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Review

Caramel color safety – An update

Thomas A. Vollmuth

Vollmuth and Associates, LLC, 6 Goldenclub Lane, Okatie, SC, 29909, United States

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ABSTRACT

Caramel color has been used in foods and beverages for over 150 years and is globally regulated as a color additive. The four distinct classes of caramel color (Plain Caramel, Sulfite Caramel, Ammonia Caramel, and Sulfite Ammonia Caramel) are well characterized and each have specifications that take into account processing variables including reactants that can give rise to low molecular weight constituents (e.g., 4-MeI and THI) that may have toxicological significance for evaluating safety.

Extensive safety testing has been conducted with the different classes of caramel color and its constituents, including toxicokinetics, genotoxicity, subchronic toxicity, carcinogenicity, and reproductive/developmental toxicity studies. In addition, data is available on uses and use levels that have been used to estimate intakes of caramel colors and their constituents. No Observable Adverse Effect Levels (NOAEL) have been identified for all classes and Acceptable Daily Intakes have been established to ensure safety of use. Available studies support a conclusion that caramel colors are not genotoxic or carcinogenic, and exposure estimates indicate that intake of caramel colors and constituents do not pose undue safety risks. This update summarizes available relevant safety studies and authoritative reviews on caramel colors and its toxicologically important constituents, 4-MeI and THI.

1. Introduction

Caramel colors (CAS No. 8028-89-5) refer to a family of distinct red to dark-brown liquids or powders that are used as color additives in a variety of food and beverage products. In addition, other product types make use of caramel colors as ingredients not covered in this review including animal feeds, cosmetics, and pharmaceuticals. Caramel colors are a complex mixture of compounds produced by heating carbohydrates under controlled heat (caramelization) and processing conditions, and are divided into four classes: Class I - Plain Caramel; Class II – Sulfite Caramel; Class III - Ammonia Caramel; and, Class IV – Sulfite Ammonia Caramel according to the ingredients used in their manufacture (Kamuf et al., 2003; Sengar and Sharma, 2014). They are characterized by a burnt sugar odor and pleasant, slightly bitter taste, but at usual levels required in most food applications they impart no notable flavor to the finished product.

These coloring substances, each class with a unique set of functional properties, are used specifically for coloring purposes and can be distinguished from caramel confectionery, caramelized sugar syrups and flavor which are made from caramelized sugars and other ingredients (Sengar and Sharma, 2014). Although intended for color use, these substances may also have other functional effects in foods. For example, they are known to stabilize colloidal systems and have emulsifying properties, thus facilitating flavor retention and the dispersion of water-insoluble material like essential flavor oils. Caramel color is also known to retard flavor changes that occur in some beverages as a result of sunlight (Chappel and Howell, 1992).

Caramel colors have been used in foods for well over a century and a half. The first commercially available caramel colors were manufactured in the United States in 1863. Cola beverages, root beers, dark beers and dark breads are familiar foods containing caramel color, but numerous other beverages, including cider, distilled spirits, dairy- and fruit-based desserts, jams and jellies, cereals, breads, fine bakery wares, soups and broths, barbeque sauces, meats, soy sauce, seasoning, and confectionery and chewing gum are all foods where caramel color is used. In fact, there are provisions for the use of caramel colors in over 80 food categories in the CODEX General Standard for Food Additives (GSFA) (CODEX, 2017).

Caramel colors have been regulated color additives in the United States since the 1940's, and following the passage of the 1958 Food Additives Amendment, were included on the Food and Drug Administration's (FDA) list of generally recognized as safe (GRAS) substances (CFR 182.1235). In 1963, with the passage of the 1960 Color Additive Amendments, FDA permanently listed caramel color (21CFR 73.85) as a color additive exempt from certification (i.e., not needing FDA review or analysis of all newly manufactured batches). FDA makes no distinction in their regulations regarding the type or class of caramel color, nor places any limitations on use (FDA, 2016). Likewise, the Food

E-mail address: Vollmuthassociates@gmail.com.

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Chemicals Codex (FCC), a listing of standards for the identity, purity and quality of food ingredients, makes note of the four different classes in its caramel color monograph, but for more than 20 years has had a single specification that covers all classes.

Caramel color is regulated in a similar manner in a few other countries, but many now recognize the four distinct classes and specify food categories and levels whereby each class can be used. For example, European Union legislation recognizes the 4 classes of caramel color and lays down use levels for each in specific food categories (European Commission, 2011; European Union, 2012). The CODEX GSFA has also established numerous provisions for Classes III and IV, and new provisions for Class II are under review; Class I caramel color is listed in Table Three of the Standard which permits use in all foods at Good Manufacturing Practice (GMP) levels. To support these uses, safety studies have been conducted and expert safety opinions expressed by national and global regulatory and safety bodies, including the Joint FAO/WHO Expert Committee on Food Additives (JECFA), over the course of several decades. Acceptable Daily Intakes (ADIs) for each class have been allocated and robust specifications established that limit the amounts of some known constituents that may be formed during manufacture.

Caramel colors were first reviewed by JECFA in 1965 and in more depth in 1970 (JECFA, 1965; JECFA, 1970). At that time, specifications could not be developed and a need for more compositional and toxicological data was requested. Nonetheless, JECFA concluded that with the exception of caramel prepared by processes using ammonia and ammonium salts, caramel colors could be considered natural constituents of the diet and acceptable as additives. At a subsequent meeting, JECFA classified caramel color into two broad categories – one made with ammonium compounds and one without – based on findings that only the caramel colors made with ammonium compounds contained measurable levels of 4-methylimidazole (4-MeI) which was known at the time to elicit neurotoxicity at high doses in animals (Nishie et al., 1969; Weiss et al., 1986; Wiggins, 1956). The Committee, at its 15th meeting in 1972 established a temporary ADI of 100 mg/kg body weight (bw) for caramel color made by the ammonia process and requested that additional safety studies be performed. In particular, additional information was requested on the potential immunotoxicity of 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), a reaction product formed in Class III ammonia caramel. As additional information became available about the safety and characterization of caramel colors and based on a classification and specification system developed by the International Technical Caramel Association (ITCA), JECFA ultimately recognized four classes of caramel color (JECFA, 1980). Limits were established for 4-MeI in Class III and Class IV caramel color and for THI in Class III caramel color (See Table 1). Further characterization and safety research by ITCA and others resulted in the establishment of permanent specifications for the four classes and ADIs for three classes (II, III, and IV). An ADI of “not specified” was allocated for Class I caramel and an ADI of 200 mg/kg bw (150 mg/kg bw on a solids basis) was established for Class III and IV caramel colors (JECFA, 1986; JECFA, 1987). At the 55th JECFA meeting in 2000, an ADI of 160 mg/kg bw was established for Class II caramel color, and specifications have been further updated in more subsequent years (JECFA, 2001; JECFA, 2011a,b).

Concurrent with JECFA's reviews and in the course of establishing regulations for the use of coloring matter in food, the Scientific Committee for Food (SCF) of the European Union evaluated and established ADIs and purity criteria for caramel colors (SCF, 1997). More recently, the European Food Safety Authority (EFSA) issued a scientific opinion on the re-evaluation of caramel color (EFSA, 2011) in which they acknowledged many aspects of the most recent JECFA evaluation and reviewed the safety data of the four caramel classes. EFSA determined that the available toxicological data was sufficient to establish an overall group ADI of 300 mg/kg bw for Classes I, II, III, and IV. Based on questions associated with the immunotoxicity of THI at the time, the Panel established an individual ADI of 100 mg/kg bw for Class III caramel and sought any information that might further clarify the immunotoxic effects of THI observed in rodents. Because of concerns associated with human exposure to 4-MeI through the manufacture of electronics and pharmaceuticals and its occurrence in many foods, the National Toxicology Program (NTP) evaluated 4-MeI in 2-year bioassays in mice and rats. Those studies reported “clear evidence of carcinogenic activity” in male and female mice based on increased incidence of alveolar/bronchiolar neoplasms (NTP, 2007). Although the results are unusual and have been contested, this study was the basis for a listing of 4-MeI on California's Proposition 65 list of carcinogens (OEHHA, 2011) and a 2B classification by the International Agency for Research on Cancer (IARC) (IARC, 2013).

There is consensus that caramel colors meeting existing specifications can be safely used in foods. Some stakeholders though have concerns and call for further research and regulatory restrictions, especially in Class III and IV caramel colors due to potential cancer risks from exposure to 4-MeI. Two citizen's petitions were filed in the United States by the Center for Science in the Public Interest (CSPI) and Consumers Union. CSPI requested that the U.S. FDA revoke the use of caramel colors produced with ammonia (Class III) or ammonia sulfite (Class IV) because they contain 4-MeI and 2-MeI (CSPI, 2011). The Consumer Union petition called for regulatory changes that included: 1) a prohibition on the use of ammonium compounds to produce caramel color until such time that manufacturers can show that caramel color produced with these compounds contains no or negligible levels of 4-MeI; 2) limits on 4-MeI in specifications in 21 CFR §73.85(b); 3) the certification of every batch of caramel colors Class II and IV produced with ammonium compounds to ensure finished products containing these products lead to no detectable or negligible levels of 4-MeI, and; 4) distinguish in its regulations between the four classes of caramel color (Consumer Union, 2014) Although FDA has not responded to these petitions with any regulatory actions or guidelines yet, they responded with information to consumers indicating that dietary changes are not needed based on the occurrence of 4-MeI currently estimated in the diet (FDA, 2014).

New information and research continues to appear in the public domain that brings clarity but sometimes confusion on the safety of caramel colors. This review will summarize existing safety data, scientific opinions, and risk assessments related to the intake of caramel colors and constituents.

2. Classes and chemistry of caramel colors

Caramel color is produced in a controlled cooking process by heating food-grade carbohydrates to various temperatures and pressures with different reactants (e.g., acids, alkalis, sulfite-containing compounds, ammonium-containing compounds) to assist the caramelization process and produce greater color intensity. In the U.S., high dextrose corn syrup is a commonly used carbohydrate in the manufacture of caramel color, but invert sugar, glucose, glucose syrups, fructose, dextrose, sucrose, honey and molasses have also been used as starting carbohydrates. Four different classes of caramel color are produced, each with slightly different chemical and functional properties that ensure compatibility with the food being colored and eliminate undesirable interactions (e.g., haze, flocculation, separation). Certified organic and non-GM0 caramel colors are now commercially available with cane sugar being the preferred starting sugar (Kamuf et al., 2003).

The acids used in manufacturing caramel colors are food-grade sulfuric or citric acids, although phosphoric, acetic, or carbonic acids may also be used. Sodium, potassium or calcium hydroxides or mixtures of these are commonly used alkalis. Salts used are carbonate, hydrogen carbonates, sulfates, and ammonium, sodium, potassium or calcium phosphate. The ammonium compounds utilized are ammonium hydroxide, ammonium carbonate and ammonium hydrogen carbonate,

Table 1
Chemical characterization and specifications of caramel colors.

	Caramel Color I	Caramel Color II	Caramel Color III	Caramel Color IV
	Plain Caramel; Caustic Caramel	Caustic Sulfite Caramel	Ammonia Caramel	Sulfite Ammonia Caramel
	E150a; INS 150a	E150b; INS 150b	E150c; INS 150c	E150d; INS 150d
Reactants Used in the Maufacturing Process	Acids and alkalis only; no ammonium or sulfite compounds during manufacture	Acids and alkalis; sulfite compounds but no ammonium compounds added	Acids and alkalis; ammonium compounds but no sulfite compounds added	Acids and alkalis; Both ammonium and sulfite compounds added during manufacture
Specifications[a]				
Solid content	62-77%	65-72%	53-83%	40-75%
Color Intensity [b]	0.01–0.12	0.06–0.10; 0.05–0.13 (EU)	0.08–0.36	0.10–0.60
Total Nitrogen[b c]	max 0.1%	max 0.2%; max 0.3%[d] (EU)	1.3–6.8; 0.7–3.3%[d] (EU)	0.5–7.5%; 0.3–1.7%[d] (EU)
Total Sulfur[b]	max 0.3%; max 0.2% (EU)	1.3–2.5%; 0.3–3.5%[d] (EU)	max 0.3%; max 0.2%[d] (EU)	1.4–10%; 0.8–2.5%[d] (EU)
Sulfur Dioxide[b]	–	max 0.2%	–	max 0.5%; max 0.2%[d] (EU)
Ammoniacal Nitrogen[b]	–	–	max 0.4%; max 0.3%[d]	max 2.8%; max 0.6%[d]
4-MeI[b]	–	–	max 200 mg/kg[d](JECFA); max 250 mg/kg[d](EU)	max 250 mg/kg[d] (EU, JECFA)
THI[b]	–	–	max 25 mg/kg[d] (JECFA); max 10 mg/kg[d] (EU)	–
Arsenic (as As)	not more than 1 mg/kg[e];	not more than 1 mg/kg[e]	not more than 1 mg/kg[e]	not more than 1 mg/kg[e]
Lead (as Pb)	not more than 2 mg/kg[e]	not more than 2 mg/kg[e]	not more than 2 mg/kg[e]	not more than 2 mg/kg[e]
Mercury (as Hg)[f]	not more than 0.1 mg/kg (FCC); not more than 1.0 mg/kg (EU)	not more than 0.1 mg/kg (FCC); not more than 1.0 mg/kg (EU)	not more than 0.1 mg/kg (FCC); not more than 1.0 mg/kg (EU)	not more than 0.1 mg/kg (FCC); not more than 1.0 mg/kg (EU)
Cadmium[g]	not more than 1 mg/kg	not more than 1 mg/kg	not more than 1 mg/kg	not more than 1 mg/kg
Typical Applications	Cereals, nutrition bars, rice cakes, croutons and stuffing	Distilled spirits (cognac, sherry)	Dark beer, breads, cake mixes, muffins, cereals, cookies, baked goods, frostings	Soft drinks, baked goods, snack foods, cereals, confectionery
Constituents Levels[h]				
5-HMF	700 - 27,300 mg/kg	3300 - 33,700 mg/kg	10 - 3900 mg/kg	4900 - 21,400 mg/kg
Furan	151 μg/kg	52 μg/kg	177 μg/kg	59 μg/kg
THI	No data; Not expected to be present	No data; Not expected to present	2.4–10 mg/kg	No data; Not expected to present
4-MeI	No Data	No Data	5–140 mg/kg	48 - 183 mg/kg (single strength) 23 - 147 mg/kg (double strength)
Sulfur Dioxide[i]	Trace	0.1–0.2%	Trace	0.02–0.13%
Acrylamide	Not expected to be present	Not expected to be present	Not detected (LOD = 100 μg/kg)	Not detected (LOD = 1 μgTHI/kg

[a] From published JECFA, EU and FCC Specifications.
[b] Expressed on a solids basis.
[c] Kjeldahl Method.
[d] Equivalent color basis; To express a parameter on a color equivalent basis, the parameter is determined for the caramel color and is expressed in terms of a product having a color intensity of 0.10 absorbance units.
[e] Food Chemical Codex (FCC) Specification: As: not more than 3 mg/kg; Pb: not more that 10 mg/kg.
[f] JECFA specifications do not set limits for Hg.
[g] EU Purity Criterial only; FCC and JECFA have not specification for Cd.
[h] From EFSA, 2011.
[i] From Licht et al., 1992a.

ammonium phosphate, ammonium sulfate, ammonium sulfite and ammonium hydrogen sulfite. Sulfite compounds used are sulfurous acid, hydrogen sulfites, and sulfites and bisulfites of potassium, sodium and ammonium. Polyglycerols esters of fatty acids may be are used as antifoaming agents. (Kamuf et al., 2003; JECFA, 2011b; Food Chemical Codex, 2016). Detailed reviews on the chemical characterization and specifications of caramel color are available (Kamuf et al., 2003; Licht et al., 1992b, 1992c; Sengar and Sharma, 2014; Kuhnert, 2016).

The physical and chemical differences between the four classes are notable and have implications for the evaluation of their safety. Commercially representative and well-characterized colors are essential for safety studies. In the 1980's, based on recommendations from JECFA and other regulatory agencies, the International Technical Caramel Association embarked on an extensive program to characterize the different classes of caramel color and validate analytical methods for testing. The results of this program provided the basis for an aligned characterization and specification system (Myers and Howell, 1992; Licht et al., 1992a, 1992b, 1992c). A brief description of the four classes of caramel along with existing specifications of the Food Chemicals Codex (2016); JECFA (2011b); and European Union (2012), and constituents are presented in Table 1.

During the production process, a number of chemical reactions take place, some which are responsible for the formation of high molecular weight (HMW) constituents responsible for the distinctive colors associated with caramel colors, but others that result in the formation of low molecular weight (LMW) substances such as 4-MeI, THI, 5-hydroxymethylfurfual (5-HMF) and furan; low levels of residual sulfites are also found in some caramel colors (Table 1). The choice of ingredients and production conditions (e.g., temperature, pressure, time, pH) impact the chemical and physical properties including color shade and intensity associated with the HMW constituents and the type and quantity of LMW substances formed. The type and quantity of LMW substances formed can influence the considerations needed to demonstrate the safety of caramel colors and finished food products; therefore, it essential that caramel colors meet specifications to control the formation and exposure to such substances. In caramel color I, both

nitrogen and sulfur are detected at low levels - less than 0.1% and 0.3% respectively - which is consistent with the fact that ammonium and sulfite compounds are not used in manufacturing. However, low and highly variable concentrations of 5-HMF, a marker substance of the LMW fraction are detectable in this class and all other classes of caramel color. Caramel color II has very little nitrogen, but sulfur accounts for approximately 0.3–3.5%. Most of the sulfur in the reactants is incorporated into compounds with no detectable sulfur dioxide. Caramel color III contains approximately 1–6.8% nitrogen and both 4-MEI and THI are consistently present. Caramel color IV, the most widely used color, has both nitrogen and sulfur, as high as 7.5% and 10%, respectively, and the presence of 4-MeI in this class of caramel color has raised the most concern in recent years for regulatory agencies. The EFSA Panel reviewing caramel color safety noted that caramel colors are complex and poorly characterized, and it was still not entirely clear whether the processing controls in place are sufficient to minimize batch-to-batch variability, particularly with respect to LMW constituents (EFSA, 2011). The Panel also noted that there was limited information about the detailed relationship between process parameters and the formation and nature of heat-derived constituents, although they were able to conclude that adequate safety information is available and that existing uses are safe. Many methods are available for quantification of 5-HMF, furan, and 4-MeI, in caramel colors and foods, and in recent years more highly sensitive methods have been developed and used to quantify very low levels of these constituents (Cunha et al., 2011; Petruci et al., 2013; Wang and Schnute, 2012; Li et al., 2013; Elsinghorst et al., 2013; Sengar and Sharma, 2014; Xu et al., 2015a; Xu et al., 2015b; Wang et al., 2015; Wu et al., 2015, 2016; Cho et al., 2015). Regardless of any uncertainties in the formation of LMW substances during the manufacturing processes, there is general uniformity of composition within each caramel class and specifications exist to ensure that unintended substances are minimized. In addition, sufficient data exists on the levels of LMW substances in caramel colors and foods to adequately assess risk and ensure safe use (EFSA, 2012).

2.1. 5-Hydroxymethylfurfural (5-HMF) and furan

Licht et al. (1992c) found 5-HMF in all samples of the four classes of caramel color analyzed; the range of reported values was highly variable [700 mg/kg (ppm) −33,700 mg/kg (ppm)] within the different classes, with Class III caramel having the overall lowest concentrations (see Table 1). Concentrations of 5-HMF in batches of caramel colors III and IV produced and analyzed over a two-year period were provided by industry to EFSA for their 2004 re-evaluation. The mean level in Class II caramel was 41.6 mg/kg (range of 5–140 mg/kg); the mean in single and double strength Class IV color was 102 mg/kg (range of 48–183 mg/kg) and 88.3 mg/kg (range of 22.7–147 mg/kg), respectively (EFSA, 2012).

The occurrence of furnanic compounds has been detected in all classes of caramel color (Myers and Howell, 1992; Bononi and Tateo, 2007; EFSA, 2011). Based on single samples for Class I, II, and III caramel, levels ranged from 0.05 to 0.18 mg/kg (ppm); furan levels in Class IV caramel averaged 0.02 mg/kg with a maximum level of 0.06 mg/kg (based on 28 samples).

5-HMF is formed in the Maillard reaction as well as during caramelization, and found in a variety of foods and beverages (Murkovic and Pichler, 2006). Genotoxicity has been demonstrated *in vitro* under certain conditions, but 5-HMF is not genotoxic when tested *in vivo*. NTP studies concluded some evidence of carcinogenic activity in female B6C3F1 mice based on elevated incidences of liver tumors, but these were not considered relevant for human risk assessment. No carcinogenicity was found in rats, and whether or not 5-HMF poses a human cancer risk at typical levels of exposure may be questionable (NTP, 2010; EFSA, 2011; Abraham et al., 2011). EFSA in their review of caramel colors did not perform an exposure assessment for 5-HMF in caramel color or specifically address the risk to humans. The Panel did, however, indicate that given the potential concern, specifications should be updated to include maximum levels for this constituent.

2.2. 4-Methylimidazole (4-MeI)

4-methylimidazole can be formed by the interaction of ammonia with reducing sugars (Moon and Shibamoto, 2011). Since ammonia and reducing sugars are common components of foods, and roasting and heating are common in food processing, 4-MeI has been found many foods. It can also be found in Class III and IV caramel colors and in products containing these colors such as soy sauce, wine, dark beers, soft drinks and other foods and from the production processes used in some products such as, coffee, breads and baked goods. Current FCC, JECFA, and EU specifications limit 4-MeI to 250 mg/kg (ppm) in these classes; however, JECFA further limits 4-MeI in Class III to 200 mg/kg (JECFA, 2011b). A number of studies have been published in recent years on detection of low part per billion and sub ppb levels of 4-MeI in caramel colors and food and beverages. Wu et al., 2015,2016, using a fluorescent-based immunochromatographic assay, tested three commercial samples of caramel color of Class III and two commercial samples of Class IV. Class III caramel color contained 88.2–168.6 mg/kg 4-MeI and the Class IV samples contained 43.2 and 120.1 mg/kg 4-MeI. Levels of 4-MeI in batches of caramel colors III and IV produced over a two-year period were provided by industry to EFSA for their 2011 re-evaluation. The mean level in class III caramel was 41.6 mg/kg (range of 5–140 mg/kg); the mean in Class IV single and double color intensity strength caramel color was 102 mg/kg (range of 48–183 mg/kg) and 88.3 mg/kg (range of 22.7–147 mg/kg), respectively (EFSA, 2011). Manufacturers now have the ability to optimize the production process to consistently produce caramel colors with much lower levels of 4-MeI, some with specified limits < 15 to a maximum of 60 mg/kg, are available.

Another imidazole compound, 2-Methylimidazole (2-MeI) has also been analyzed in caramel colors and soft drinks (Petruci et al., 2013; Xu et al., 2015a, 2015b; Schlee et al., 2013). Petruci et al. (2013) reported finding no 2-MeI in commercial samples of caramel colors using capillary electrophoresis with a limit of detection of 0.22 mg/L (ppm). Likewise Schlee et al. (2013) detected no 2- MeI in 97 cola samples and 13 caramel colors from Germany and France using LC//MS/MS with a detection limit of 0.2 mg/L, and a more recent analysis by Xu et al. (2015a) of soft drinks with caramel color reported levels below the limit of quantification of 0.03 mg/L using a solid-phase extraction and amino trap column coupled with pulsed amperometric detection. Collectively, these results indicate that 2-MeI is unlikely to be present in caramel colors and beverages. As such it is not considered to pose a safety or regulatory risk in beverages or foods with caramel colors and will not be covered in this review.

2.3. 2-Acetyl-4-tetrahydroxy-butylimidazole (THI)

THI is only found in Class III caramel color. A recent study by Elsinghorst et al. (2013), reported that THI is not present in caramel color Class IV at levels above the limit of quantification of 80 μg/kg (ppb) caramel in a stable isotope dilution assay (SIDA) with a labeled ($^{13}C_6$)THI analogue. Wang et al., 2015 reported THI levels in caramel color III ranging from 1.0 to 74.3 mg/kg (ppm). Industry-provided data on THI levels in Class III caramel ranged from 2.4 to 10 mg/kg with a mean of 4.5 g/kg (EFSA, 2011).

2.4. Acrylamide

Caramel color production relies upon the Maillard reaction and acrylamide has been noted to occur as a constituent in some food and beverage products produced utilizing Maillard reactions of protein and sugars. There is a small amount of protein in starting sugars so it is not surprising that a small amount of acrylamide - less than 37 μg/kg (ppb)

- was reported in caramel color III prepared with varying amounts of ascorbic acid (Chen and Gu, 2014). However, acrylamide was not detected in one sample of Caramel color III when analyzed at a detection level of 100 μg/kg nor was it detectable in 16 commercial samples of caramel color IV at a detection level of 1 μg/kg (data provided to EFSA by the European Technical Caramel Association in EFSA, 2011), suggesting acrylamide formation is minimal in caramel colors. No data are available on acrylamide in caramel colors I and II, but the formation of acrylamide would not be expected based on the lack of nitrogen sources in the manufacturing process (EFSA, 2011). Because acrylamide was inconsistently reported to be found, and when found occurred at such low levels, acrylamide has not been identified as a safety concern with caramel colors, and it will not be discussed further in this review.

2.5. Sulfur dioxide (SO_2)

Sulfiting ingredients are used in the production of Class II and Class IV caramel colors. Sulfite in these caramel colors combines with various molecules and becomes integrated and irreversibly bound to the polymer that makes up the HMW color bodies of caramel color. For compliance to specifications, total sulfite (bound and unbound) is determined using digestive methods that break down the polymer releasing the sulfite. When a non-digestive method is used, “free” or “unbound” sulfite (SO2) is not detected. Su Sulfite-sensitive asthmatics appear to be more sensitive to residues of unbound sulfites in foods than they are to sulfited foods that contain bound sulfites. (Taylor et al., 1988). As sulfites in caramel color are bound they should have no safety implications, especially given their levels in caramel colors and finished foods; therefore sulfites are not covered in this review.

3. Intake of caramel colors and constituents

3.1. Intake of caramel colors

Only a few reports on intake of caramels colors have been published. In 2011, EFSA calculated exposures for the different classes of caramel color and noted at that time that anticipated combined exposures to all classes exceeded the group ADI of 300 mg/kg bw at the mean intake in children and at the 95th and 97.5th percentile for both adults and children and (EFSA, 2011). Anticipated exposure exceeded the group ADI at the upper percentile intakes (95th/97.5th) in the adults and children for Class I and IV colors. The anticipated dietary exposure for class II caramel was below the group ADI for both adults and children. For caramel color III, the individual ADI of 100 mg/kg bw was also exceeded for in some age groups. .EFSA, however, completed a more refined exposure assessment in 2012 based on data submitted by industry on the actual uses of caramel colors in foods as consumed and consumption data available from five countries in the EFSA Comprehensive European Food Consumption Database. This refined assessment did not include intakes for Class II since anticipated exposures were low and did not exceed the group ADI in their previous assessment (EFSA, 2012). The refined exposure intakes are summarized in Table 2. On the basis of the above estimates, high dietary exposure (95th percentile) to caramel color Class III was still found to exceed the ADI of 100 mg/kg bw in toddlers and adults in one and five countries, respectively. However the estimated combined exposure to all four caramel colors was below the group ADI of 300 mg/kg bw for all population groups both at the mean and at the higher exposure level. The results of the refined exposure estimates for Class I, III, and IV caramel colors and combined exposure to the four caramel colors were considerably lower – in some cases as much as 20 times lower - than those reported in the original EFSA opinion (EFSA, 2011).

3.2. Intake of constituents

A number of published studies have provided intake estimates for 4-MeI and THI. EFSA estimated 4-MeI exposures in children and adults from intakes of Class III and IV caramel colors based on maximum levels (MLs) permitted in the color [(250 mg/kg)(ppm)] and maximum reported levels (MRLs) reported by industry (140 mg/kg in Class III and 183 mg/kg in Class IV) and intake data available at the time of their 2011 reevaluation of caramel colors. THI intakes were also estimated using the ML of 10 mg THI/kg caramel color III. For this publication, 4-MeI and THI exposures were recalculated based on the updated intakes published in EFSA's refined exposure assessment of caramel colors (EFSA, 2012). To simplify the calculations, the upper level of the range of caramel color intake presented in Table 2 were used. For toddlers and children (1–9 years old), a mean and 95th percentile intake of 60 mg/kg bw/day and 106 mg/kg bw/day was used for Class III; a mean and 95th percentile intake of 86 mg/kg bw/day and 136 mg/kg bw/day was used for Class IV. For adults (18–64 years old), a mean and 95th percentile intake of 43 and 151 mg/kg bw/day was used for Class III; a mean and 95th percentile intake of 36 and 101 mg/kg bw/day was used for Class IV caramel color. The EFSA intake data estimated in 2011 and revised intakes are presented in Table 3. Intakes based on more refined usage data are clearly lower than were previously reported by EFSA in 2011.

The U.S. FDA recently completed an examination of 400 different foods containing caramel color for the presence of 4-MeI using liquid chromatography-tandem mass spectrometry (LC-MS/MS); the method had a limit of detection of 0.001 mg/kg (ppm) in beverages and 0.02 mg/kg for other foods (Folmer et al., 2014). This data was then used to estimate exposure to 4-MeI from foods containing caramel color based on 2007–2010 NHANES 2-Day dietary intake surveys and 2007–2008 NPD-NET-NID 10–14 day food diaries for over 5000 respondents. Depending on the survey data and exposure scenarios used, mean and 90th percentile intake ranges in 1–12 year old children were 0.03–0.06 mg/person/day and 0.08–0.13 mg/person/day, respectively. For teenage boys (12–18 years old), mean and 90th percentile intake ranges were 0.06–0.09 mg/person/day and 0.10–0.18 mg/person/day, respectively. For the U.S.population (> 2 years old), mean and 90th percentile ranges were 0.06–0.09 mg/person/day and 0.12–0.19 mg/person/day, respectively. Caramel color-containing carbonated beverages contributed to approximately 25% of 4-MEI intake in the US population over age 2, accounting for more exposure than any other source. Breads and rolls and dairy-based desserts (ice cream, pudding, yogurt) contributed 17% and 13% of the cumulative dietary exposure to 4-MeI for the U.S population aged 2 years or more (Folmer et al., 2014).

In a follow-up study, Folmer et al. (2015) estimated background intake levels of 4-MEI formed in a select group of 80 foods and beverages as a result of cooking and processing, and not from the addition of caramel colors. As in the previous assessment, 4-MeI intake was estimated using 2007–2010 NHANES and 2007–2008 NPD-NET-NID intake survey data. Depending on the survey data and exposure scenarios used, mean and 90th percentile intake ranges for the U.S. population aged 2 years or more were 0.006–0.02 mg/person/day and 0.02–0.05 mg/person/day, respectively. Coffee was the predominant source of 4-MeI exposure, and exposure to 4-MeI from cooking and processing was found to be approximately 1/3 of the exposure from food containing added caramel color.

Smith et al. (2015) estimated exposure to 4-MeI in soft drinks to assess potential cancer risk using combined NHANES data from 2003 to 2010 and analysis data of 12 beverage types sampled in California and New York. Most all beverages tested resulted in 4-MeI intakes below 29 μg/day, which is the No Significant Risk Level (NSRL) set by OEHHA under Prop 65. While some soft drinks were found to contain levels of 4-MeI that exceeded this level when these analysis were conducted in 2013, most all soft drink manufacturers are now using caramel colors with significantly reduced levels of 4-MeI (ABA, 2014; Folmer et al., 2014), and daily exposure to 4-MeI from beverages today would be expected to be lower and continue to not pose a regulatory risk.

Table 2
Summary of intake for caramel colors.

Source	Age Range	Class I	Class II	Class III	Class IV	Combined – All Classes
		(150a) mg/kg bw day	(150b) mg/kg bw day	(150c)mg/kg bw day	(150d) mg/kg bw day	mg/kg bw day
EFSA, 2012 Data presented is range of intakes from individual EU country surveys	Toddlers (12–35 mo)	Mean: 11-45 95th: 32-79	Individual color intake not estimated	Mean: 10-60 95th: 36-106	Mean: 11-86 95th: 38-127	Mean: 19-105 95th: 73-158
	Children (3–9 y)	Mean: 15-39 95th: 36-81		Mean: 20-56 95th: 43-67	Mean: 19-62 95th: 49-136	Mean: 31-83 95th: 68-160
	Adolescents (10–17 y)	Mean: 6-24 95th: 16-56		Mean: 9-33 95th: 20-86	Mean: 8-44 95th: 21-122	Mean: 12-56 95th: 28-144
	Adults (18–64 y)	Mean: 6-18 95th: 16-42		Mean: 10-43 95th: 27-151	Mean: 9-36 95th: 26-101	Mean: 15-57 95th: 41-165
	Elderly (> 65y)	Mean: 4-12 95th: 13-32		Mean: 6-30 95th: 14-82	Mean: 6-22 95th: 19-60	Mean: 9-35 95th: 24-87

Table 3
Intake of 4-MeI and THI based on EFSA intake surveys.

Constituent	MLs[a] or MRLs[b] used for intake assessment	Adults (> 18 years)		Children (1–10 years)		Source
		Mean Exposure	97.5th Percentile	Mean Exposure	95th or 97.5th Percentile[c]	
		mg/kg bw/day	mg/kg bw/day	mg/kg bw/day	mg/kg bw/day	
4-MeI						
Class III	ML - 250 mg/kg	0.015	0.074	0.005–0.076	0.027–0.189	EFSA, 2011
	MRL- 140 mg/kg	0.009	0.041	0.003–0.042	0.015–0.106	
Class IV	ML - 250 mg/kg	0.022	0.092	0.006–0.127	0.032–0.370	
	MRL-183 mg/kg	0.016	0.068	0.004–0.093	0.024–0.271	
4-MeI						
Class III	ML - 250 mg/kg	0.011	0.038	0.015	0.027	Based on refined intake data (EFSA, 2012)[d]
	MRL- 140 mg/kg	0.006	0.021	0.008	0.015	
Class IV	ML - 250 mg/kg	0.009	0.025	0.022	0.034	
	MRL-183 mg/kg	0.007	0.018	0.016	0.025	
THI						
Class III	ML- 10 mg/kg	0.0006	0.003	0.0002–0.003	0.011–0.0076	EFSA, 2011
THI						
Class III	ML- 10 mg/kg	0.0004	0.0015	0.0006	0.011	Based on refined intake data (EFSA, 2012)[d]

[a] Maximum Level (ML) allowed in color; European Union Purity Criteria.
[b] Maximum Reported Level (MRL) based on data supplied by industry (EFSA, 2011).
[c] Range lowest to highest, based on survey location and body weight of 15–29 kg.
[d] Maximum of the range reported intake data for Class III and IV caramel color in the refined assessment were used to recalculate potential intake of 4-MeI.

Cunha et al. (2011) measured 4-MeI in soft drinks and dark beer in the United States and Europe, and reported the estimated daily intake (EDI) of 4-MeI in soft drinks was 2300 ng/kg bw/day in Europe and 5700 ng/kg bw/day in the United States. Lee and Lee (2016) also analyzed 4-MeI in 144 food and beverages containing caramel color, including coffees, sauces, caramel syrups, red ginseng juice and apricot fruit juice. Reported levels were 47.6–1748.5 ng/g (ppb) in processed sauces, 64.1–1821.3 ng/g in coffee, 115.5–491.9 ng/g in caramel syrups, 91.0–854.1 ng/g in ginseng juice and 137.6–587.4 ng/g in apricot fruit juice. Based on this analysis the authors, estimated daily intake (EDI) and chronic daily intake (CDI) were highest for all types of foods at 1618.8 and 1256.8 ng/kg bw/day. An EDI based on cumulative intake from all sources was not done. EDIs averaged as low as 9–25.7 ng/kg bw/day for soy sauces to as high as 887 ng/kg bw/day for red ginseng juice; the average EDI for caramel syrup was 91.7 ng/kg bw/day.

4. Toxicology of caramel colors

4.1. Absorption, distribution, metabolism, and elimination (ADME)

4.1.1. ADME of caramel colors

No toxicokinetic studies have been published on caramel color I, II, and III, except one study with caramel color III cited in a JECFA review for which no conclusions could be drawn (Haldi and Wynn, 1951; JECFA, 1987; EFSA, 2011). However, a study with caramel color IV is available. The absorption, distribution, metabolism and elimination of a C14 radiolabeled high-molecular-weight color fraction (^{14}C-HMWF) of caramel color IV was evaluated in male F-344 rats after single and repeated oral dosing (Selim et al., 1992). The HMWF, which accounts for most of the coloring properties of caramel color, was isolated from low molecular weight constituents, and had an average molecular weight of approximately 20,000 Da (range of 15,000–125,000 Da). Approximately 5% of the HMWF test material contained LMW constituents, but the 4-MeI content was not quantified. A single oral dose was administered by gavage. Urine, feces, and CO_2 were collected over a 96-h

period. Tissue distribution of radioisotopes, including blood, brain, heart, lungs, liver, kidney, spleen, thymus, mesenteric and cervical lymph nodes, stomach (contents and tissue), small intestine, caecum, large intestine, and total carcass radioactivity were examined 4, 8, 12, 24 and 96 h after dosing. The vast majority (~90%) of the radioactivity was excreted in the feces within the first 24 h, but measurable amounts were excreted over the 96-h collection period. Approximately 1% of the administered dose was excreted in the urine, mostly in the first 24 h and only a negligible amount was found in expired CO^2. Radioactivity in tissues was mostly located in the gastrointestinal tract, but low levels were found in the liver (≤0.02%), kidneys(≤0.01%), thymus, mesenteric lymph nodes, spleen and lungs. Overall, less than 0.5% of the administered dose remained in the body by Day 4.

In a multiple dose experiment, rats were administered unlabeled HMWF in drinking water for 13 days followed by a single oral gavage dose of 2.5 mg/kg ^{14}C-HMWF on day 14 (Selim et al., 1992). Urine, feces, and CO^2 were collected over the next 96 h and tissues as listed above were collected following sacrifice on day 4. As with the single dose exposure, over 99% of the administered dose was eliminated within 96 h and mostly in feces by 24 h; 1–2% and only negligible amounts were seen in urine and expired air, respectively. The gastrointestinal tract had the highest amounts of labeled material. Consistent with the single dose exposure, radioactivity was found in mesenteric lymph nodes, indicating some absorption into the lymphatic system. Discoloration of the mesenteric lymph nodes suggest that this was primarily HMWF, but the authors did not rule out the possibility that some LMW constituents were also absorbed. Measurable concentrations of labeled material in the brain in both the single and repeated dose experiments also suggested some LMW substances crossed the blood-brain barrier. Only one other toxicokinetic study (with caramel color III) by Haldi and Wynn (1951) was referenced in the JECFA (1987) and EFSA (2011) reviews but no definitive conclusions could be drawn from this study. In general, while toxicokinetic studies on caramel colors are limited, especially regarding bioavailability of LMW substances, it is generally believed that LMW constituents can be absorbed.

4.1.2. ADME of 4-MeI

Studies are available for 4-MeI in a number of animal species including rats. Uptake of 4-MeI has been shown to be rapid in several animal species with intravenous, intraperitoneal, or oral gavage reaching peak plasma concentrations in under 5 h. In rats, about 90% of a single intraperitoneal dose of 4-MeI was excreted unchanged in urine beginning about 30 min after injection; uptake was greatest in the intestines, followed by the blood, liver, stomach, and kidney (Hidaka, 1976). Likewise in ewes, half of an oral dose of 4-MeI was absorbed in under 30 min and more than 98% was excreted unchanged. Metabolites of 4-methylimidazole were not detected using high-performance liquid chromatography (Karangwa et al., 1990). In goats and heifers, the mean residence time of 20 mg/kg 4-methylimidazole administered orally or intravenously was about 5 h 4-Methylimidazole was distributed mainly to the liver, kidney, and lung. 4-Methylimidazole and its metabolites were excreted mainly in urine, but also in milk and feces. Metabolites identified included 5-methyl hydantoin, 2-methylhydantoic acid, and urea (Nielsen et al., 1993). In male F344 rats, a 50 mg/kg dose of 4-MeI by oral gavage was mainly excreted unchanged in the urine within 48 h. Only one minor hydrophilic metabolite was present in urine and in plasma, and elimination of radioactivity via fecal and biliary excretion or respiration was negligible (Yuan and Burka, 1995). NTP (2007) conducted single dose intravenous (10 mg.kg) and gavage (10, 50, and 1000 mg/kg) toxicokinetic studies with 4-MeI in male and female F344/N and B6C3F1 mice. 4-Methylimidazole was rapidly absorbed when administered by gavage. The absorption half-life values ranged from 5 to 23 min in rats and 2–5 min in mice and declined with dose. Elimination half-life values ranged from 65 to 499 min in rats and from 21 to 87 min in mice but increased with dose in both sexes of both species. In pregnant and postpartum cows and mice, 4-methylimidazole was excreted in milk following oral administration (Morgan and Edwards, 1986).

Fennell et al. (2014) investigated the metabolism of 4-MeI *in vitro* in rat and mouse lung and liver microsomes and S-9 fractions, and *in vivo* in rats and mice. 4-MeI was not oxidized and no metabolites were detected by HPLC analysis in rat or mouse lung and liver microsomes or S-9 fractions. Male and female F-344 rats and B6C3F1 mice were administered [14C] 4-MeI at doses of 50 and 150 mg/kg by gavage and urine, feces, and exhaled CO2 and volatiles were collected for 48 h. In rats, the major route of elimination was via urine, with 79–89% of the radioactivity in urine, 3–9% in cage rinse, 2–4% in feces, 1–2% exhaled as CO2, 1–2% in carcass, and less than 0.1% as exhaled volatiles. In mice, 41–70% of the administered dose was recovered in urine, 4–12% in feces, 18–34% in cage rinse, 2–4% exhaled as CO2, and 1–3% in carcass, and 0.1% or less as exhaled volatiles. The majority of the radioactivity (71–88%) in urine was present as unchanged 4-MeI in both rats and mice with only limited oxidative metabolism and conjugation. Additional radioactive peaks were detected and characterized as 4-hydroxymethylimidazole, two other oxidized products, possibly hydroxylated on the 2- and 5-positions, and a glucuronide of 4-hydroxymethylimidazole. Overall there was no indication that 4-MeI is metabolized to reactive species.

4.2. Genotoxicity of caramel colors

The current weight of evidence indicates that caramel colors do not pose a genotoxic risk to humans. Any positive genotoxic findings in bacterial cell mutation, micronucleus, mouse lymphoma and mammalian cytogenetic assays generally occurred only at high does *in vitro* where no exogenous metabolic enzymes were included in the assays. Key studies that have been used to assess potential genotoxic risk are summarized in Table 4.

Early studies that examined potential genotoxicity of caramel colors reported some positive findings including induction of mutations in *Salmonella typhimurium*, unscheduled DNA synthesis in human cells, induction of sister chromatic exchange in diploid human fibroblasts and CHO cells, and chromosomal damage in Chinese hamster lung and ovary cells (Aeschhbacher, 1986; Stich et al., 1981; Ishidate et al., 1984; Kawachi et al., 1980; Yu et al., 1984, 1984; Jensen et al., 1983; Stich et al., 1982; Ishidate and Yoshikawa, 1980; Sasaki et al., 1980). These were mostly weakly positive results at high concentration levels that were eliminated in the presence of metabolic activation. Artifactual effects due to the use of high concentrations of colors in culture media and/or uncharacterized colors may also have contributed to some to the effects observed in some assays. Other reports at the time found no mutagenicity or clastogenicity in a range of caramel colors tested (Ashoor and Monte, 1983; Kawachi et al., 1980), which further complicated the overall interpretation of the genotoxicity findings.

A number of *in vitro* and *in vivo* genotoxicity studies have since been published in well characterized caramel colors that provide sufficient evidence to conclude that caramel colors are of no concern with regards to potential genotoxicity. Allen et al. (1992) tested 15 batches of caramel color representing all four classes in 5 standard strains of *Salmonella typmhimuruim* in a bacterial mutation assay (Ames test) with and without S9 activation and none of the caramels demonstrated mutagenic activity. In this study, two batches of Class II and III caramels were also tested *in vitro* for chromosomal damage in Chinese Hamster Ovary (CHO) cells. No clastogenic activity was reported under the conditions in this study. Adams et al. (1992) reported on the results of three short-term *in vitro* tests and one *in vivo* test in Caramel color I. The results of the bacterial mutation assay and *in vivo* mouse micronucleus assay showed no evidence of genotoxic activity. Results from both a chromosome aberration and mouse lymphoma assay indicated some genotoxic activity but only in the absence of S-9 at high dose levels. Brusick et al. (1992) also tested Caramel colors III and IV in a bacterial mutation assay, a yeast gene conversion assay (*Saccharomyes cerevisiae*),

Table 4
Summary of genotoxicity and cytotoxicity studies of caramel colors.

Caramel Color	Test System	Test Objects	Maximum Concentration	Result	Reference
Class I	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	20 mg/plate	Negative[a]	Allen et al., 1992
	Ames (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	10 mg/plate	Negative[a]	Adams et al., 1992
	Ames (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, Wp2uvrA	5 mg/plate	Negative[a]	Harigae et al., 2000a
	Chromosome Aberration (*in vitro*)	Chinese hamster ovary (CHO)	10 mg/ml	Positive - significant increase in chromosomal damage only at highest dose without S-9 activation; no effects with S-9 and at lower doses	Adams et al., 1992
	Cell Mutation (*in vitro*)	Mouse lymphoma L5178Y cells	9/8 mg/ml	Positive dose-related increases in mutant frequency without activation; metabolic activation eliminated mutations at lower doses with reduced, but significant increases at highest levels, only	Adams et al., 1992
	Mouse CD-1 micronucleus (*in vivo*)	Bone marrow cells (erythrocytes)	5000 mg/kg bw dose	Negative	Adams et al., 1992
Class II	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	20/5 mg/plate	Negative[a]	Allen et al., 1992
	Chromosome Aberration (*in vitro*)	Chinese hamster ovary (CHO)	5 mg/ml	Negative[a]	Allen et al., 1992
Class III	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	20 mg/plate	Negative[a]	Allen et al., 1992
	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	50 mg/plate	Negative[a]	Brusick et al, 1992
	Gene conversion assay	*Saccharomyes cerevisiae Strain D4*	20 mg/plate	Negative[a]	Brusick et al, 1992
	Chromosome Aberration (*in vitro*)	Chinese hamster ovary (CHO)	5 mg/ml	Negative[a]	Allen et al., 1992
	Chromosome Aberration *(in vitro*)	Chinese hamster ovary (CHO)	5 mg/ml	Positive - significant increase in chromosomal damage without S-9 metabolic activation only; abolished with S-9 activation	Brusick et al., 1992
	Mouse (CD-1) micronucleus (*in vivo*)	Bone marrow cells (erythrocytes)	1.05/3.5 g/kg	Negative	Brusick et al., 1992
Class IV	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	10 mg/plate	Negative[a]	Allen et al., 1992
	Ames test (*in vitro*)	*Salmonella typhimurium* TA 98, TA100, TA1535, TA1537, TA1538	50 mg/plate	Negative[a]	Brusick et al, 1992
	Gene conversion assay	*Saccharomyes cerevisiae Strain D4*	20 mg/plate	Negative[a]	Brusick et al, 1992
	Chromosome Aberration (*in vitro*)	Chinese hamster ovary (CHO)	5 mg/ml	Negative without S-9 metabolic activation	Brusick et al., 1992

[a] With and without metabolic activation with rat S-9.

a chromosome aberration assay in CHO cells and in an *in vivo* mouse bone marrow micronucleus assay. Negative results were seen in the Ames and yeast assays in both classes. A weak clastogenic effect was seen in Caramel color III in CHO cells but this was abolished with S-9 activation; this caramel color was also negative in the micronucleus assay. Caramel color IV was not clastogenic in CHO cells, with or without activation.

While the results of some studies reported in the literature have found sporadic positive results for some samples of caramel color *in vitro*, *in vivo* assays have produced negative findings. Positive results with *in vitro* bacterial cell mutation, mouse lymphoma, and chromosomal aberration assays occurred mainly at high doses where no exogenous metabolic enzymes were included. The ability of normal metabolism to deactivate potential genotoxic activity of caramel color minimizes or eliminates carcinongenic potential, which is further supported by negative results in *in vivo* genotoxicity studies and carcinogenicity studies in animals. Overall evidence indicates that caramel colors are non-genotoxic, a conclusion also supported by the EFSA Panel in their re-evaluation of caramel colors (EFSA, 2011).

4.3. Caramel color I: toxicological studies and authoritative reviews

4.3.1. Toxicological studies

Only a few studies have examined the short-term and sub-chronic toxicology of caramel color I. Female Wistar rats were fed class I caramel color in the diet at 0, 15, or 30% for 8 weeks followed by a 4 week recovery period; the highest dose was equivalent to 30 g/kg body weight. Diarrhea was observed in all caramel treated groups. Food efficiency was decreased (dose not specified) but growth rate was normal compared to controls. No effects were seen on hematological endpoints. Relative caecal weights increased but were normal after the 4-week recovery. Discoloration of the mesenteric lymph nodes was observed but diminished over the course of the recovery period. No other gross or microscopic pathological changes were reported (Sinkeldam and van der Heyden, 1976 unpublished report cited in JECFA, 1986).

Harigae et al. (2000b) reported on a 28-day study with a caramel sample meeting the specifications of Class I caramel color. Doses of 0 (water), 0.5, 1, and 2 ml/kg bw/day (equivalent to 0.67, 1.33, and 2.66 g/kg bw/day, respectively) were orally administered to 5 male and female Crj:CD (SD) IGS strain rats for 28 days. No deaths or clinical signs of toxicity were noted in either sex in any treatment group, and no treatment-related effects were reported on body weight, food consumption, urinalysis, ophthalmology, blood chemical analysis or organ weights. Females in all groups showed a decrease in Activated Partial Thromoplastin Time (APTT) when compared to control groups; these decreases in APTT, a measure of blood clotting time, were within historical ranges and not attributable by the authors to caramel color exposure. Increases in lymphocytes were also significantly elevated in females at the 0.5 ml/kg bw/day and 2 ml/kg bw/day groups, but all values were within historical ranges and not attributable to exposure. Occasional histopathological findings were reported (e.g., vitreous inclusions in proximal renal tubular epithelial cells), but the authors concluded the none of the effects observed were related to caramel color exposure and the non-toxic dose was 2 ml/kg bw/day (equivalent 2.66 g/kg bw/day). The EFSA review panel in their re-evaluation of caramel colors agreed with these findings (EFSA, 2011). No chronic/carcinogencity or reproductive and/or developmental toxicity studies have been published.

4.3.2. Authoritative reviews

At the thirteenth and fifteenth meetings of JECFA (1970, 1972), the Committee concluded that caramel color I was a natural constituent of the diet and was acceptable as an additive. An ADI “not limited” was allocated at the fifteenth meeting. In subsequent meetings based on the available safety data and chemical characterization, JECFA reiterated their opinion that a numerical ADI was not needed, and an ADI of “not specified” continues to be been in place. In the 2011 EFSA reevaluation of caramel colors, while a specific ADI was not allocated to Class I caramel, it was included in the Group ADI of 300 mg/kg bw set for all classes of caramel color.

4.4. Caramel color II: toxicological studies and authoritative reviews

4.4.1. Toxicological studies

The pivotal study to establish a safe daily intake of Caramel color II was conducted in 1985 and published by MacKenzie et al. (1992a). Five groups of 20 male and 20 female weanling Fischer F-344 rats were given caramel color II in drinking water at dose levels of 0, 4, 8, 12, or 16 g/kg bw/day for 13 weeks. Body weights and food and water intakes were recorded weekly. Hematological examinations, blood chemistry, and urinalysis were performed during the seventh week and at terminal sacrifice. At necropsy, organs were weighed and examined macroscopically. Histopathological examinations were conducted on all animals in the control and high-dose groups and on any animals from the low- and mid-dose groups that died or were sacrificed during the course of the study.

A dose-related decrease in body weight and food consumption was observed in all treatment groups, most prominently at the 12 and 16 g/kg bw/day dose levels between week 7 and 8, but these body weight decreases were attributed to food withdrawal, anesthesia, and blood sampling at week 8. Males receiving 12 and 16 g/kg bw/day had significantly lower mean body weights than controls by weeks 7–13 and weeks 4–13, respectively. Females treated with 8, 12, and 16 g/kg bw/day had significantly lower mean body weights than controls by weeks 7–13. In addition, caramel color II caused significant dose-related reductions in fluid intake in both sexes.

Some treatment-related clinical pathology changes were noted, but these were sporadic, within normal control ranges, and not considered to be of toxicological importance. There was a dose-related reduction in urinary volume and pH, and an increase in urine specific gravity; however it has been shown in previous short- and long-term studies, that when rats are given high doses of caramel color in drinking water, they conserve body water by excreting reduced quantities of urine with increased solute concentrations (MacKenzie et al., 1992a).

Slight, dose-related increases in absolute and relative kidney weights and full and empty caecum weights, and pigment staining was observed in the gastrointestinal tract and mesenteric lymph nodes, but no significant histopathological changes were observed in these or any other tissues. The investigators concluded that the reduced body weights, food and fluid intakes, and increased kidney weights were due to water imbalance reflecting poor palatability of the caramel solutions rather than toxic effects of caramel color itself, and determined the NOAEL for Class II was 16 g/kg bw/day. MacKenzie et al. (1992a) discussed the palatability issue of caramel color in drinking water, noting that caramel colors in concentrated form have a bitter taste which rats likely find unpalatable when these colors are added at high concentrations in drinking water. This results in an immediate reduction in water intake that can be sustained over time. Generally, rats fed caramel color in drinking water fail to increase their fluid intake as they grow compared to controls. To maintain constant dose levels per unit body weight, an increase in concentration of caramel color in drinking fluid is necessary which further augments the palatability issue. Both JECFA and EFSA agreed with this conclusion in their independent reviews (JECFA, 1986; EFSA, 2011).

No chronic/carcinogenicity studies have been published with Caramel color II. Likewise, no studies on reproductive and/or developmental toxicity have been published, but based on developmental and reproductive studies conducted on Class III and IV colors, the risk of reproductive harm with this class of caramel color is not a concern.

4.4.2. Authoritative reviews

At the 29th meeting of JECFA (1986), the Committee concluded

that caramel color II was sufficiently different than the other classes and warranted a separate evaluation to establish an ADI, but the data at the time was insufficient. In a later review, JECFA allocated an ADI of 160 mg/kg bw based on the 13-week subchronic study No Observed Adverse Effect Level (NOAEL) of 16 g/kg bw/day and a safety factor of 100 and supporting genotoxicity data (JECFA, 2000). At the time, the JECFA committee was informed that the volume of production of Class II caramel color represented less than 1% of all the total production of caramel colors, and mainly used in distilled spirits (e.g., rum, whiskey, and brandy), although uses in other foods was known. The Committee concluded that the decreased body weight gain and renal hypertrophy observed in rats were a consequence of the reduced consumption of food and fluids attributed to poor palatability of caramel drinking water solutions. This and the pigmentation of the mesenteric lymph nodes and enlargement of the caecum were not of toxicological significance. Although the EU Scientific Committee for Food (SCF) included Class II caramel within the ADI of 200 mg/kg bw it had established for Class IV caramel, the 2011 EFSA reevaluation of caramel colors included Class II caramel color in the Group ADI of 300 mg/kg bw set for all classes of caramel color. No new safety data has emerged since the last JECFA and EFSA evaluations that would impact the conclusions and ADI's established by these groups.

4.5. Caramel color III: toxicological studies and authoritative reviews

4.5.1. Toxicological studies

Acute and short-term studies have been conducted on Caramel color III and summarized by JECFA (JECFA, 1986). The caramel colors used in many of these studies were not always well characterized chemically or consistent with the specifications of this class of caramel as commercially produced today. Nonetheless, early studies demonstrated that Class III caramel color induced lymphocytopenia in rats and mice (Evans et al., 1977; Gaunt et al., 1977) when ingested at high levels ($\geq$3%) in the diet. Additional studies, including subchronic and chronic studies, were conducted to investigate this effect and the possible underlying mechanism(s), and it was subsequently determined that 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), an imidazole component in Caramel color III, was responsible for this effect (Kroplein et al., 1985; Conway and Paine, 1988; Sinkeldam et al., 1988). Many more studies are now available on caramel color III and comprehensive summaries can be found elsewhere (JECFA, 1986; EFSA, 2011; Houben and Penninks, 1994). This update will focus only on the key studies and findings related to the mechanism by which THI affects the immune system and other studies relevant to establishing safe intakes of Class III caramel.

Two batches of Caramel color III were tested in a 13-week subchronic study in male and female F-344 rats (MacKenzie et al., 1992b). One batch of caramel color contained THI at a levels of 10 mg/kg color (Low-THI CC) and the other a THI level of 200 mg/kg color (High-THI CC); the low THI caramel was representative of a commercially available Class III caramel. Based on a 4-week range-finding study, doses of 0, 10, 15, and 20 mg/kg bw/day Low-THI CC or 20 mg/kg bw/day of High-THI CC were given in drinking water. Males receiving 15 or 20 g/kg bw/day Low-THI CC or 20 g/kg High-THI CC and females given 20 g/kg of either Low- or High-THI CC had lower body weights than controls. Body weight gains were affected in some groups, and water and food intake were reduced in all treatment groups, effects that were attributed to poor palatability of the treatment solutions. A 13-week paired feeding study of caramel color III in drinking water supported the conclusion that poor palatability was the contributing factor in the decreases in body weight gain, fluid and food consumption observed (Sinkeldam et al., 1988 referenced in JECFA, 1986). Urinalysis findings were inconsistent across dose groups and sex but decreased urinary volume and increased specific gravity were found in all treated groups. There were no consistent treatment-related alterations in blood chemistry or other urinalysis endpoints and any changes noted were not associated with macroscopic or microscopic pathological alterations. Hematological changes included lower total white blood cell and lymphocyte (absolute and percentage) counts, and higher absolute and percentage neutrophil counts at 2 and/or 6 week in animals given 20 g/kg bw High-THI CC but these changes returned to normal by the termination of the study. Total white blood cell counts, lymphocyte counts, and neutrophil counts were not significantly different in the 20 g/kg Low THI CC or at lower dose levels, with the exception of transient but statistically significant lower white blood cell and lymphocyte counts in male rats receiving 10 g/kg bw/day – an effect not seen at 15 or 20 g/kg bw/day.

Dose-related increases were observed in absolute and relative caecum and kidney weights in animals of both sexes that were not associated with any histopathological changes, and attributed to reduced water intake and osmotic effects of high caramel color intake. There was also an accumulation of caramel color III in the intestinal tract and mesenteric lymph node with no concurrent tissue damage. A dose-related decrease in the absolute weight of the thymus was also observed with both Low- and High-THI CC groups, which reached statistical significance at the top-dose in males treated with Low- and High-THI CC and at the top-dose in females given with High-THI CC. These organ weight changes and any other differences in absolute and relative organ weights were considered consequences of body weight decreases and were not considered to be of toxicological significance by the authors, a conclusion reinforced by experts at JECFA or EFSA in their independent reviews. Based on these results, the no-observed-adverse-effect level (NOAEL) for Low-THI CC was considered to be 20 g/kg body weight (MacKenzie et al., 1992b).

Longer-term studies with caramel color III are also available, which include complete histopathology of treated animals, and based on these chronic studies, it can be concluded that this class of caramel color does not pose a carcinogenic risk. Wistar rats were fed diets containing 0, 1, 3, or 6% caramel color III (equivalent to 0, 0.5, 1.5, and 3.0 g/kg bw/day) for 2 years (Evans et al., 1977). The test material was described as straight ammonia catalyzed “half open-half closed caramel and specifications were not available, including the level of THI and 4 MeI. Significant decreases in body weight and body weight gain were observed in males at all dose levels; similar decreases were seen in females but were not statistically significant. Contrary to what has been reported in shorter-term studies of caramel colors administered in drinking water, water intake was not affected in this study where caramel color was administered in the diet. Most organ weights were not different from control when corrected for body weight. Relative caecal, brain, thyroid, and kidney weights showed various statistical differences from control in some male and female treatment groups. Lymphocytopenia was reported at week 13 in both males and females receiving 3% caramel, but only in males at this dose level by week 52. A significant reduction in leucocyte counts compared with controls in both males and females receiving 6% Class III caramel color was associated with lymphocytopenia in the early part of the study. This effect was sustained until week 80 and 52 in males and females, respectively. There was no evidence of a carcinogenic effect based on histopathology.

The potential for carcinogenicity was examined by Maekawa et al. (1983) in male and female F344 rats after 2 years of treatment of an ammonia-process caramel in drinking water at concentration levels of 0%, 1%, and 4% (equivalent to 0, 0.5, and 2.0 mg/kg bw/day); THI levels were reported to be less than 25 mg/kg caramel color. A variety of tumors developed in both treatment groups and the control group. The incidence of tumors in the treatment groups were not significantly higher than controls, except for pituitary tumors in males at 4%; however, this was likely attributed to the normal variability of incidence of spontaneous pituitary tumors and not directly to treatment with caramel color III.

Three groups of B6C3F1 mice (50 animals/sex/group) were given drinking water containing 0, 1.25, or 5% caramel color III (equivalent to approximately 0, 2.1, or 8.4 g/kg bw/day) for 96 weeks followed by

an 8 week recovery period without caramel color (Hagiwara et al., 1983). THI levels were below 25 mg THI/kg color. No consistent differences in body weight, body weight gain, or water intake were noted compared with controls, nor were any clear treatment related differences in urinalysis or organ pathology. Statistically significant elevations of the total leucocyte counts were observed in males, but were considered by the authors to be within the normal range for B6C3F1 mice. No significant differences in tumor incidence were found compared to controls.

4.5.2. Reproductive and developmental studies

Developmental toxicity studies have been conducted in CD-1 mice, Wistar rats, and Dutch-belted rabbits given caramel color III by gavage at doses of 0, 16, 74, 345, or 1600 mg/kg bw on days 6–15 of gestation (mice and rats) or 6–18 days of gestation (rabbits). Dams were caesarean sectioned on Days 17, 20, and 29 in mice, rats, and rabbits, respectively. No treatment-related effects were observed in the number of implantation sites and resorption sites, or on maternal and fetal survival in any species. The urogenital sites of dams were examined and found normal compared to controls. No treatment-related effects were noted on fetal weights and the number of abnormalities of either soft or skeletal tissues of the test groups did not differ from those occurring spontaneously in controls (Morgareidge, 1974). Class III caramel color does not pose a developmental or reproductive risk based on these study results (Tierney, 1980).

4.5.3. Immunotoxicity of caramel color III and THI

The toxicity studies conducted with Class III caramel color reviewed above showed a clear reduction in total white blood cell counts in rats and mice due to reduced lymphocyte counts, which was attributed to the effects of THI. In a 1994 review, Houben and Penninks (1994) provided an excellent overview of the research that lead to the discovery of THI as the component responsible for this effect, and summarized animal and human research on the effects of Caramel color III and THI on the immune system, including studies on immune function and potential mechanism(s) of action. Early studies demonstrated that the reduction in lymphocyte counts observed in rats and mice generally occurred when the animals were fed diets low in Vitamin B6 (Evans et al., 1977; Gaunt et al., 1977; Conway and Paine, 1988). This lymphopenia was shown to be prevented by the addition of pyridoxine in the diet (Sinkeldam et al., 1988). Caramel color III was also shown *in vitro* to inhibit pyridoxal kinase, an enzyme in the brain and other tissues that is critical to both the accumulation and release of non-phosphorylated vitamin B6 vitamers (pyridoxine, pyridoxal, and pyridoximine). These non-phosphorylated vitamers enter the cell where they are phosphorylated and trapped intracellularly as Vitamin B6. Inhibition of pyridoxal kinase not only impacts cellular vitamin B6 levels but also accelerates the release of non-phophoryated vitamers. Spector and Huntoon (1982) further demonstrated that Caramel color III contained an inhibitor of pyridoxal kinase that could be removed from Caramel color III under certain conditions with dialysis or addition of activated charcoal. The addition of Caramel color III to culture medium increased the release of [H_3]Vitamin B6 from brain slices. The antipyroxidine factor that competitively inhibits pyroxidine kinase and impacts retention of B6 in the brain resulting in lymphopenia was later isolated and identified by Kroplien et al. (1985) as THI, an imidazole component in Class III caramel color.

Subsequently it was shown that orally administered THI can cause a reduction in the number of circulating B- and T-lymphocytes in rats (by a single dose within 16 h), but the effect was reversible when THI exposure ceased (Phillips and Paine, 1990). Similarly other studies demonstrated that THI can reduce the number of $CD4^+$ (T-helper cells) and $CD8^+$ (T-suppressor/cytotoxic cells) lymphocytes in the peripheral blood in mice (Gugasyan et al., 1995). THI increased the number of mature $CD4^+CD8^-$and $CD4^-CD8^+$ (single positive) cells and decreased $CD4^+CD8^+$ (double positive) cells in rats leading to the hypothesis that THI had an effect on the migration of T-cells from the thymus which has the important role of providing mature T-cells to peripheral blood. A later study by Gugasyan et al. (1998) examined the ability of THI to inhibit cell export from the thymus. Female BALB/c mice were exposed to THI in drinking water (50 mg/L) for 5 days. Marked changes in thymus were reported as evidenced by effects on thymocyte subsets (i.e., increase in number of single positive and decrease in number of double positive thymocytes) as previously reported. The finding that THI prevented the final step in T-cell release from the thymus provided more convincing evidence that this was a key contributory factor to the immunotoxic effects seen with high doses of Caramel color III and THI.

A 4-week study of THI in drinking water was conducted by MacKenzie et al. (1992b) in which groups of 20 rats/sex were dosed for 4 weeks with 0, 8, 64 ppm THI (equivalent to 0, 0.9, 7.2 mg THI/kg bw/day); 10 rats/sex were allowed a 2-week recovery period after 4 weeks of treatment. Other groups (10 rats/sex/group) received, 0, 1, 2, 4, 16, 32 ppm THI (equivalent to 0, 0.1, 0.2, 0.5, 1.9, 3.7 mg THI/kg bw/day). Decreases in body weight and body weight gains occurred across all treatment and control groups and were attributable to fasting for blood and urine collection. No consistent treatment-related alterations in blood chemistry or urinalysis were found and any changes were not associated with any macroscopic or microscopic pathological changes. Hematological changes noted in treated animals included lower total white blood cell and lymphocyte (absolute and percentage) counts, and higher absolute and percentage neutrophil counts at 2 and 4 weeks; however, these changes returned to normal by the third day post-treatment recovery period (8 and 64 mg/kg bw/day recovery groups) and remained normal at study termination. A NOAEL for THI was not reported by the authors based on this 4-week study; however, based on a 13 weeks study with Caramel color III with known levels of THI, the authors reported an NOAEL of 0.38 mg THI/kg bw/day and 0.12 mg THI/kg bw/day for male and females rats, respectively.

Studies have also been conducted on the effects on the overall functionality of the immune system in rats exposed to Caramel color III and THI. Houben et al. (1992a, 1993) exposed rats to caramel color III (0.4 or 4%) or THI (5.72 ppm) in drinking water for 28 days; rats were fed a diet low, but not deficient, in Vitamin B6. Rats had impaired resistance in a *Trichinella sprialis* infection model, decreased natural killer cell activity of splenic cells, and impaired antibody responses to T-cell-dependent antigens. In addition, caramel color exposed rats had an increased capacity to clear *Listeria monocytogenes* bacteria after intravenous infection. The effects of Caramel color III on the immune system of mice with an adequate Vitamin B6 status (~12 ppm in diet) was also examined (Thuvander and Oskarrsson, 1994). In this study, female BALB/C mice were exposed to a commercial caramel color III preparation with a low THI content (< 25 ppm) at 0%, 2 or 10% concentrations in drinking water for 9 weeks. This corresponded to caramel color intakes equivalent to 0, 3 or 15 mg/kg bw/day, respectively and THI dose levels of 0, 110 and 380 μg/kg bw/day, respectively. Lymphopenia was not observed but there were significant reductions in relative numbers and proportions of $CD4^+$ and $CD8^+$ cells at both dose levels. The proliferative response of splenocytes to the mitogens LPS and con A was significantly reduced at the 2% dose but not the 10% dose. The reason for the lack of effect at the higher dose level was not clear but may have been related to disturbances in the subset populations. No changes were observed in interleukin-2 activity, natural killer cell activity in splenocytes, or humoral antibody response to the influenza antigen PR8. The authors concluded that the 2% dose level (~3 mg/kg bw/day caramel color equivalent to 110 μg/kg bw/day of THI) was the lowest observable adverse effect level in this study. For perspective, this level of THI is approximately 20 times higher than the current ADI for Caramel color III of 200 mg/kg (JECFA, 1986), which leads to a daily THI exposure of 5 μg/kg body weight if the caramel color meets the JECFA specification for THI of 25 mg/kg or roughly 100 times higher when considering the updated EFSA ADI of

100 mg color/kg assuming the color meets the EU specification of THI of 10 mg/kg.

Gobin and Phillips (1991) examined the biological significance of a decrease in circulating lymphocytes resulting from THI treatment. They exposed F344 rats to THI at 0, 1, 10 and 50 mg/L in drinking water (equivalent to 0.1, 1, and 5 mg/kg bw) along with a vitamin B deficient diet. No changes in thymus or adrenal weight were observed, but there was a dose dependent increase in spleen weight that was not attributable to the total number of lymphocytes. There was a slight thinning of the thymic cortex with an increased number pyknotic cells, mainly engulfed by macrophages. A 1 mg/kg bw dose was the lowest that caused a decrease in peripheral blood lymphocyte (B and T) counts but not in the spleen in Vitamin B deficient rats. Immune functional assays after 7 days of treatment at 1 mg/kg bw were examined. Natural killer (NK) cell activity against YAC-1 target cells (a murine T cell lymphoma) in the spleen was not affected but NK activity increased in peripheral blood due to an increase in large granular lymphocytes. B cell function as measured by responsiveness to lipopolysaccharide (LPS) was inhibited although there was no effect on serum antibody IgG and IgA titers to KLH production. T-cell function was impaired as measured by 3 separate assays: mitogen-induced proliferation; delayed-type hypersensitivity; and host versus graft (popliteal lymph node) reaction. These results clearly further demonstrated some immunosuppressive effects of THI in Vitamin B6 deficient rats.

Houben et al. (1992b, 1992c) conducted a human study with 24 healthy males 65 years of age or older with a marginally deficient Vitamin B6 status. Blood pyri-doxal-5-phosphate (PLP) levels and their erythrocyte glutamate-oxaloacetate transaminase stimulation were used to determine Vitamin B6 status. Baseline B6 levels were stabilized prior to administration caramel color III in pudding at a total daily dose of 200 mg caramel color/kg body weight (i.e., the JECFA ADI). Two caramel batches were tested, one with a THI content of 143 ppm (sufficiently higher than the JECFA specification limit of 25 ppm for THI) and the other with a THI content of 23 ppm (i.e., just below the current JECFA specification limit). These levels, according to the authors, were sufficient to justify conclusions for Class III caramel color. A control group consumed pudding with no caramel color. The test materials were consumed for 7 day and blood was collected for hematology and B6 status on Days 3 and 7. A washout period followed where blood was collected on Day 10, 14, 21, and 33. All subjects were considered marginally deficient in Vitamin B6 during the course of the study. There were no significant differences in mean blood lymphocyte numbers between the control group and the test groups on any day during the study. In addition, no differences were seen in lymphocyte numbers over the course of the study in each individual. The authors compared their results in humans to doses of THI that induce lymphopenia in rats with low, but not deficient levels of B6. The lowest level exposure of Caramel color III in rats that produces lymphopenia corresponds to an estimated daily THI intake of 20 μg/kg bw day. In the current study, a THI intake of 28.6 μg/kg bw/day was estimated for the high-THI caramel color III group; this level of intake did not impact the number of lymphocyte in marginally B6- deficient men. Based on these results the authors concluded that humans may be less sensitive to the effect of THI compared to rats, and these differences in sensitivity may in part be due to toxicokinetics.

The underlying mechanism by which THI causes immunosuppression is now believed to be primarily mediated by the immunomodulatory molecule, sphingosine-1-phosphate (S1P) (Hla, 2004; Schwab et al., 2005; Yu et al., 2010; Ohtoyo et al., 2016). S1P, a lysophopholipid, acts through G-protein coupled receptors on cell surfaces to regulate immune cell movement between lymphoid tissues and blood and other body tissues. Mice receiving 7.5 mg THI/kg bw/day in drinking water for 3 days caused lymphopenia and suppressed immunity concurrent with a 100-fold increase in S1P levels in lymphoid tissues. This was found to be due to sphingosine-1-phosphate lyase (S1PL) inhibition, an enzyme that is involved in the degradation of S1P in the cell (Schwab et al., 2005). A single dose of THI, administered by gavage to male rats at levels of 10 and 100 mg/kg bw/day decreased blood lymphocyte counts in a dose-related manner relative to increased splenic S1P and plasma THI concentrations. These studies demonstrate that increases in cellular S1P can inhibit the release of immunocompetent cells from lymphoid organs leading to immunosuppression. Recently, Ohtoyo et al. (2016) identified a bioactive THI derivative (A6770) that is a smaller molecule than THI but has the same lymphopenic effect via S1PL inhibition. Its detection in plasma following oral administration of THI suggests that A6770 may be an important metabolic intermediate of THI.

4.5.4. Authoritative reviews

JECFA last reviewed Caramel color III at their 29th meeting in 1985 (JECFA, 1986). At that time, mutagenicity studies were negative or equivocal and there was no evidence of carcinogenicity based on long-term studies. The Committee based their evaluation on the 90-day study by MacKenzie et al. (1992b) with a no observed adverse effect levels of 20 g/kg bw that contained 10 mg/kg THI (15 mg/kg color on a solids basis). An ADI of 200 mg/kg bw (150 mg/kg bw on a solids basis) was allocated for Caramel color III.

EFSA in their most recent evaluation (EFSA, 2011) noted that the pattern of toxicity in 90-day studies was similar to that seen in longer-term studies, and there was no evidence of genotoxicity or carcinogenicity. More specifically, the lymphocytopenic and immunomodulatory effects observed in the subchronic studies were due to THI together a deficiency of pyridoxine (Vitamin B6), and these effects appeared to be transient in nature. As with the JECFA review, in the pivotal study by MacKenzie et al. (1992b) rats dosed with up to 20 g/kg bw/day Caramel color containing either 15 mg THI/kg caramel or 295 mg THI/kg caramel showed no dose-related lymphocytopenia at the lower THI level. The Panel did note that effects on the immune system were seen in mice with adequate vitamin B6 status fed caramel color with THI levels less than 25 mg THI/kg color (Thuvander and Oskarrsson, 1994). Based on this and the effects seen in other studies conducted by MacKenzie et al. (1992b), Sinkeldam et al. (1988) and Houben et al. (1992a), the Panel concluded that a NOAEL for the lymphocytopenic effects of THI in vitamin B6 rats lies in the rang of 120–400 μg THI/kg body weight. The Panel also acknowledged and concurred that humans are less sensitive to Class III caramel and THI based on the human study conducted by Houben et al. (1992c). While the Panel considered that the Tuvander and Oskarsenn study should not be used as a pivotal study for the purposes of risk assessment, they 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 established, within the group ADI for all caramel colors of 300 mg/kg bw, an ADI of 100 mg/kg bw/day for Class III caramel color.

The NOAEL for THI was found to be 380 μg/kg bw for male rats and 120 μg/kg body weight for females (MacKenzie et al., 1992b) The maximum concentration of THI allowed in Caramel color III by JECFA specifications is 25 mg/kg color (JECFA, 1986). On the basis of the 200 mg/kg ADI for Caramel color III; this is equivalent to 5 μg THI/kg body weight providing a margin of safety for THI based on this study (NOAEL/ADI) ranging from 24 to 76. Since some studies indicate humans may be less sensitive than the rat to the effects of THI (Houben et al., 1992b; Houben and Penninks, 1994), this margin of safety would expected to be adequate.

4.6. Caramel color IV: toxicological studies

Class IV caramel color has been extensively tested in short-term, subchronic and chronic studies (JECFA, 1986; EFSA, 2011). The assessment of safety has always acknowledged the presence of trace amounts of the imidazole compound 4-MeI. 4-MeI, specifically had been shown in a series of studies to have appreciable neurotoxicity in

animals, notably tremorogenic and convulsive effects (Nishie et al., 1969, 1970; Patey et al., 1985). Based on these studies and studies with Class IV caramel, safe intake levels were established and specification were put in place limiting the concentration of 4-MeI in the caramel color IV. Although Class IV caramel has been safety used in foods for decades, an NTP study of 4-MeI (NTP, 2007) that found neoplasias in the lungs of mice re-opened questions on safety. This review will focus on critical studies conducted for assessing the safety of caramel color IV, including studies on 4-MeI that are especially relevant to this class of caramel color.

4.6.1. Subchronic and chronic studies

Two 13 week subchronic studies conducted in F344 rats independently determined a no observable adverse effect level for Caramel color IV of 30 g/kg bw/day. In the first study by Heidt and Rao (1980), males and female rats were give 0, 15, 20, 25, or 30 g/kg bw/day in drinking water. Dose related decreases in body weight gain, food consumption and water consumption were found at all dose levels, effects that were attributed to the palatability of the drinking water solution. All treatment groups had significantly lower blood urea nitrogen (BUN) and alkaline phosphatase levels at 45 and 90 days, and total serum protein was reduced in all treated groups by 90 day. Reduced urine volumes and increased urine specific gravity was also seen in treated animals. Treatment related changes in absolute and relative organ weights in males and females were noted, notably decreases in thymus and spleen and increases in kidneys and caecum. None of these organ weight changes were accompanied by histopathological changes except for pigmentation in the mesenteric lymph nodes commonly seen with treatment of caramel colors and thickening of the caecal mucosa and tunical muscalaris.

In a second study by MacKenzie et al. (1992c) similar doses of 0, 15, 20, 25, and 30 g/kg bw/day were administered. Not unexpectedly, water consumption, food consumption and body weights were decreased in a dose-related manner. Similar changes were seen in blood chemistry (i.e., decrease in BUN, alkaline phosphatase and total serum protein), urinalysis endpoints (i.e., decreases in urine volume and increases in specific gravity), and organ weights. All of these changes in both studies were attributed to the administration of large amounts of caramel color and were considered compensatory changes of no toxicological relevance. Similar effects were seen in other studies with various classes of caramel color and both JECFA and EFSA experts in their reviews have attributed the effects to administration of large doses of caramel color and poor palatability of test solutions. Decreases in BUN may have been due to residual unreacted glucose (~30%) typically found in the caramel color; similar effects have been documented in animals studies given high amounts glucose or other sugars.

Caramel color IV was administered to male and female F344 rats in drinking water at doses of 0, 2.5, 5, 7.5, and 10 g/kg bw/day for 1 year or 0, 2.5, 5, and 10 g/kg bw/day for 2 years (MacKenzie et al., 1992c). Results of the 1 and 2 year studies in rats produced consistent findings with the 13-week studies previously reported. Males but not females had significantly lower body weights at 10 g/kg bw/day in the 1 year study, but significantly lower body weights were seen in both males and females at the 5 and 10 g/kg bw/day dose groups in the 2-year study. Dose related decreases often significant in food and water consumption in both sexes were seen throughout the studies. Also consistent with the 13 week studies were changes is serum chemistry, more specifically decreases in BUN, serum creatinine, and alkaline phosphatase levels in both sexes at levels of 5 g/kg bw/day and higher. Likewise urine volumes were decreased with concomitant increases in urine specific gravity. None of these changes were accompanied by histopathological changes in the kidney or liver. Changes in organ weights were seen in kidneys and caecum that were significant in both males and females at doses of 5 g/kg bw/day and higher. By two years, chronic progressive nephropathy and bile duct hyperplasia was frequently observed in all treated groups at comparable incidences. Reduced water intake is known to affect renal function with compensatory hypertrophy as were seen in these studies. Also observed in the 1 year chronic and 2 -year carcinogenicity studies were pigmentation at dose levels of 5 g/kg bw/day and higher in the mucosa of the caecum and colon and macrophages of the mesenteric lymph nodes. By 2 years of exposure, pigmentation was also seen in the perineum lymph nodes and submucosa of the ilieum, and colon in addition to caecum. No tissue reactions or hyperplasia were seen microscopically in any of these tissues where pigmentation occurred in either studies. Chronic progressive nephropathy and bile duct hyperplasia in the 2-year study were seen across all treatment and control groups and considered incidental. Chronic progressive nephropathy is a spontaneous age-related disease that occurs in high incidence in the strains of rat commonly used in preclinical toxicology studies, exhibiting a male predisposition (Hard and Khan, 2004). Commonly occurring spontaneous neoplasms of F-344 rats were observed all groups but there were no differences in incidence rates of benign or malignant tumors between treated and control groups and no rare or unusual tumors were found. The observed pigmentation, and other spurious changes in hematological and urinary values, clinical chemistry, organ weights and pathology were considered common incidental findings consistent with high dose caramel exposure and not considered to be of toxicological important. The NOAEL in both the 1- and 2-year studies was considered to be 10 g/kg bw/day.

In a 2 year mouse study, Caramel color IV was administered to male and female B6C3F1 mice in drinking water at doses of 0, 2.5, 5, and 10 g/kg bw/day (MacKenzie et al., 1992c). No consistent differences in mean body weights were observed although food consumption was lower for males at 10 g/kg bw/day. Mice in the 5 and 10 g/kg bw/day groups consumed less water but this was statistically significant only at 10 mg/kg bw/day. Treatment-related gross and microscopic changes consisted of staining in the gastrointestinal tract (ileum, caecum, and colon) and diffusely red mesenteric lymph nodes at gross autopsy that appeared congested on microscopic examination in both treated and control mice that died during the study or were killed at the end of the study; the incidence of congestion was only significantly increased at the highest dose compared to controls in male mice that died or were killed during the study or females killed at study termination. A variety of benign and malignant tumors were found in both treated and control mice, typical of what occur spontaneously in B6C3F1 mice. There were no treatment-related neoplastic changes attributed to Caramel color IV and no rare or unusual tumors were found. As in rats, the changes observed were typical of what has been observed previously with Caramel color IV and other classes of caramel color at high dose levels. These included reduced body weight, reduced fluid and food consumption, decreased urine volume, increased urine specific gravity, increased kidney weights, caecal enlargement and pigmentation in the lower gastrointestinal tract and mesenteric lymph nodes. They were all considered to be adaptive changes to feeding high concentrations of caramel color in drinking water, which is associated with palatability. The NOAEL in the study was 10 g/kg bw/day.

4.6.2. Developmental and reproductive studies

Three reproductive studies were cited as part of the safety assessments conducted by JECFA and EFSA (JECFA, 1987; EFSA, 2011). Fifteen male and female Wistar rats were given 0 or 10% solutions of Class IV caramel for 100 days and then mated. Animals of the F1 generation (25 males and 25 females) were weaned and again administered 0 or 10% caramel color until day 100. Caramel color IV did not affect the number of litters born and the number of pups/litter. No influence food consumption, growth, hematology, gross pathology, or histopathology of the F1-generation at 100 days of age was observed (Haldi and Wynn, 1951).

Six different samples of Class IV caramel color, three double strength and three single strength colors each containing a different level of 4-MEI (ranging between 200 and 850 mg/kg) were tested in a reproduction study in rats (Til and Spanjers, 1973). [Note: The

maximum intake of 4-MEI in this study was estimated by the EFSA Panel to be approximately 6 mg/kg bw/day, based on consumption of a diet containing 10% single strength Class IV caramel with a 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.] Ten male and 20 female weanling Wistar rats were assigned to each of 12 treatment groups that included 2 control groups fed two different stock diets. At week 12, the rats were mated for a 3-week mating period after which 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 clubfeet, cleft palate and hydrocephalus. After weaning, the dams were sacrificed 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% single color intensity strength + 600 mg 4-MEI/kg) there was a slight increase in mortality at birth. No developmental effects were found (Til and Spanjers, 1973).

In a third reproduction study conducted with F344 rats (12 animals/sex/group), Class IV caramel color with concentrations of 0, 10, 15, 20, or 25% in drinking water (equivalent to doses of 8–28 g/kg bw/day) was administered for 21 days prior to mating until lactation (Tierney, 1980). The two highest dose groups of both generations had a higher incidence of soft stools compared to controls, and dose-related decreases in body-weight gain were observed in animals of the F0 and F1 generation, that were typical of effects seen in studies with high doses of caramel color, but not considered to be adverse. Mating, pregnancy, and fertility rates were comparable for all groups. Pups in the 25% dose group showed a higher incidence of alopecia compared with controls, and generalized poor condition during the last 7 days of suckling. Alopecia was also seen in the pups in the 20% group, and in the F0 animals in these two dose groups, which was attributed by the authors to the increased grooming activity of the animals in an effort to remove test substance/fecal material. The number of implantation sites and of pups alive at days 0, 4, and 21 of lactation in the 20% dose groups were significantly lower than control values. Litter size was decreased at the 15, 20, and 25% dose groups, but there was no dose-related trend as these parameters were not statistically different at the 25% dose group when compared to control. Some hematological changes, including decreased blood urea nitrogen and dose-related increases in absolute and relative weights of the liver, kidneys, and caecum were observed at doses of 15% and higher dose groups, not unlike incidental high-dose effects considered non-toxic in other subchronic and chronic studies. Since the findings in the pups in the 25% dose group were attributable to the general condition of the dams and body contamination with fecal/test material rather than a specific developmental or toxic response, and since the decreases in implantation sites and numbers of live pups seen in the 20% group did not show a dose response relationship, the highest dose level tested - 25% equivalent to 25–30 g/kg bw/day for female rats - was considered a NOAEL (EFSA, 2011).

Developmental studies in mice, rats and rabbits were conducted by Morgareidge (1974) and reported in JECFA (1987). The doses employed were 0, 16, 74.3, 345, and 1600 mg/kg bw in all 3 species. Pregnant albino CD1 mice were administered Class IV caramel color 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. Pregnant Wistar rats were gavaged with doses of Caramel color IV on days 6–15 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. The number of gross external, visceral, or skeletal abnormalities did not differ from those occurring spontaneously in controls. Lastly, Pregnant Dutch-belted female rabbits were administered Class IV caramel color by gavage on days 6–18 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, 1974). Collectively, these studies indicate that Caramel color IV does not pose a reproductive or developmental risk at maximally tolerated doses.

4.6.3. Studies with 4-Methylimidazole (4-MeI)

4-MeI has been known for some time to be a constituent of both Caramel color III and IV. As previously stated, 4- MEI was shown to cause convulsions at acutely toxic doses in various species of animals by different routes of exposure. 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). For many years, 4-MeI was not believed to be of concern for humans since any potential levels in food were far below known neurotoxic levels and limits on 4-MeI concentrations in Caramel color IV assured that levels in foods would be at safe levels. However in the early 2000's, NTP published studies on toxicology and carcinogenicity of 4-MeI that opened up new questions on the safety of use of Caramel color IV that required further investigation. The key science and current risk associated with these constituents is summarized below.

4.6.3.1. Genotoxicity and cytotoxicity. The National Toxicology Program completed a battery of genotoxicity assays with 4-MeI (NTP, 2004). 4-MeI was negative in the *Salmonella typhimurium* mutation assay when tested in strains TA97, TA98, TA100, and TA1535, with and without exogenous activation with rat or hamster liver S-9 at doses up to 10 mg/plate. In vivo bone marrow micronucleus assays were conducted in male F344/N and B6C3F1 mice injected intraperitoneally (3 times at 24 h intervals) with 25, 50, and 100 mg/kg bw. Bone marrow cells were collected and polychromatic erythrocytes (PCEs) were scored for frequency of micronucleated cells. Micronucleus assays were also conducted in peripheral blood cells collected from male and female B6C3F1 mice treated for 14 weeks at doses of 4-MeI ranging from 625 to 10,000 mg/kg bw/day in feed. 4-MeI was negative in both the short-term *in vivo* bone marrow microcleus test and 14-day peripheral blood micronucleus test.

Norizadeh et al. (2016a,b) also investigated genotoxic and cytotoxic effects of 4-MeI using a chromosomal aberration assay and mitotic index (MI) in Swiss albino mice. Mice were treated with 4-MeI at 0 (untreated control), 100, 130, and 160 mg/kg bw for 12 and 24 h treatment periods and bone marrow cells were analyzed. A dose dependent increase in the percentage of chromosome aberrations was found at all concentration levels after 12 exposures and at the highest level after a 24 h treatment period. 4-MeI was decreased compared to controls at the highest concentration at 12 h of exposure and at all levels at 24 h of exposure. They concluded 4-MeI is genotoxic and cytotoxic in the mouse. These investigators also investigated the potential anti-genotoxic and anti-cytotoxic effects of 4-MeI (100, 130, and 160 mg/kg) against a 240 mg/kg dose of ethyl methanesulfonate (EMS) using chromosome aberration and mitotic index assays in bone marrow cells of Swiss albino mice. Contrary to the results reported above, 4-MeI at all concentrations for 12 h treatments reduced chromosomal aberrations and at 130 and 160 mg/kg concentrations for 24 h treatment period increased chromosomal aberrations induced by EMS; the reductions and increases however were not statistically significant. An intraperitoneal injection of 4-MeI at doses of 100, 130, and 160 mg/kg combined with EMS (240 mg/kg dose) showed mitotic index was decreased at 100 and 130 mg/kg for 12 h and 130 mg/kg for 24 h treatment periods when compared to EMS positive controls, but again

the differences were not statistically significant. Because 4-MeI was not able to reduce chromosomal aberrations induced by EMS, the authors concluded that 4-MeI may not be anti-genotoxic and anti-cytoxic in bone marrow cells.

Bu et al. (2015) investigated the effects of 4-MeI on bone marrow mesenchymal stem cells (MSCs) *in vitro*. MSCs have the potential to differentiate into osteoblasts, chondroblasts, adipocytes and myoblasts and thereby play an important role in hematopoiesis. Tumor cells are known to secrete cytokines, chemokines and growth factors that recruit and activate MSCs to the site of tumors *in vivo*. The authors speculate that 4-MeI can have effects on MSCs and cancer progression. They measured cell proliferation in osteoblast and adipocytes, apoptosis, cell cycle, gene expression of hematopoietic cytokines, migration and differentiation in control and treated cell, *in vitro*. High concentration of 4 MeI (≥150 μg/ml) affected bone marrow MSCs viability but lower concentrations (≤100 μg/ml} had no effect on viability or on the other endpoints examined. Overall concentrations ≤100 μg/ml had no significant effects on the biological characteristics, and the authors concluded that low concentrations of 4-MeI may not have toxic effects on bone marrow MSCs and the anemia and weight loss seen in animals exposed to 4-MeI is not likely due to an effect on these cells. Further investigation of long term exposure of 4-MeI *in vivo* on cytotoxic effects on MSCs would be needed to validate this finding.

Because alveolar/bronchiolar tumors were found in mice in the NTP studies, there was a possibility that DNA reactive intermediates could be formed by lung specific metabolites through metabolism directly in the lung. Beevers and Adamson (2016) evaluated 4-MeI in a bacterial reverse mutation (Ames) assay using five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535, TA 1537) with both rat (F344N) and mouse (B6C3F1) liver and lung S9 as a source of exogenous activation. A lung S-9 source was included to examine the possibility that DNA reactive intermediates could be formed by lung specific metabolites. No induction of mutation was observed using either rat or mouse liver or lung S-9 when tested at concentrations of 5–5000 μg/plate. These results are consistent with most other genotoxicity assays which support a conclusion that 4-MeI is not genotoxic, and a non-genotoxic mechanism for the carcinogenicity of 4-MeI in mouse lung is likely. However, the precise mechanism by which lung tumors are formed in mice is still unknown.

4.6.3.2. 4-MeI carcinogenicity. In the NTP carcinogenesis studies (NTP, 2004, NTP, 2007, Chan et al., 2008), male and female F344/N rats were exposed to 4-methylimidazole in feed for 2 years at doses equivalent to approximately 0, 625, 1250, or 2500 ppm (male rats) and 0, 1250, 2500, or 5000 ppm (female rats) equivalent to an average daily dose of 30, 55, or 115 mg/kg bw/day to males and 60, 120, or 260 mg/kg to females. Mean body weights of males in the 1250 and 2500 ppm groups and females in the 2500 and 5000 ppm groups were less than those of the control groups throughout the study. Clonic seizures, excitability, hyperactivity, and impaired gait were observed primarily in 2500 and 5000 ppm females. The incidence of mononuclear cell leukemia in 5000 ppm 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 ppm males and 5000 ppm females.

Male and female B6C3F1 mice were also fed diets containing 0, 312, 625, or 1250 ppm (male and female mice) equivalent average daily doses of approximately 40, 80, and 170 mg/kg bw/day to males and females for 2 years. Mean body weights of males and females decreased over the course of the study at all doses were lower by the end of the study period. The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in 1250 ppm males, and alveolar/bronchiolar adenoma or carcinoma (combined) in 1250 ppm males and 625 and 1250 ppm females were significantly greater than those in the control groups. The incidence of alveolar epithelium hyperplasia was significantly increased in 1250 ppm females.

Under the conditions of these 2-year studies, NTP concluded there was no evidence of carcinogenic activity of 4-MeI in male F344/N rats exposed to 625, 1250, or 2500 ppm. 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. This conclusion has been challenged by some scientists (Murray, 2011; Haseman, 2013), especially in light of the overall lack of genotoxicity found in *in vitro* and *in vivo* studies and a possibility that the observed tumors may be forming through a non-genotoxic mode of action.

One interesting observation seen in the NTP studies was a significantly decreased incidence of 1) pituitary (par distalis) gland adenoma, 2) benign complex or malignant pheochromocytoma (combined) of the adrenal gland in males, 3) pituitary (pars distalis) gland and clitoral gland adenoma, 4) mammary gland fibroadenoma, and 5) uterine stromal polyps in females. Notably, 4-MeI was associated with a 25-fold decrease in the incidence of mammary tumors among high dose females. These decreases, noted by NTP, could not be attributed to loss of body weight alone. Murray (2011) speculated that 4-MeI, while showing increases in certain tumor types also exhibits tumor preventive activity in the rat based on the NTP results. Reduced body weight offers a partial explanation for the reduction in tumors, but does not appear to be the primary cause of the decreased tumor incidences, indicating that 4-MeI itself may possess an ability to prevent tumor formation.

Haseman (2013) conducted a detailed analysis of all NPT mouse-lung tumor-only carcinogens with regards to the category of evidence, i.e., “no evidence of carcinogenicity”, “clear evidence of carcinogenic activity”, “some evidence of carcinogenicity”, and “equivocal evidence of carcinogenicity”, that NTP assigns to each carcinogen based on tumor type, incidence, and other factors. The purpose of the analysis was two-fold: 1) to examine whether of not the ”clear evidence” interpretation made by the NTP for lung tumors in the 4-MeI study was consistent with the call made by NTP for lung tumor effects in mice in other similar studies; and 2) to present suggestions for the NTP to consider in the preparation of future Technical Reports to enhance consistency in the interpretation of experimental results. NTP defines “clear evidence of carcinogenic activity is demonstrated by studies showing a dose -related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.” NTP defines “some evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related incidence of neoplasm (malignant, benign or combined) in which the strength of the response is less than that required for clear evidence”. The evaluation found six “clear evidence” mouse lung carcinogens – five were inhalation studies and one was a gavage study. Based on an overall evaluation of lung tumor effects and genotoxicity data, Hasemen found the lung tumor effect observed in male mice in the 4-MeI study was weaker than those seen for male mice in any of the other “clear evidence” lung-only carcinogens classified by the NTP. These compounds clearly showed much stronger lung tumor effects (in both males and females) than did 4-MeI. Similarly, the lung tumor effects observed in female mice in the 4-MeI study were weaker than those seen for female mice in any of the six “clear evidence” NTP lung-only carcinogens in females classified by NTP (tetranitromethane, cobalt sulfate heptahydrate, vanadium pentoxide, cumene, 1-bromopropane, and ridelline). An evaluation of the incidence of lung tumors in male B6C3F1 mice in NTP studies concluding “some evidence” of carcinogenic activity compared to the incidences of tumors in the 4-MeI study showed the tumor response was more matched to these

compounds. Genotoxicity data was also examined with these compounds, but the author noted there was no obvious differences between the genotoxic potential of compounds showing “clear evidence” versus “some evidence” of lung carcinogenicity. However, 4-MeI is not genotoxic, whereas several compounds showing “clear evidence” of lung carcinogenicity were assigned overall positive or mixed (positive and negative) designations for genotoxicity. Based on his analysis, Hasemen concluded that the tumor effects of 4-MeI were more consistent with NTP's past classifications of “some evidence” and were weaker than all of the NTP “clear evidence” calls for male or female mouse lung carcinogens. In the author's opinion, the proper category of evidence in the 4-MeI study should have been “some evidence” rather than “clear evidence”.

4.6.4. Authoritative reviews

JECFA reviewed long term/carcinogenicity studies in rats and mice with caramel color IV that conformed to current specifications, including limits of 4-MeI, and established an ADI of 200 mg/kg bw based on a NOAELs of 10 g/kg bw/day and a safety factor of 50. A smaller safety factor was applied in view of ancillary human data, which demonstrated no adverse effects other than laxation with exposure up to 18 g/day (JECFA, 1986). JECFA has not reviewed caramel color since the publication of the NTP carcinogenicity study of 4-MeI.

EFSA, however, did conduct a comprehensive review of Class IV caramel color fully considering the results of the NTP carcinogenicity studies with 4-MeI. The EFSA Panel noted that Class IV caramel color exhibited a similar toxicological profile to that of other caramel color classes and long-term toxicity and carcinogenicity were of not concern. With regards to 4-MeI, acute toxicity was not of concern at the maximum restricted level in Class IV caramel color of 250 mg/kg. The Panel also concluded that the carcinogenic effect of 4-MeI in mice was thresholded, based on the lack of genotoxicity of 4-MEI and the high incidence of alveolar/bronchiolar neoplasms that occur spontaneously in B6C3F1 mice. Based on 90 day and 2 years studies of Class !V and other classes of caramel color, as well as a reproductive study in Caramel color IV providing a NOAEL of 25–30 g/kg bw/day, EFSA included Class IV caramel color within the Group ADI of 300 mg/kg bw/day for all classes of caramel color.

The International Agency for Research on Cancer (IARC) has classified 4-MeI as a group 2B human carcinogen – “possibly carcinogenic to human” – based on sufficient evidence in experimental animals from the NTP studies (IARC, 2013). Although NTP has concluded that there was “clear evidence of carcinogenic activity” in male and female mice based on an increased incidence of lung tumors, and the substance is under consideration for review for the Report on Carcinogens (RoC) (Federal Register, 2013), to date they have not included 4-MeI in the RoC (NTP, 2013). The Office of Environmental Health Hazard Assessment (OEHHA) in California listed 4-MEI as a carcinogen in January 2011 with a proposed no significant risk level of 29 μg per person per day (OEHHA, 2011). The listing was based on NTP's findings of clear evidence of carcinogenicity in its studies showing the development of lung cancer in male mice and female mice from 4-MEI exposure. Human cancer potency was estimated from dose-response data for lung tumors in male mice exposed to 4-MEI and took into account body-size differences between humans and experimental animals. The NSRL was derived from the human cancer potency.

The United States Food and Drug Administration has stated that it has “no reason to believe that there is any immediate or short term danger presented by 4-MeI at levels expected in food from the use of caramel coloring”, based analytical testing and intake assessments for 4-MeI. The FDA stated the NTP carcinogenicity study as having been conducted at doses that “far exceed” current estimates of human exposure from the consumption of caramel coloring in foods and soft drinks, and has not recommended consumers change their diets based on concerns about 4-MeI (FDA, 2014).

5. Conclusions

Caramel color has been used in foods and beverages for over 150 years, and is globally regulated as a color additive. The 4 distinct classes of caramel color (I, II, III, and IV) are well characterized and each have specifications that take into account manufacturing process parameters including reactants such as ammonia and sulfites that can give rise to constituents that have toxicological significance for evaluating safety. Among these are low molecular compounds, including THI, 4-MeI, furan, and 5-hydroxymethyl-2-furfural (5-HMF).

Toxicokinetic data shows that the majority of ingested color is not absorbed, but excreted in feces within 24 h. Only negligible amounts (< 1%) are seen in urine and expired air, and radiolabeled material found in mesenteric lymph nodes indicates some absorption into the lymphatic system. There is further evidence that a small fraction of some constituents of the low molecular weight fraction of caramel color is absorbed. Studies with 4-MeI, in particular, indicate the major route of elimination is via urine as unchanged 4-MeI. Caramel colors, including 4-MeI, have been extensively tested for genotoxic potential in a variety of *in vitro* and *in vivo* assays. While the results of some studies reported in the literature have found sporadic positive results *in vitro* where no exogenous metabolic enzymes are present, normal metabolism appears to deactivate any potential genotoxic potential. Negative results in *in vivo* assays support a conclusion caramel colors are not genotoxic.

When tested at high levels, all of the caramel color classes have a similar effect on body weight that is due mainly to reduced water intake caused by poor palatability. Other effects observed at high doses, including changes in organ weights, clinical chemistry, urinalysis, and hematology, are considered compensatory changes of no toxicological relevance. No Observable Adverse Effect Levels in subchronic, chronic, and reproductive and developmental toxicity studies are generally seen at the highest doses tested. An Acceptable Daily Intake (ADI) of “not specified” has been established by JECFA for Class I, and ADIs of 160 mg/kg bw for Class II caramel color and 200 mg/kg bw/day for both Class III and IV caramels have also been established. One notable exception is the lymphocytopenic and immunomodulatory effects observed in the subchronic studies of Class III Ammonia Caramel which are due to THI together a deficiency of pyridoxine (Vitamin B6); however, these effects are transient in nature, and a NOAEL has been established for THI. Contrary to the conclusions of JECFA, an EFSA Panel established a higher group ADI of 300 mg/kg bw/day for all caramel colors; but, for the purposes of their risk assessment, applied an additional safety factor or 2 along with the default factor of 100 to the NOAEL of 20 mg/kg bw/day identified in a 90-day study with Caramel color III to establish an ADI of 100 mg/kg bw for this caramel class. According to the EFSA report, this means that within the group ADI of 300 mg/kg bw established for the four caramel colors, only 100 mg/kg bw of this 300 mg/kg bw/day can be made up by Class III Ammonia Caramel. Current restrictions on the level of THI in Class III caramel color as well as exposure estimates that indicate intakes are generally within the ADI provide further assurance of the safe use of this color.

The exposure to 4-MeI, a potential carcinogenic constituent, at levels present in Class III and IV Caramel colors are not expected to be of concern for the reasons that: 1) 4-MeI does not appear to be genotoxic or metabolized to a reactive metabolite, and; 2) doses of 4-MeI that produce carcinogenicity in mouse lungs far exceed current estimates of human exposure from the consumption of caramel coloring and 4-MeI in foods and soft drinks by several thousand-fold. Likewise, SO_2 and sulfites as found in a bound form in Class II and IV caramel colors do not pose a safety risk. Other constituents identified in caramel colors, namely acrylamide, furan, and 5-HMF, do not appear to pose a carcinogenic risk at levels present in caramel colors as long-term studies support a conclusion that caramel colors are not carcinogenic. In summary, safe usage levels have been established for caramel colors, as manufactured under current specifications and consumed for their

intended use in foods and beverages. Normal intakes of caramel colors are below established ADI's across age groups, and estimated combined exposure to the four classes of caramel color are below the group ADI of 300 mg/kg/day established by EFSA. On-going monitoring of constituents of concern in caramel color is recommended to ensure safe use levels and exposure intakes are maintained.

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