100 mg/kg bw/day of this 300 mg/kg bw/day can be made up by Class III Ammonia Caramel.

The exposure assessment 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. As caramel colours Class I, II, III and IV are authorised *quantum satis* in almost all categories, the refined exposure estimates have been performed only for Tier 3 using the maximum reported use levels or when no usages were reported to EFSA, values defined by decision rules for *quantum satis* usages were used (Table 6).

Exposure estimates for children (1-14 years old) have been done by the Panel for 11 European countries (Belgium, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Denmark, Cyprus, Greece) based on detailed individual food consumption data provided by the EXPOCHI consortium (Huybrechts et al., 2010). As the UK is not part of the EXPOCHI consortium, estimates for UK children (aged 1.5- 4.5 years) were made by the Panel with the use of detailed individual food consumption data (UK NDNS, 1992-1993) available from the UNESDA report (Tennant, 2008). For the adult population, the Panel has selected the UK population as representative of the EU consumers for estimates of exposure.

The mean dietary exposure of European children including UK pre-school children ranged from 76.9 to 427.2 mg/kg bw/day for Class I Plain Caramel, from 8.7 to 34.6 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 21.7 to 302.4 mg/kg bw/day for Class III Ammonia Caramel, and from 23.2 to 506.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel. At the 95th or 97.5th percentile, estimates ranged from 179.6 to 882.2 mg/kg bw/day for Class I Plain Caramel, from 18.5 to 117.3 mg/kg bw/day for Class II Caustic Sulphite Caramel, from 107.9 to 757.3 mg/kg bw/day for Class III Ammonia Caramel, and from 129.7 to 1480.2 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors (>10% in all or several countries) to the total anticipated exposure of children were for Class I Plain Caramel: non alcoholic flavoured drinks (12% to 55%), fine bakery wares (15% to 32%), desserts including flavoured milk products (11% to 48%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 56%), soups (11% to 32%) and malt bread (16% to 49%). For Class II Caustic Sulphite Caramel the main contributors were fine bakery wares (12% to 53%), desserts including flavoured milk products (11% to 41%), edibles ices (11% to 22%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 45%), soups (18% to 54%) and malt bread (19% to 55%). For Class III Ammonia Caramel the main contributors were fine bakery wares (13% to 45%), desserts including flavoured milk products (12% to 44%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (12% to 79%), and vinegar (12% to 45%), while in one country non alcoholic flavoured drink, malt bread confectionery, and sausages, pates and terrines contributed

29%, 15%, 13% and 10%, respectively. For Class IV Sulphite Ammonia Caramel the main contributors were non alcoholic flavoured drinks (13% to 51%), confectionery (20% to 81%), fine bakery wares (10% to 29%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (10% to 24%), and malt bread (10% to 34%).

The anticipated dietary exposure reported for the UK adult population gives a mean of 136.6 mg/kg bw/day and a 97.5th percentile of 429.3 mg/kg bw/day for Class I Plain Caramel; a mean of 21.7 mg/kg bw/day and a 97.5th percentile of 109.5 mg/kg bw/day for Class II Caustic Sulphite Caramel; a mean of 60.8 mg/kg bw/day and a 97.5th percentile of 295.0 mg/kg bw/day for Class III Ammonia Caramel; and a mean of 89.4 mg/kg bw/day and 97.5th percentile of 368.9 mg/kg bw/day for Class IV Sulphite Ammonia Caramel.

The main contributors to the total anticipated exposure of adults were for Class I Plain Caramel non alcoholic flavoured drinks (30%), beer and cidre bouché (27%), soups (16%), and sauces, seasonings (e.g. curry powder, tandoori) and pickles (10%). For Class II Caustic Sulphite Caramel the main contributors were beer and cidre bouché (50%) and soups (20%). For Class III Ammonia Caramel the were beer and cidre bouché (48%) and sauces, seasonings (e.g. curry powder, tandoori) and pickles (22%). For Class IV Sulphite Ammonia Caramel the main contributors were confectionery (65%) and non alcoholic flavoured drinks (23%).

The Panel also evaluated combined anticipated dietary exposure to all four classes of caramel colours, taking into account the highest maximum reported level for all caramel classes, described in Table 8, from each food category. When considering this scenario, as presented in Table 10, anticipated mean combined dietary exposure reported for European children, including UK pre-school children, ranged from 83.5 to 698.3 mg/kg bw/day. At the 95th/97.5th percentile, estimates ranged from 224.8 to 1672.3 mg/kg bw/day. For the UK adult population this scenario gave a range of exposures of 194.8 and 474.3 mg/kg bw/day for the mean and the 97.5th percentile, respectively.

The main contributors to the total combined anticipated exposure to caramel colours for children were non alcoholic flavoured drinks (11% to 28%), confectionery (19% to 58%), fine bakery wares (15% to 29%), desserts including flavoured milk products (10% to 31%), sauces, seasonings (e.g. curry powder, tandoori) and pickles (14% to 44%), and malt bread (16% to 46%). Soups were estimated to contribute from 25% to 28% in two countries and vinegar was estimated to contribute 20% in one country. For the adult population the main contributors were confectionery (30%), non alcoholic flavoured drinks (21%), beer, cidre bouché (19%) and soups (11%).

The Panel noted that the anticipated dietary exposure of the adult population at the 97.5th percentile to Class I Plain Caramel exceeds the group ADI of 300 mg/kg bw/day proposed for the caramel colours. Similarly, the anticipated dietary exposure of the adult population at the 97.5th percentile to Class IV Ammonia Caramel exceeds this group ADI. For children, the upper end of both the mean intake ranges and also the 95th/97.5th percentile intakes for Class I Plain Caramel exceed the group ADI of 300mg/kg bw/day. Similarly, for children the upper end of both the mean intake ranges and also the 95th/97.5th percentile intakes for Class IV Sulphite Ammonia Caramel exceed the group ADI of 300 mg/kg bw/day.

The anticipated dietary exposure to Class II Sulphite Caramel for both adults and children was below the group ADI of 300 mg/kg bw/day.

For Class III Ammonia Caramel the upper end of the mean intake range for children exceeds the individual ADI of 100 mg/kg bw/day established for this colour within the group ADI, while the 97.5th percentile anticipated dietary exposures of both the child and adult populations are above this ADI of 100 mg/kg bw/day.

Anticipated combined dietary exposures of both adults and children to all caramel colours exceed the group ADI of 300 mg/kg bw/day at the 95th/97.5th percentile, while the ADI is also exceeded by the

combined mean intake for children. In the case of children, this exceedance applies to the upper end of the exposure range only.

Reflecting the concerns regarding the immunotoxicity seen in a number of studies with either Class III Ammonia Caramel or with THI alone, found as a constituent in Class III Ammonia Caramel, and the carcinogenicity of 4-MEI, found as a constituent in Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel, the Panel has estimated exposure to THI and 4-MEI as a result of consumption of these caramel colours in the diet (Table 11). Additionally the Panel has also estimated exposure to sulphur dioxide, present in Class II and Class IV caramels as a result of the production method (Table 11). The Panel considered overall that the outcome of these exposure estimates did not give rise to concern and that the maximum levels established for THI, 4-MEI and sulphur dioxide in the specifications in the relevant caramel classes are sufficiently protective. The Panel noted in particular that for THI the reduction of the ADI for Class III Ammonia Caramel from 200 mg/kg bw/day (as established by the SCF in 1987) to 100 mg/kg bw/day, together with the outcome of the exposure estimates indicating that this ADI is likely to be exceeded by the upper end of the mean intake range for children and by the 97.5th percentile intakes of both the child and adult populations may result in lower intakes of THI in the future than those indicated in Table 11.

The Panel additionally considered the possible risks of exposure to furan and 5-hydroxymethylfurfural (5-HMF), present as constituents of the caramel colours as a result of the heat-treatment processes involved in the manufacture of the caramel colours. The Panel noted that furan has carcinogenic effects in experimental animals, probably produced by a genotoxic mechanism. The Panel noted that while 5-HMF is genotoxic in *in vitro* studies, the conclusion from the toxicological database on 5-HMF is that overall no *in vivo* genotoxic or relevant carcinogenic response is seen in studies in rodents and that no genotoxicity or carcinogenicity is expected in humans.

The Panel noted that variations in the manufacturing processes of the caramel colours may result in a wide variability in the nature and levels of the various constituents, including constituents of toxicological concern such as 5-HMF and furan. Given this likely variability, the Panel considered that in order to further guarantee the safety of caramel colours with respect to their minor constituents, such as THI, 4-MEI, 5-HMF and furan, it would be prudent to reduce their levels as much as technologically feasible. The Panel considers therefore that the specifications for the caramel colours should be updated and extended to also include maximum levels for constituents ofpossible concern not yet included in the specifications, such as for example 5-HMF and furan.

CONCLUSIONS

Caramel colours are colouring substances authorised as food additives in the EU, and are complex mixtures of compounds produced by heating carbohydrates under controlled heat and chemical processing conditions. The caramel colours are classified according to the reactants used in their manufacture as follows: Class I Plain Caramel or Caustic Caramel (E1 50a); Class II Caustic Sulphite Caramel (E 150b); Class III Ammonia Caramel (E 150c) and Class IV Sulphite Ammonia Caramel (E 150d).

Both JECFA and SCF have concluded that a numerical ADI was not necessary for Class I Plain Caramel, while both bodies have established an ADI of 200 mg/kg bw/day for Class III Ammonia Caramel and Class IV Sulphite Ammonia Caramel. This is also the ADI established by the SCF for Class II Caustic Sulphite Caramel, while JECFA have established an ADI of 160 mg/kg bw/day for this class.

The Panel concludes that, given the toxicological similarity of all four classes of caramel colours and the consistency in the toxicological database, the caramel colours can be considered as a single group in terms of assessing their safety, and that a group ADI can be derived. The Panel establishes a group ADI of 300 mg/kg bw/day for the caramel colours. Given, however, concerns regarding the immunotoxicity of THI, present in Class III Ammonia Caramel, the Panel decided to define an

individual ADI of 100 mg/kg bw/day for this caramel within the group ADI, based on the currently available database. This means that within the group ADI of 300 mg/kg bw/day, only 100 mg/kg bw/day of this 300 mg/kg bw/day can be made up by Class III Ammonia Caramel.

The Panel notes that the anticipated dietary exposure of the adult population at the 97.5th percentile to Class I Plain Caramel exceeds the group ADI of 300 mg/kg bw/day proposed for the caramel colours. Similarly, the anticipated dietary exposure of the adult population at the 97.5th percentile to Class IV Ammonia Caramel exceeds this group ADI. For children, the upper end of both the mean intake ranges and also the 95th/97.5th percentile intakes for Class I Plain Caramel exceed the group ADI of 300mg/kg bw/day. Similarly, for children the upper end of both the mean intake ranges and also the 95th/97.5th percentile intakes for Class IV Sulphite Ammonia Caramel exceed the group ADI of 300 mg/kg bw/day.

The anticipated dietary exposure to Class II Sulphite Caramel for both adults and children was below the group ADI of 300 mg/kg be/day.

For Class III Ammonia Caramel the upper end of the mean intake range for children exceeds the individual ADI of 100 mg/kg bw/day established for this colour within the group ADI, while the 97.5th percentile anticipated dietary exposures of both the child and adult populations are above this ADI of 100 mg/kg bw/day.

Anticipated combined dietary exposures of both adults and children to all caramel colours exceed the group ADI of 300 mg/kg bw/day at the 95th/97.5th percentile, while the ADI is also exceeded by the combined mean intake for children. In the case of children, this exceedance applies to the upper end of the exposure range only.

The Panel concludes overall that the exposure estimates for THI, 4-MEI or sulphur dioxide are not of concern, but notes remaining uncertainties regarding the effects of THI on the immune system. The Panel would welcome additional studies to clarify these effects .

The Panel notes that variations in the manufacturing processes of the caramel colours may result in a wide variability in the nature and levels of the various constituents, including constituents of toxicological concern such as 5-HMF and furan. Given this likely variability, the Panel considers that in order to further guarantee the safety of caramel colours with respect to their minor constituents, such as THI, 4-MEI, 5-HMF and furan, it would be prudent to reduce their levels as much as technologically feasible. The Panel considers therefore that the specifications for the caramel colours should be updated and extended to also include maximum levels for constituents of possible concern not yet included in the specifications, such as for example 5-HMF and furan.

The Panel additionally concludes that there is limited information about the relationship between processing parameters for the caramel colours and the formation and nature of heat-derived constituents which is also relevant for the control of manufacturing processes. Future research work is recommended in this respect.

DOCUMENTATION PROVIDED TO EFSA

1. Pre-evaluation document prepared by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

2. Caramel colours (E150a-d): preliminary EUTECA input containing info on use levels, production methods, safety evaluation studies. EUTECA, Secretariat, Brussels, March, 2007.

3. A report "Screening potential intake of Caramel Colours" (D. Tennant). EUTECA, Secretariat, Brussels, July, 2007.

4. CIAA (Confederation of the Food and Drink Industries of the EU), 2009. CIAA data in response to the Commission request for data: "EFSA re-evaluation of food colours" - Southampton study colours) (SANCO/E3/OS/km D 53007, May 22, 2009).

5. MacKenzie, K.M. (1985). 90-day toxicity study of caramel color (caustic sulfite process) in rats. Vols. I & II. Unpublished report No. 6154-105 from Hazleton Laboratories America Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association. Unpublished report from report provided by EUTECA (2010).

6. MacKenzie, K. M. (1985). 90-day toxicity study of caramel color (ammonia process) in rats. Vols. I and II. Unpublished report No. 6154-107 from Hazleton Laboratories America, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association. (MacKenzie, K.M. (1985c). Carcinogenicity and chronic toxicity study of caramel color (sulfite ammonia process) type SAC2 in rats (Chronic toxicity portion, Vols. I-V; Carcinogenicity portion, Vols. I-VIII). Unpublished study No. 81184 from Hazleton Laboratories America, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association. Unpublished report from report provided by EUTECA (2010).

7. MacKenzie, K.M. (1985). Carcinogenicity and chronic toxicity study of caramel color (sulfite ammonia process) type SAC2 in rats (Chronic toxicity portion, Vols. I-V; Carcinogenicity portion, Vols. I-VIII). Unpublished study No. 81184 from Hazleton Laboratories America, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association Unpublished report from report provided by EUTECA (2010).

8. MacKenzie, K.M. (1985). Carcinogenicity study of caramel color (sulfite ammonia process) type SAC2 in mice. Vols. I-VI. Unpublished study No. 81185 from Hazleton Laboratories America, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association. Unpublished report from report provided by EUTECA (2010).

9. Evans, J. G., Butterworth, K. R., Gaunt, I. F. and Grasso, P. (1976). Long-term toxicity study in the rat of a caramel produced by the "half-open-half closed pan" ammonia process. Unpublished report No. 6/1976 from the British Industrial Biological Research Association, Carshalton, Surrey, England. Unpublished report from report provided by EUTECA (2010).

10. Re-evaluation of caramel colours. Additional data follow-up to Email sent by EFSA on 24 May 2010. EUTECA, Secretariat, Brussels, June, 2010.

11. Til HP and Spanjers M, 1973. Reproduction study in rats with six different Ammonia Caramels. Unpublished report No. R 4068 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherland. Unpublished report provided by EUTECA (2010).

12. Tierney WJ, 1980. An in-utero range-finding study of caramel colour no. 3, sample 3-1, in rats. Unpublished project No. 79-2405 from Bio/dynamics Inc., East Millstone, NJ, USA. Submitted to WHO by International Technical Caramel Association. Unpublished report provided by EUTECA (2010).

13. Heidt M and Rao GN, 1980. 90-day subacute toxicity study of caramel color (sulfite ammonia process) type SAC2 in rats. Unpublished report No. 79028 from Raltech Scientific Services, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association. Unpublished report provided by EUTECA (2010).

14. Sinkeldam EJ, van der Heyden CA and Beems RB, 1976. Chronic (two year) feeding study in rats with six different Ammonia Caramels. Unpublished report No. R 4961 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Unpublished report provided by EUTECA (2010).

15. Level of use of caramel colours provided by EUTECA to EFSA on 05.11.2010 a follow up of Email sent on 21 September 2010

16. Re-evaluation of caramel colours_Additional data follow-up to email sent on 14 December 2010. Data provided on 21 January 2011.

17. Re-evaluation of caramel colours_Additional data follow-up to email sent on 24 January 2011. Data provided on 28 January 2011

REFERENCES

Aeschbacher HU, 1986. Genetic toxicology of browning and caramelizing products. In Progress in Clinical and Biological Research, Vol. 206, 133-144. Genetic Toxicology of the Diet. Edited by I. Knudsen. Alan R. Liss, New York (as referred to by SCF, 1997).

Adams K, Allen JA, Brooker PC, Jones E and Proudlock RJ, 1992. Assessment of the genotoxic potential of Caramel Colour I in four short-term tests. Food and Chemical Toxicology 30, 397-403 (as referred to by SCF, 1997).

Adams ML, Meyer ER and Cicero TJ, 1998. Imidazoles suppress rat testosterone secretion and testicular interstitial fluid formation In vivo. Biology of Reproduction 59(2), 248-54.

Allen JA, Brooker I C, Jones E, Adams K and Richold M, 1992. Absence of mutagenic activity in Salmonella and of clastogenic activity in CHO cells of caramel colours I, II, III and IV. Food and Chemical Toxicology, 30, 389-395 (as referred to by SCF, 1997).

Allen JA, Brooker PC, Birt DM and McCaffrey KJ, 1984. Analysis of metaphase chromosomes obtained from CHO cells cultured in vitro and treated with caramel colour II. Unpublished report No. ITC 3B/84966 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Allen JA and Proudlock RJ, 1984. Autoradiographic assessment of DNA repair in mammalian cells after exposure to caramel colour II. Unpublished report from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Ashoor SH and Monte WC, 1983. Mutagenicity of commercial caramels. Cancer Letters 18, 187-190 (as referred to by JECFA, 1987).

Bartlett S and Broster WH, 1957. Feeding trials with ammoniated molasses in the diet of young dairy cattle. Journal of Agricultural Science 50, 60-63.

Battelle Memorial Institute, 1971. Unpublished report dated 4 May 1971 (as referred to by JECFA, 1977).

BIBRA (British Industrial Biological Research Association), 1976. A study of the haematological effects of caramel in human volunteers. Unpublished report No. 1/172/76 from the British Industrial Biological Research Association, Carshalton, Surrey, England (as referred to by JECFA, 1987).

BIBRA (British Industrial Biological Research Association), 1977. The short-term (10 week) feeding study with three caramels in rats. Unpublished report No. 177/2/77 from the British Industrial Biological Research Association, Carshalton, Surrey, England.

BIBRA (British Industrial Biological Research Association), 1978. The evaluation of the effect of sulfite Ammonia Caramels on the lymphocytes of the rat. Unpublished report No. 238/2/78 from the British Industrial Biological Research Association, Carshalton, Surrey, England.

Bononi M and Tateo F, 2007. Determination of furan by headspace solid-phase microextraction-gas chromatography-mass spectrometry in balsamic vinegars of Modena. Journal of Food Composition and Analysis 22, 79-82.

Bradbury MG, Doherty KV, Parish CR and Lyons AB, 1996.The immunosuppressive compound 2-acetyl-4-tetrahydroxybutyl imidazole inhibits the allogeneic mixed lymphocyte reaction by sequestration of a recirculating subpopulation of T cells. Immunology, 87(1), 80-5.

Bradbury MG, Qiu MR and Parish CR, 1997. The immunomodulatory compound 2-acetyl-4-tetrahydroxybutyl imidazole causes sequestration of lymphocytes in non-lymphoid tissues. Immunology and Cell Biology 75(5), 497-502.

Brusick D, 1974. Mutagenic Evaluation of Compound FDA 71-83, Caramel. Unpublished report submitted to the Food and Drug Administration.

Brusick DJ, Jagannath DR and Galloway SM, 1992. Genotoxicity hazard assessment of caramel colours III and IV. Food and Chemical Toxicology 30, 403-410 (as referred to by SCF, 1997).

CFR (Code of Federal Regulation), 2006. Title 21: Food and Drug. Charper I: Food and Drug Administration, Department of health and human services. Part 182: Substances generally regonized as safe. Sec 182.1235: Caramel. Available at: http://www.grokfood.com/regulations/182.1235.htm

Chacharonis P, 1960. Acute and chronic toxicity studies on caramel colours A and B. Unpublished report No. S.A. 54219 from Scientific Associates Inc., St. Louis, MO, USA (as referred to by JECFA, 1987).

Chacharonis P, 1963. Acute oral toxicity study in rats on caramel colorings 25A-1, 30B-0and 30F-1. Unpublished report No. S.A. 79105 from Scientific Associates Inc., St. Louis, MO, USA (as referred to by JECFA, 1987).

CIAA (Confederation of the Food and Drink Industries of the EU), 2009. CIAA data in response to the Commission request for data: "EFSA re-evaluation of food colours" - Southampton study colours) (SANCO/E3/OS/km D 53007, May 22, 2009)

Cimino MC and Brusick DJ, 1981. Mutagenicity evaluation of ETA 48-IH caramel color in the mouse micronucleus test. Unpublished report No. 22129 from Litton Bionetics Inc., Kensington, MD, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Ciolino LA, 1998. Determination and classification of added caramel color in adulterated acerola juice formulations. Journal of Agricultural and Food Chemistry 46, 1746-1753.

Code of Federal Regulations (CFR), 2006. Caramel. Title 21, Volume 3, Sec. 182.1235. e-CFR Data as of May 20, 2010.

De Heer C, Schuurman HJ, Houben GF, Pieters RH, Penninks AH and van Loveren H, 1995. The SCID-hu mouse as a tool in immunotoxicological risk assessment: effects of 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI) and di-n-butyltin dichloride (DBTC) on the human thymus in SCID-hu mice. Toxicology. 1995 Jun 26;100(1-3):203-11.

Dross A and Baltes W, 1989. Űber die Fraktionierung von Zuckercouleur-Inhaltsstoffen nach iher Molmasse. Z Lebensm Unters Forsch 188, 540-544.

EC (European Commission), 1998. Report on Methodologies for the Monitoring of Food Additive Intake Across the European Union. Final Report Submitted by the Task Coordinator, 16 January 1998. Reports of a Working Group on Scientific Cooperation on Questions Relating to Food, Task 4.2. SCOOP/INT/REPORT/2 (Brussels: European Commission Directorate General I11 Industry).

EC (European Commission), 2001. Commission of the European Communities (COM). 542 final. Report from the commission on dietary food additive intake in the European Union. Brussels, 01.10.2001.Available at:

http://eur-lex.europa.eu/LexUriServ/site/en/com/2001/com2001_0542en01.pdf

EFSA (European Food Safety Authority), 2004a. Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the evaluation of allergenic foods for labelling purposes . The EFSA Journal (2004) 32, 1-197.
Available at: http://www.efsa.europa.eu/en/efsajournal/doc/opinion_nda_04_en1,1.pdf

EFSA (European Food Safety Authority), 2004b. Report of the Scientific Panel on Contaminants in Food Chain on provisional findings on furan in food. The EFSA Journal (2004) 137, 1-20.
Available at: http://www.efsa.europa.eu/it/efsajournal/doc/137.pdf

EFSA (European Food Safety Authority), 20010. Scientific opinion of the EFSA Panel on Food Conctact Material, Enzymes, Flavourings and Processing Aids (CEF) on Flavouring Group Evaluation 13, Revision 1 (FGE.13Rev1): Furfuryl and furan derivatives with and without additional side-chain substituents and heteroatoms from chemical group 14. EFSA Journal 2010;8(4):1403, 1-112.
Available at: http://www.efsa.europa.eu/en/scdocs/doc/1403.pdf

Emerton V (Editor), 2008. Ingredients Handbook: Food Colours. Leatherhead Ingredients Handbook series. Wiley, John and Sons, Incorporated. ISBN: 1905224443.

EUTECA, 2007a. European Technical Committee on Caramels, Caramel colour – production method (general description). Personal communication to EFSA, March 2007.

EUTECA, 2007b. Screening Potential Intakes of Caramel Colours. Report provided for the European Technical Committee on Caramels, July 2007.

EUTECA, 2010a. Revaluation of caramel colours – Additional data. Letter from EUTECA to EFSA on 17.06.2010 a follow up of Email sent on 24 May 2010.

EUTECA, 2010b. Revaluation of caramel colours –EUTECA/ITCA joint response to email sent by EFSA on 1st October. 27 October 2010.

EUTECA, 2010c. Revaluation of caramel colours –Level of use of caramel colours provided by EUTECA to EFSA on 05.11.2010 a follow up of Email sent on 21 September 2010.

EUTECA, 2011a. Revaluation of caramel colours –Additional data provided by EUTECA to EFSA on 21.01.2011 a follow up of Email sent on 14 December 2010.

EUTECA, 2011b. Revaluation of caramel colours –Additional data provided by EUTECA to EFSA on 28.01.2011 a follow up of Email sent on 24 January 2011.

Evans JG, Butterworth KR, Gaunt IF and Grasso P, 1976. Long-term toxicity study in the rat of a caramel produced by the "half-open-half closed pan" ammonia process. Unpublished report No. 6/1976 from the British Industrial Biological Research Association, Carshalton, Surrey, England.

Foote WL, Robinson RF and Davidson RS, 1958. Toxicity of caramel color products. Unpublished report of Battelle Memorial Institute, Columbus, OH, USA.

Galloway SM and Brusick DJ, 1981a. Mutagenicity evaluation of ETA-48-IH in an in vitro cytogenetic assay measuring chromosome aberration frequencies in Chinese hamster ovary (CHO) cells. Unpublished report No. 20990 from Litton Bionetics Inc., Kensington, MD, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Galloway SM and Brusick DJ, 1981b. Mutagenicity evaluation of ETA-48-IH in an in vitro cytogenetic assay measuring chromosome aberration frequencies in Chinese hamster ovary (CHO) cells. Part II. Unpublished report No. 20990 from Litton Bionetics Inc., Kensington, MD, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Gaunt IF, Lloyd AG, Grasso P, Gangolli SP and Butterworth KR, 1977. A short-term study in the rat on two caramels produced by variations of the “ammonia process”. Food and. Cosmetics Toxicology 15, 509-521.

Gobin SJ, Legg RF, Paine AJ and Phillips JA, 1989. The effect of 2-acetyl-4-tetrahydroxybutylimidazole on lymphocyte subsets in peripheral blood of the rat. International Journal of Immunopharmacology 11(8), 937-46.

Gobin SJ and Phillips JA, 1991. Immunosuppressive effects of 2-acetyl-4-tetrahydroxybutyl imidazole (THI) in the rat. Clinical and Experimental Immunology 1991 Aug;85(2), 335-40.

Gobin and Paine AJ, 1992. Effects of 2-acetyl-4-tetrahydroxybutyl imidazole (THI) on the thymus of rats. Thymus 20(1), 17-30.

Gobin SJ, Legg RF and Paine AJ, 1992. The role of lymphocyte production and migration in the lymphocytopenia caused by 2-acetyl-4-tetrahydroxybutyl imidazole. International Journal of Immunopharmacology 14(4), 687-97.

Gugasyan R, Losinno C and Mandel T, 1995. The effect of 2-acetyl-4-tetrahydroxybutylimidazole on lymphocyte subsets during a contact hypersensitivity response in the NOD mouse. Immunology Letters 46(3), 221-7.

Gugasyan R, Clouston D, Mandel T and Wicks I, 1997. Prevention of splenic granuloma formation in adjuvant arthritis by 2-acetyl-4-tetrahydroxybutylimidazole (THI). Immunology Letters 58(3), 133-8.

Gugasyan R, Coward A, O'Connor L, Shortman K and Scollay R, 1998. Emigration of mature T cells from the thymus is inhibited by the imidazole-based compound 2-acetyl-4-tetrahydroxybutylimidazole. Immunology 93(3), 398-404.

Haldi J and Wynn W, 1951. A study to determine whether or not caramel has any harmful physiological effect. I. Unpublished report from Emory University, Atlanta, GA, USA (as referred to by JECFA, 1987).

Haldi J and Wynn W, 1958. A study to determine whether or not caramel has any harmful physiological effect. II. Unpublished report from Emory University, Atlanta, GA, USA.

Hagiwara A, Shibata M, Kurata Y, Seki, K, Furushima S and Ito N, 1983. Long-term toxicity and carcinogenicity test of ammonia-process caramel colouring given to $B_6C_3F_1$ mice in the drinking water. Food and Chemical Toxicology 21, 701-706.

Harigae Y, Takimoto H, Tohi S, Hata S, Kondo J, Ohta T, Naito K, Nagase T, Kimura H and Okada M, 2000a. Repeated dose toxicity study of caramel I by 28-day oral administration in rats. Nippon Shokuhin Kagaku Gakkaishi 7, 35-40.

Harigae Y, Takimoto H, Tohia S, Hata S, Kondo J, Miwa Y and Nagasawa Y, 2000b. Mutagenicity studies of caramel I. Nippon Shokuhin Kagaku Gakkaishi 7, 28-34.

Hagiwara A, Shibata M, Kurata Y, Seki K, Furushima S and Ito N, 1983. Long-term toxicity and carcinogenicity test of ammonia-process caramel colouring given to B6C3F1 mice in the drinking water. Food and Chemical Toxicology 21, 701-706 (as referred to by JECFA, 1987).

Heidt M and Rao GN, 1980. 90-day subacute toxicity study of caramel color (sulfite ammonia process) type SAC2 in rats. Unpublished report No. 79028 from Raltech Scientific Services, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association.

Heidt M and Rao GN, 1981. Subchronic toxicity study of Ammonia Caramel color type AC2 in rats. Unpublished report No. 80036 from Raltech Scientific Services, Inc., Madison, WI, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Heyns K, 1971. Unpublished report of Technical Caramel Committee (as referred to by JECFA, 1977).

Hla T, 2004. Physiological and pathological actions of sphingosine-1-phosphate. Semin. Cell Dev. Biol. 15(5), 513-520.

Hla T, 2005. Dietary factors and immunological consequences. Science 309, 1682-1683.

Houben GF, 1992. Vitamin B, status dependent immunomodulation by caramel III. ISBN 90-393-0181-6. University of Utrecht, Utrecht.

Houben GF, van den Berg H, Kuijpers MH, Lam BW, van Loveren H, Seinen W and Penninks AH, 1992a. Effects of the color additive caramel color III and 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) on the immune system of rats. Toxicology and Applied Pharmacology 113(1), 43-54.

Houben GF, van Dokkum W, van Loveren H, Penninks AH, Seinen W, Spanhaak S and Ockhnizen T, 1992b. Effects of caramel colour III on the number of blood lymphocytes: A human study on caramel colour III immunotoxicity and a comparison of the results with data from rat studies. Food and Chemical Toxicology 30**,** 427-430.

Houben GF, Abma PMH, van den Berg H, van Dokkum W, van Loveren H, Penninks AH, Seinen W, Spanhaak S, Voss JG and Ockhnizen T, 1992c. Effects of the colour additive caramel colour III on the human system: A study with human volunteers. Food and Chemical Toxicology 30, 749-758 (as referred to by SCF, 1997).

Houben GF, Penninks AH, Seinen W, Vos JG and van Loveren H, 1993. lmmunotoxic effects of the color additive Caramel Color III and 2-acetyl-4(5)-tetrahydroxybutyl-imidazole (THI); immune function studies in rats. Fundamental and Applied Toxicology 20, 30.

Houben GF and Penninks AH, 1994. Immunotoxicity of the colour additive Caramel Colour III: a review ofn complicated issues in the safety evaluation of a food additive. Toxicology 91, 289-302.

Huybrechts I, Sioena I, Boon PE, De Neve M, Amiano P, Arganini C, Bower E, Busk L, Christensen T, Hilbig A, Hirvonen T, Kafatos A, Koulouridaki S, Lafay H, Liukkonen K-H, Papoutsou S, Lourdes R-B, Ruprich J, Rehurkova I, Mathilde K, Serra-Majem L, Turrini A, Verger E, Westerlund A, Tornaritis M, vanKlaverenb JD and De Henauw S, 2010. Long-term dietary exposure to different food colours in young children living in different European countries. Scientific report submitted to EFSA. EFSA-Q-2010-00787. Available from: http://www.efsa.europa.eu/en/scdocs/doc/53e.pdf

Iscaro A, Mackay IR and O'Brien C, 1988. Lymphopenic effects on mice of a component of Ammonia Caramel, 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI). Immunology and Cell Biology 6 (Pt 5-6), 395-402.

Ishidate M Jr and Yoshikawa K, 1980. Chromosome aberration tests with Chinese hamster cells in vitro with and without metabolic activation - a comparative study on mutagens and carcinogens. Archives of Toxicology Supplements 4, 41-44 (as referred to by JECFA, 1987).

Ishidate M Jr, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M and Matsuoka A, 1984. Primary mutagenicity screening of food additives currently used in Japan. Food and Chemical Toxicology 22, 623-636.

ITCA, 1990. Caramel Colour II. Toxicological and chemical properties. Monograph prepared by the International caramel Association (ITCA, Washington DC, August 1990) and submitted to the European Commission by the European Technical Caramel Association, 3 September 1990) (document not available to the Panel).

Jagannath DR and Brusick D, 1978a. Mutagenicity evaluation of ETA 4-10, ETA 4-11, ETA 4-15 in the Ames Salmonella/microsome plate test. Unpublished report No. 20838 from Litton Bionetics Inc., Kensington, MD, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Jagannath DR and Brusick D, 1978b. Mutagenicity evaluation of ETA 4-8, ETA 4-9, ETA 4-12, ETA 4-13, ETA 4-14 in the Ames Salmonella/microsome plate test. Unpublished report No. 20838 from Litton Bionetics Inc., Kensington, MD, USA. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1970. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers,

stabilizers, anticaking agents, and certain other substances. 13th Report of JECFA. WHO Technical Report Series, No. 445, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1972a. Evaluation of food additives: some enzymes,, modified starches, and certain other substances: toxicological evaluations and specifications and a review of the technological evaluations and specifications and a review of the technological efficacy of some antioxidants. 14th Report of JECFA. WHO Technical Report Series, No. 488, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1972b. Toxicological evaluation of some enzymes, modified starches, and certain other substances. 15th Report of JECFA. WHO Food Additives Series, No 1.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1977. Summary of toxicological data of certain food additives. 21st Meeting of ~JECFA. WHO Food Additives Series, No 12. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1978. Evaluation of certain food additives. 21st report of JECFA. WHO Technical Report Series 617. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1980. Evaluation of certain food additives. 24th report of JECFA. WHO Technical Report Series, No. 653, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1986. Evaluation of certain food additives and contaminants. 29th report of JECFA. WHO Technical Report Series, No. 733, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1987. Toxicological evaluation of certain food additives and contaminants. 29th Meeting of JECFA. WHO Food Additives Series, No 20.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2001. Evaluation of certain food additives and contaminants. 55th report of JECFA. WHO Technical Report Series, No 901, 8-10.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2006. Combined compendium of food additive specifications. Monographs 1. Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-102.pdf

Jensen NJ, Willumsen D and Knudsen I, 1983. Mutagenic activity at different stages of an industrial Ammonia Caramel process detected in Salmonella typhimurium TA 100 following pre-incubation. Food and Chemical Toxicology 21, 527-530 (as referred to by JECFA, 1987; SCF, 1997).

Kamuf W, Nixon A, Parker O and Barnum GC, 2003. Overview of Caramel Colors. Cereal foods world, Vol. 42, No. 2, 64-69. Available at: http://www.aaccnet.org/cerealfoodsworld/pdfs/W03-0205-01F.pdf

Kawachi T, Yahagi T, Kada T, Tazima Y, Ishidate M, Sasaki M and Sugiyama T, 1980. Cooperative programme on short-term assays for carcinogenicity in Japan. In Molecular and Cellular Aspects of Carcinogen Screening Tests, IARC Scientific Publication No. 27, 323-330. International Agency for Research on Cancer, Lyon, France (as referred to by JECFA, 1987).

Kawana K, Akema R, Nakaoka T, Ikeda H, Takimoto T and Kawauchi S, 1980. Studies on mutagenicities of natural food additives. II. Mutagenicities and antibacterial activities of caramels. Eisei Kagaku 26, 259-263 (as referred to by JECFA, 1987).

Kay JH and Calandra JC, 1962a. Subacute oral toxicity of caramel colorings (coded Sample A) - albino rats. Unpublished report from Industrial Bio-test Laboratories, Inc., Northbrook, IL, USA.

Kay JH and Calandra JC, 1962b. Ninety-day subacute oral toxicity of caramel coloring (coded Sample B) - albino rats. Unpublished report from Industrial Bio-test Laboratories, Inc., Northbrook, IL, USA.

Kay JH and Calandra JC, 1962c. Subacute oral toxicity of caramel coloring (coded Sample A) - dogs. Unpublished report from Industrial Bio-test Laboratories, Inc., Northbrook, IL, USA.

Kitts DD, wu CH, Kopec A and Nagasawa T, 2006. Chemistry and genotoxicity of caramelized sucrose. Mol Nutr. Food Res 50, 1180-1190.

Komoto M, 1962. Journal of Agricultural and Food Chemistry 36, 305 (as referred to by JECFA, 1977).

Kroplien U, 1984. Letter dated 17 December 1984 to International Technical Caramel Association, submitted to WHO (as referred to by JECFA, 1987).

Kroplien U, Rosdorfer J, van der Greef J, Long RC Jr and Goldstein JH, 1985. 2-Acetyl-4(5)-(tetrahydroxybutyl)-imidazole: Detection in commercial caramel colour III and preparation by a model browning reaction. The Journal of Organic Chemistry 50, 1131-1133 (as referred to by JECFA, 1987).

Licht BH, Shaw K, Smith C, Mendoza M, Orr J and Myers DV, 1992a. Development of specifications for caramel colours. Food and Chemical Toxicology 30(5), 383-387.

Licht BH, Shaw K, Smith C, Mendoza M, Orr J and Myers DV, 1992b. Characterization of caramel colours I, II and III. Food and Chemical Toxicology 30(5), 375-382.

Licht BH, Shaw K, Smith C, Mendoza M, Orr J and Myers DV, 1992c. Characterization of caramel colour IV. Food and Chemical Toxicology 30(5), 365-373.

MacKenzie KM, 1985a. 90-day toxicity study of caramel color (caustic sulfite process) in rats. Study No. 6154-105. Volumes I and II. Hazleton Laboratories America Inc., Madison, WI, USA. 11 January 1985. Unpublished report. Submitted to WHO by International Technical Caramel Association.

MacKenzie KM, 1985b. 90-day toxicity study of caramel color (ammonia process) in rats. Study No. 6154-107. Volumes I and II. Hazleton Laboratories America, Inc., Madison, WI, USA. 11 January 1985. Unpublished report. Submitted to WHO by International Technical Caramel Association.

MacKenzie KM, 1985c. Carcinogenicity and chronic toxicity study of caramel color (sulfite ammonia process) type SAC2 in rats (Chronic toxicity portion, Volumes I-V; Carcinogenicity portion, Volumes I-VIII). Study No. 81184. Hazleton Laboratories America, Inc., Madison, WI, USA. 11 January 1985. Unpublished report. Submitted to WHO by International Technical Caramel Association.

MacKenzie KM, 1985d. Carcinogenicity study of caramel color (sulfite ammonia process) type SAC2 in mice. Volumes I-VI. Study No. 81185. Hazleton Laboratories America, Inc., Madison, WI, USA. 14 January 1985. Unpublished report. Submitted to WHO by International Technical Caramel Association.

MacKenzie KM, Carter JL, Petsel SR, Chappel CI, Emerson JL and Stanley J, 1992a. Subchronic toxicity study of Caramel Colour II in F344 rats. Food and Chemical Toxicology 30(5), 411-6.

MacKenzie KM, Boysen BG, Field WE, Petsel SRW, Chappel CI, Emerson JL and Stanley J, 1992b. Toxicity studies of caramel colour III and 2-acetyl-4(5)-tetrahydroxybutyl imidazole in F344 rats. Food and Chemical Toxicology 30, 417-425.

MacKenzie KM, Boysen BG, Field WE, Petsel SR, Chappel CI, Emerson JL and Stanley J, 1992c. Toxicity and carcinogenicity studies of Caramel Colour IV in F344 rats and B6C3F1`mice. Food and Chemical Toxicology 30(5), 431-443.

Maekawa A, Ogiu T, Matsuoka C, Onodera H, Furuta K, Tanigawa H, Hayashi Y and Odashima S, 1983. Carcinogenicity study of ammonia-process caramel in F344 rats. Food and Chemical Toxicology 21, 237-244 (as referred to by JECFA, 1987).

Mandel TE, Koulmanda M and Mackay IR, 1992. Prevention of spontaneous and cyclophosphamide-induced diabetes in non-obese diabetic (NOD) mice with oral 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel colouring III. Clinical and Experimental Immunology 88(3), 414-9.

Marier G, Berry G, and Orr JM, 1977a. Tolerance study of single strength ammonia sulfite caramel in human volunteers. Unpublished project No. 5612, report No. 2, from Bio- Research Laboratories Ltd., Pointe Claire, Quebec, Canada.

Marier G, Berry G, and Orr JM, 1977b. Tolerance study of double strength ammonia sulfite caramel in human volunteers. Unpublished project No. 5711, report No. 2, from Bio-Research Laboratories Ltd., Pointe Claire, Quebec, Canada.

Morgan SE and Edwards WC, 1986a. Bovine bonkers: new terminology for an old problem. A review of toxicity associated with ammoniated feeds. Vet Hum Toxicol 28, 240–242.

Morgareidge K, 1974a. Teratologic evaluation of FDA 71-82 (caramel, bakers and confectioners) in mice, rats and rabbits. Unpublished report No. PB 234-870 from Food and Drug Research Laboratories Inc., Waverly, NY; USA (as referred to by JECFA, 1987).

Morgareidge K, 1974b. Teratologic evaluation of FDA 71-83 (caramel beverage) in mice, rats and rabbits. Unpublished report No. PB 234-867 from Food and Drug Research Laboratories Inc., Waverly, NY; USA.

Motoi Y, Yoshioka M, Hirose H, Ishino S, Nakajima Y, Miyazaki S, Miyamoto S, Sudou M, Monda T and Murai M, 1997. Central nervous disorder of calves consuming colostrums containing 4 methylimidazole or colostrums from cows fed excess ammoniated hay. Japan Agricultural Research Quarterly 31, 225-231.

Murkovic M and Pichler N, 2006. Analysis of 5-hydroxymethylfurfural in coffee, dried fruits and urine. Mol Nutr. Food Res 50, 842-846.

Myers DV and Howell JC, 1992. Characterization and specifications of caramel colours: an overview. Food Chemistry Toxicology 30, 359-363.

Nees PO, 1964. Toxicological feeding study of caramel E. Unpublished report from Wisconsin Alumni Research Foundation, Madison, WI, USA.

Nishie K, Waiss AC and Keyl AC, 1969. Toxicity of methylimidazoles. Toxicology and Applied Pharmacology 14, 301-307 (as referred to by JECFA, 1987).

Nishie K, Waiss AC and Keyl AC, 1970. Pharmacology of alkyl and hydroxyalklpyrazines. Toxicology and Applied Pharmacology 17, 1 (as referred to by JECFA, 1987).

NTP, 2004. National Toxicology Program Toxicity Report Series Number 67 NTP Technical Report on the Toxicity Studies of 2- and 4-Methylimidazole (CAS No. 693-98-1 and 822-36-6) Administered in Feed to F344/N Rats and B6C3F1 Mice April 2004 NIH Publication No. 04-4409 U.S. Department of Health and Human Services Public Health Service National Institutes of Health

NTP (National Toxicology Program), 2007. NTP Technical Report 535 on the Toxicology and Carcinogenesis Studies of 4-Methylimidazole (CAS No. 822-36-6) in F344/N Rats and B6C3F1 Mice. NIH Publication No. 07-4471. U.S. Department of Health and Human Services.

NTP, 2008. National Toxicology Program. Toxicology and carcinogenesis studies of 5-(hydroxymethyl)-2-furfural (CAS No. 67-47-0) in F344/N rats and B6C3F1 mice (gavage studies) (Draft). National Toxicology Program, Research Triangle, NC, USA. TR-554. NIH Publication No. 08-5895. [Online] http://ntp.niehs.nih.gov/.

O'Brien J, Nursten HE, James M and Crabbe MJC, 1998. The Maillard reaction in foods and medicine. Published by the Royal Society of Chemistry, Cambridge, UK. ISBN 0-85404-733-6.

OEHHA, 2011. OEHHA Proposition 65 - Request for Relevant information - Dibromoacetic acid, 4-Methylimidazole. http://www.oehha.ca.gov/prop65/prop65_list/010711list.html

Oser BL, 1963. Toxicological feeding study of "acid-proof" caramel. Unpublished report No. 83911 from Food and Drug Research Laboratories, Inc., Waverly, NY, USA

Phillips JA and Paine AJ, 1990. Effect of pyridoxine on the disposition and lymphopenic effects of 2-acetyl-4(5)-tetrahydroxybutyl imidazole in the rat. Xenobiotica 20(6), 555-62.

Patey AL, Shearer G, Knowles ME and Denner WHB, 1985. Ammonia caramels: specifications and analysis. Food Additives and Contaminants 2 (2), 107-112.

Patey AL, Startin JR, Rowbottom PM and Shearer G, 1987. Identification of substituted hydroxypyridines and hydroxypyrazines in caramel food colourings. Food Additives and Contaminants 4, 9-15.

Prier RF, 1960. The toxicity of double strength acid proof caramel in rats - 12 week feeding test. Unpublished report No. 9070599 from Wisconsin Alumni Research Foundation, Madison, WI, USA.

Procter BG, 1976. A preliminary evaluation of the potential toxicological effects of Ammonia Caramel in mice. Unpublished report No. 5705 from Bio-Research Laboratories Ltd., Pointe Claire, Quebec, Canada (as referred to by JECFA, 1987).

Procter BG, Berry G and Chappel CI, 1976. A toxicological evaluation of various caramels fed to albino rats. Unpublished report No. 4244 from Bio-Research Laboratories Ltd., Pointe Claire, Quebec, Canada (as referred to by JECFA, 1987).

Procter BG, 1977. A preliminary evaluation of the potential toxicological effects of Ammonia Caramel (U.S. origin) in rats. Unpublished report No. 5617 from Bio-Research Laboratories Ltd., Pointe Claire, Quebec, Canada. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Ray AC, Raisor MJ, Herd DB, Murphy MJ and Reagor JC, 1984. Methylimidazole content of ammoniated forages associated with toxicity in cattle. In American Association of Veterinary Laboratory Diagnosticians 27th Annual Proceedings, pp. 337-348.

Richold M and Jones E, 1980a. Ames metabolic activation test to assess the potential mutagenic effect of ETA-38-1W. Unpublished report No. FDC 8/80361 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association.

Richold M and Jones E, 1980b. Ames metabolic activation test to assess the potential mutagenic effect of ETA-38-2H. Unpublished report No. FDC 8/80359 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association

Richold M and Jones E, 1980c. Ames metabolic activation test to assess the potential mutagenic effect of ETA-38-3N. Unpublished report No. FDC 8/80356 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association.

Richold M and Jones E, 1980d. Ames metabolic activation test to assess the potential mutagenic effect of ETA-48-1H. Unpublished report No. FDC 8/80358 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Richold M, Jones E and Fenner LA, 1984a. Ames metabolic activation test to assess the potential mutagenic effect of caramel colour II. Unpublished report No. ITC 1B/84709.

Richold M, Jones E and Fenner LA, 1984b. Ames metabolic activation test to assess the potential mutagenic effect of caramel colour III. Unpublished report No. ITC 1B/84709.

Rivera J, Prola RL, Olivera A, 2008. The alliance of sphingosine-1-phosphate and its receptos in immunity. Nat. Rev. Immunol. 8(10), 753-763.

Royle L and Radcliffe CM, 1999. Analysis of caramels by capillary electrophoresis and ultrafiltration. Journal of the Science of Food and Agriculture 79, 1709-1714.

Royle L, Ames JM, Castle L and Nursten HE, 2002. Analysis of malts by capillary electrophoresis. Journal of the Science of Food and Agriculture 82, 443-451.

Sádecká J and Tóthová J, 2010. Determination of caramel in non-aged mixed wine spirits by synchronous fluorecence spectroscopy. European Food and Research Technology , 230;797-802.

SCF (Scientific Committee on Food), 1975. Reports of the Scientific Committee for Food, 1st series. Opinion expressed 31 December 1975, 17-20.

SCF (Scientific Committee on Food), 1984. Reports of the Scientific Committee for Food,14th series. EUR 8752. Luxembourg: Office for Official Publications of the European Communities, 1983, ISBN 92-825-3893-1. Opinion expressed 1983, 56-57.

SCF (Scientific Committee on Food), 1989. Reports of the Scientific Committee for Food, 21st series. EUR 11617. Luxembourg: Office for Official Publications of the European Communities, 1998. ISBN 92-826-0823-9. Opinion expressed 1987, 3-8.

SCF (Scientific Committee on Food), 1997. Reports of the Scientific Committee for Food, 36st series. Opinion of the SCF on Causitic sulphite caramel and Ammonia caramel. Luxembourg: Office for Official Publications of the European Communities, 1997, ISBN 92-827-9580-2. 1-8.

Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y and Cyster JG, 2005. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science 309(5741), 1682-3.

Selim S, Chappel CI and Schoenig GP, 1992. Absorption, distribution and excretion of the colour fraction of Caramel Colour IV in the rat. Food and Chemical Toxicology 1992 May, 30(5), 445-51.

Sharratt M, 1971. Unpublished report (as referred to by JECFA, 1987).

Sinkeldam EJ and van der Heyden CA, 1975. Short-term feeding study with caramel SS 202 in albino rats. Unpublished report No. R 4777 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands.

Sinkeldam EJ, Willems MI and van der Heyden CA, 1975. One year feeding study in rats with six different Ammonia Caramels. Unpublished report No. R 4767 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands.

Sinkeldam EJ and van der Heyden CA, 1976a. Short-term feeding test with three types of caramels in albino rats. Unpublished report No. R4789 from CIVO/TNO, Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association.

Sinkeldam EJ and van der Heyden CA, 1976b. Short-term (10 week) feeding study in rats with three different Ammonia Caramels. Unpublished report No. R 5120 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands (as referred to by JECFA, 1987).

Sinkeldam EJ, van der Heyden CA and Beems RB, 1976. Chronic (two year) feeding study in rats with six different ammonia caramels. Unpublished report No. R 4961 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands.

Sinkeldam EJ, 1979. Paired feeding test in rats with caramel (AC3) in drinking water. Unpublished report No. R 6157 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, Bruyntjes JP and Kuper CF, 1980a. Sub-chronic (13-week) oral toxicity study with a modified Ammonia Caramel (ETA-26-1) in rats. Unpublished report No. R 6483 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, Roverts WG and Kuper CF, 1980b. Sub-chronic (13-week) oral toxicity study with Ammonia Caramel (AC2) in rats. Unpublished report No. R 6121 from Centraal Instituut voor

Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, 1981. Quantitative relationship between dietary pyridoxine content and lymphocyte counts in rats fed Ammonia Caramel. Unpublished report No. V81.390/211704 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, 1982a. Quantitive relationship between dietary pyridoxine content and lymphocyte counts in mature rats fed Ammonia Caramel. Unpublished report No. V82.102/220102 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, 1982b. Short-term (7 day) bioassay in rats with three dose levels of 2-acetyl-4(5)-tetrahydroxybutylimidazole (13-A-82). Unpublished report No. V82.291/221153 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands. Submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, de Groot AP, van den Berg H and Chappel CI, 1984. The effect of vitamin B6 on the number of lymphocytes in blood of rats fed caramel colour (ammonia process). Unpublished report submitted to WHO by International Technical Caramel Association (as referred to by JECFA, 1987).

Sinkeldam EJ, de Groot AP, van den Berg H and Chappel CI, 1988. The effect of pyridoxine on the number of lymphocytes in blood of rats fed Caramel Colour III. Food and Chemical Toxicology 26(3), 195-203.

TemaNord, 2002. Food additives in Europe 2000; Status of safety assessments of food additives presently permitted in the EU. TemaNord 2002, 560, 145-148.

Tennant, D, 2007. Screening potential intakes of caramel colours. Report provided for the European Technical Committee on Caramels (EUTECA).

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

Thuvander A and Oskarsson A, 1994. Effects of subchronic exposure to caramel colour III on the immune system in mice. Food and Chemical Toxicology 32, 7-13 (as referred to by TemaNord, 2002).

Tierney WJ, 1979. A four-week dose range-finding study of caramel colour no. 3, sample 3-1, in mice. Unpublished project No. 79-2380 from Bio/dynamics Inc., East Millstone, NJ, USA. Submitted to WHO by International Technical Caramel Association.

Tierney WJ, 1980. An in-utero range-finding study of caramel colour no. 3, sample 3-1, in rats. Unpublished project No. 79-2405 from Bio/dynamics Inc., East Millstone, NJ, USA. Submitted to WHO by International Technical Caramel Association.

Til HP and Spanjers M, 1973. Reproduction study in rats with six different Ammonia Caramels. Unpublished report No. R 4068 from Centraal Instituut voor Voedingsonderzoek (CIVO/TNO), Zeist, The Netherlands.

UNEP (United Nations Environment Programme), 2005. SIDS Initial Assessment Report for imidazole. OECD SIAM meeting 17, Italy, 2003. http: //www.chem.unep.ch/irptc/sids/OECDSIDS/288324.pdf

Vinggaard AM, Hass U, Dalgaard M, Andersen HR, Bonefeld-Jørgensen E, Christiansen S, Laier P and Poulsen M E, 2006. Prochloraz: an imidazole fungicide with multiple mechanisms of action. International Journal of Andrology, 29: 186–192.

Weiss WP, Conrad HR, Martin CM, Cross RF and Shockey WL, 1986. Etiology of ammoniated hay toxicosis. Journal of Animal Sciences 63, 525-532. Sugar Journal 18, 18-20.

Wiggins LF, 1956a. The ammoniation of molasses. Proceedings of the International Society of Sugar Chemistry Technology 2, 525-529.

Wiggins, L.F., 1956b. Some recent studies on ammoniated molasses. Sugar Journal 18, 18-20.

Yu YN, Chen XR, Ding C, Cai ZN and Li QG, 1984. Genotoxic activity of caramel on Salmonella and cultured mammalian cells. Mutation Research 139, 161-165 (as referred to by SCF, 1997).

Yu XQ, Kramer J, Moran L, O'Neill E, Nouraldeen A, Oravecz T, Wilson AGE (2010). Pharmacokinetic/pharmacodynamic modelling of 2-acetyl-4(5)-tetrahydroxybutyl imidazole-induced peripheral lymphocyte sequestration through increasing lymphoid sphingosine 1-phosphate. Xenobiotica 40(5), 350-356.

Yuan JH and Burka LT, 1995. Toxicokinetics of 4-methylimidazole in the male F344 rat. Xenobiotica 25(8), 885-94.

ANNEX A.

Rules defined by the Panel to deal with quantum satis (QS) authorisation, usage data or observed analytical data for all regulated food additives to be re-evaluated and procedures for estimating intakes using these rules

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

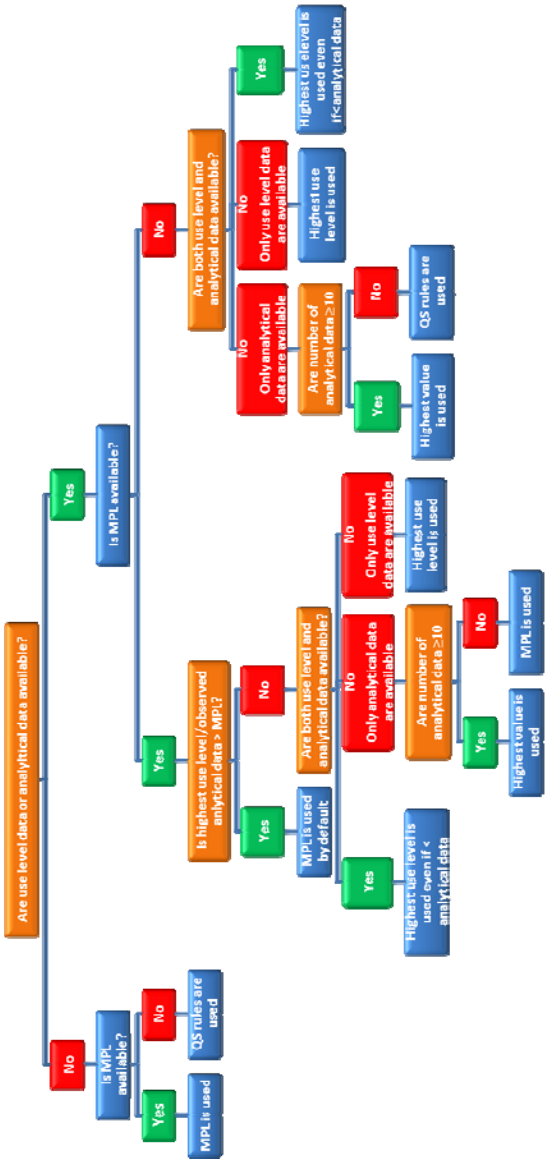
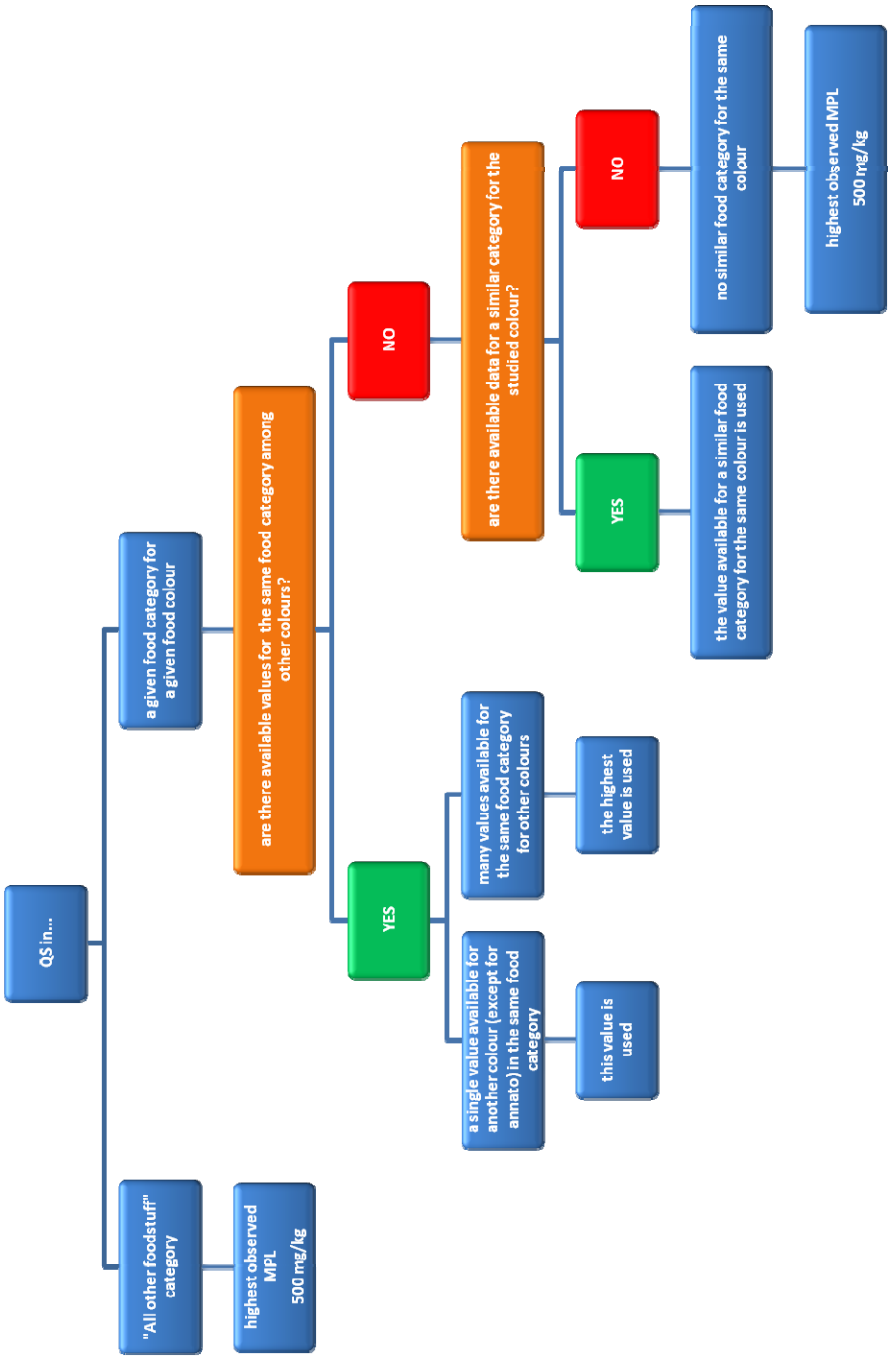


Figure 2: Rules defined by the Panel to deal with *quantum satis* (QS) authorisation.



ANNEX B

Summaries of short-term toxicity studies focussing on toxicity of Class III Ammonia Caramel including studies investigating the influence of 2-acetyl-4-tetrahydroxy-butylimidazole (THI) and pyridoxine on the toxic effects seen.

Four groups of rats (10 animals/sex) received either 0 or 10% of 2 different samples of Class III Ammonia Caramel in their diet (equivalent to 0 or 5 g/kg bw/day) for 90 days. (No information on the THI content of the sample or dietary pyridoxine (vitamin B_6) levels was provided). Weight gains were slightly reduced whereas food consumption was normal in all groups. No abnormalities were noted regarding haematology, urinalysis, gross pathology, or histopathology (Chacharonis, 1963).

Four groups of rats (no detail on group size or sex ratio) received 0, 4, 8, or 16% Class III Ammonia Caramel in their diet (equivalent to 0, 2, 4, or 8 g/kg bw/day) for 3 months. No convulsions or other behavioural abnormality or signs of neurological damage were seen. No macroscopic pathological abnormalities were found in the central nervous system (Sharratt, 1971).

Groups of Sprague-Dawley (CFE strain) rats (15 animals/sex; 25 animals/sex in the control group) were fed either "open" or "closed" pan process Class III Ammonia Caramel at dietary levels of 0, 4, 8, or 16% (equivalent to 0, 2, 4, or 8 g/kg bw/day) for 13 weeks (no information on the THI content of the sample or dietary pyridoxine levels is given). Both caramel colours decreased body-weight gain at all dietary levels. At necropsy, thymus and spleen weights were decreased at the 8 and 16% dose levels. At the same dose levels the caecal weights were increased. Relative liver and kidney weights were also increased suggesting an effect on these organs. Histopathological examination did not reveal treatment-related changes. Numbers of leucocytes and lymphocytes were significantly decreased at all dose levels after 6 and 10 weeks; after 13 weeks these changes were observed only in males. Haemoglobin concentrations were reduced at the highest dietary levels after 6 weeks, while at the lower levels this effect was less clear. At week 13, significantly decreased haemoglobin concentrations were noted at all dose levels in males but only at the 8 and 16% levels in females. In some groups there was a (less consistent) decrease in the total number of red blood cells after 6 and 13 weeks (Gaunt et al., 1977).

Female Wistar rats (20/group) were fed diets containing 0, 15, or 30% of Class III Ammonia Caramel (equivalent to 0, 7.5 or 15 g/kg bw/day) for 8 weeks followed by a 4-week recovery period (no information on the THI content of the sample or dietary pyridoxine levels was given). The colour caused dose-related decreases in body weights and food efficiency. The relative weights of the caecae of animals receiving Class III Ammonia Caramel, both filled and empty, were increased at 4 and 8 weeks but reverted to normal after the 4-week recovery period. Diarrhoea was observed at the 30% dietary level. Gross examination at autopsy after 8 weeks revealed a slight, dose-related brown discolouration of the mesenteric lymph nodes in a few animals of each test group. After recovery periods of 2 or 4 weeks the discolouration was less intensive, but still visible. Microscopically, the lymph nodes of the test rats showed accumulation of pigment-laden macrophages, which was not noticeably diminished after a 2 or 4 weeks recovery period. White blood cell counts were significantly increased at the highest dose level after 4 weeks but not after 8, 10, and 12 weeks (Sinkeldam and van der Heyden, 1976a).

Groups of Wistar rats (10 animals/sex/group) received diets containing Class III Ammonia Caramel at concentrations of 0, 1.25, 2.5, 5.0, 10.0, or 15.0% (equivalent to approximately 0, 0.6, 1.3, 2.5, 5, or 7.5 g/kg bw/day) for 10 weeks (no information on the THI content of the sample or dietary pyridoxine levels was provided). Class III Ammonia Caramel caused slight decreases in body weights at the 5% dietary level. Loose stools were observed, particularly at the 5% dietary level. The relative weight of the caecum (both full and empty) was increased by feeding Class III Ammonia Caramel. Minimal amounts of pigment were observed in mesenteric lymph nodes of several rats at dose levels of 1.25% and higher. White blood cell counts and lymphocyte counts were decreased in both sexes at a dose

level of 15%; at lower dose levels this effect occurred only in females (Sinkeldam and van der Heyden, 1976b).

Groups of Sprague-Dawley rats (15 animals/sex/group; 20 animals/sex in the control group) were fed diets containing 0, 10 or 15% Class III Ammonia Caramel (equivalent to 0, 5 or 7.5 g/kg bw/day) for 4 weeks (no information on the THI content of the sample or dietary pyridoxine levels was provided). There were no consistent differences in body-weight gain or food consumption and no mortality occurred. During the course of the study rats receiving the food colour had soft dark-coloured faeces, particularly at the highest dietary level. At necropsy, caecal weights were increased in both sexes. In addition, males showed a statistically significant increase in relative thymus weights. Histopathological studies were not conducted. Haematological studies were conducted prior to feeding the colour and after 2 and 4 weeks. At both dose levels a significant depression of differential lymphocytes (expressed as %), and a concomitant increase in segmented neutrophils were observed at both intervals in males and after 4 weeks in females. After 4 weeks female rats also displayed a significant depression of total white cell and lymphocyte counts at both dose levels (Procter et al., 1976).

Groups of Sprague-Dawley rats (15 animals/sex/group) were fed diets containing Class III Ammonia Caramel at concentrations of 0, 1.25, 2.5, 5, 10, or 15% (equivalent to approximately 0, 0.6, 1.3, 2.5, 5, or 7.5 g/kg bw/day) for 10 weeks (no information on the THI content of the sample or dietary pyridoxine levels was provided). Animals fed Class III Ammonia Caramel generally displayed reduced body-weight gains, particularly during the last 2-4 weeks of the study. Faeces became soft and contained increased water content at levels of 5% or higher. An increase in empty caecal weight was consistently evident at dose levels of 5, 10, and 15%. Histopathological examination did not reveal changes in the structure of the ileal or caecal mucosa nor in the reticuloendothelial components of the central or peripheral systems. No macroscopic or histopathological evidence of abnormal pigmentation of the mesenteric lymph nodes was found. Lymphocyte counts were significantly reduced whereas numbers of segmented neutrophils were increased at all dose levels. No changes were observed in haemoglobin levels or erythrocyte counts (Procter et al., 1977).

Six groups of weanling Wistar rats (15 animals/sex in the 0-4%-groups; 10 animals/sex in the 16%-group; 60 animals/sex in the control group) were fed Class III Ammonia Caramel in the diet at levels of 0, 0.5, 1, 2, 4, or 16% (equivalent to 0, 0.25, 0.5, 1, 2, or 8 g/kg bw/day) for 10 weeks. Another 2 groups of 10 rats received a diet containing 0 or 16% of the colour for 10 weeks followed by a 28-day recovery period (no information on the THI content of the sample or dietary pyridoxine levels was provided). Class III Ammonia Caramel depressed body-weight gain at dietary levels greater than 1%. Spleen weights were decreased and caecal weights were increased and at the highest dose level; the relative weights of the liver and kidney were increased at dietary levels of 2% and higher. All organ weight deviations returned to normal during the recovery phase, except for the kidney weights which only partially recovered. Microscopically, pigment was observed in the mesenteric lymph nodes of male and female rats at this dose level. Total white blood cell counts were decreased in the 2, 4, and 16% groups in males and the 4 and 16% groups in females. However, the lymphocyte/neutrophil ratio was significantly decreased in both sexes at all dose levels. In both sexes white cell counts, cell ratios, and total numbers of lymphocytes had returned to normal within 7 days of recovery (BIBRA, 1977).

Weanling Wistar rats (10 animals/sex/group) received drinking water containing concentrations of 0, 4, 6, 8, or 10% Class III Ammonia Caramel (equivalent to 0, 2, 3, 4, or 5 g/kg bw/day) for 13 weeks (the caramel sample used contained 78 mg THI/kg on an 'as is' basis, or 105 mg THI/kg on a solids basis; the diet fed to the rats contained approximately 13 mg pyridoxine/kg). There was a dose-related decrease in fluid consumption, decreased urinary output of more concentrated urine, decreased food consumption, and decreased body-weight gain in both sexes. These changes were related to the palatability of the drinking fluid. Necropsy revealed an increase in the relative weight of the caecum and a dose-dependent increase in the relative weight of the kidneys in several groups receiving the colour (not specified). No treatment-related histopathological changes were seen in the caecum, kidney, or a range of additional organs examined. The enlargement of the kidneys was attributed to the

decreased fluid consumption. No effects on the relative weights or histology of spleen or thymus were observed. Statistically significant decreases in lymphocyte counts were observed in males in all treatment groups after 29/30 days, but only in the 4 and 8% groups after 57/58 days. In females decreases were statistically significant only in the 8% group after 29/30 days. For both sexes the decreases in lymphocyte counts were not clearly dose-dependent and after 13 weeks there were no significant differences among any of the treatment groups compared to controls. No outstanding differences were observed in red blood cell analyses between test groups and controls (Sinkeldam et al., 1980b).

Class III Ammonia Caramel was given to weanling Wistar rats (10 animals/sex/group) in the drinking water at concentrations of 0, 5, 10, 15, or 20% (equivalent to 2.5, 5, 7.5, or 10 g/kg bw/day) for 13 weeks. (The caramel sample contained 0-3 mg THI/kg on an 'as is' basis; the diet fed to the rats contained 13.5 mg pyridoxine/kg.). Food intake was generally lower in all treated groups. Body-weights were decreased in a dose-related manner only in males. Treated animals of both sexes excreted less and more concentrated urine which was attributed to a dose-related decrease in water intake. The urine was darker-coloured at the 15 and 20% dose levels although urine composition was essentially normal. Although the relative weights of the caecum, liver, brain, kidneys, and testes were increased in several test groups (not specified), there were no pathological changes in any of these organs. Microscopic examination revealed a treatment-related increase in numbers of macrophages containing a yellow-brown pigment in the mesenteric lymph nodes. Lymphocyte counts of both sexes were in general relatively low in all test groups but only statistically significant in males of the 20%-group after 30 days and males of all treatment groups after 57 days. No significant differences in lymphocyte counts were observed in males of any group after 80 days, nor in females at any dose level at any time interval. Mean neutrophil counts were significantly increased in females receiving 20% Class III Ammonia Caramel for 81 days (Sinkeldam et al., 1980a).

Groups of weanling F344 rats (10 animals/sex/group) were given Class III Ammonia Caramel in the drinking water at concentrations of 0, 0.5, 1, 2, 4, or 8% (equivalent to 0, 0.25, 0.5, 1, 2, or 4 g/kg bw/day) for 4 weeks (the sample of Class III Ammonia Caramel used contained 70 mg THI/kg on an 'as is' basis; the diet used in this study contained 17 mg pyridoxine/kg.) No differences in body-weight gain were noted for any of the test groups, although food consumption of the males was significantly lower than controls throughout the study. At necropsy no differences in organ weights were noted and there were no gross or microscopic pathological changes related to treatment. As decreases in lymphocyte counts were noted among the treatment groups at the midpoint of the study but not at 1 month, these findings were considered transient. Other haematological and clinical chemistry parameters were normal for all groups (Heidt and Rao, 1981).

In order to determine appropriate dose levels for a chronic toxicity study, groups of F344 rats (10 animals/sex/group) were given 20 ml per day of solutions containing Class III Ammonia Caramel at concentrations of 0, 1.25, 2.5, 5, 10, or 20% (equivalent to 0, 0.5, 1, 2, 4, or 8 g/kg bw/day) for 13 weeks (the Class III Ammonia Caramel used contained 78 mg THI/kg on an 'as is' basis; the diet (*ad libitum*) contained 11-12 mg pyridoxine/kg.) Weight gains were reduced in all exposed groups from week 1. At week 13 weight gains compared to controls were 89*, 94, 84*, 76*, and 76%* for males, and 96, 98, 80*, 84*, and 92% for females, according to rising doses (asterisks denotate significance). At necropsy, no pronounced macroscopic changes were observed in any animals, although a few rats in the experimental groups were very emaciated. No histological changes related to administration of the colour were found in any experimental groups. No haematological changes were observed during or at the end of the experimental period (Maekawa et al., 1983).

Groups of mice (10 animals/sex) were fed Class III Ammonia Caramel in the diet at levels of 0, 1, 2, 4, 8, or 16% (equivalent to approximately 0, 1.4, 2.9, 5.7, 11.4, or 22.9 g/kg bw/day) for 4- to 6-weeks (the caramel sample contained 830 mg 4-MEI/kg); no information on the THI content of the sample or dietary pyridoxine levels are given.) In the highest dose group growth was decreased (especially in the third and fourth weeks) although no influence on food intake was observed. The faeces of the animals fed the higher dose levels were soft, tarry in appearance, poorly-formed, and sticky or pasty in

consistency. The mean relative weights of the caecae (full and empty) were increased at the 4, 8, and 16% dietary levels (no sex distinction). In the males fed 16% Class III Ammonia Caramel, an increase of neutrophilic leucocytes and a decrease in lymphocytes were observed. No other remarkable findings were observed on appearance, behaviour, or gross examination; histopathological examinations were not conducted (Procter, 1976).

In a study in female Balb/c mice, the animals received a diet containing 0, 2 or 10% (equivalent to approximately 0, 3.3, or 16.7 g/kg bw/day) of a commercial Class III Ammonia Caramel preparation in the drinking water for 9 weeks (the sample of Class III Ammonia Caramel used contained < 25 mg/kg THI; the diet contained relatively high vitamin B_6 content (not specified)). Although a treatment related lymphocytopenia was not evident, flow cytometric analysis demonstrated reductions in the $CD4^+$ and $CD8^+$ lymphocyte subpopulations in exposed mice. In addition, the proliferative response of spleen cells to B and T cell mitogens was significantly reduced in the mice exposed to 2% Class III Ammonia Caramel. No changes were observed in natural killer cell activity or in the humoral antibody response to a viral antigen (Thuvander and Oskarsson, 1994).

To investigate the effect of pyridoxine on lymphocyte counts, two groups of male weanling rats (10 per group) received a Spratt's diet containing 0 (basal diet; containing 2.3 mg pyridoxine/kg) or 8% Class III Ammonia Caramel (equivalent to 4 g/kg bw/day) for 12 days. A third group received a diet containing 8% Class III Ammonia Caramel supplemented with 10 mg pyridoxine/kg hydrochloride. After 12 days, rats receiving Spratt's diet with 8% Class III Ammonia Caramel displayed a marked reduction in total white blood cells and lymphocytes and an increase in neutrophil counts. Rats receiving the caramel diet supplemented with pyridoxine had white blood cells and lymphocytes in numbers similar to those fed the basal diet (Sinkeldam, 1981).

In a further study rats received a CIVO (Zeiss) stock diet with 0 (basal diet, containing 3 mg pyridoxine/kg) or 8% Class III Ammonia Caramel (no detail on duration). The colour induced a reduction in total white blood cells and lymphocyte numbers which were ameliorated by the addition of 10 mg pyridoxine/kg in the diet. In this study, neutrophils were not affected by the Class III Ammonia Caramel treatment, but the plasma pyridoxal phosphate levels were reduced by treatment (Sinkeldam et al., 1984).

In another study by Sinkeldam (1982a), groups of weanling and mature Wistar rats (10 males per group) were fed diets containing approximately 0, 2.5, 6, 12, or 24 mg/kg of pyridoxine (equivalent to 0, 0.13, 0.3, 0.6, or 1.2 mg/kg bw/day) for 14-days. At each dietary level, groups were given Class III Ammonia Caramel in the drinking water at levels of 0, 1, 4, or 8%. equivalent to 0, 0.5, 2, or 4 g/kg bw/day (the caramel sample contained 107 mg THI/kg on a colour equivalent basis, 204 mg THI/kg on an 'as is' basis, and 295 mg THI/kg on a solids basis). In Table 12 the effects on lymphocytes in weanling and mature rats are summarized:

Table 12. Lymphocyte reduction in weanling and mature rats after combined administration of pyridoxine and Class III Ammonia Caramel.

	Weanling rats								Mature rats							
	Day 6				Day 13				Day 6				Day 13			
pyr. / THI	2.5	6	12	24	2.5	6	12	24	2.5	6	12	24	2.5	6	12	24
1	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
4	+	-	-	-	+	+	-	-	+	+	+	-	+	-	-	-
8	+	-	-	-	+	+	+	-	+	+	+	-	+	-	-	-

pyr. = pyridoxine
\+ = statistically significant lymphocyte reduction
\- = no (statistically significant) lymphocyte reduction

Based on these findings, Sinkeldam concluded that a clear inverse dose relationship exists between the severity of lymphocyte depression and the pyridoxine level of the diet.

Sinkeldam and co-workers also examined the effect of vitamin E, folic acid, pyridoxine and choline on the reduction in circulating lymphocytes in the blood of rats administered Class III Ammonia Caramel in drinking water and maintained on normal or pyridoxine-deficient diets (Sinkeldam et al., 1988). The authors found that the reduction in the number of circulating lymphocytes in rats fed Class III Ammonia Caramel could be prevented by the addition of pyridoxine to the diet. The activity of Class III Ammonia Caramel in reducing the number of circulating lymphocytes also closely resembled that of the pyridoxine antagonist 4'-deoxypyridoxine. After the isolation and identification of THI, comparative studies indicated that THI was the constituent of Class III Ammonia Caramel responsible for reducing the number of circulating lymphocytes. Although the effect of Class III Ammonia Caramel was reduced or prevented by increasing dietary pyridoxine, the lymphocyte reduction associated with the administration of THI was not materially affected by the dietary level of pyridoxine. The authors demonstrated significant reductions in lymphocytes and total leucocytes at a dose level of 1% Class III Ammonia Caramel in drinking water, equivalent to 1 g caramel/kg bw/day and estimated to be equivalent to 200 µg THI/ kg bw/day. Similar statistically significant reductions in lymphocytes were observed when the rats were given 200 µg, 500 µg or 2 mg THI/kg bw/day alone in drinking water. The authors reported decreases in lymphocyte counts in rats receiving as little as 0.1% Class III Ammonia Caramel in drinking water for 1 week and maintained on a low-pyridoxine diet (2-3 mg/kg). Class III Ammonia Caramel intake at this level in drinking water was 0.1 g/kg bw/day, equivalent to 20 µg THI/ kg bw/day. Statistical analysis was not reported for this aspect of the study, but evaluation of the data provided would indicate that while lymphocytopenia was evident at this dose level, the reduction was unlikely to be statistically significant.

Houben et al.(1992b) found that effects induced by Class III Ammonia Caramel and/or THI were less pronounced or absent in rats fed a diet high in pyridoxine. Furthermore, THI did not induce obvious effects on vitamin B_6 status. Similar effects of THI and Class III Ammonia Caramel as those observed in rats were observed in mice on a basic diet with 2-3 mg/kg vitamin B_6 (pyridoxine) (equivalent to 0.3 to 0.45 mg/kg bw/day) (no further details) (Houben et al., 1992b).

ANNEX C.

Summaries of toxicity studies focussing on toxicity of Class IV Caramel (Sulphite Ammonia Caramel) not included in the main body of the opinion

Rats (5/group) received either 10 or 20% Class IV Sulphite Ammonia Caramel solution (equivalent to about 10 or 20 g/kg bw/day) as their sole source of fluid for up to 127 days. Only dark faeces and very mild diarrhoea were noted. No adverse effects were noted regarding general health, body weight, food and fluid consumption, haematology, gross pathology, or histopathology (Haldi and Wynn, 1951).

Weanling rats (5 animals/sex/group) received 0 or 10% Class IV Sulphite Ammonia Caramel solution as their sole fluid source for 100, 200, or 300 days. No adverse effects were noted regarding growth, food and fluid intake, haematology, gross pathology, or histopathology (Haldi and Wynn, 1951).

Rats (20 animals/sex/group) received either 0 or 11-14 g/kg bw of Class IV Sulphite Ammonia Caramel solutions for 100 days. Growth and food intake did not differ significantly between test and control animals. Gross pathology and histopathology showed no abnormal findings related to administration of the test compound (Haldi and Wynn, 1958).

Rats (5 animals/sex/group) were given 1 ml/kg bw/day of concentrated caramel colour (unspecified) for 21 days. Some diarrhoea was induced in all animals, but no other abnormalities were noted. Gross pathology and histopathology revealed no significant changes due to administration of the test compound (Foote et al., 1958).

Sprague-Dawley rats (10 animals/sex/group) received 0, 0.1, 1.0 or 10% Class IV Sulphite Ammonia Caramel in their diet for 12 weeks. No adverse effects were noted on growth, food consumption, urinalysis, haematology, gross pathology or histopathology that were related to administration of the caramel colour (Prier, 1960).

Rats (10 animals/sex/group) received 0, 5, or 10 g/kg (not specified whether per kg food or bw) Class IV Sulphite Ammonia Caramel in their diet for 3 months. Weight gain was normal in all groups. Food consumption, haematology, and urinalysis were comparable in all groups. Gross pathology and histopathology showed no test-related adverse findings (Chacharonis, 1960).

Sprague-Dawley rats (10 animals/sex/group) received 0, 5, 10, or 20% low or high colour intensity Class IV Sulphite Ammonia Caramel in their diet for 90 days. In addition, a paired-feeding study involving 5 male rats in 2 groups was run for 23 days (one sample was at the 20% level), and there were no differences in the rate of growth. The only effects attributable to treatment were a mild depression in growth of male rats at the 10 and 20% levels due to unpalatability of the test diet. No other adverse findings were noted in growth, behaviour, mortality, haematology, urinalysis, gross pathology, organ weights, or histopathology (Kay and Calandra, 1962a; 1962b).

Sprague-Dawley rats (10 animals/sex/group) received either 0 or 10% of 3 different caramel colours (one of which was a sample of Class IV Sulphite Ammonia Caramel) in their diet for 90 days. Weight gains showed a slight reduction compared with controls but food consumption was normal for all groups. No abnormalities were noted with regard to haematology, urinalysis, gross pathology, or histopathology (Chacharonis, 1963).

Rats (15 animals/sex/group) received 0, 5, 10, or 20% Class IV Sulphite Ammonia Caramel in their diet for 90 days. No adverse effects were noted on appearance, behaviour, survival, body weights, food intake, haematology, blood chemistry, urinalysis, organ weights, gross pathology, or histopathology (Oser, 1963).

Rats (10 animals/sex/group) received 0, 0.015, 0.3, or 3.0% Class IV Sulphite Ammonia Caramel in their diet for 90 days. No differences between test and control animals were noted regarding body weight, food consumption, haematology, urinalysis, gross pathology, or histopathology (Nees, 1964).

Four groups of rats received 0, 4, 8, or 16% Class IV Sulphite Ammonia Caramel in their diet for 3 months. No convulsions or other behavioural abnormalities or signs of neurological damage were seen. No macroscopic or microscopic pathological abnormalities were found in the central nervous system (Sharratt, 1971).

B6C3F1 mice (10 animals/sex/group) were given concentrations of 0, 10, 15, 20, or 30% Class IV Sulphite Ammonia Caramel in drinking water for 4 weeks. At 28 days the body weights of male animals at the 30%-dose level were significantly decreased compared to the controls, and transient depressions of body weights were noted at the highest dose level in females. Fluid consumption was depressed throughout the study among all treatment groups. However, these changes were not consistent with time or dosage. There were no statistically significant differences in food consumption between treated animals and controls. At necropsy, the only treatment-related effect reported was enlargement of the caecum (Tierney, 1979).

In addition, one special study on haematology is described.

A study was conducted in rats to determine whether haematological changes were associated with the feeding of high dietary concentrations of high (16 or 22%) or low intensity (34 or 47%) Class IV Sulphite Ammonia Caramel. Male rats (10/group), mean initial body weights approximately 173 g, were fed Class IV Sulphite Ammonia Caramel in the diet for 28 days. Sixteen rats were assigned to a control group. Rats were bled from the retro-orbital sinus on days 16, 9, and 2 before treatment and again on days 7, 14, and 28 during treatment. Feeding of these high dietary levels of Class IV Sulphite Ammonia Caramel led to reduced rates of body-weight gain. No effect on relative lymphocyte counts was observed. Caecal weights were increased in all treatment groups (BIBRA, 1978).

ANNEX D.

Summaries of toxicity studies on 2-acetyl-4-tetrahydroxy-butylimidazole (THI) and 4-methylimidazole (4-MEI)

D.1 Studies on 2-acetyl-4-tetrahydroxy-butylimidazole (THI)

THI was administered to male weanling Wistar rats (10 per group) at levels of 0, 2, 5, or 20 mg/l in drinking water (equivalent to 0, 0.1, 0.25, or 1 mg/kg bw/day) for 7 days. A marked depression of lymphocyte counts at all dose levels was observed. The lymphocyte-depressing potency of 0.1 mg THI/kg bw/day was comparable to a positive control group that received 1% Class III Ammonia Caramel in the drinking water (equivalent to 500 mg/kg bw/day), indicating a level of approximately 200 mg THI/kg in the caramel sample (which compares with a value of 204 mg THI/kg on an 'as is' basis). In addition to lymphocyte depression, THI induced a dose-dependent increase in neutrophils (Sinkeldam, 1982b; Kroplien, 1984).

In a study in which the lymphocyte-depressing fraction of Class III Ammonia Caramel was isolated, THI was identified as the sole responsible constituent (no further details) (Kroplien et al., 1985).

The haematological effects were studied in mice of oral administration in drinking water of THI, the constituent of Class III Ammonia Caramel responsible for lymphocytopenia (Iscaro et al., 1988). Initially five groups of BALB/c mice (five mice per group) were given doses of THI ranging from 0 to 200 parts/10(6) and bled weekly. Doses of THI from 5 to 100 parts/10(6) had no effect on circulating leucocytes over 6 weeks, but lymphocytopenia occurred with 200 parts/10(6). An increase in the concentration of THI to 400 parts/10(6) in the group on the lowest dose resulted in lymphocytopenia. An increase in dosage in two groups of mice, to 1000 and 2000 parts/10(6), resulted in marked lymphocytopenia. The number of neutrophils, eosinophils and monocytes remained unchanged throughout the experiment. Measurement of the proportions of CD4(L3T4)+ and CD8(Ly2)+ lymphocytes in lymph nodes from mice on high doses of THI did not show a selective depression of either subset, although both were increased relative to non-T cells. The authors concluded that THI causes a selective lymphocytopenia in mice, as in rats, but at relatively higher doses, and merits investigation in mice as an experimental treatment for states of lymphocyte excess or overactivity (Iscaro et al., 1988).

Gobin and co-workers examined the effect of giving THI at a level of 1 mg/kg bw/day by gavage for 7 days on the numbers of lymphocytes in subsets in peripheral blood (Gobin et al., 1989). Both immunoglobulin light chain-bearing B-cells (MARK-1+) and CD5 marker-bearing T-cells (OX-19+) were reduced in number within 1 day of treatment. Within the pan-T-cell population, Class II MHC reactive helper T-lymphocytes (W3/25-) were more reduced than the Class I MHC reactive cytotoxic/suppressor T cells (OX-8+). The number of null cells (MARK-1-, OX-19-) was not affected; the majority of these cells appeared to be large granular lymphocytes.

In a further study by the same group, THI was given in the drinking water at doses of 1, 10 and 50 mg/l (equivalent to 0.1, 1 and 5 mg/kg per day) to animals on a vitamin B_6-deficient diet (Gobin and Phillips, 1991). After 1 week, the immune competence of the animals was assessed under continued THI treatment. No marked changes in thymus or spleen weight were observed after THI treatment, although there was an increased number of pyknotic cells in the thymic cortex, mainly engulfed by macrophages and there appeared to be a slight thinning of the cortex area. THI produced a significant loss in T and B lymphocytes in peripheral blood but not in the spleen. No change in Natural Killer (NK) cell activity against YAC-1 target cells was observed in the spleen. The observed increase in NK cell activity in peripheral blood was due to an increase in circulating Large Granular Lymphocytes (LGL). Although the serum antibody titre against Keyhole Limpet Haemocyanin (KLH) was not affected by THI treatment, B cells showed less proliferation when cultured with lipopolysaccharide. T cell function was impeded as measured in mitogen-induced proliferation assay, delayed-type hypersensitivity assay and host versus graft (popliteal lymph node) assay. The authors concluded that

their results indicate that THI is an immunosuppressor in the rat, in whom it can produce profound lymphocytopenia and suppression of cell-mediated immunity (Gobin and Phillips, 1991).

In further investigations, THI (1 mg/kg bw/day) was given for up to 7 days in the drinking water to Fischer 344 rats on a vitamin B_6 deficient diet (Gobin and Paine, 1992; Gobin et al., 1992). THI rapidly produced profound lymphocytopenia resulting in depletion of T- and B-lymphocytes in peripheral blood but not in bone marrow and spleen. No marked change in thymus weight was found but the cellularity was marginally decreased and flow cytometric analysis of the lymphocyte subsets revealed an increase in the number of CD4+CD8- and CD4-CD8+ single positive (SP) cells and a reduction in the number of CD4+CD8+ double positive (DP) thymocytes. This reduction was in agreement with histological findings of increased numbers of pyknotic cells in the cortex, mainly engulfed by macrophages. Mitogen-induced proliferation of thymocytes prepared from THI-treated animals was increased, concordant with the gradual increase in the percentage of mature SP cells. No change in normal proliferation of thymocytes cultured *in vitro*, or, in proliferation *in vivo*, detected as 5-bromo-2'-deoxyuridine (BrdU) incorporation, was found. It is concluded that THI produced an increase in death of immature DP cells. However, THI did not affect thymocyte proliferation or their differentiation into mature SP cells in the thymus, but rather impairs their migration into the circulation. The authors concluded that the mechanism of action of THI appears to be indirect, but that THI does not act through increasing the release of adrenal corticosteroids to supra-physiological levels as the same histopathological changes in the thymus were found in adrenalectomised rats (Gobin and Paine, 1992). The authors also concluded that collectively these results indicate that the lymphocytopenia in the peripheral blood compartment after THI treatment, is caused by a rapid sequestration of lymphocytes into the spleen and bone marrow rather than by a reduced lymphocyte production and release into the periphery. The fact that THI also caused lymphocytopenia in splenectomised rats indicates that the spleen does not play an active part in the change in migrational behaviour of lymphocytes after THI treatment. Finally, as there was no increase in the absolute number of lymphocytes found in the spleen or bone marrow it seems they are rapidly degraded (Gobin et al., 1992).

THI administered orally to rats markedly decreased the peripheral blood lymphocyte count within 16-24 hours (Phillips and Paine, 1990). The lymphocytopenic effect of THI was prevented by co-administration of pyridoxine. ^{14}C-THI, administered orally, was absorbed in a dose-dependent manner and excreted unmetabolised. These processes were not affected by co-administration of pyridoxine. In contrast, the rate of excretion of ^{14}C-THI administered i.v. was increased by both dietary and parenterally-administered pyridoxine, and pyridoxine decreased the amount of radiolabel associated with lymphoid tissues. The authors propose that their results show that the lymphocytopenic effect of THI is not sustained once it is excreted, and indicate that pyridoxine and THI may compete for the same binding site in lymphoid tissues (Phillips and Paine, 1990).

The effect of oral administration of THI was studied in spontaneous and induced murine diabetes mellitus (Mandel et al., 1992). Continuous administration of THI at 400 mg/kg in drinking water reduced the prevalence of spontaneous diabetes in female NOD/Lt mice from 63% in untreated controls to 8% in treated animals. Since cyclophosphamide (CP) accelerates and intensifies diabetes in NOD mice, the authors also studied the effect of THI in this model. Diabetes incidence was reduced from 100% in mice given only CP to 13-14% in mice given THI either concurrently or from 14 days previously. Histologically, THI greatly reduced the severity of insulitis. As measured by flow cytometry, all THI-treated mice had a 60-80% reduction in splenic CD4+ and CD8+ T cells. THI-treated mice showed no untoward effects and specifically no weight loss, or pathological changes in their livers, kidneys or lungs. However, there was moderate atrophy of the thymus cortex. The authors concluded that THI could be a useful immunosuppressive agent (Mandel et al., 1992).

Groups of 10 rats per sex were given 0, 1, 2, 4, 16 or 32 mg THI/l in drinking water for 4 weeks (equivalent to approximately 0.1, 0.2, 0.5, 1.9 and 3.7 mg THI/kg bw/day in the treated groups), and groups of 20 rats per sex were given 0, 8 or 64 mg THI/l, equivalent to 0, 0.9, or 7.2 mg THI/kg bw/day. The rats were maintained on a normal rat chow, with adequate levels of pyridoxine. After 4

weeks 10 rats per sex of the 0, 0.9 and 7.2 mg/kg groups were given water without THI for 2 weeks (recovery phase). Decreased mean body weights in all test groups after 2 and 4 weeks were noted but ascribed to fasting for blood sampling and urinalysis. Gross and microscopic pathology in a large range of organs and tissues revealed no treatment-related effects. Treatment-related decreases in white blood cell and lymphocyte counts, and increases in neutrophil counts were observed. These changes returned to normal after 3 days recovery phase and remained normal. The NOAEL for the reduction in total lymphocytes was determined to be 4 mg THI/l in drinking water, equivalent to 380 µg/kg bw/day, in males and 1 mg THI/l in drinking water, equivalent to 120 µg/kg bw/day, in females (MacKenzie et al., 1992b).

In another study in rats, Class III Ammonia Caramel and THI (no details on doses) both induced a reduction in cell numbers in blood, spleen and lymph nodes, which were ascribed to decreases in T and B lymphocyte numbers. Increases in the number of mature medullary thymocytes (T cell precursors) in the thymus, and decreases in the number of $ER4^+$ (recent thymus emigrants) in the spleen, were considered to indicate a diminished migration of mature cells from the thymus to the periphery. The increase in the number of mature cells was also indicated by an increase in mitogen-induced thymidine incorporation by thymocytes from THI exposed rats (Houben, 1992b). Further evidence for a change in the function of accessory cells was demonstrated in *ex vivo* studies, as splenic cells from THI exposed rats did not show adequate response upon mitogenic stimulation (no further detail) (Houben, 1992b).

SCID-hu mice were exposed to THI, Class III Ammonia Caramel, or the organotin compound, di-n-butyltin dichloride (DBTC). Histopathological examination of the human thymus grafts of SCID-hu mice either exposed to THI or to DBTC showed a reduction in the relative size of the thymus cortex, an effect also described in rodents. The authors conclude that the human thymus is a target for the immunotoxic action of both THI and DBTC (de Heer et al., 1995).

In a study investigating the effects of THI on the murine immune system, Bradbury et al. (1996) showed that splenic T lymphocytes from mice treated with 50 mg/l THI in their drinking water were unable to launch a Mixed Lymphocyte Reaction (MLR) against allogeneic stimulator cells, and had decreased and delayed interleukin-2 (IL-2) production. However, these T cells exhibited a normal proliferative response to concanavalin A (Con A), immobilized anti-CD3 monoclonal antibody (mAb) and anti-CD3 plus anti-CD28 mAb. Furthermore, the MLR response could be restored by the addition of IL-2 to the MLR culture. Homing studies using intravenous injection of fluorescence-labelled splenocytes showed that THI treatment decreased absolute numbers of labelled T and B lymphocytes in the blood and the spleen. Furthermore, these labelled cells reappeared in the blood and the spleen when mice were taken off THI, indicating that lymphocyte recirculation and splenic homing were modified reversibly by THI treatment. Cessation of THI treatment also resulted in a rapid reappearance of MLR responsiveness in the spleen, indicating that THI treatment does not functionally impair recirculating T cells. Collectively the authors suggested that the data were collectively compatible with the concept that a rapidly recirculating population of T cells, which produce IL-2 in an allogeneic MLR, are lost from the blood and spleen following THI treatment, and are sequestered in other, yet to be identified, tissues (Bradbury et al., 1996).

The fate of cells lost from the blood and the spleen during THI treatment was followed via injection of fluorescently labelled splenocytes into THI-pretreated mice (Bradbury et al.1997). The results showed that THI increased labelled cells in the liver, lungs and kidneys of THI-treated mice. Furthermore, the sequestration in the liver occurred just 1.5 hours after injection of labelled cells with the increase still being present at 24 hours after injection. Microscopic examination of liver sections indicated that fluorescent lymphocytes were clustered within the liver sinusoids in THI-treated mice, possibly associated with endothelial cells. The liver retention of lymphocytes was confirmed by immunohistochemical studies which showed a significant increase of T cells in the liver of THI-treated mice. To determine the subset of lymphocytes which are lost from the spleen and sequestered in non-lymphoid organs, lymphocytes remaining in the spleen after THI treatment were characterized. The

results of this study confirmed that THI reduced B cells, CD4+ and CD8+ T cells and cells expressing CD62L, CD44 and IL-2R in the spleen.

The ability of THI to inhibit T-cell export from the thymus was examined in a study in BALB/c mice maintained on drinking water containing THI for 5 days (Gugasyan et al., 1998). The mice showed a two-fold increase in the total number of mature medullary thymocytes (CD4+CD8- and CD4-CD8+) as well as a slight decrease in the total number of immature double-positive cells (CD4+CD8+). The mature single-positive thymocytes were found to express high levels of the homing molecule L-selectin, suggesting that these potential emigrants were prevented from leaving the thymus. To confirm this, THI-treated mice were injected intrathymically with fluorescein isothiocyanate and the number of labelled T cells appearing in the lymph nodes and spleen was determined 16 hours later. A 10-fold decrease in the number of CD4+ and CD8+ recent thymic emigrants in the lymph nodes and spleen of THI-treated mice was observed. Previous studies have shown that THI does not affect other aspects of thymocyte development, such as proliferation and differentiation. Taken together, the authors proposed that their results suggest that the immunosuppressive effects of THI may be due, in part, to preventing of the final step of T-cell export out of the thymus (Gugasyan et al., 1998). These authors also reported that continuous oral treatment of non-obese diabetic (NOD) mice with THI reduced the number of CD4+ and CD8+ T cells in the peripheral blood (Gugasyan et al., 1995). In the draining lymph nodes THI treatment had no effect on lymphocyte subsets prior to contact sensitization, but subsequent sensitization and elicitation with OX could not stimulate a significant increase in the number of CD4+ T cells in the treated mice, whereas untreated control mice showed elevated numbers of these lymphocytes. The authors suggested that THI can inhibit a contact hypersensitivity response by preventing the recruitment of CD4+ T cells in the draining lymph nodes through an unknown mechanism (Gugasyan et al., 1995). In a study by the same group, THI marginally delayed the development of adjuvant-induced arthritis (AA) in Lewis rats, a widely used model of chronic inflammatory arthritis. However, THI had no effect on the incidence or severity of disease, although it totally prevented granuloma formation in the spleen and associated splenomegaly. The authors concluded that THI may be a useful adjunctive agent for some inflammatory diseases (Gugasyan et al., 1997).

Schwab et al. (2005) have shown that in mice (age not indicated) receiving 1 mg/kg bw/day THI (50 mg/l drinking water) for 3 consecutive days, THI induced a marked decrease in the number of circulating lymphocytes and suppressed immunity. The mean THI plasma level was 135 ng/mL. The migration of lymphocytes from the thymus and peripheral lymphoid organs depends on their membrane sphingosine 1–phosphate (S1P) receptor-1 and is thought to occur in response to elevated concentrations of circulatory S1P. S1P is a lysophospholipid that acts through G-protein-coupled receptors in the cell, and THI mediates the increase in S1P via inhibition of S1P lyase, the enzyme involved in degradation of S1P in the cell (Schwab et al., 2005). The authors have found that S1P levels in lymphoid tissues of mice are normally low but increase more than 100-fold after THI treatment. This treatment inhibited the S1P degrading enzyme S1P lyase, an intracellular degrading enzyme which is expressed by hematopoietic cells. Disruption of the S1P gradient caused mature T cells to remain stored in immune tissues such as the thymus and peripheral lymphoid organs and thus blocked lymphocyte trafficking.

D.2 Studies on 4-methylimidazole (4-MEI)

The toxicokinetics and metabolism of 4-MEI have been studied in the male F344 rat using ^{14}C radiolabelled compound (Yuan and Burka, 1995). Radioactivity in plasma and urine was profiled by HPLC. After gavage administration of 50 mg/kg, about 85% of the administered radioactivity was recovered in urine within 48 hour. The majority of the radioactivity in urine or plasma was associated with the parent compound and only one minor hydrophilic metabolite was present in urine and in plasma. Elimination of radioactivity via faecal, biliary or respiration was negligible. Elimination of 4-MEI after an i.v. dose of 5 mg/kg can be described by a two-compartment process with an estimated half-life of 1.8 hours and an estimated apparent volume of distribution of 2.3 litre/kg. After gavage at doses of 5, 50 and 150 mg/kg, 4-MEI was readily absorbed with a estimated bioavailability of 60-70%.

Urinary excretion data indicated that renal clearance of 4-MEI accounted for about 80% of total body plasma clearance. Based on the estimated AUC of metabolite and the estimated renal clearance of 4-MEI, the formation of metabolite and the renal clearance of 4-MEI appeared to be a saturable process (Yuan and Burka, 1995).

Imidazole, 1-methylimidazole, 4-MEI, and ketoconazole, an oral imidazole antifungal agent, caused dose-dependent decreases in testosterone secretion and Testicular Interstitial Fluid (TIF) formation (Adams et al., 1998). Imidazole, 2-methylimidazole (2-MEI), and 4-MEI decreased Luteinizing Hormone (LH) secretion. 4-MEI decreased testosterone secretion 1-6 hours after injection, increased testosterone at 8-16 hours, decreased LH secretion at 4 hours, decreased TIF volumes at 1-8 hours, and slightly increased TIF volumes at 24 hour. 4-MEI blocked the stimulatory effects of human Chorionic Gonadotrophin (hCG) on testosterone secretion and prevented an expected increase in LH secretion after the 4-MEI-induced decrease in testosterone secretion. 4-MEI also reversed the effects of three other stimulants of testosterone secretion that presumably act through three different testicular regulatory systems: *N*-methyl-D,L-aspartate, an excitatory amino acid; NG-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor; and naltrexone, an opioid antagonist. The authors suggested that these results support the hypothesis that imidazoles inhibit testicular function and male reproductive function through inhibition of testosterone secretion, TIF formation, and LH secretion regulatory systems (Adams et al., 1998).

D.2.1 NTP studies on the toxicity and carcinogenicity of 4-MEI (text reproduced from NTP, 2004, and NTP 2007)

Groups of five male and five female rats and mice were fed diets containing 0, 300, 800, or 2500 mg/kg 4-MEI (equivalent to average daily doses of approximately 30, 80, or 220 mg/kg for rats and 65, 170, or 500 mg/kg for mice) for 15 days. All animals survived to the end of the studies, and there were no significant differences in mean body weights, clinical findings, organ weights, or gross or microscopic lesions between exposed and control groups (NTP, 2004).

Groups of 10 male and 10 female rats and mice were fed diets containing 0, 625, 1250, 2500, 5000, or 10 000 mg/kg 2- or 4-MEI (equivalent to average daily doses of approximately 40, 80, 160, 300, or 560 mg/kg 2- or 4-MEI to rats; and 100, 165, 360, 780, or 1740 mg/kg 2-MEI or 100, 240, 440, 915, or 1840 mg/kg 4-MEI to male mice; and 90, 190, 400, 800, or 1860 mg/kg 2-MEI or 110, 240, 540, 1130, or 3180 mg/kg 4-MEI to female mice for 14 weeks. In the 14-week 4-MEI studies, one 10 000 mg/kg male mouse was found dead during week 4, and seven 10 000 mg/kg female mice were found dead during weeks 1 and 2. Mean body weights were significantly less than those of the controls for male rats exposed to 2500 mg/kg or greater, 5000 and 10 000 mg/kg female rats, male mice exposed to 1250 mg/kg or greater, and all exposed groups of female mice. Reduced feed consumption was observed in 5000 and 10 000 mg/kg male and female rats. Clinical findings included nasal/eye discharge, ruffled fur, thinness, ataxia, and abnormal breathing in rats, and ruffled fur and dull coats in female mice. On days 29 and 82, functional observations in 5000 and 10 000 mg/kg rats included labored or increased respiration, mild tremors, walking on tiptoes, hunched posture, piloerection, crouching over, impaired coordination of movement, ataxia, and pupillary constriction. 4-MEI induced a transient erythrocytosis and a minimal, exposure concentration-related, microcytic, normochromic, nonresponsive anaemia in male and female rats. Clinical chemistry evaluations generally showed a cholestatic effect in exposed male and female rats. At week 14, there was a significant decrease in total protein and albumin concentrations of female rats exposed to 5000 or 10 000 mg/kg (NTP, 2004).

In mice, 4-MEI induced a macrocytic, hyperchromic, responsive anemia and, particularly in males, increases in triiodothyronine concentrations and transient decreases in thyroxine concentrations. In the 4-MEI studies, the liver weights of male rats exposed to 2500 mg/kg or greater were significantly increased; spleen weights of female rats exposed to 2500 mg/kg or greater were decreased. The absolute liver weight was decreased in 10 000 mg/kg male mice, and relative weights were significantly increased in all exposed groups of mice. In female mice, there was a significant decrease in the absolute weights and increase in the relative weights of the heart, right kidney, and liver in

groups exposed to 2500 mg/kg or greater. The epididymal spermatozoal concentration was significantly increased in 5000 mg/kg male rats. Gross pathology observations included pale livers in male rats exposed to 2500 mg/kg or greater and small testes and uteri in 10 000 mg/kg male and female rats. Microscopic analysis identified significantly increased incidences of cytoplasmic hepatocyte vacuolization of the liver of male rats exposed to 2500 mg/kg or greater and 10 000 mg/kg female rats, hypospermia of the epididymis in 10 000 mg/kg male rats, atrophy and inflammation of the prostate gland in 10 000 mg/kg male rats, and degeneration of the testes in 5000 and 10 000 mg/kg male rats (NTP, 2004).

4-MEI was negative in the *S. typhimurium* mutation assay when tested in strains TA97, TA98, TA100, and TA1535, with and without S9 activation enzymes. *In vivo*, no consistent or significant increases in the frequencies of micronucleated erythrocytes were seen in the bone marrow of male rats or mice treated with 4-MEI by intraperitoneal injection, or in peripheral blood samples from male and female mice administered the compound in dosed feed for 14 weeks (NTP, 2007).

Groups of 50 male and 50 female rats were fed diets containing 0, 625, 1250, or 2500 mg/kg 4-MEI (males) or 0, 1250, 2500, or 5000 mg/kg 4-MEI (females) (equivalent to average daily doses of approximately 30, 55, or 115 mg 4-MEI/kg bw to males and 60, 120 or 260 mg 4-MEI/kg bw to females) for 106 weeks. Survival of all exposed groups of male and female rats was similar to that of the control groups. Mean body weights of males in the 1250 and 2500 mg/kg groups and females in the 2500 and 5000 mg/kg groups were less than those of the control groups throughout the study; mean body weights of 1250 mg/kg females were less after week 41. Feed consumption by 5000 mg/kg females was less than that by the controls. Clonic seizures, excitability, hyperactivity, and impaired gait were observed primarily in 2500 and 5000 mg/kg females. The incidence of mononuclear cell leukemia in 5000 mg/kg females was significantly greater than that in the controls, and the incidence exceeded the historical range in feed study controls. The incidences of hepatic histiocytosis, chronic inflammation, and focal fatty change were generally significantly increased in all exposed groups of male and female rats. The incidences of hepatocellular eosinophilic and mixed cell focus were significantly increased in 2500 mg/kg males and 5000 mg/kg females (NTP, 2007).

Groups of 50 male and 50 female mice were fed diets containing 0, 312, 625, or 1250 mg/kg 4-MEI (equivalent to average daily doses of approximately 40, 80, 170 mg 4-MEI/kg bw to males and females) for 106 weeks. Survival of all exposed groups of male and female mice was similar to that of the control groups. Mean body weights of males and females in the 1250 mg/kg groups were less than those of the control groups after weeks 17 and 12, respectively. Mean body weights of 312 and 625 mg/kg females were less after weeks 85 and 65, respectively. Feed consumption by exposed groups of male and female mice was generally similar to that by the controls. The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in 1250 mg/kg males, and alveolar/bronchiolar adenoma or carcinoma (combined) in 1250 mg/kg males and 625 and 1250 mg/kg females were significantly greater than those in the control groups. The incidence of alveolar epithelium hyperplasia was significantly increased in 1250 mg/kg females (NTP, 2007).

D.2.2 NTP conclusions on carcinogenicity of 4-MEI (NTP, 2007)

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity* of 4-MEI in male F344/N rats exposed to 625, 1250, or 2500 mg/kg. There was *equivocal evidence of carcinogenic activity* of 4-MEI in female F344/N rats based on increased incidences of mononuclear cell leukemia. There was *clear evidence of carcinogenic activity* of 4-MEI in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms.

Exposure to 4-MEI resulted in non-neoplastic lesions in the liver of male and female rats and the lung of female mice and in clinical findings of neurotoxicity in female rats.

GLOSSARY AND/OR ABBREVIATIONS

ADI	Acceptable Daily Intake
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
APTT	Activated Partial Thromboplastin Time
BIBRA	British Industrial Biological Research Association
CAS	Chemical Abstracts Service
CE	Capillary Electrophoresis
CEF	EFSA Scientific Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary
CIAA	Confederation of the Food and Drink Industries of the EU
CONTAM	EFSA Scientific Panel on Contaminants in the Food Chain
CP	Cyclophosphamide
CPY	Cytochrome P450
DB	Double positive
DBTC	Di-n-butyltin dichloride
DEAE	Diethylamino Ethyl
EC	European Commission
EFSA	European Food Safety Authority
EUTECA	European Technical Committee on Caramels
EXPOCHI	Refers to EFSA Article 36 2008 call for Proposals Focused on Children and Food Consumption
FDA	Food and Drug Administration
GC	Gas chromatography
GS/MS	Gas Chromatography/Mass Spectrometry
HMCF	High Molecular weight Colour Fraction
HMF	5-hydroxymethyl-2-furfural
HPLC	High-performance liquid chromatography
IC_{50}	50% cell growth inhibition concentration

IL-2	Interleukin-2
ITCA	International Technical Caramel Association
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KLH	Keyhole limpet haemocyanin
LD_{50}	Lethal Dose, 50% i.e. dose that causes death among 50% of treated animals
LGL	Large granular lymphocytes
LGL	Large granular lymphocytes
LH	Luteinizing Hormone
LMW	Low Molecular Weight
LOAEL	Low-Observed-Adverse-Effect-Level
MEI	Methylimidazole
MW	Molecular Weight
NCE/PCE	Normochromatic erythrocytes/Polychromatic erythrocytes
NK	Natural killer
ML	Maximum level
MLR	Mixed lymphocyte reaction
NOD	Non-Obese Diabetic
MRL	Maximum Reported Levels
MS	Mass spectrometry
NATCOL	Natural Food Colours Association
NOAEL	No-Observed-Adverse-Effect-Level
NTP	National Toxicology Programme
OECD	Organisation for Economic Co-operation and Development
PAPS	3'-phosphoadenosine-5´-phosphosulphate
PCR	Polymerase Chain Reaction
SCF	Scientific Committee on Food
SCOOP	A scientific cooperation (SCOOP) task involves coordination amongst Member States to provide pooled data from across the EU on particular issues of concern regarding food safety
SFFCC	Syndicat des Fabricants Français de Caramels Colorants
SMF	5-sulphoxymethyl-2-furfural

SP	Single positive
SP1	Sphingosine 1–phosphate
SULT	Sulphotransferase
UNEP	United Nations Environment Programme
UNESDA	Union of European Beverage Associations
TemaNord	Nordic Council of Ministers
THI	2-acetyl-4-tetrahydroxy-butylimidazole
TIF	Testicular Interstitial Fluid
WHO/FAO	World Health Organization/Food and Agriculture Organization