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SCIENTIFIC OPINION

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Flavouring Group Evaluation 76 Revision 2 (FGE.76Rev2): Consideration of sulfur-containing heterocyclic compounds, evaluated by JECFA, structurally related to thiazoles, thiophenes, thiazoline and thienyl derivatives from chemical group 29 and miscellaneous substances from chemical group 30 evaluated by EFSA in FGE.21Rev5

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Abstract

The Panel on Food Additives and Flavourings (FAF) was requested to consider the JECFA evaluations of 28 flavouring substances in the Flavouring Group Evaluation 76 (FGE.76Rev2). Twenty-one of these substances have been considered in FGE.76Rev1. Seven substances could not be evaluated, because of concerns with respect to genotoxicity. New genotoxicity data have been provided for 4-methyl-5-vinylthiazole [FL-no: 15.018] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032], which are representative substances of [FL-no: 15.005] and [FL-no: 15.029, 15.030, 15.130 and 15.131], respectively. The Panel concluded that the concern for genotoxicity is ruled out for [FL-no: 15.018 and 15.005]. The concerns for gene mutations and clastogenicity are ruled out for [FL-no: 15.032, 15.029, 15.030, 15.130 and 15.131]. *In vitro*, [FL-no: 15.032] induced micronuclei through an aneugenic mode of action. The available *in vivo* micronucleus study was not adequate to rule out the concern for potential aneugenicity *in vivo.* The Panel compared the lowest concentration resulting in aneugenicity *in vitro* with the use levels reported for [FL-no: 15.032]. Based on this comparison, the Panel concluded that the use of [FL-no: 15.032] at the maximum reported use levels does not raise a concern for aneugenicity. Based on structural similarity, for the remaining four substances [FL-no: 15.029, 15.030, 15.130 and 15.131], an aneugenic potential may also be anticipated. Individual genotoxicity data are needed to establish whether they have aneugenic potential. The Panel agrees with JECFA conclusions for 24 flavouring substances 'No safety concern at estimated levels of intake as flavouring substances' when based on the MSDI approach. For six substances, more reliable information on uses and use levels are needed to refine the mTAMDI estimates. For 15 substances, use levels are needed to calculate the mTAMDIs. For [FL-no: 15.109 and 15.113], information on the actual stereochemical composition is inadequate and the conclusion reached for the named substances cannot be applied to the materials of commerce.

Keywords: Sulfur-containing heterocyclic compounds, thiazoles, thiophenes, thiazoline and thienyl derivatives, FGE.21, FGE.76, FGE.93

Requestor: European Commission

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1. Introduction

1.1. Background and terms of reference as provided by the requestor

The use of flavourings in food is regulated under Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008[1] on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union list of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[2]. The list includes flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[3].

On 24 October 2013, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and processing Aids (CEF) adopted the following three opinions:

- an opinion on Flavouring Group Evaluation 76Rev1 (FGE.76 Rev1)
- an opinion on Flavouring Group Evaluation 21Rev4 (FGE.21 Rev4)
- and an opinion on Flavouring Group Evaluation 93Rev1 (FGE.93 Rev1)

In the opinion on FGE.76Rev1, the Panel requested *in vivo* genotoxicity data concerning three thiazolines [FL-no: 15.029, 15.030 and 15.032] as the representative flavouring substance 15.032 is considered to have genotoxic potential *in vitro*. Additionally, the Panel noted the presence of a terminal conjugated bond in the substances [FL-no: 15.018] and [FL-no: 15.005] which raises genotoxicity concern and therefore requested additional data on them.

In the opinion on FGE.21Rev4, the Panel concluded that for two substances [FL-no: 15.060] and [FL-no: 15.119] additional genotoxicity data were required.

In the opinion on FGE.93Rev1, the Panel concluded that for two substances [FL-no: 15.130 and 15.131] additional genotoxicity data were required.

The applicant has submitted additional data in response to these three EFSA evaluations.

Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information and, depending on the outcome, proceed to the full evaluation on these flavouring substances in accordance with Commission Regulation (EC) No 1565/2000.

1.2. Interpretation of the terms of reference

In FGE.76Rev1 (EFSA CEF Panel, 2013a), 3-thiazolines: 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029], 4,5-dimethyl-2-ethyl-3-thiazoline [FL-no: 15.030] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] were considered by the CEF Panel to have genotoxic potential *in vitro*, and therefore, the CEF Panel decided that the Procedure should not be applied to these three flavouring substances until adequate *in vivo* genotoxicity data become available.

Additionally, the CEF Panel noted the presence of a terminal conjugated double bond in the substances 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018] which raised concern for genotoxicity. The CEF Panel concluded that the Procedure should not be applied to these two substances either until additional data become available.

The three substances [FL-no: 15.029, 15.030, 15.032] evaluated in FGE.76 are supporting substances for [FL-no: 15.060 and 15.119] in FGE.21 and for [FL-no: 15.130 and 15.131] in FGE.93.

For the 3-thiazolines in subgroup B-II (2,4-dimethyl-3-thiazoline [FL-no: 15.060] and 2-isobutyl-3-thiazoline [FL-no: 15.119]), the CEF Panel concluded in FGE.21Rev4 that, in the absence of further genotoxicity data, the Procedure could not be applied to these substances (EFSA CEF Panel, 2013e).

[1] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, pp. 34–50.

[2] Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, pp. 1–161.

[3] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, pp. 8–16.

In FGE.93Rev1, the CEF Panel concluded that 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131], which are 3-thiazolines, are structurally similar to two 3-thiazolines in FGE.21 [FL-no: 15.060, 15.119] for which the CEF Panel has expressed a concern for genotoxicity, and accordingly the Procedure should not be applied to these two substances until adequate genotoxicity data become available (EFSA CEF Panel, 2013b). In the same opinion (FGE.93Rev1), the CEF Panel identified 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] from FGE.76 as a supporting substance with respect to genotoxicity, for the group of 3-thiazolines.

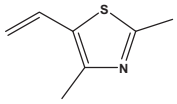
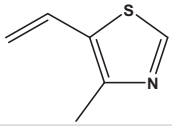
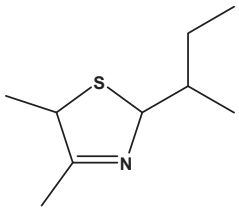
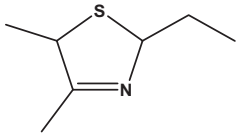
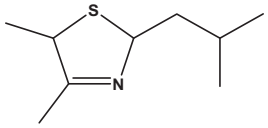
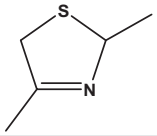
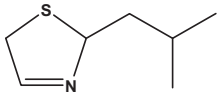
Since genotoxicity data have been submitted for [FL-no: 15.032] and based on structural similarity, the Panel considered that the substances 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131] originally allocated to FGE.93 can be evaluated in the present revision of FGE.76 (FGE.76Rev2). Therefore, the genotoxicity data for [FL-no: 15.032] can support the evaluation of [FL-no: 15.029, 15.030, 15.130, 15.131] in FGE.76Rev2 and of [FL-no: 15.119, 15.060] in FGE.21Rev6.

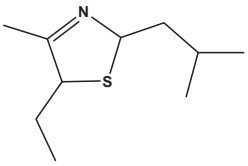
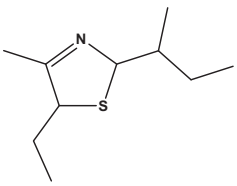
Genotoxicity data have been submitted also for 4-methyl-5-vinylthiazole [FL-no: 15.018], representative substance for [FL-no: 15.005] in FGE.76Rev2.

Following the submission of these new data, the European Commission requests EFSA to carry out a safety assessment in accordance with Commission Regulation (EC) No 1565/2000[3].

Table 1 summarises the flavouring substances whose genotoxicity evaluation is covered by the two representative substances [FL-no: 15.018 and 15.032] in FGE. 76Rev2, FGE.21Rev6 and FGE.93Rev1.

Table 1: Substances in FGE.76Rev2, FGE.21Rev6 and FGE.93Rev1 for which the genotoxicity evaluation is based on the representative substances [FL-no: 15.018] or [FL-no: 15.032]

FGE	FL-no JECFA no	Chemical name	Structural formula	Representative substance	Group
FGE.76	15.005 1039	2,4-Dimethyl-5-vinylthiazole			Thiazoles
FGE.76	15.018 1038	4-Methyl-5-vinylthiazole		REPRESENTATIVE	Thiazoles
FGE.76	15.029 1059	2-(sec-Butyl)-4,5-dimethyl-3-thiazoline			3-Thiazolines
FGE.76	15.030 1058	4,5-Dimethyl-2-ethyl-3-thiazoline			3-Thiazolines
FGE.76	15.032 1045	4,5-Dimethyl-2-isobutyl-3-thiazoline		REPRESENTATIVE	3-Thiazolines
FGE.21	15.060	2,4-Dimethyl-3-thiazoline			3-Thiazolines
FGE.21	15.119	2-Isobutyl-3-thiazoline			3-Thiazolines

FGE	FL-no JECFA no	Chemical name	Structural formula	Representative substance	Group
FGE.93	15.130 1761	5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline			3-Thiazolines
FGE.93	15.131 1762	5-Ethyl-4-methyl-2-(2-butyl)-thiazoline			3-Thiazolines

1.3. History of the evaluation of the substances in the present FGE

The JECFA has evaluated a group of 30 flavouring substances consisting of sulfur-containing heterocyclic compounds (JECFA, 2002a, 2003).

FGE.76

In FGE.76, which covered a group of 26 of the 30 JECFA-evaluated substances, the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) concluded that for six substances [FL-no: 15.005, 15.018, 15.028, 15.029, 15.030 and 15.032], the Procedure should not be applied until adequate genotoxicity data become available (EFSA, 2008).

Therefore, only the remaining 20 substances were evaluated through the Procedure.

For three substances [FL-no: 15.014, 15.015 and 16.027], expected to be metabolised to innocuous products (A-side), the AFC Panel agreed with the JECFA evaluation.

For the remaining 17 substances, the AFC Panel agreed with the JECFA that they cannot be expected to be metabolised to innocuous products. The 17 substances were allocated to one of the 10 structural subgroups identified in FGE.21.

For all 20 substances evaluated through the Procedure use levels were needed to calculate the modified theoretical added maximum daily intake (mTAMDI) in order to identify those flavouring substances that needed more refined exposure assessment and to finalise the evaluation.

For eight substances, EU production volumes were not available [FL-no: 15.002, 15.005, 15.008, 15.027, 15.029, 15.030, 15.109 and 15.113]. In addition, the AFC Panel considered that for the substances [FL-no: 15.109 and 15.113], there were insufficient data available to provide margins of safety from their use as flavouring substances and that additional toxicity data were needed.

Adequate specifications including complete purity criteria and identity were available for 20 of the 26 JECFA evaluated substances. For six substances [FL-no: 15.022, 15.029, 15.030, 15.032, 15.109 and 15.113], information on the stereoisomeric composition was lacking.

Thus, for 12 substances [FL-no: 15.002, 15.005, 15.008, 15.018, 15.022, 15.027, 15.028, 15.029, 15.030, 15.032, 15.109 and 15.113], data were not sufficient to complete the safety evaluation.

For the remaining 14 of the JECFA evaluated sulfur-containing heterocyclic compounds [FL-no: 15.001, 15.011, 15.013, 15.014, 15.015, 15.016, 15.017, 15.019, 15.020, 15.021, 15.026,[4] 15.033, 15.035 and 16.027], the AFC Panel agreed with the JECFA conclusion 'no safety concern at estimated levels of intake as flavouring substances' based on the MSDI approach.

FGE.76 Rev1

Since the publication of FGE.76, industry has informed that thiazole [FL-no: 15.028] is no longer of interest for use as flavouring substances in Europe (DG SANCO, 2012) and no further data were submitted. Therefore, [FL-no: 15.028] was not considered any further in this revision.

In FGE.76Rev1, the consideration of one additional substance, 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] was included. This substance is an α,β-unsaturated aldehyde for which genotoxicity

[4] Industry informed that genotoxicity studies are ongoing on the substance [FL-no: 15.026] (EFFA /IOFI letter to DG SANTE on additional data collection for 2-isopropyl-4-methylthiazole [FL-no: 15.026], September 2022).

data were submitted and evaluated in FGE.224 (EFSA CEF Panel, 2013c), where the CEF Panel concluded that the genotoxicity concern for [FL-no: 15.004] is ruled out. This substance was added to the group of the 17 substances already evaluated via the B-side of the Procedure, in FGE.76.

Additionally, 14-day and 90-day toxicity studies were submitted for 5,6-dihydro-2,4,6-tris(2-methylpropyl)-4H-1,3,5-dithiazine [FL-no: 15.113]. A no observed adverse effect level (NOAEL) of 9.3 mg/kg body weight (bw) per day was derived from the 90-day toxicity study, which was used to support also the evaluation of the structurally related substance 2,4,6-trimethyldihydro-1,3,5(4H)-dithiazine [FL-no: 15.109]. For both substances [FL-no: 15.113] and [FL-no: 15.109], an adequate margin of safety was calculated based on the MSDI approach (EFSA CEF Panel, 2013a).

Information on European poundage figures were provided by EFFA for eight substances: [FL-no: 15.002, 15.005, 15.008, 15.027, 15.029, 15.030, 15.109 and 15.113] (EFFA, 2010, 2012, 2013a) and accordingly the 'maximised survey-derived daily intake' (MSDI) has been estimated for these substances. Furthermore, information from industry on missing stereoisomeric composition for [FL-no: 15.022, 15.029, 15.030, 15.032, 15.109 and 15.113] and information on solubility in water for [FL-no: 15.005, 15.008, 15.017, 15.018, 15.019 and 15.113] (EFFA, 2013b) were included in FGE.76Rev1 (EFSA CEF Panel, 2013a).

For 21 out of 26 JECFA-evaluated substances [FL-no: 15.001, 15.002, 15.004, 15.008, 15.011, 15.013, 15.014, 15.015, 15.016, 15.017, 15.019, 15.020, 15.021, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113 and 16.027] evaluated in FGE.76Rev1, the CEF Panel agreed with the JECFA conclusion 'No safety concern at estimated levels of intake as flavouring substances' based on the MSDI approach (EFSA CEF Panel, 2013a).

The CEF Panel indicated that for all 21 substances evaluated through the Procedure, use levels are needed to calculate the modified theoretical added maximum daily intake (mTAMDI) in order to identify those flavouring substances that need more refined exposure assessment and to finalise the evaluation.

Moreover, the CEF Panel confirmed that three 3-thiazolines, 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029], 4,5-dimethyl-2-ethyl-3-thiazoline [FL-no: 15.030] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] are structurally related to 2-methylthiazolidine [FL-no: 15.090] and 2-propylthiazolidine [FL-no: 15.099], evaluated by the CEF Panel in FGE.21Rev3 (subgroup B-III) and reported to be positive in the Ames test (TA98 and TA100). Considering the structural similarities between these two thiazolidines [FL-no: 15.090 and 15.099][5] in subgroup B-III and the three thiazolines in subgroup B-II (2-methyl-2-thiazoline [FL-no: 15.086], 2,4-dimethyl-3-thiazoline [FL-no: 15.060] and 2-isobutyl-3-thiazoline [FL-no: 15.119]), the CEF Panel concluded, in FGE.21Rev3, that in the absence of further genotoxicity data, the Procedure could not be applied to these five substances from subgroup B-II and B-III. In parallel with its conclusion on the subgroup B-II (thiazolines) in FGE.21Rev3 (EFSA CEF Panel, 2012), the CEF Panel concluded that the Procedure could not be applied to these three thiazolines [FL-no: 15.029, 15.030 and 15.032], until adequate *in vivo* genotoxicity data become available.

Furthermore, the CEF Panel noted the presence of a terminal conjugated double bond in the substances 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018], which raised concern for genotoxicity. The CEF Panel confirmed that the Procedure should not be applied to these two substances until genotoxicity data become available due to the possibility of formation of reactive metabolites via epoxidation. Therefore, the evaluation through the Procedure was not applied to these five substances [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032].

The present revision 2 of FGE.76 (FGE.76Rev2) deals with the assessment of the genotoxicity potential, of seven flavouring substances: five substances from FGE.76 [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032] and two substances previously allocated to FGE.93 [FL-no: 15.130 and 15.131] (see Sections 1.2 and 1.4).

Industry has provided *in vitro* and *in vivo* genotoxicity data on the representative substances 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] and 4-methyl-5-vinylthiazole [FL-no: 15.018], which are representative for [FL-no: 15.029, 15.030, 15.130 and 15.131] and for [FL-no: 15.005], respectively (see Table 1).

[5] The use of 2-methylthiazolidine [FL-no: 15.090] and 2-propylthiazolidine [FL-no: 15.099] as flavouring substances was no longer of interest for industry and no further data were provided. Therefore, these substances were not included in the Union List, i.e. Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC.

Additionally, for the substances [FL-no: 15.001, 15.002, 15.005, 15.008, 15.011, 15.016, 15.018, 15.021, 15.029, 15.030 and 15.032], already considered in FGE.76Rev1, information on uses and use levels have been provided (DG SANCO, 2014; EFFA, 2022). Similar information was provided for [FL-no: 15.130 and 15.131], which were initially in FGE.93 (EFFA, 2022). Accordingly, the mTAMDI estimates, for these substances, are included in this opinion.

The present revision FGE.76Rev2, contains 28 substances, including 21 substances that were already considered in FGE.76Rev1. These 21 flavouring substances will not be discussed further. FGE.76Rev2 will thus consider the safety assessment of seven substances [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032, 15.130 and 15.131]. In addition, it will consider the information on uses and use levels and mTAMDIs for six substances [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016 and 15.021], already dealt with in FGE.76Rev1. Nevertheless, for the sake of completeness, for all 28 substances in this FGE, the information on the specifications, evaluation status and intake are maintained in the respective tables. For more details on the previously evaluated flavouring substances, the former versions of this FGE (FGE.76 and FGE.76Rev1) should be consulted.

Table 2 gives information on adoption dates and links to the published scientific opinions.

Table 2: Adoption dates and links to the published versions of FGE.76

FGE	Adopted	Link	Substances
FGE.76	31 January 2008	http://www.efsa.europa.eu/en/efsajournal/pub/875	26
FGE.76Rev1	24 October 2013	http://www.efsa.europa.eu/en/efsajournal/pub/3455	26
FGE.76Rev2	13 December 2022	http://www.efsa.europa.eu/en/efsajournal/pub/7784	28

A summary of the history of the evaluation of the substances in FGE.76 is presented in Figure 1.

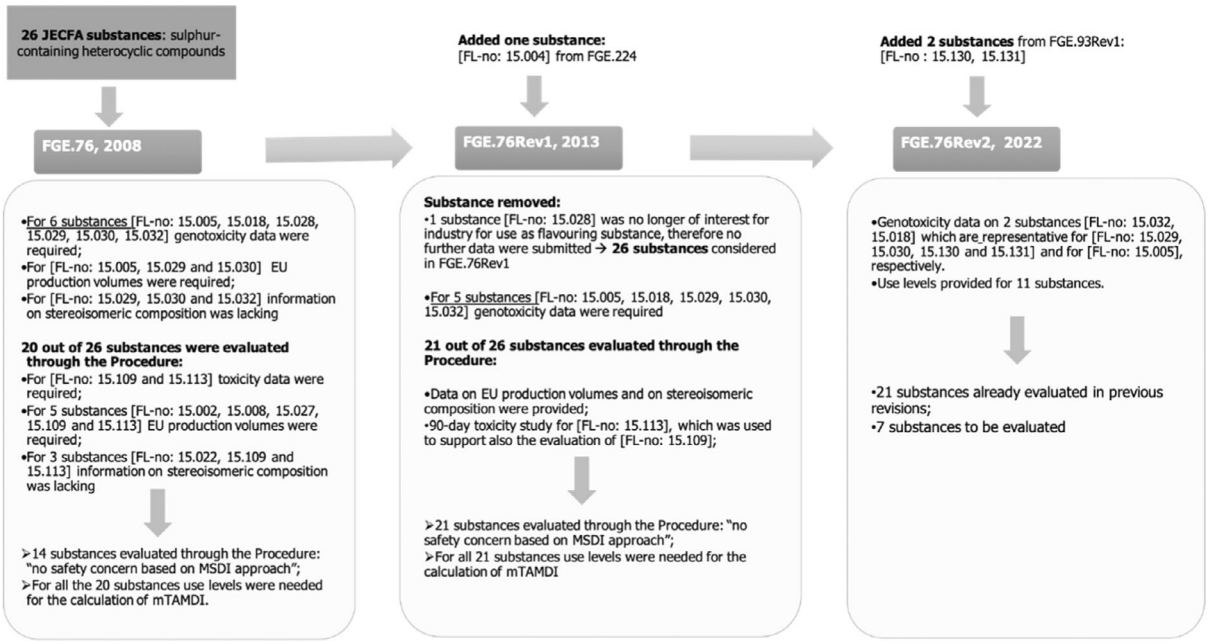


Figure 1: Summary of the history of evaluation of the substances in FGE.76

1.4. Presentation of the substances in the JECFA flavouring group

JECFA status (FGE.76)

The JECFA has at its 59th meeting in 2002 evaluated a group of 30 flavouring substances consisting of sulfur-containing heterocyclic compounds (JECFA, 2002a, 2003).

JECFA status (FGE.93)

The JECFA has evaluated a group of 17 flavouring substances consisting of sulfur-containing heterocyclic substances at its 68th meeting (JECFA, 2007, 2008a).

EFSA considerations

Two of the 30 sulfur-containing heterocyclic compounds from the JECFA 59th meeting had not been in the Register of flavouring substances [2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-dimethyldihydro-1,3,5-dithiazine (mixture) (JECFA-no: 1046) and 2-isopropyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isopropyl-2,6-dimethyldihydro-1,3,5-dithiazine (mixture) (JECFA-no: 1047)]. One of the substances, 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004], is an α,β-unsaturated aldehyde and one substance, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024], is an α,β-unsaturated ketone. These two substances were evaluated in FGE.224 (EFSA CEF Panel, 2013c), where the substance [FL-no: 15.004] was considered not to be of concern with respect to genotoxicity. Therefore, [FL-no: 15.004] was included in FGE.76Rev1 to complete the evaluation through the Procedure. On the contrary, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024] was mutagenic *in vitro* and *in vivo* and the CEF Panel concluded that its use as flavouring substance raises a safety concern (EFSA CEF Panel, 2013d). The substance [FL-no: 15.024] was deleted from the Union list.[6]

Thiazole [FL-no: 15.028] was no longer of interest for industry for use as a flavouring substance in Europe and no further data were submitted. Therefore, [FL-no: 15.028] was not included in the Union List.[2] Consequently, this substance was not considered any further in the evaluation.

The CEF Panel concluded that all the 26 substances in the JECFA flavouring group of sulfur-containing heterocyclic compounds are structurally related to the group of 59 thiazole, thiophene, thiazoline and thienyl derivatives from chemical group 29 and miscellaneous substances from chemical group 30 evaluated by EFSA in FGE.21Rev3 (EFSA CEF Panel, 2012). The substances in FGE.21Rev3 were subdivided into a number of subgroups, and the substances in this JECFA evaluated group were considered in relation to their corresponding subgroup in FGE.21Rev3.

In FGE.93Rev1, the CEF Panel evaluated five substances [FL-no: 15.010, 15.126, 15.128, 15.130, 15.131] of the 17 substances evaluated by the JECFA (JECFA, 2007, 2008a). The CEF Panel, in FGE.93Rev1, agreed with the JECFA conclusions 'No safety concern at estimated levels of intake as flavouring substances' based on the MSDI approach for the substances [FL-no: 15.010, 15.126, 15.128] (EFSA CEF Panel, 2013b).

In the same opinion, the CEF Panel considered that the two substances 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131], which are 3-thiazolines, are structural similar to two other 3-thiazolines in FGE.21Rev1 (EFSA CEF Panel, 2009) for which there is a genotoxicity concern; therefore, the Procedure was not applied because genotoxicity data were needed. Since genotoxicity data have been submitted for [FL-no: 15.032], representative substance for the group of 3-thiazolines and considering the structural similarity, the 2 substances [FL-no: 15.130 and 15.131] from FGE.93 will be evaluated in the present revision (FGE.76Rev2).

The present opinion will therefore deal with 28 JECFA substances.

2. Data and methodologies

2.1. Data

Following the potential concern for genotoxicity for the substances [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032, 15.130, 15.131] expressed by the CEF Panel in FGE.76Rev1 (EFSA CEF Panel, 2013a) and in FGE.93Rev1 (EFSA CEF Panel, 2013b), industry submitted genotoxicity data for both [FL-no: 15.018 and 15.032].

For [FL-no: 15.018], the following studies were submitted: an *in vitro* reverse gene mutation assay in bacteria (Covance, 2012a), an *in vitro* micronucleus assay (Covance, 2013a), an *in vivo* combined bone marrow micronucleus test and comet assay in liver and duodenum (Covance, 2014a).

A 90-day toxicity study was submitted for 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] (Posternak et al., 1969).

For [FL-no: 15.032], the following studies were submitted: an *in vitro* reverse gene mutation assay in bacteria (Covance, 2012b), an *in vitro* micronucleus assay (Covance, 2013b), an *in vivo* combined bone marrow micronucleus test and comet assay in liver (Covance, 2014b).

[6] Commission Regulation (EU) No 545/2013 of 14 June 2013 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards the flavouring substance 3-acetyl-2,5-dimethylthiophene.

A 90-day toxicity study was submitted for 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029] (Food and Drug research laboratories, 1978).[7]

Moreover, industry provided updated poundage data (EFFA, 2018).

Additional information was provided by the applicant during the assessment process in response to requests from EFSA sent on 19 February 2015, 11 June 2018 and 11 August 2021 (BioReliance, 2018a,b; Charles River Laboratories, 2020; Covance, 2015; EFFA, 2018, 2022). Information requested is summarised below.

In the *in vivo* micronucleus studies in bone marrow, the exposure of the bone marrow had not been demonstrated; therefore, the applicant was requested to provide evidence of bone marrow exposure to 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] and 4-methyl-5-vinylthiazole [FL-no: 15.018] by plasma analysis as recommended in the OECD Test Guideline (TG) 474 (EFSA letter dated 19/02/2015).

Following this request, a technical hearing was held with the applicant on 19 January 2016 (EFSA, 2016) to clarify the challenges observed by the applicant in performing the plasma analysis and demonstrating bone marrow exposure.

As follow-up of the technical hearing, additional information on the plasma analysis already performed (EFSA letter dated 2 February 2016) was requested. After reviewing these data, the CEF Panel suggested to suspend the activities related to plasma analysis and requested to test both representative substances in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity) (EFSA letter dated 29 April 2016).

Following this request (EFSA letter dated 29 April 2016), a second technical hearing was held with the applicant on 24 January 2017 (EFSA, 2017) to clarify the applicant's proposal to test the representative substances with a new method instead of the *in vitro* micronucleus with centromere analysis.

Following the second technical hearing, the CEF Panel reiterated the request for an *in vitro* micronucleus assay with centromere analysis for investigating the mode of action of the representative substances (EFSA letter dated 10 April 2017). The applicant provided the requested data on 10 April 2018 (BioReliance, 2018a,b; EFFA, 2018) that are listed in Table 3 and evaluated in the present revision of FGE.76 (FGE.76Rev2).

Since the clarification of the mechanism of action would allow to identify the most appropriate follow-up study, industry submitted an *in vitro* micronucleus assay in TK6 cells with kinetochores staining (CREST), for both the representative flavouring substances [FL-no: 15.018] and [FL-no: 15.032]. In the new *in vitro* micronucleus test on 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (BioReliance, 2018b), an increase in micronucleated cell frequency was not observed. These results are in contrast to the previous submitted *in vitro* micronucleus study on human peripheral blood lymphocytes (Covance, 2013b). Therefore, a new *in vitro* micronucleus assay in cultured peripheral blood lymphocytes with fluorescence *in situ* hybridisation (FISH) analysis, complying with OECD TG 487 (OECD, 2016) was requested (EFSA letter dated 6/11/2018). This study was submitted on 20 February 2020 (Charles River Laboratories, 2020).

A further request for additional data was sent by EFSA on 8 November 2021 and data were provided on 25 February 2022 (EFFA, 2022).

After the publication of FGE.76Rev1 (EFSA CEF Panel, 2013a), use levels data have been provided for the substances [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016, 15.021] (DG SANCO, 2014). These data are considered in FGE.76Rev2.

The new available data considered in the present revision of FGE.76 (FGE.76Rev2) are summarised in Table 3.

[7] Study previously reported as Babish and Re, 1978 in FGE.21Rev5 and former revisions.

Table 3: Data evaluated in FGE.76Rev2

FL-no JECFA no	Chemical name	Data provided for the current revision 2 of FGE.76	Appendix (Table nr) and relevant section of the opinion	Documentation provided to EFSA/ Reference
15.001 1052	2-Mercaptothiophene	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.002 1057	2-Methyl-5-methoxythiazole	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.005 1039	2,4-dimethyl-5-vinylthiazole	90-day toxicity study Use levels and poundage data	Appendix F (Table F.1); Appendix C (Tables C.2 and C.5); Sections 3.2 and 3.3	Posternak et al., 1969; EFFA, 2018, 2022
15.008 1053	2-Thienyl disulfide	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.011 1055	5-Acetyl-2,4-dimethylthiazole	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.016 1040	Benzothiazole	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.018 1038	4-Methyl-5-vinylthiazole	Genotoxicity data Use levels and poundage data	Appendix E (Tables E.1 and E.2); Appendix C (Tables C.2 and C.5); Sections 3.2 and 3.3	Covance, 2012a; Covance, 2013a; Covance, 2014a; BioReliance, 2018a EFFA, 2018, 2022
15.021 1056	2-Ethoxythiazole	Use levels data	Appendix C (Tables C.1 and C.5); Section 3.2	DG SANCO, 2014
15.029 1059	2-(sec-butyl)-4,5-dimethyl-3-thiazoline	90-day toxicity study Use levels and poundage data	Appendix F (Table F.1); Appendix C (Tables C.2 and C.5); Sections 3.2 and 3.3	Food and Drug research laboratories, 1978; EFFA, 2018, 2022
15.030 1058	4,5-dimethyl-2-ethyl-3-thiazoline	Use levels and poundage data	Appendix C (Tables C.2 and C.5); Section 3.2	EFFA, 2018, 2022
15.032 1045	4,5-Dimethyl-2-isobutyl-3-thiazoline	Genotoxicity data Use levels and poundage data	Appendix E (Tables E.1 and E.2); Appendix C (Tables C.2 and C.5); Sections 3.2 and 3.3	Covance, 2012b; Covance, 2013b; Covance, 2014b, 2015; BioReliance, 2018b; Charles River Laboratories, 2020 EFFA, 2018, 2022
15.130 1761	5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	Use levels and poundage data	Appendix C (Tables C.2 and C.5); Section 3.2	EFFA, 2018, 2022
15.131 1762	5-Ethyl-4-methyl-2-(2-butyl) thiazoline	Use levels and poundage data	Appendix C (Tables C.2 and C.5); Section 3.2	EFFA, 2018, 2022

In addition, the following references were used:

- JECFA specifications for seven flavouring substances [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032, 15.130 and 15.131] (JECFA, 2002b, 2008b).
- EFSA scientific opinion on FGE.76Rev1 (EFSA CEF Panel, 2013a)
- EFSA scientific opinion on FGE.21Rev5 (EFSA CEF Panel, 2015)
- EFSA scientific opinion on FGE.93Rev1 (EFSA CEF Panel, 2013b)

2.2. Methodologies

This opinion was prepared following the principles described in the EFSA Guidance on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidelines from the EFSA Scientific Committee. The assessment strategy applied for the evaluation programme of flavouring substances, as laid down in Commission Regulation (EC)

No 1565/2000[3], is based on the Opinion on a Programme for the Evaluation of Flavouring substances of the Scientific Committee on Food (SCF, 1999).

2.2.1. Procedure for the safety evaluation of flavouring substances

The approach for safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000[3], named the 'Procedure', is described in Appendix A.

2.2.2. Approach used for the calculation of exposure

The approach used for calculation of the intake of the flavouring substances is described in Appendix A (point 'a) *Intake*') and in Appendix C (Section C.2 'mTAMDI calculation').

3. Assessment

3.1. Specifications

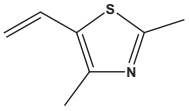
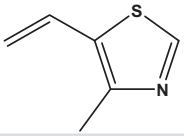
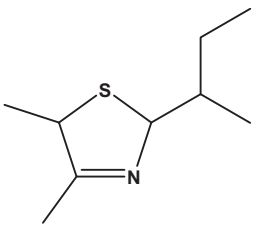
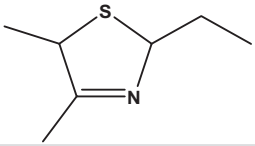
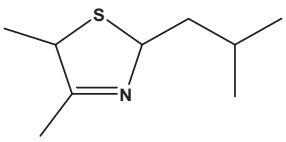
JECFA status

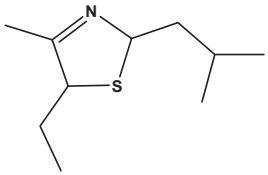
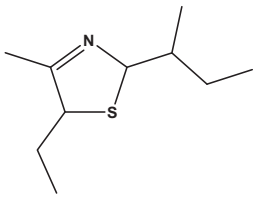
The JECFA specifications are available for all the 28 candidate flavouring substances [FL-no: 15.001, 15.002, 15.004, 15.008, 15.011, 15.013, 15.014, 15.015, 15.016, 15.017, 15.019, 15.020, 15.021, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113, 15.130, 15.131, 16.027, 15.005, 15.018, 15.029, 15.030 and 15.032] (JECFA, 2002b, 2008b).

EFSA considerations

The chemical structures of the flavouring substances considered in FGE.76Rev2 are reported in Table 4.

Table 4: Chemical structures of flavouring substances under evaluation in FGE.76Rev2

FL-no JECFA no	Structural formula	Chemical name	Structural class[(a)]
15.005 1039		2,4-Dimethyl-5-vinylthiazole	III
15.018 1038		4-Methyl-5-vinylthiazole	III
15.029 1059		2-(sec-Butyl)-4,5-dimethyl-3-thiazoline	III
15.030 1058		4,5-Dimethyl-2-ethyl-3-thiazoline	III
15.032 1045		4,5-Dimethyl-2-isobutyl-3-thiazoline	III

FL-no JECFA no	Structural formula	Chemical name	Structural class[(a)]
15.130 1761		5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	III
15.131 1762		5-Ethyl-4-methyl-2-(2-butyl)-thiazoline	III

FL-No: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives.
(a): Determined with OECD Toolbox (version 4.4.1 available at https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm).

Eight substances [FL-no: 15.022, 15.029, 15.030, 15.032, 15.109, 15.113, 15.130 and 15.131] in the group of JECFA evaluated sulfur-containing heterocyclic compounds have one or more chiral centres. Industry informed that all these substances are racemates (EFFA, 2013b). Five of these substances can furthermore exist as geometrical stereoisomers [FL-no: 15.029, 15.030, 15.032, 15.130 and 15.131] and adequate information on their stereoisomeric composition has been provided (EFFA, 2013b).

Regarding [FL-no: 15.109 and 15.113], theoretical options for the proportions of stereoisomers have been reported by industry (non-chiral meso-form: 50%; two chiral stereoisomers, 25% each). For both flavouring substances, no information on the actual percentages of the stereoisomers in the material of commerce has been provided. However, for [FL-no: 15.109], the CAS-no corresponds to the all-*cis* configuration.

In conclusion, the Panel considered the available specifications adequate for all substances, except [FL-no: 15.109 and 15.113] for which the information on the stereochemical composition is inadequate.

The most recent specifications data for substances in FGE.76Rev2 are summarised in Appendix B, Table B.1.

3.2. Estimation of intake

JECFA status

For 20 substances evaluated through the JECFA Procedure intake data were available for the EU. For the remaining eight substances [FL-no: 15.002, 15.005, 15.008, 15.027, 15.029, 15.030, 15.109, 15.113], production figures were only available for the USA (JECFA, 2002a, 2003, 2007, 2008a).

EFSA considerations

Updated poundage data have been provided for [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032, 15.130 and 15.131] (EFFA, 2018), which are considered in the present opinion (Appendix C). For these substances also updated data on use and use levels have been provided (EFFA, 2022).

For the 28 JECFA evaluated substances, production volumes are available for the EU and accordingly MSDI figures for the EU can be calculated, see Appendix C, Table C.5.

Updated use levels have been submitted also for flavouring substances [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016, 15.021] (DG SANCO, 2014). Therefore, the mTAMDI intake values can be calculated for 13 substances (see Tables C.1 and C.2 – Appendix C).

The Panel noted that the normal and maximum use levels reported for the food category 7.1 for [FL-no: 15.018] and for the food categories 9.2, 9.3, 9.4 and 12.6 for [FL-no: 15.032] are not plausible, because maximum use levels are lower than the respective normal use levels. The lower value was provisionally used by the Panel for the calculation of mTAMDI. However, clarification on these discrepancies should be provided.

The mTAMDI intake estimates for flavouring substances [FL-no: 15.002, 15.016, 15.018, 15.032, 15.130 and 15.131] are above the threshold of toxicological concern (TTC) for their structural class (i.e. structural class III, 90 μg/person per day) and more reliable data on uses and use levels are required to finalise their evaluation.

The mTAMDI intake estimates for seven substances [FL-no: 15.001, 15.005, 15.008, 15.011 15.021, 15.029 and 15.030] are below the TTC for their structural class (also class III).

No normal and maximum use levels have been provided for the remaining 15 substances in the flavouring group [FL-no: 15.004, 15.013, 15.014, 15.015, 15.017, 15.019, 15.020, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113, 16.027].

The MSDI figures and mTAMDI intake estimates for the flavouring substances in FGE.76Rev2 are shown in Table C.5 – Appendix C.

Natural occurrence

Data on natural occurrence were reported by JECFA (2002a, 2003) and provided by industry (EFFA, 2018). This information is not considered for this evaluation, but it is included in Appendix C.

3.3. Biological and toxicological data

3.3.1. ADME data

JECFA

JECFA (2002a, 2003) reported that 'thiazole and its derivatives are metabolized primarily by side-chain oxidation or oxidation of the ring sulfur or nitrogen atoms; however, other routes of metabolism, involving ring cleavage, are possible. Derivatives of dithiazine, which are cyclic sulfides, are expected to be metabolized primarily by S-oxidation to yield the corresponding sulfoxides and sulfones. Thiazoline is predicted to be similarly metabolized'.

JECFA (2007, 2008a) reported that for substances [FL-no: 15.130 and 15.131], data were insufficient to allow conclusions about their probable metabolic fate.

EFSA considerations

The Panel agreed with the options for metabolism outlined by JECFA and noted that there is no further information available.

3.3.2. Genotoxicity studies

JECFA

JECFA did not present any information pertaining to the flavouring substances under consideration in this revision of FGE.76.

EFSA

In FGE.76 (EFSA, 2008), the AFC Panel concluded that three 3-thiazolines, 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029], 4,5-dimethyl-2-ethyl-3-thiazoline [FL-no: 15.030] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] are structurally related to 2-methylthiazolidine [FL-no: 15.090][5] and 2-propylthiazolidine [FL-no: 15.099][5], evaluated by the AFC Panel in FGE.21 (EFSA, 2007) and reported to be positive in the Ames test (TA98 and TA100) (Appendix D). In concordance with its conclusion on the subgroup B-II (thiazolines) in FGE.21, the AFC Panel concluded that the Procedure could not be applied to these three thiazolines [FL-no: 15.029, 15.030 and 15.032], until adequate *in vivo* genotoxicity data become available (EFSA, 2008). Similarly, in FGE.93Rev1 (EFSA CEF Panel, 2013b), the CEF Panel noted that 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131], which are 3-thiazolines, are structurally similar to two other 3-thiazolines in FGE.21Rev1 (EFSA CEF Panel, 2009) for which the CEF Panel has expressed a genotoxicity concern, and accordingly, the Procedure should not be applied to these two substances until adequate genotoxicity data become available.

Additionally, in FGE.76Rev1, the CEF Panel noted the presence of a terminal conjugated double bond in the substances 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018], which raised concern for genotoxicity. The CEF Panel decided that the Procedure should

not be applied to these two substances until adequate genotoxicity data become available due to the possibility of formation of reactive metabolites via epoxidation.

Industry has provided *in vitro* and *in vivo* genotoxicity studies for the representative substances 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (covering the evaluation of [FL-no: 15.029, 15.030, 15.130 and 15.131]) and 4-methyl-5-vinylthiazole [FL-no: 15.018] (covering the evaluation of [FL-no: 15.005]), listed in Table 5.

Table 5: New genotoxicity studies evaluated in FGE.76Rev2

Substance name [FL-no]	Study
4-Methyl-5-vinylthiazole [15.018]	Bacterial reverse mutation assay (Covance, 2012a)
	In vitro micronucleus assay in human lymphocytes (Covance, 2013a)
	In vitro micronucleus assay with CREST staining in TK6 cells (BioReliance, 2018a)
	In vivo combined micronucleus (bone marrow) and comet assay (in liver and duodenum) (Covance, 2014a)
4,5-Dimethyl-2-isobutyl-3-thiazoline [15.032]	Bacterial reverse mutation assay (Covance, 2012b)
	In vitro micronucleus assay in human lymphocytes (Covance, 2013b)
	In vitro micronucleus assay with CREST staining in TK6 cells (BioReliance, 2018b)
	In vitro micronucleus assay in human lymphocytes with FISH analysis (Charles River Laboratories, 2020)
	In vivo combined micronucleus (bone marrow) and comet assay (in liver and duodenum) (Covance, 2014b, 2015)

3.3.3. Data on 4-methyl-5-vinylthiazole [FL-no: 15.018]

***In vitro* studies**

3.3.3.1. 4-Methyl-5-vinylthiazole [FL-no: 15.018] - Reverse bacterial mutation assay

In order to investigate the potential of 4-methyl-5-vinylthiazole [FL-no: 15.018] (purity 99.2%) and/or its metabolites to induce gene mutations in bacteria, an Ames test was performed according to OECD TG 471 (OECD, 1997a) and following good laboratory practice (GLP) in tester strains of Salmonella Typhimurium (TA98, TA100, TA1535, TA1537 and TA102), in the presence or absence of metabolic activation (Aroclor 1254-induced rat liver post-mitochondrial fraction (S9-mix)), in three separate experiments (Covance, 2012a).

In the first experiment, 4-methyl-5-vinylthiazole was tested at concentrations from 5 to 5000 μg/plate, with the plate incorporation method in the absence and presence of S9-mix. Following these treatments, no increase of revertants was observed.

As the results of experiment 1 were negative, treatments in the presence of S9-mix in experiment 2 and 3 included a pre-incubation step. Moreover, narrowed concentration intervals were employed in experiment 2 (156.3–5,000 μg/plate) and in experiment 3 (62.5–2,000 μg/plate).

No increase in the mean number of revertant colonies was observed in any tester strains, in any testing conditions. Therefore, the Panel concluded that 4-methyl-5-vinylthiazole [FL-no: 15.018] did not induce gene mutation in S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) in the absence or presence of metabolic activation.

Study details and summary of results are reported in Appendix E, Table E.1.

3.3.3.2. 4-Methyl-5-vinylthiazole [FL-no: 15.018] – *In vitro* Micronucleus Assay in human lymphocytes

The *in vitro* micronucleus assay was carried out according to OECD TG 487 (OECD, 2010) and GLP principles. Human peripheral blood lymphocytes from healthy donors were treated with 4-methyl-5-vinylthiazole [FL-no: 15.018] (purity 99.2%) in a concentration range finding assay at concentrations ranging from 4.54 to 1,252 μg/mL for 3 + 21 h, with and without S9-mix (from Aroclor 1254-induced rats), or for 24 h without S9-mix (Covance, 2013a). Based on the results of the concentration range-finding study, duplicate cultures of lymphocytes were treated with 4-methyl-5-vinylthiazole at concentrations ranging from 200 to 1,252 μg/mL for short-term treatment (3 h +21 h recovery period) in the absence or presence of S9-mix. Concentrations ranging from 10 to 200 μg/mL were applied in the 24 h continuous treatment in the absence of S9-mix.

Based on the level of cytotoxicity observed, three concentration levels were selected for MN analysis for each experimental condition: (i) 700, 900 and 950 μg/mL with the 3-h treatment without S9-mix (0%, 33% and 54% cytotoxicity, respectively); (ii) 600, 700 and 800 μg/mL with the 3-h treatment with S9-mix (2%, 7% and 53% cytotoxicity, respectively); and (iii) 70, 110 and 130 μg/mL with the 24-h treatment (9%, 39% and 50% cytotoxicity, respectively).

A statistically significant increase in micronucleated cell frequency was observed at the highest concentration tested (950 μg/mL) for 3 + 21 h in the absence of S9-mix. Since the micronucleated cell frequency slightly exceeded the range of historical vehicle control (0 to 0.95%), additional binucleated cells were scored for MN. At the same concentration of 950 μg/mL, a micronucleated cell frequency of 1% still marginally exceeded the historical vehicle control range (0–0.95%).

4-Methyl-5-vinylthiazole did not increase the micronucleated cell frequency at 3 + 21 h in the presence of S9-mix or at 24 h in the absence of S9-mix.

The Panel concluded that 4-methyl-5-vinylthiazole induced a weak, but statistically significant, increase in micronucleated cell frequency when tested for 3 + 21 h in the absence of S9-mix.

Study details and summary of results are reported in Appendix E, Table E.1.

3.3.3.3. 4-Methyl-5-vinylthiazole [FL-no: 15.018] – *In vitro* Micronucleus Assay with CREST staining in TK6 cells

4-Methyl-5-vinylthiazole [FL-no: 15.018] (purity 98%) was tested in an *in vitro* MN assay with kinetochore staining (CREST) in human lymphoblastoid cell line TK6 (BioReliance, 2018a), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. The study was performed according to GLP and OECD TG 487 (OECD, 2016).

Based on the results of the preliminary cytotoxicity test, concentrations from 100 to 1000 μg/mL were tested for the treatment 4 + 23 h both in the absence or presence of S9-mix; and concentrations from 25 to 500 μg/mL were tested for the 27 h treatment in the absence of metabolic activation. In the main experiment, duplicate cultures of TK6 cells were treated for short-term treatment (4 + 23 h recovery period) in the absence or presence of S9-mix or for 27 h continuous treatment in the absence of S9-mix.

4-Methyl-5-vinylthiazole did not induce an increase in micronucleated cell frequency both at 4 + 23 h treatment and at 27 h treatment in the absence of S9-mix.

4-Methyl-5-vinylthiazole induced a statistically significant increase in micronucleated cell frequency (1.15%) at 4 + 23 h in the presence of S9-mix, which was outside the historical control range (95% reference range 0.37–0.74%).

In order to confirm this positive result in the 4 + 23 h treatment with S9-mix, the micronucleus assay was repeated with a narrower range of concentrations from 100 to 700 μg/mL. In this repeated experiment, 4-methyl-5-vinylthiazole induced a statistically significant increase in micronucleated cell frequency (1.15% and 1.20%) at 300 and 675 μg/mL, the highest concentrations analysed for MN (cytotoxicity of 23% and 54%, respectively). The increase in micronucleated cell frequency was outside the historical vehicle control range (95% reference range 0.37–0.74%).

Since positive responses were observed in the 4 + 23 h treatment with S9-mix, kinetochore staining (CREST staining) was applied to the slides of the highest concentration analysed for MN (675 μg/mL) and to vehicle and positive controls in order to determine the mechanism of chromosomal damage (aneugenicity or clastogenicity).

This analysis showed that, in the sample treated with 4-methyl-5-vinylthiazole, the percentage of MN positive for the CREST antibody (K + MN) was 43%. A comparison of this result with the percentages of K + MN observed with the positive controls (clastogen cyclophosphamide (CPA): 30% K + MN and aneugen vinblastine (VB): 87% K + MN) indicates that 4-methyl-5-vinylthiazole induced MN mainly via a clastogenic mechanism.

The Panel noted that the statistically significant increase in micronucleated cell frequency observed in TK6 cells for the 4 + 23 h treatment only in the presence of metabolic activation is not consistent with the results obtained in the *in vitro* MN study in human peripheral blood lymphocytes (Covance, 2013a), where a statistically significant increase in micronucleated cell frequency was observed at 3 + 21 h in the absence of metabolic activation.

Study details and summary of results are reported in Appendix E, Table E.1.

In vivo studies

3.3.3.4. 4-Methyl-5-vinylthiazole [FL-no: 15.018] – *In vivo* combined comet and micronucleus assay

4-Methyl-5-vinylthiazole [FL-no: 15.018] (purity 99.9%) was tested *in vivo* using the bone marrow micronucleus assay combined with the comet assay in liver and duodenum of rats (Covance, 2014a). The micronucleus assay was conducted in accordance with GLP and OECD TG 474 (OECD, 1997b). The comet assay was conducted before the publication of the first relevant OECD test guideline (OECD TG 489, 2014b), but it was based on the guidance provided by the Comet Workshop (Tice et al., 2000; Hartmann et al., 2003), International Workshops on Genotoxicity Testing (Burlinson et al., 2007), the international validation of the *in vivo* comet assay by the JaCVAM and literature (Hartmann et al., 2004; Smith et al., 2008) available at that time.

In a dose range-finding assay, groups of three male and three female Han Wistar rats were given three administrations by gavage (at 0, 24 and 45 h) of 4-methyl-5-vinylthiazole at doses of 180, 250 and 500 mg/kg bw per day.

Following the first administration at 500 mg/kg bw, one female rat was killed in extremis due to the severity of clinical signs observed (prone, ptosis (semi-closed eyes), increased lachrymation, cold to touch and laboured breathing). All other animals at this dose level showed signs of lethargy, ataxia, increased lachrymation, ptosis and piloerection. Necropsy identified no obvious cause of death. Due to the severity of the observations, no further dosing at 500 mg/kg bw was performed.

At 250 mg/kg bw per day, signs of toxicity (including decreased activity, increased lachrymation, closed eyelids, piloerection and/or hunched posture) were observed after dosing on Day 1 and Day 2. On Day 3, decreased activity was observed.

Rats dosed at 180 mg/kg bw per day showed signs of decreased activity, piloerection and ptosis, with increased lachrymation also apparent in two females. Reductions in the group mean body weight were noted in male and female animals at 180 and 250 mg/kg bw per day.

Based on the clinical signs of toxicity and/or morbidity observed at 250 and 500 mg/kg bw per day, 180 mg/kg bw per day was considered an appropriate estimate of the maximum tolerated dose (MTD) and was chosen for testing in the main experiment. As no sex-specific effects were seen, only male rats were used in the main study.

In the main experiment, groups of six male Han Wistar rats were treated by oral gavage with 4-methyl-5-vinylthiazole at doses of 45, 90 or 180 mg/kg bw per day, including a vehicle control (corn oil) and a positive control (ethyl methanesulfonate (EMS), 150 mg/kg bw per day). Animals were dosed at 0, 24 and 45 h.

Clinical signs of toxicity were reduced activity, semi-closed eyes and/or piloerection in animals dosed at 180 mg/kg bw per day. Clinical chemistry results showed a small increase of the levels of serum aspartate aminotransferase (AST, +29%) and cholesterol (+29%) following dosing at 180 mg/kg bw per day compared to control values. No findings were observed at the microscopic or macroscopic level.

Micronucleus assay

4-Methyl-5-vinylthiazole did not increase mean frequencies of MNPCE in any treated groups, but did not reduce the percentage of PCE with no indication of bone marrow toxicity, although clinical signs of toxicity observed in the main experiment indicate some systemic exposure after oral administration. Overall, the Panel noted that 4-methyl-5-vinylthiazole [FL-no: 15.018] did not induce MN in bone marrow. However, the Panel considered that the available data do not allow to clarify if the bone marrow was exposed to [FL-no: 15.018]. Therefore, results from this *in vivo* MN assay are not sufficiently conclusive to rule out a concern for clastogenicity.

Comet assay

Liver and duodenum cells collected in the main study were analysed in the comet assay.

Although there is no direct evidence of liver exposure, the signs of toxicity observed suggest that 4-methyl-5-vinylthiazole was absorbed.

Both in duodenum and in liver, 4-methyl-5-vinylthiazole did not induce any statistically significant increase in mean tail intensity and tail moment values compared to the vehicle control treatment group.

No dose-related increase in %clouds was observed following treatment with 4-methyl-5-vinylthiazole demonstrating that the treatment did not cause excessive DNA damage that could have interfered with comet analysis.

The Panel concluded that 4-methyl-5-vinylthiazole [FL-no: 15.018] did not induce DNA single strand breaks in duodenum and liver of treated rats. Therefore, it does not show clastogenic effects *in vivo*.

Study details and summary of results are reported in Appendix E, Table E.2.

3.3.4. Data on 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032]

***In vitro* studies**

3.3.4.1. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032]- Reverse bacterial mutation assay

In order to investigate the potential of 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (purity 97.2%) and/or its metabolites to induce gene mutations in bacteria, an Ames test was performed according to OECD TG 471 (OECD, 1997a) and following GLP in tester strains of S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102), both in the absence and presence of metabolic activation (Aroclor 1254-induced rat liver post-mitochondrial fraction (S9-mix)), in three separate experiments (Covance, 2012b).

In the first experiment, 4,5-dimethyl-2-isobutyl-3-thiazoline was tested at concentrations from 5 to 5000 μg/plate with the plate incorporation method in the absence and in the presence of S9-mix. Following these treatments, evidence of toxicity was observed at 1581 and/or 5000 μg/plate in all strains in the absence and presence of S9-mix.

As the results of experiment 1 were negative, treatments in the presence of S9-mix in experiment 2 and 3 included a pre-incubation step. Moreover, narrowed concentration intervals were employed in experiment 2 (78–5,000 μg/plate in TA100 in the absence of S9-mix; 156–5,000 μg/plate in TA100 in the presence of S9-mix and in all other strains both in the presence and in the absence of S9-mix) and in experiment 3 (39–1,250 μg/plate).

No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains in the absence or presence of metabolic activation. Therefore, the Panel concluded that 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] did not induce gene mutations in S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) in the absence or in the presence of metabolic activation.

Study details and summary of results are reported in Appendix E, Table E.1.

3.3.4.2. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] – *In vitro* Micronucleus Assay in human lymphocytes

An *in vitro* cytokinesis-block micronucleus assay was carried out according to OECD TG 487 (OECD, 2010) and GLP principles. Human peripheral blood lymphocytes from healthy donors were treated with 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (purity 97.2%) in a concentration range finding assay at concentrations ranging from 3.62 to 1,000 μg/mL for 3 + 21 h, with and without S9-mix (from Aroclor 1254-induced rats), or for 24 h without S9-mix (Covance, 2013b).

Based on the results of the concentration range-finding test, duplicate cultures of lymphocytes were treated with 4,5-dimethyl-2-isobutyl-3-thiazoline at concentrations ranging from 10 to 250 μg/mL for 3 h + 21 h in the absence of S9-mix; at concentrations ranging from 40 to 300 μg/mL for 3 + 21 h in the presence of S9-mix; and at concentrations ranging from 4 to 40 μg/mL for 24 h in the absence of S9-mix.

Based on the level of cytotoxicity observed, the highest concentration was selected for MN analysis in each experimental condition: (i) 4, 8 and 9 μg/mL with the 24 h treatment (6%, 15% and 52% cytotoxicity, respectively); (ii) 40, 160 and 210 μg/mL with the 3 h treatment with S9-mix (1%, 25% and 52% cytotoxicity, respectively); and (iii) 10, 20, 60 and 80 μg/mL with the 3 h treatment without S9-mix (8%, 17%, 40% and 53% cytotoxicity, respectively).

4,5-Dimethyl-2-isobutyl-3-thiazoline did not increase the micronucleated cell frequency at 24 h in the absence of S9-mix.

At 3 + 21 h in the absence of S9-mix, 4,5-dimethyl-2-isobutyl-3-thiazoline induced a statistically significant increase in micronucleated cell frequency at 60 and 80 μg/mL, which slightly exceeded the historical vehicle control range (0.1–1.00%) only at 60 μg/mL (1.05%).

At 3 + 21 h in the presence of S9-mix, 4,5-dimethyl-2-isobutyl-3-thiazoline induced a statistically significant increase in micronucleated cell frequency at 160 and 210 μg/mL, which exceeded the historical vehicle control range (0.1–1.10%) only at 160 μg/mL (1.60%).

The Panel concluded that 4,5-dimethyl-2-isobutyl-3-thiazoline induced a statistically significant increase in micronucleated cell frequency at 3 + 21 h both in the presence and in the absence of S9-mix (3.5-fold and 2.4-fold increase, respectively).

Study details and summary of results are reported in Appendix E, Table E.1.

3.3.4.3. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] – *In vitro* Micronucleus Assay with CREST staining in TK6 cells

4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (purity 97%) was tested in an *in vitro* MN assay with kinetochore staining (CREST) in human lymphoblastoid cell line TK6 (BioReliance, 2018b), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

Duplicate cultures of TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence or presence of S9-mix (from Aroclor 1254-induced rat liver) or 27 h in the absence of S9-mix.

Based on the results of the preliminary cytotoxicity test, concentrations from 5 to 150 μg/mL were tested for the treatment 4 + 23 h in the absence or in the presence of S9-mix; and concentrations from 0.5 to 50 μg/mL were tested for the treatment at 27 h in the absence of S9-mix.

4,5-Dimethyl-2-isobutyl-3-thiazoline did not increase the micronucleated cell frequency at any testing condition. Consequently, the CREST analysis was not carried out.

The Panel noted that the negative results observed in this study in TK6 cells for all treatment conditions are not consistent with results obtained in the *in vitro* micronucleus study in human peripheral blood lymphocytes (Covance, 2013b), where 4,5-dimethyl-2-isobutyl-3-thiazoline increased the micronucleated cell frequency at 3 + 21 h treatment.

Because of inconsistency in results between the two cell types and the inappropriate design of the study in TK6 cells, the Panel requested for 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] a new *in vitro* micronucleus assay in cultured human peripheral blood lymphocytes with cytochalasin B block protocol. The Panel considered that the use of FISH analysis is preferable to the kinetochore staining (CREST) to evaluate the mechanism of chromosomal damage because the *in situ* hybridisation with DNA probes would allow to overcome potential false-negative results due to damages induced to the kinetochore proteins, altering the availability of the antigens to be detected by CREST antibodies. Therefore, the Panel requested to apply FISH analysis with pancentromeric DNA probes for the elucidation of the mechanism of MN formation.

3.3.4.4. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] – *In vitro* Micronucleus Assay in human lymphocytes with FISH analysis

An *in vitro* micronucleus assay was carried out according to OECD TG 487 (OECD, 2016) and GLP principles using the cytochalasin B-induced cytokinesis block. Human peripheral blood lymphocytes from healthy donors were treated with 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (purity 99%) in a concentration range finding assay at concentrations ranging from 3.36 to 1,720 μg/mL (10 mM, the highest concentration recommended by OECD TG 487) for 4 + 20 h, with and without S9-mix (from Aroclor 1254-induced rats), or for 24 h without S9-mix (Charles River Laboratories, 2020).

Based on the results of the concentration range-finding study, duplicate cultures of lymphocytes were treated with 4,5-dimethyl-2-isobutyl-3-thiazoline at concentrations ranging from 52 to 430 μg/mL for 4 + 20 h both in the presence and in the absence of S9-mix; at concentrations ranging from 30 to 108 μg/mL for 24 h in the absence of S9-mix.

Since adequate cytotoxicity (50–60%) was not achieved for the 4 h treatment both in the absence and in the presence of S9-mix, the experiment was repeated testing concentrations from 167 to 348 μg/mL. Also in this second experiment, adequate cytotoxicity was not achieved. Therefore, the experiment was repeated with concentrations from 129 to 250 μg/mL in the 4-h treatment without S9-mix and from 202 to 300 μg/mL in the 4-h treatment with S9-mix.

Three concentration levels were selected for MN analysis in each experimental condition, based on the level of cytotoxicity observed (as reported in parentheses): 4-h treatment without S9-mix, 129 μg/mL (6%), 196 μg/mL (24%) and 228 μg/mL (49%); 24 h treatment 33.9 μg/mL (3%), 70.9 μg/mL (26%) and 97.2 μg/mL (53%); and 4-h treatment with S9-mix, 222 μg/mL (5%), 263 μg/mL (37%) and 272 μg/mL (63%). The micronucleated cell frequency of the vehicle control (1%) in the 24 h treatment was slightly outside the range for historical vehicle control (0.22–0.87) of the laboratory.

4,5-Dimethyl-2-isobutyl-3-thiazoline induced a statistically significant and concentration-related increase (positive test for trend $p < 0.01$) in micronucleated cell frequency at 4 + 20 h (1.60% MN

cells and up to 2.40 %MN cells in the absence and in the presence of S9-mix, respectively) and at 24 h (2.40% MN cells). The micronucleated cell frequency increase was outside the range of historical controls for each treatment conditions (range at 95% confidence interval: 0.05–1.11% MN at 4 h without S9-mix, 0.00–1.21% MN at 4 h with S9-mix, 0.22–0.87% MN at 24 h without S9-mix).

Slides from 97.2 μg/mL cultures of the 24 h treatment were investigated with FISH analysis for centromere positive MN (C+ MN). Slides from cultures treated with CPA or VB were scored as positive controls for clastogenic or aneugenic mode of action, respectively. This analysis showed that, in the sample treated with 4,5-dimethyl-2-isobutyl-3-thiazoline, the percentage of centromere positive MN (C+ MN) was 81%. A comparison of this result with the percentages of C+ MN observed with the positive controls (CPA: 28% C+ MN; VB: 83% C+ MN) indicates that 4,5-dimethyl-2-isobutyl-3-thiazoline induced MN via an aneugenic mechanism.

Study details and summary of results are reported in Appendix E, Table E.1.

In vivo studies

3.3.4.5. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032]- *In vivo* combined comet and micronucleus assay

4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] (purity 97%) was tested *in vivo* using the bone marrow micronucleus assay combined with the comet assay in liver and duodenum of rats (Covance, 2014b). The micronucleus assay was conducted in accordance with GLP and OECD TG 474 (OECD, 1997b). The comet assay was conducted before the publication of the first relevant OECD test guideline (OECD TG 489, 2014b), but it was based on the guidance provided by the Comet Workshop (Tice et al., 2000; Hartmann et al., 2003), International Workshops on Genotoxicity Testing (Burlinson et al., 2007), the international validation of the *in vivo* comet assay by the JaCVAM and literature (Hartmann et al., 2004; Smith et al., 2008) available at that time.

In a dose range-finding assay, groups of three male and three female animals were given three administrations (at 0, 24 and 45 h via gavage) of 4,5-dimethyl-2-isobutyl-3-thiazoline at 350, 500, 1,000 or 2,000 mg/kg bw per day.

In the range-finding assay, marked toxicity was observed at 2,000 and 1,000 mg/kg bw per day, which resulted in morbidity and early termination of the animals at 2,000 mg/kg bw per day. Clinical signs included piloerection, decreased activity, ataxia, mouth rubbing, prone, gasping, unconscious, loss of righting reflex, hypothermia, lethargy, ptosis, laboured breathing, tremors and hunched posture. Reductions in body weights were also noted in both sexes.

At 500 mg/kg bw per day, decreased activity and piloerection were observed in both sexes. Slight reductions in body weights were also noted in males (group mean reduction of approximately 3%).

At 350 mg/kg bw per day, clinical signs of toxicity were observed, including decreased activity, hunched posture and piloerection. Slight losses in body weight were seen in both sexes.

Based on the clinical signs of toxicity observed, the applicant considered 350 mg/kg bw per day to be an appropriate estimate of the MTD and was chosen for testing in the main experiment. As no sex-specific effects were seen, only male rats were used in the main study.

In the main experiment, groups of six male Han Wistar rats were treated by oral gavage with 4,5-dimethyl-2-isobutyl-3-thiazoline at doses of 87.5, 175 and 350 mg/kg bw per day, including a vehicle control (corn oil) and a positive control (EMS, 150 mg/kg bw per day). Animals were dosed at 0, 24 and 45 h. Clinical signs of toxicity (decreased activity and piloerection) were observed only at the highest dose tested. No changes in clinical chemistry parameters were observed. No findings were observed at microscopic or macroscopic level.

Micronucleus assay

4,5-Dimethyl-2-isobutyl-3-thiazoline did not increase mean frequencies of MNPCE in any treated groups and did not reduce the percentage of PCE, therefore with no indication of bone marrow toxicity.

In the dose-range finding study, severe toxicity was observed and some of the effects may be indicative of systemic exposure. However, only limited toxic effects were observed, in the main study, which was performed at much lower dose levels.

The Panel considered that the available data do not allow to clarify if the bone marrow was exposed to 4,5-dimethyl-2-isobutyl-3-thiazoline. Therefore, results from this *in vivo* MN assay are not sufficiently conclusive to rule out a concern for aneugenicity.

Comet assay

Liver and duodenum cells collected in the main study were analysed in the comet assay (Covance 2014b, 2015).

No dose-related increase in %clouds was observed following treatment with 4,5-dimethyl-2-isobutyl-3-thiazoline demonstrating that the treatment did not cause excessive DNA damage that could have interfered with comet analysis in the liver and duodenum cells.

In the analysis of duodenum, slides of two animals from the mid-dose group (175 mg/kg bw per day) were not scored due to irregular cellular morphology or diffused cells. Therefore, the statistical analysis was based on four animals instead of at least five, as recommended by OECD TG 489. In this mid-dose group, the study authors noted the presence of one animal showing a higher tail intensity (2.21%) compared to all other animals in this group and compared to the vehicle control. However, since the mean tail intensity was inside the 95% reference range for historical vehicle control (0.43–5.50%), the authors considered the higher tail intensity value as not biologically relevant.

The Panel noted that in the analysis of duodenum slides, two animals of the mid-dose group of 175 mg/kg bw per day were excluded from the comet analysis. Although the statistical power in the analysis of this mid-dose group is reduced, in the low- and high-dose group, the statistical analysis was based on data from six animals showing that 4,5-dimethyl-2-isobutyl-3-thiazoline did not induce DNA damage.

In the liver, 4,5-dimethyl-2-isobutyl-3-thiazoline did not induce any statistically significant increase in mean tail intensity and tail moment values compared to the vehicle control treatment group. Although there is no direct evidence of liver exposure, the signs of toxicity observed suggest that 4,5-dimethyl-2-isobutyl-3-thiazoline was absorbed.

Therefore, the Panel concluded that 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] did not induce DNA damage in duodenum and liver of treated rats.

Study details and summary of results are reported in Appendix E, Table E.2.

3.3.5. Discussion on genotoxicity data

3.3.5.1. 4-Methyl-5-vinylthiazole [FL-no: 15.018]

4-Methyl-5-vinylthiazole [FL-no: 15.018] did not induce gene mutations in S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) in the absence or presence of metabolic activation.

In an *in vitro* MN assay in human peripheral blood lymphocytes, 4-methyl-5-vinylthiazole induced a weak, but statistically significant, increase in micronucleated cell frequency when tested for 3 + 21 h in the absence of S9-mix.

On the contrary, in the *in vitro* MN assay in TK6 cells, 4-methyl-5-vinylthiazole [FL-no: 15.018] induced a statistically significant increase in micronucleated cell frequency in the presence of metabolic activation. The CREST analysis indicates a prevalence of clastogenicity.

An *in vivo* combined bone marrow micronucleus test and comet assay in liver and duodenum of rats was performed to follow up the observed *in vitro* chromosomal damage. No increase in the mean frequencies of MNPCE was observed in any treated groups. There were limited clinical signs of toxicity, but these were not sufficient to conclude that bone marrow exposure had occurred.

However, based on the results of the *in vivo* comet assay, the Panel concluded that 4-methyl-5-vinylthiazole [FL-no: 15.018] did not induce DNA damage in the liver and duodenum of rats after oral administration; therefore, the potential clastogenicity in the presence and in the absence of metabolic activation was ruled out.

3.3.5.2. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032]

4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] did not induce gene mutations in *S. Typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) in the absence or in the presence of metabolic activation.

In an *in vitro* MN assay in human peripheral blood lymphocytes, 4,5-dimethyl-2-isobutyl-3-thiazoline induced a statistically significant increase in micronucleated cell frequency at 3 + 21 h in the presence of metabolic activation and a weak, but statistically significant, increase in micronucleated cell frequency when tested for 3 + 21 h in the absence of S9-mix.

In TK6 cells, 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] did not induce an increase in micronucleated cell frequency at any testing conditions. Since this result is in contrast to the positive results observed in primary cultures of human lymphocytes, the Panel requested to repeat the assay in

conditions similar to the *in vitro* MN assay performed in human lymphocytes and applying FISH analysis instead of CREST staining.

An *in vitro* micronucleus assay combined with FISH analysis was provided by the applicant to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity). The test was applied in human peripheral blood lymphocytes using the cytokinesis-block method. 4,5-Dimethyl-2-isobutyl-3-thiazoline was tested up to 10 mM, the highest concentration recommended by OECD TG 487 with a short-term treatment with and without S9-mix and with 24 h continuous treatment. A statistically significant and concentration-related increase in percent of cells with MN was detected at all the conditions of treatment. The FISH analysis applied on the samples from the 24 h treatment showed 81% of MN positive for the DNA probe demonstrating the aneugenic mechanism of the compound.

An *in vivo* combined bone marrow micronucleus test and comet assay in liver and duodenum of rats was performed to follow up the observed *in vitro* chromosomal damage. The results of the *in vivo* micronucleus assay in bone marrow were negative. The data in the range-finding study showed severe toxicity indicative of systemic exposure. However, only limited toxic effects were observed, in the main study, which was performed at much lower dose levels (87.5, 175 and 350 mg/kg bw per day). The Panel noted that there was insufficient evidence of adequate target tissue exposure and considered these results, therefore, as inconclusive.

The most appropriate *in vivo* follow-up for compounds detected to be aneugenic *in vitro* in the presence or absence of S9-mix would be the application of the MN assay in liver and in gastrointestinal tract (GIT), respectively. However, the Panel noted that these tests still need further development and validation.

Following the recommendations for risk assessment in the EFSA Scientific Committee guidance on aneugenicity (EFSA Scientific Committee, 2021), the Panel compared the concentration resulting in aneugenicity *in vitro* (97.2 μg/mL) with the estimated concentration of the substance in the GIT following ingestion of food or beverage. Since the dilution in the upper parts of the GIT can be considered to be small, the estimated concentration of a substance in this part of the GIT would be in the same order of magnitude as its concentration in food and beverages.

The Panel noted that for 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032], for all food categories the normal use levels and/or the maximum use levels (highest normal use level is 0.49 mg/kg (i.e. 0.49 μg/g) and highest maximum use level is 2 mg/kg (i.e. 2 μg/g), see Appendix C) are more than one order of magnitude below the concentration for which an aneugenic effect of this flavouring substance was observed in the *in vitro* MN assay (i.e. 97.2 μg/mL). Therefore, for the application in the respective food categories, the use of the flavouring substance [FL-no: 15.032] at the reported use levels would not raise a concern for aneugenicity. Accordingly, this substance can be evaluated following the Procedure for evaluation of flavouring substances.

Based on structural similarity, for the remaining four 3-thiazoline substances in this FGE [FL-no: 15.029, 15.030, 15.130, 15.131], an aneugenic potential may also be anticipated. However, the lowest concentration resulting in aneugenicity *in vitro* for [FL no: 15.032] cannot be used for the safety assessment for these four substances, because quantitative extrapolation of this concentration to estimate the aneugenic potency of these four substances would be connected to a high level of uncertainty. Therefore, for these four substances, individual data are needed to establish whether they have aneugenic potential. These four substances should be screened in an *in vitro* MN test preferably with human lymphocytes and application of a FISH technique for centromere analysis. In case of an increase in micronucleated cell frequency, an appropriate follow-up test should be done, according to the EFSA guidance on genotoxicity testing strategy (EFSA Scientific Committee, 2011) and the EFSA guidance on aneugenicity (EFSA Scientific Committee, 2021).

Accordingly, the substances [FL-no: 15.029, 15.030, 15.130, 15.131] cannot currently be evaluated following the Procedure for evaluation of flavouring substances.

3.3.6. Conclusions on genotoxicity data

3.3.6.1. 4-Methyl-5-vinylthiazole [FL-no: 15.018]

The Panel concluded that the concern for genotoxicity is ruled out for the representative substance 4-methyl-5-vinylthiazole [FL-no: 15.018] and thus also for the structurally related substance 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005]. Both substances can be evaluated through the Procedure.

3.3.6.2. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032]

The Panel concluded that the concern for gene mutation and clastogenicity is ruled out for the representative substance 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] and thus also for the structurally related substances [FL-no: 15.029, 15.030, 15.130, 15.131].

4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] induced MN *in vitro* through an aneugenic mode of action. The available *in vivo* MN study was inconclusive and cannot be used to rule out the potential aneugenicity of [FL-no: 15.032] *in vivo*. Therefore, the Panel compared the lowest concentration resulting in aneugenicity *in vitro* with the use levels reported for this substance. These use levels were about 50 times lower than (i.e. at least one order of magnitude below) the lowest concentration resulting in aneugenicity *in vitro*. The Panel concluded that the use of the substance [FL-no: 15.032] at the maximum reported use levels does not raise a concern for aneugenicity. Accordingly, 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] can be evaluated through the Procedure.

Based on structural similarity, for the remaining four 3-thiazoline substances in this FGE [FL-no: 15.029, 15.030, 15.130, 15.131], an aneugenic potential may also be anticipated. However, the lowest concentration resulting in aneugenicity *in vitro* for [FL no: 15.032] cannot be used for the safety assessment for these four substances, because quantitative extrapolation of this concentration to estimate the aneugenic potency of these four substances would be connected to a high level of uncertainty. Therefore, for these four substances, individual data are needed to establish whether they have aneugenic potential. These four substances should be screened in an *in vitro* MN test preferably with human lymphocytes and application of a FISH technique for centromere analysis. In case of an increase in micronucleated cell frequency, an appropriate follow-up test should be done, according to the EFSA guidance on genotoxicity testing strategy (EFSA Scientific Committee, 2011) and the EFSA guidance on aneugenicity (EFSA Scientific Committee, 2021).

3.3.7. Toxicity studies

JECFA status

For the group of 3-thiazolines, JECFA (2002a, 2003, 2007, 2008a) considered in the evaluation a 90-day dietary study in rats on the substance 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029] (Food and Drug Research Laboratories, 1978). The NOAEL of 1.2 mg/kg bw per day from this study was considered appropriate also for the structurally related substances [FL-no: 15.030, 15.032, 15.130, 15.131].

JECFA (2002a, 2003) considered in the evaluation a 90-day dietary study in rats on the substance 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] (Posternak et al., 1969). This study reported a NOAEL of 0.92 mg/kg bw per day, which was considered appropriate also for the structurally related substance 4-methyl-5-vinylthiazole [FL-no: 15.018].

EFSA considerations

The same 90-day toxicity studies evaluated by JECFA were provided by industry for the present evaluation of thiazoles and 3-thiazolines substances in FGE.76Rev2 (Table 6).

For the group of 3-thiazolines, industry submitted a 90-day toxicity study for 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029] (Food and Drug Research Laboratories, 1978).

2-(Sec-butyl)-4,5-dimethyl-3-thiazoline was administered via diet to male and female Sprague Dawley rats (15/sex) for 90 days at one-dose level of 1.2 mg/kg bw per day. Body weight changes, food consumption, limited haematological and clinical chemistry parameters were assessed, and urinalysis was undertaken.

A significant increase in the percentage of eosinophils was observed in treated males; however, according to the study report, the value was within the normal percentage range for 'the rat', with a general reference to scientific data from the public domain (from the report of the study with [FL-no: 15.029], it is not clear whether this reference is strain specific). No other differences were observed. The authors stated that 'based on the data evaluated the material produced no overt toxicity'.

The Panel noted that the study had several shortcomings, e.g. only one dose level, purity of test material was not specified. Pathology and histopathology were studied, but detailed results were not included in the study report.

For the thiazoles (4-methyl-5-vinylthiazole [FL-no: 15.018] and 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005]), industry submitted a summary (Posternak et al., 1969) of toxicological data. This publication (Posternak et al., 1969) is a summary of 90-day studies on 42 flavouring compounds. The publication

states that 'of these, 29 were at least 98% pure and (...) the purity of the remainder was 90% or more'; but it is not clear how this relates to 2,4-dimethyl-5-vinylthiazole. The only available information on that study is a brief description of the method employed and a few general remarks. No study report or any details are available.

According to Posternak et al. (1969), a NOAEL of 0.92 mg/kg bw per day could be established for 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] in a 90-day study in rats. A control and a single treatment group of the same sex were housed in pairs and given ad libitum access to water and food. The concentration of the test material in the diet was adjusted during the study to maintain a dietary intake of 0.92 mg/kg bw per day and 1.0 mg/kg bw per day in males and females, respectively. Food consumption and body weights were determined weekly. Limited haematology and blood urea analyses were conducted during the weeks 7 and 13 of the study, on half of the animals. At the end of the study, animals were sacrificed, liver and kidney weights were measured and 'gross and histological examinations were carried out on a wide range of organs'.

The authors reported that at the exposure of 0.92 mg/kg bw per day in males and 1.0 mg/kg bw per day in females, 2,4-dimethyl-5-vinylthiazole did not induce any effects in the parameters measured. However, the Panel noted that the design and number of parameters of the 90-day study deviated significantly from the current requirements for 90-day study according to OECD TG 408; moreover, the reporting was deficient.

Table 6: Toxicity studies considered in FGE.76Rev2

FL-no JECFA no	Chemical name	Group	Study
15.005 1039	2,4-dimethyl-5-vinylthiazole	Thiazoles	[FL-no: 15.005] tested in a 90-day oral toxicity study in rats (Posternak et al., 1969) NOAEL of 0.92 mg/kg bw per day
15.018 1038	4-Methyl-5-vinylthiazole		
15.029 1059	2-(sec-butyl)-4,5-dimethyl-3-thiazoline	3-Thiazolines	[FL-no: 15.029] tested in a 90-day oral toxicity study in rats (Food and Drug Research Laboratories, 1978) NOAEL of 1.2 mg/kg bw per day
15.030 1058	4,5-dimethyl-2-ethyl-3-thiazoline		
15.032 1045	4,5-Dimethyl-2-isobutyl-3-thiazoline		
15.130 1761	5-ethyl-4-methyl-2-(2-methylpropyl) thiazoline		
15.131 1762	5-ethyl-4-methyl-2-(2-butyl)thiazoline		

Conclusions on toxicity studies

The Panel considered that despite the limitations of the toxicity data available, the NOAEL of 1.2 mg/kg bw per day, from the study on [FL-no: 15.029], can be used for the calculation of a margin of exposure (MOE) for the structurally related substance [FL-no: 15.032].

Similarly, the NOAEL of 0.92 mg/kg bw per day from the study on [FL-no: 15.005] can be used for the calculation of an MOE for [FL-no: 15.005] and for the structurally related substance [FL-no: 15.018].

4. Application of the procedure

4.1. Application of the procedure to 26 Sulfur-Containing heterocyclic compounds by the JECFA (59th meeting)

According to the JECFA (2002a, 2003), 17 of the substances belong to structural class II and nine to structural class III using the decision tree approach presented by Cramer et al. (Cramer et al., 1978).

The JECFA concluded three sulfur-containing heterocyclic compounds [FL-no: 15.014, 15.015 and 16.027] at step A3 in the JECFA Procedure, i.e. the substances are expected to be metabolised to innocuous products (step 2) and that the intakes for two of the substances are below the thresholds for their structural class II (step A3). For one substance, thiamine hydrochloride [FL no: 16.027], the

intake was above the threshold for structural class II and the substance is considered not to occur endogenously in humans, therefore the evaluation proceeded to step A5, where it was considered as of no safety concern at the estimated level of intake based on a 90-day dietary study in rats in which a no observed adverse effect level (NOAEL) of 36 mg/kg bw per day provides a margin of safety of more than 500.

Twenty-three substances were concluded at step B4 in the JECFA Procedure, i.e. the substances are not expected to be metabolised to innocuous products and the estimated intakes are below the thresholds for their structural classes II and III. An adequate NOAEL was available for all 23 substances and the JECFA concluded that the substances are therefore not expected to be of safety concern when used as flavouring substances.

In conclusion, the JECFA evaluated all 26 substances to be of no safety concern at the estimated levels of intake as flavouring substances based on the MSDI approach.

In particular regarding the substances under evaluation through the Procedure in FGE.76Rev2 [FL-no: 15.005, 15.018 and 15.032], the following steps of the Procedure were applied by JECFA:

Step 1

JECFA reported that 4-methyl-5-vinylthiazole [FL-no: 15.018] and 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] are 'structurally closely related to common constituents of food and were therefore placed in structural class II'. 4,5-Dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] was classified in structural class III.

Step 2

JECFA reported that data on metabolism are insufficient. The substances [FL-no: 15.005, 15.018, 15.032] were evaluated via the B-side of the Procedure.

Step B3

The estimated daily intakes for [FL-no: 15.005 and 15.018] and for [FL-no: 15.032] were below the TTC for structural class II (540 μg/person per day) and for structural class III (90 μg/person per day), respectively. Therefore, the evaluation of these substances proceeded at step B4.

Step B4

The no observed effect level (NOEL) of 0.92 mg/kg bw per day from a 90-day study on [FL-no: 15.005] was considered appropriate also for the structurally related substance [FL-no: 15.018]. Based on this NOEL and on exposure estimated according to MSDI, the margin of safety was above 9 million and above 20,000 for [FL-no: 15.005] and [FL-no: 15.018], respectively.

The NOEL of 1.2 mg/kg bw per day from a 90-day study on [FL-no: 15.029] was considered appropriate also for the structurally related substance [FL-no: 15.032]. Based on this NOEL and on exposure estimated according to MSDI, the margin of safety was above 10,000 for [FL-no: 15.032].

The evaluations by JECFA of the 26 sulfur-containing heterocyclic compounds are summarised in Appendix G.

4.2. Application of the procedure to Sulfur containing heterocyclic compounds by the JECFA (68th meeting)

The substances evaluated by JECFA at the 68th meeting (JECFA, 2007, 2008a) were initially allocated to FGE.93. In FGE.93Rev1, five substances were evaluated (2-acetyl-2-thiazoline [FL-no: 15.010], 3-(methylthio)-methylthiophene [FL-no: 15.126], 2-propionyl-2-thiazoline [FL-no: 15.128], 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131]). According to JECFA, two of the substances belong to structural class II [FL-no: 15.010 and 15.128], and three to structural class III [FL-no: 15.126, 15.130 and 15.131] using the decision tree approach presented by Cramer et al. (Cramer et al., 1978). All five substances were concluded at step B4 in the JECFA Procedure – i.e. that the substances are not expected to be metabolised to innocuous products and that the estimated intakes are below the thresholds for their structural classes II and III. An adequate NOAEL was available for relevant structurally related substances for all five substances and the JECFA concluded that the substances are therefore not expected to be of safety concern when used as flavouring substances. In conclusion, the JECFA evaluated all five substances to be of no safety concern at the estimated levels of intake as flavouring substances based on the MSDI approach. The evaluations by JECFA of

the two sulfur containing heterocyclic substances [FL-no: 15.130, 15.131] are summarised in Appendix G (Table G.1).

Because of a better structural similarity of substances [FL-no: 15.130 and 15.131] with candidate substances in FGE.76, these two substances will not be further evaluated in FGE.93, but will be included in FGE.76Rev2 (see also Section 1.2).

4.3. Application of the procedure to thiazoles, thiophenes, thiazoline and thienyl derivatives from chemical groups 29 and 30 (FGE.21Rev5)

Since the publication of FGE.21Rev3, 18 substances [FL-no: 15.037, 15.042, 15.043, 15.064, 15.070, 15.072, 15.077, 15.088, 15.090, 15.091, 15.092, 15.094, 15.099, 15.106, 15.107, 15.114, 15.129 and 15.133] of the 59 candidate substances are no longer of interest for industry for use as flavouring substances in Europe and no further data were submitted. Therefore, 41 candidate substances were evaluated in FGE.21Rev5 (EFSA CEF Panel, 2015). Thirty-three substances were classified into structural class II and eight into structural class III using the decision tree approach presented by Cramer et al. (1978).

For two substances, the Procedure could not be applied due to indication of genotoxic potential *in vitro* [FL-no: 15.060, 15.119].

The substances were divided into subgroups based on the nature of the ring (aromatic (clustered in subgroups A-Ib, A-Ic and A-II) vs. non-aromatic (clustered in subgroups B-II and B-IV)), on type and number of ring heteroatoms (sulfur or sulfur with nitrogen) and the degree of saturation in the non-aromatic rings (for description, see FGE.21Rev3 and FGE.21Rev5).

All the 39 substances were evaluated at step B4 of the Procedure, i.e. the substances are not expected to be metabolised to innocuous products and the estimated intakes are below the thresholds for their structural classes II and III.

None of the 39 candidate substances evaluated through the Procedure is of safety concern at their estimated levels of intake, when based on the MSDI approach.

The stepwise evaluations of the supporting substances relevant for the current evaluation are summarised in Appendix H (Table H.1).

4.4. EFSA considerations

In FGE.76Rev1, the CEF Panel agreed with the application of the Procedure, as performed by the JECFA (JECFA, 2002a, 2003), for 21 of the 26 substances in the group of sulfur-containing heterocyclic compounds.

Three of the 26 substances evaluated by the JECFA, 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029], 4,5-dimethyl-2-ethyl-3-thiazoline [FL-no: 15.030] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] were considered by the CEF Panel to have genotoxic potential *in vitro*, and therefore, the CEF Panel concluded that the Procedure should not be applied to these three flavouring substances until adequate *in vivo* genotoxicity data become available. Additionally, the CEF Panel noted the presence of a terminal conjugated double bond in the substances 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018] which raised concern for genotoxicity. The CEF Panel concluded, contrary to the JECFA, that the Procedure should not be applied to these two substances until genotoxicity data become available (EFSA CEF Panel, 2013a).

In FGE.93Rev1, the CEF Panel agreed with the application of the Procedure, as performed by the JECFA (JECFA, 2007, 2008a), for three [FL-no: 15.010, 15.126, 15.128] of the five substances in the group of sulfur-containing heterocyclic substances.

The remaining two substances, 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131], are structurally similar to two other 3-thiazolines in FGE.21Rev1 for which the CEF Panel has expressed a genotoxicity concern. Therefore, the CEF Panel concluded that the Procedure should not be applied to these two substances until adequate genotoxicity data become available (EFSA CEF Panel, 2013b).

In the present revision of FGE.76 (FGE.76Rev2), the Panel concluded that the concern for aneugenicity cannot be ruled out for the substances [FL-no: 15.029, 15.030, 15.130 and 15.131]. Therefore, the Procedure cannot be applied to the evaluation of these four substances.

Since the additional genotoxicity data allow to rule out the genotoxicity concern for [FL-no: 15.005, 15.018 and 15.032], these substances can be evaluated through the Procedure:

Step 1. Contrary to JECFA, both 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018] were assigned to structural class III[8] (TTC of 90 μg/person per day). The substance 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] was confirmed as structural class III.

Step 2. The Panel agreed with JECFA that the substances [FL-no: 15.005, 15.018, 15.032] cannot be predicted to be metabolised to innocuous products; therefore, the evaluation proceeded via the B-side of the Procedure.

Step B3. The estimated MSDI values are 0.01, 5 and 4.3 μg/capita per day for [FL-no: 15.005, 15.018 and 15.032], respectively. The MSDI values are below the TTC for class III.

Step B4. Based on a NOAEL of 0.92 mg/kg bw per day from a 90-day toxicity study on [FL-no: 15.005], adequate margins of exposure approximately of 46×10^5 and 11,000 are calculated for [FL-no: 15.005 and 15.018], respectively.

Based on a NOAEL of 1.2 mg/kg bw per day from a 90-day toxicity study on [FL-no: 15.029], an adequate margin of exposure of approximately 17,000 is calculated for [FL-no: 15.032].

Thus, the Panel concluded that the substances [FL-no: 15.005, 15.018, 15.032] are not of safety concern, when based on the MSDI approach.

Based on the use levels provided, mTAMDI values of 70, 171 and 213 μg/person per day are estimated for 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005], 4-methyl-5-vinylthiazole [FL-no: 15.018] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032], respectively. The mTAMDI value for [FL-no: 15.005] is below the TTC for structural class III, but the mTAMDI values for [FL-no: 15.018 and 15.032] are above the TTC for class III.

In addition, industry provided use levels for six structural class III substances, allowing the mTAMDI estimation for [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016, 15.021]. The mTAMDIs were estimated as 0.02, 550, 15, 6.2, 650 and 4 μg/person per day, respectively. The mTAMDI values for [FL-no: 15.002 and 15.016] are above the TTC for structural class III substances.

The Panel concluded that more reliable data on use and use levels are needed for [FL-no: 15.002, 15.016, 15.018 and 15.032] as the mTAMDI exposure estimates are above the TTC for their structural class III (90 μg/person per day). When these data become available, the assessment for these flavouring substances should be updated accordingly and expanded if necessary (i.e. request of additional toxicology data).

For the substances [FL-no: 15.004, 15.013, 15.014, 15.015, 15.017, 15.019, 15.020, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113 and 16.027] already evaluated through the Procedure in FGE.76Rev1, the Panel reiterates that use levels are needed to calculate the mTAMDI in order to identify those flavouring substances that need more refined exposure assessment and to finalise the evaluation.

5. Discussion

The 28 substances in the JECFA flavouring group of sulfur-containing heterocyclic compounds are structurally related to the 59 thiazoles, thiophenes, thiazoline and thienyl derivatives from chemical group 29 and miscellaneous substances from chemical group 30 evaluated by EFSA in FGE.21Rev5 (EFSA CEF Panel, 2015).

Based on data on structurally related substances of subgroup B-II (thiazolines, [FL-no: 15.060, 15.119]) and B-III (thiazolidines, [FL-no: 15.090, 15.099][5]) of FGE.21Rev3, the CEF Panel considered that 2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029], 4,5-dimethyl-2-ethyl-3-thiazoline [FL-no: 15.030] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032] have genotoxic potential *in vitro*, and therefore, the Procedure should not be applied to these three flavouring substances until adequate *in vivo* genotoxicity data become available (EFSA, 2008; EFSA CEF Panel, 2013a).

Moreover, the CEF Panel did not agree with the application of the Procedure as performed by JECFA for 4-methyl-5-vinylthiazole [FL-no: 15.018] and 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005], because the CEF Panel noted the presence of a terminal conjugated double bond in the substances 2,4-dimethyl-5-vinylthiazole [FL-no: 15.005] and 4-methyl-5-vinylthiazole [FL-no: 15.018] which raised concern for genotoxicity (EFSA, 2008; EFSA CEF Panel, 2013a).

New genotoxicity data have been provided for the representative substances 4-methyl-5-vinylthiazole [FL-no: 15.018] and 4,5-dimethyl-2-isobutyl-3-thiazoline [FL-no: 15.032], which are evaluated in the present revision of FGE.76 (FGE.76Rev2).

This revision 2 of FGE.76 comprises 26 sulfur-containing heterocyclic compounds evaluated by JECFA at its 59th meeting (JECFA, 2002a, 2003) and two substances [FL-no: 15.130, 15.131]

[8] OECD QSAR Toolbox v.4.4.1.

evaluated by JECFA at its 68th meeting (JECFA, 2007, 2008a). Twenty-one of these substances have already been considered in FGE.76Rev1. The remaining seven substances could not be evaluated in FGE.76Rev1 [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032] and in FGE.93Rev1 [FL-no: 15.130, 15.131], because of concerns with respect to genotoxicity.

Based on the new available data, the concern for genotoxicity is ruled out for [FL-no: 15.018] and also for the structurally related substance [FL-no: 15.005].

The concerns for gene mutations and clastogenicity are ruled out for the representative substance [FL-no: 15.032] and thus also for the structurally related substances [FL-no: 15.029, 15.030, 15.130, 15.131].

The flavouring substance [FL-no: 15.032] induced MN *in vitro* through an aneugenic mode of action. The available *in vivo* MN study in bone marrow was not adequate to rule out the potential aneugenicity of [FL-no: 15.032] *in vivo*, because there was insufficient evidence of adequate target tissue exposure. However, applying the recommendations for risk assessment of aneugenic substances (EFSA Scientific Committee, 2021), the Panel noted that the use levels for [FL-no: 15.032] are about 50 times lower (i.e. at least one order of magnitude below) than the lowest concentration resulting in aneugenicity *in vitro* (97.2 μg/mL); therefore, the Panel concluded that the use of the substance [FL-no: 15.032] at the maximum reported use levels does not raise a concern for aneugenicity.

Based on structural similarity, for the remaining four 3-thiazoline substances in this FGE [FL-no: 15.029, 15.030, 15.130, 15.131], an aneugenic potential may also be anticipated. However, the lowest concentration resulting in aneugenicity *in vitro* for [FL-no: 15.032] cannot be used for the safety assessment for these four substances, because quantitative extrapolation of this concentration to estimate the aneugenic potency of these four substances would be connected to a high level of uncertainty. Therefore, for [FL-no: 15.029, 15.030, 15.130, 15.131], individual data are needed to establish whether they have aneugenic potential; consequently, these substances have not been evaluated through the Procedure in FGE.76Rev2.

Since the concern for genotoxicity has been ruled out for [FL-no: 15.005, 15.018 and 15.032], these substances have been evaluated through the Procedure.

Based on considerations of structural class, metabolism data, absence of genotoxic potential *in vivo* and the MSDI exposure estimates, the FAF Panel concluded that [FL-no: 15.005, 15.018 and 15.032] do not raise a safety concern at step B4 of the Procedure, when based on MSDI approach.

Normal and maximum use levels for different food categories are available for the substances evaluated in the present revision and for six substances [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016, 15.021] already considered in FGE.76Rev1 (Appendix C). These data on use levels allowed the calculation of mTAMDI intake estimates.

For seven substances [FL-no: 15.001, 15.005, 15.008, 15.011, 15.021, 15.029 and 15.030], the mTAMDI values are below the TTC for structural class III.

For six substances [FL-no: 15.002, 15.016, 15.018, 15.032, 15.130 and 15.131], the mTAMDI exposure estimates are above the TTC for structural class III; therefore, more reliable data on uses and use levels should be provided in order to refine the exposure assessment and to finalise their safety evaluation.

For 15 substances [FL-no: 15.004, 15.013, 15.014, 15.015, 15.017, 15.019, 15.020, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113 and 16.027], evaluated through the Procedure in FGE.76 and FGE.76Rev1, use levels are needed to calculate the mTAMDIs in order to identify those flavouring substances that need more refined exposure assessment and to finalise the evaluation.

For 24 JECFA-evaluated sulfur-containing heterocyclic compounds [FL-no: 15.001, 15.002, 15.004, 15.005, 15.008, 15.011, 15.013, 15.014, 15.015, 15.016, 15.017, 15.018, 15.019, 15.020, 15.021, 15.022, 15.026, 15.027, 15.032, 15.033, 15.035, 15.109, 15.113 and 16.027] the Panel agreed with the JECFA conclusion 'No safety concern at estimated levels of intake as flavouring substances' when based on the MSDI approach.

This conclusion for the named substances cannot be applied to the material of commerce for the substances [FL-no: 15.109 and 15.113], since for these flavouring substances, the information on the stereochemical composition is inadequate.

6. Conclusions

The Panel evaluated 24 flavouring substances [FL-no: 15.001, 15.002, 15.004, 15.005, 15.008, 15.011, 15.013, 15.014, 15.015, 15.016, 15.017, 15.018, 15.019, 15.020, 15.021, 15.022, 15.026, 15.027, 15.032, 15.033, 15.035, 15.109, 15.113 and 16.027] through the Procedure and agreed with the JECFA conclusion 'No safety concern at estimated levels of intake as flavouring substances' when based on the MSDI approach.

The conclusion on [FL-no: 15.032] is restricted to uses and use levels for this substance that are more than one order of magnitude lower than the concentration inducing aneugenicity *in vitro*.

For the remaining four substances [FL-no: 15.029, 15.030, 15.130, 15.131], individual data are needed to determine whether they have aneugenic potential according to the EFSA guidance on genotoxicity testing strategy (EFSA Scientific Committee, 2011) and the EFSA guidance on aneugenicity (EFSA Scientific Committee, 2021).

For seven substances, [FL-no: 15.001, 15.005, 15.008, 15.011, 15.021, 15.029 and 15.030], there is no concern when the exposure was estimated based on the mTAMDI approach.

For six substances [FL-no: 15.002, 15.016, 15.018 15.032, 15.130 and 15.131], more reliable information on uses and normal and maximum use levels are needed to refine the mTAMDI estimates in order to finalise their evaluation. For 15 substances [FL-no: 15.004, 15.013, 15.014, 15.015, 15.017, 15.019, 15.020, 15.022, 15.026, 15.027, 15.033, 15.035, 15.109, 15.113 and 16.027], use levels are needed to calculate the mTAMDIs. Upon submission of such data, additional data on toxicity may become necessary.

Adequate specifications including purity criteria and identity have been provided for 26 flavouring substances. For substances [FL-no: 15.109 and 15.113], information on the actual stereochemical composition is inadequate and the conclusion reached for the named substances cannot be applied to the material of commerce.

7. Recommendations

The Panel recommends the European Commission to consider the following:

- In order to underline that 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131] belong to the group of 3-thiazolines, their names in the Union List[1] should be changed to 5-ethyl-4-methyl-2-(2-methylpropyl)-3-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-3-thiazoline [FL-no: 15.131].
- To request clarification of those cases where maximum use levels are reported to be lower than normal use levels for [FL-no: 15.018 and 15.032].

Documentation provided to EFSA

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Abbreviations

AFC	Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
CREST	Calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia
CPA	Cyclophosphamide
DNA	Deoxyribonucleic acid
EFFA	European Flavour Association
EMS	Ethyl methanesulfonate
FAF	Food Additives and Flavourings
FAO	Food and Agriculture Organisation of the United Nations
FEMA	Flavour and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FISH	Fluorescence *in situ* hybridisation
FLAVIS (FL)	Flavour Information System (database)
GIT	Gastrointestinal Tract
GLP	Good Laboratory Practice
HPRT	Hypoxanthine Phosphoribosyl transferase
ID	Identity
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MNBN	Micronucleated Binucleate cells
MOE	Margin of Exposure
MSDI	Maximised Survey-derived Daily Intake
MTD	Maximum Tolerated Dose
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NCE	Normochromatic erythrocyte
No	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
OECD	Organisation for Economic Cooperation and Development
PCE	Polychromatic erythrocyte
SCF	Scientific Committee on Food
TTC	Threshold of toxicological concern
VB	Vinblastine
WHO	World Health Organisation

Appendix A – Procedure for the safety evaluation

The approach for a safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000[3], named the 'Procedure', is shown in schematic form in Figure A.1. The Procedure is based on the Opinion of the Scientific Committee on Food expressed on 2 December 1999 (SCF, 1999), which is derived from the evaluation Procedure developed by the Joint FAO/WHO Expert Committee on Food Additives at its 44th, 46th and 49th meetings (JECFA, 1995, 1996, 1997, 1999), hereafter named the 'JECFA Procedure'.[9]

The Procedure is a stepwise approach that integrates information on intake from current uses, structure–activity relationships, metabolism and, when needed, toxicity. One of the key elements in the Procedure is the subdivision of flavourings into three structural classes (I, II and III) for which toxicological thresholds of concern (TTCs) (human exposure thresholds) have been specified. Exposures below these TTCs are not considered to present a safety concern.

Class I contains flavourings that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of oral toxicity. Class II contains flavourings that have structural features that are less innocuous but are not suggestive of toxicity. Class III comprises flavourings that have structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity (Cramer et al., 1978). The TTCs for these structural classes of 1,800, 540 or 90 μg/person per day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996).

In step 1 of the Procedure, the flavourings are assigned to one of the structural classes. The further steps address the following questions:

- Can the flavourings be predicted to be metabolised to innocuous[10] products (step 2)?
- Do their exposures exceed the TTC for the structural class (steps A3 and B3)?
- Are the flavourings or their metabolites endogenous[10] (step A4)?
- Does a NOAEL exist on the flavourings or on structurally related substances (steps A5 and B4)?

In addition to the data provided for the flavouring substances to be evaluated (candidate substances), toxicological background information available for compounds structurally related to the candidate substances is considered (supporting substances), in order to assure that these data are consistent with the results obtained after application of the Procedure.

The Procedure is not to be applied to flavourings with existing unresolved problems of toxicity. Therefore, the right is reserved to use alternative approaches if data on specific flavourings warranted such actions.

[9] The FAF Panel is aware that a Revised Procedure for the Safety Evaluation of Flavouring agents has been agreed by JECFA (JECFA, 2016). Also, the EFSA Scientific Committee has developed a modified procedure for evaluation of substances based on the TTC approach (EFSA Scientific Committee, 2019). However, these developments have no impact on the present evaluation, which should follow the requirements as set out in Commission Regulation (EC) No 1565/2000.

[10] *Innocuous products*: products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent (JECFA, 1997).
Endogenous substances: intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included (JECFA, 1997).

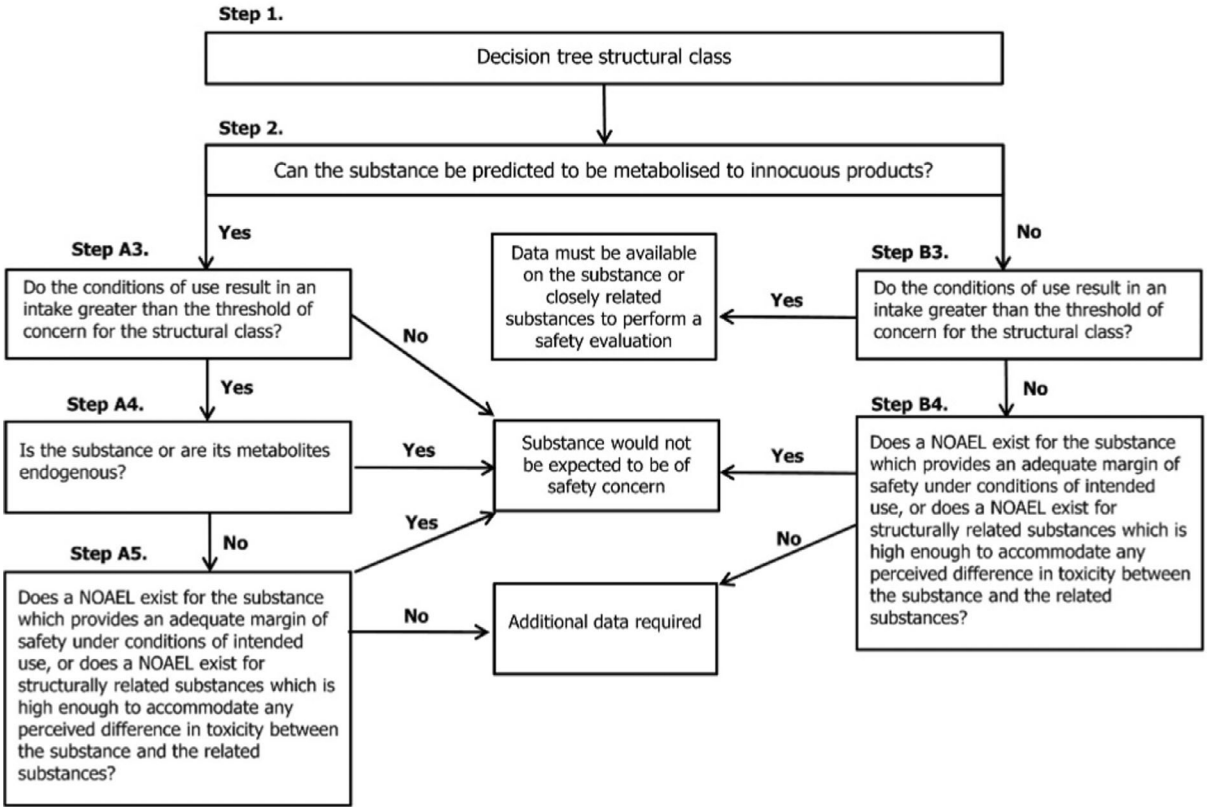


Figure A.1: Procedure for the safety evaluation of chemically defined flavouring substances

For the flavouring substances considered in this FGE, the FAF Panel compares the JECFA evaluation of structurally related substances with the result of a corresponding EFSA evaluation, focussing on specifications, intake estimations and toxicity data, especially genotoxicity data. The considerations by EFSA will conclude whether the flavouring substances are of no safety concern at their estimated levels of intake, whether additional data are required or whether certain substances should not be evaluated through the EFSA Procedure.

The following issues are of special importance:

a) Intake

In its evaluation, the Panel as a default uses the 'maximised survey-derived daily intake' (MSDI)[11] approach to estimate the per capita intakes of the flavouring substances in Europe.

In its evaluation, the JECFA includes intake estimates based on the MSDI approach derived from both European and USA production figures. The highest of the two MSDI figures is used in the evaluation by the JECFA. It is noted that in several cases, only the MSDI figures from the USA were available, meaning that certain flavouring substances have been evaluated by the JECFA only on the basis of these figures. For substances in the Union List of flavouring substances[12] for which this is the case, the Panel will need European Union (EU) production figures in order to finalise the evaluation.

When the Panel examined the information provided by the European Flavour Industry on the use levels in various foods, it appeared obvious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavoured at the use levels reported by the industry, especially in those cases where the annual production values were reported to be small. In consequence, the Panel had reservations about the data on use and use levels provided and the intake estimates obtained by the MSDI approach. It is noted that the JECFA, at its 65th meeting,

[11] EU MSDI: Amount added to food as flavour in (kg/year) × 10^9/(0.1 × population in Europe (= 375 × 10^6) × 0.6 × 365) = μg/capita per day.

[12] Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, pp. 1–161.

considered how to improve the identification and assessment of flavouring agents, for which the MSDI estimates may be substantially lower than the dietary exposures that would be estimated from the anticipated average use levels in foods (JECFA, 2006).

In the absence of more accurate information that would enable the Panel to make a more realistic estimate of the intakes of the flavouring substances, the Panel has decided also to perform an estimate of the daily intakes per person using a modified theoretical added maximum daily intake (mTAMDI) approach based on the normal use levels reported by Industry (see Appendix C).

As information on use levels for the flavouring substances has not been requested by the JECFA or has not otherwise been provided to the Panel, it is not possible to estimate the daily intakes using the mTAMDI approach for many of the substances evaluated by JECFA. The Panel will need information on use levels in order to finalise the evaluation.

b) Threshold of 1.5 μg/person per day (step B5) used by the JECFA

JECFA uses the threshold of concern of 1.5 μg/person per day as part of the evaluation procedure:

'The Committee noted that this value was based on a risk analysis of known carcinogens which involved several conservative assumptions. The use of this value was supported by additional information on developmental toxicity, neurotoxicity and immunotoxicity. In the judgement of the Committee, flavouring substances for which insufficient data are available for them to be evaluated using earlier steps in the Procedure, but for which the intake would not exceed 1.5 μg/person per day would not be expected to present a safety concern. The Committee recommended that the Procedure for the Safety Evaluation of Flavouring Agents used at the forty-sixth meeting be amended to include the last step on the right-hand side of the original procedure ('Do the condition of use result in an intake greater than 1.5 μg per day?')' (JECFA, 1999).

In line with the opinion expressed by the Scientific Committee on Food (SCF, 1999), the Panel does not make use of this threshold of 1.5 μg/person per day.

c) Genotoxicity

As reflected in the opinion of SCF (SCF, 1999), the Panel has in its evaluation focussed on a possible genotoxic potential of the flavouring substances or of structurally related substances. Generally, substances for which the Panel has concluded that there is an indication of genotoxic potential *in vitro*, will not be evaluated using the EFSA Procedure until further genotoxicity data are provided. Substances for which a genotoxic potential *in vivo* has been concluded, will not be evaluated through the Procedure.

d) Specifications

Regarding specifications, the evaluation by the Panel could lead to a different opinion than that of JECFA, since the Panel requests information on e.g. isomerism.

e) Structural Relationship

In the consideration of the JECFA evaluated substances, the Panel will examine the structural relationship and metabolism features of the substances within the flavouring group and compare this with the corresponding Flavouring Group Evaluation (FGE).

Appendix B – Specifications

Table B.1: Summary table on specifications data for flavouring substances in FGE.76Rev2 (for chemical structures, see Appendix G) that are included in the Union List. Substance [FL-no: 15.028] which is not included in the EU Union list is referenced in the previous version, FGE.76Rev1 (EFSA CEF Panel, 2013a)

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	**EFSA Comments**
15.001 1052 3062 478 7774-74-5	2-Mercaptothiophene	(b)	Liquid $C_4H_4S_2$ 116.20	Very slightly soluble Miscible	166 n.a. NMR 98%	1.618–1.622 1.250–1.255	
15.002 1057 3192 736 38205-64-0	2-Methyl-5-methoxythiazole	(b)	Liquid C_5H_7ONS 129.18	Insoluble Miscible	117 (44 hPa) n.a. MS 98%	1.515–1.520 1.146–1.154	
15.004 1050 3209 2203 13679-70-4	5-Methyl-2-thiophenecarbaldehyde	(b)	Liquid C_6H_6OS 126.18	Practically insoluble or insoluble Miscible	113–114 (33 hPa) n.a. NMR 95%	1.574–1.586 1.168–1.172	
15.005 1039 3145 2237 65505-18-2	2,4-Dimethyl-5-vinylthiazole	(b)	Liquid C_7H_9NS 139.22	Slightly soluble Miscible	183–184 n.a. NMR 99%	1.560–1.565 1.050–1.056	

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				EFSA Comments
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	
15.008 1053 3323 2333 6911-51-9	2-Thienyl disulfide	(b)	Solid $C_8H_6S_4$ 230.39	Very soluble Soluble	n.a. 55–60 NMR 98%	n.a. n.a.	
15.011 1055 3267 2336 38205-60-6	5-Acetyl-2,4-dimethylthiazole	(b)	Liquid C_7H_9ONS 155.22	Insoluble Miscible	228–230 n.a. NMR 97%	1.536–1.547 1.147–1.152	
15.013 1034 3134 11618 18640-74-9	2-Isobutylthiazole	(b)	Liquid $C_7H_{11}NS$ 141.24	Slightly soluble Miscible	178–180 n.a. NMR 96%	1.490–1.499 0.993–0.997	
15.014 1031 3204 11621 137-00-8	5-(2-Hydroxyethyl)-4-methylthiazole	(b)	Liquid C_6H_9ONS 143.21	Soluble Miscible	135 (9 hPa) n.a. IR, NMR 96%	1.540–1.556 1.196–1.210	
15.015 1054 3205 11620 656-53-1	4-Methyl-5-(2-acetoxyethyl)thiazole	(b)	Liquid $C_8H_{11}O_2NS$ 185.25	Slightly soluble Miscible	117–118 (8 hPa) n.a. NMR 97%	1.505–1.515 1.145–1.149	
15.016 1040 3256 195-16-9	Benzothiazole	(b)	Liquid C_7H_5NS 135.19	Very slightly soluble Miscible	231 n.a. NMR 96%	1.637–1.644 1.236–1.240	

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				EFSA Comments
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	
15.017 1035 3274 11606 3581-91-7	4,5-Dimethylthiazole	(b)	Liquid C_5H_7NS 113.18	Very slightly soluble Miscible	158 (965 hPa) n.a. NMR 97%	1.516–1.524 1.067–1.072	
15.018 1038 3313 11633 1759-28-0	4-Methyl-5-vinylthiazole	(b)	Liquid C_6H_7NS 125.19	Very slightly soluble Miscible	78–80 (33 hPa) n.a. NMR 97%	1.560–1.570 1.091–1.095	
15.019 1036 3325 11650 13623-11-5	2,4,5-Trimethylthiazole	(b)	Liquid C_6H_9NS 127.21	Very slightly soluble Miscible	65–67 (26 hPa) n.a. NMR 97%	1.503–1.511 1.011–1.015	
15.020 1041 3328 11726 24295-03-2	2-Acetylthiazole	(b)	Liquid C_5H_5ONS 127.17	Insoluble Miscible	89–91 (16 hPa) n.a. NMR 97%	1.543–1.550 1.225–1.229	
15.021 1056 3340 11611 15679-19-3	2-Ethoxythiazole	(b)	Liquid C_5H_7ONS 129.18	Insoluble Miscible	157–160 n.a. NMR 99%	1.498–1.502 1.131–1.135	
15.022 1033 3372 11598 18277-27-5	2-(sec-Butyl)thiazole	(b)	Liquid $C_7H_{11}NS$ 141.24	Slightly soluble Miscible	173–174 n.a. NMR 98% (racemate, EFFA, 2013b)	1.496–1.502 0.998–1.003	

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				EFSA Comments
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	
15.026 1037 3555 – 15679-13-7	2-Isopropyl-4-methylthiazole	(b)	Liquid $C_7H_{11}NS$ 141.24	Slightly soluble Miscible	92 (65 hPa) n.a. NMR MS 96%	1.480–1.502 1.001–1.006	
15.027 1042 3611 – 43039-98-1	2-Propionylthiazole	(b)	Liquid C_6H_7ONS 141.19	Insoluble Miscible	95 (1 hPa) n.a. IR NMR MS 98%	1.528–1.533 1.205–1.210	
15.029 1059 3619 – 65894-82-8	2-(sec-Butyl)-4,5-dimethyl-3-thiazoline	(b)	Liquid $C_9H_{17}NS$ 171.31	Insoluble Miscible	71 (5 hPa) n.a. IR NMR MS 98%; mixture of diastereoisomers, each of them racemic, with 60% in *cis*- and 40% in *trans*- configuration	1.483–1.488 0.950–0.955	
15.030 1058 3620 – 76788-46-0	4,5-Dimethyl-2-ethyl-3-thiazoline	(b)	Liquid $C_7H_{13}NS$ 143.25	Insoluble Miscible	50 (4 hPa) n.a. IR NMR MS 98%; mixture of diastereoisomers, each of them racemic, with 60% in *cis*- and 40% in *trans*- configuration	1.490–1.495 1.001–1.010	
15.032 1045 3621 – 65894-83-9	4,5-Dimethyl-2-isobutyl-3-thiazoline	(b)	Liquid $C_9H_{17}NS$ 171.31	Insoluble Miscible	71 (5 hPa) n.a. IR NMR MS 97%; mixture of diastereoisomers, each of them racemic, with 60% in *cis*- and 40% in trans-configuration	1.483–1.489 0.933–0.937	

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				EFSA Comments
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	
15.033 1044 3680 11612 15679-12-6	2-Ethyl 4-methylthiazole	(b)	Liquid C_6H_9NS 127.21	Slightly soluble Miscible	161–162 n.a. NMR MS 97%	1.500–1.510 1.026–1.031	
15.035 1043 3716 11627 693-95-8	4-Methylthiazole	(b)	Liquid C_4H_5NS 99.16	Slightly soluble Miscible	133–134 n.a. IR NMR MS 97%	1.519–1.528 1.088–1.092	
15.109 1049 4018 11649 638-17-5	2,4,6-Trimethyldihydro-1,3,5(4H)-dithiazine	(b)	Solid $C_6H_{13}NS_2$ 163.30	Insoluble Miscible	n.a. 48 IR NMR MS 99% According to EFFA (2013b): mixture of three stereoisomers; theoretical options: meso-form (50%), two chiral stereoisomers (25% each)	n.a. n.a.	The CAS-no corresponds to the all-*cis* configuration. Information on the actual percentages of the stereoisomers in the material of commerce is inadequate.
15.113 1048 4017 – 74595-94-1	5,6-Dihydro-2,4,6,tris(2-methylpropyl)-4H-1,3,5-dithiazine	(b)	Solid $C_{15}H_{31}NS_2$ 289.55	Slightly soluble Miscible	n.a. 33–35 IR NMR 95% According to EFFA (2013b): mixture of three stereoisomers; theoretical options: meso-form (50%), two chiral stereoisomers (25% each)	n.a. n.a.	Information on the actual percentages of the stereoisomers in the material of commerce is inadequate.

Information included in the EU Union list Regulation (EC) No. 1334/2008 as amended			Most recent available specifications data[a]				EFSA Comments
FL-no JECFA-no FEMA no CoE no CAS no	**Chemical name**	**Purity of the named compound**	**Phys. form Mol. formula Mol. weight**	**Solubilityc[c] Solubility in ethanol[d]**	**Boiling point, °C[e] Melting point, °C ID test Assay minimum (isomers distribution/secondary components)**	**Refrac. Index[f] Spec. gravity[g]**	
15.130 1761 4319 – 83418-53-5	5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	(b)	Liquid $C_{10}H_{19}NS$ 185.33	Soluble Soluble	253 n.a. NMR MS 95%; mixture of diastereoisomers, each of them racemic, with 60% in *cis*- and 40% in *trans*- configuration	1.483–1.489 0.939–0.945	The name should be changed to 5-Ethyl-4-methyl-2-(2-methylpropyl)-3-thiazoline
15.131 1762 4318 – 83418-54-6	5-Ethyl-4-methyl-2-(2-butyl)-thiazoline	(b)	Liquid $C_{10}H_{19}NS$ 185.33	Soluble Soluble	253 n.a. NMR MS 95%; mixture of diastereoisomers, each of them racemic, with 60% in *cis*- and 40% in *trans*- configuration	1.487–1.493 0.950–0.956	The name should be changed to 5-Ethyl-4-methyl-2-(2-butyl)-3-thiazoline
16.027 1030 3322 10493 67-03-8	Thiamine hydrochloride	(b)	Solid $C_{12}H_{18}ON_4S$ 337.27	Soluble Slightly soluble	n.a. 248–250 NMR 98%	n.a. n.a.	

n.a.:not applicable; FL-No: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavour and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; ID: identity; IR: infrared spectroscopy; NMR: nuclear magnetic resonance; MS: mass spectrometry.
(a): JECFA, 2002b, 2008b and documentation provided to EFSA (EFFA, 2013b).
(b): At least 95% unless otherwise specified.
(c): Solubility in water, if not otherwise stated.
(d): Solubility in 95% ethanol, if not otherwise stated.
(e): At 1013.25 hPa, if not otherwise stated.
(f): At 20°C, if not otherwise stated.
(g): At 25°C, if not otherwise stated.

Appendix C – Exposure estimate

C.1. Uses and use levels as provided by the Flavour Industry

After the publication of FGE.76Rev1, industry provided data on uses and use levels for the substances [FL-no: 15.001, 15.002, 15.008, 15.011, 15.016, 15.021] (DG SANCO, 2014) according to the different food categories reported in Annex I of Regulation (EC) 1565/2000[3]. These data are included in the present revision, FGE.76Rev2, and used for the calculation of mTAMDI (Tables C.1 and C.5).

For each of the substances under evaluation [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032, 15.130 and 15.131], use levels have been provided by industry (EFFA, 2022) according to the different food categories reported in Annex I of Regulation (EC) 1565/2000 or according to the EFSA Guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA CEF Panel, 2010). For substances 5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline [FL-no: 15.130] and 5-ethyl-4-methyl-2-(2-butyl)-thiazoline [FL-no: 15.131] use levels are reported only for the food categories according to Regulation (EC) 1565/2000. Use levels data (Table C.2) have been used to calculate the mTAMDI (Table C.5). Since the calculation of mTAMDI is based on food categories as reported in Annex I of Regulation (EC) 1565/2000 (Table C.4), for the substances [FL-no: 15.005, 15.018, 15.029, 15.030, 15.032] the highest values of the normal use levels among the subcategories have been selected.

Table C.1: Normal and maximum use levels (mg/kg food) for JECFA-evaluated substances in FGE.76Rev2 (DG SANCO, 2014)

CODEX code	Food categories	Occurrence level as added flavouring substance[(a)] (mg/kg)											
		Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:		**15.001**		**15.002**		**15.008**		**15.011**		**15.016**		**15.021**	
1.0	Dairy products, excluding products of category 02.0									1.3	9.3		
2.0	Fats and oils, and fat emulsions (type water-in-oil)									0.023	0.045		
3.0	Edible ices, including sherbet and sorbet							0.0024	–	1.3	16		
4.1	Processed fruit												
4.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds												
5.0	Confectionery									0.011	0.13	0.002	–
6.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery					0.1	–			0.016	0.073	0.005	–
7.0	Bakery wares			4	–	0.1	–			0.038	0.73	0.001	–
8.0	Meat and meat products, including poultry and game			2	–					0.0000006	–		

CODEX code	Food categories	Occurrence level as added flavouring substance[(a)] (mg/kg)											
		Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:		**15.001**		**15.002**		**15.008**		**15.011**		**15.016**		**15.021**	
9.0	Fish and fish products, including molluscs, crustaceans and echinoderms												
10.0	Eggs and egg products												
11.0	Sweeteners, including honey												
12.0	Salts, spices, soups, sauces, salads, protein products, etc.			1	–	0.1	–			–	1.2		
13.0	Foodstuffs intended for particular nutritional uses												
14.1	Non-alcoholic ('soft') beverages, excl. dairy products							0.018	0.88	1.3	6.2	0.01	–
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts									2.5	16		
15.0	Ready-to-eat savouries	0.001	0.05							0.035	12		
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0												

FL-No: FLAVIS number; '–': no value for normal or maximum use level was provided.
(a): 'Normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002).

Table C.2: Normal and maximum use levels (mg/kg food) reported by industry for the flavouring substances in FGE.76Rev2 (EFFA, 2022)

CODEX code	Food categories[a],[b]	Standard portions[c],(g)	Occurrence level as added flavouring substance (mg/kg)													
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			**15.005[f]**		**15.018**		**15.029**		**15.030**		**15.032**		**15.130[d]**		**15.131[d]**	
01.0	Dairy products and analogues, excluding products of category 02.0														0.4	0.4
01.1	Milk and dairy-based drinks	200			0.11	0.22					0.00001	0.00001				
01.6	Cheese and analogues	40			0.03	0.04					0.0009	0.0009				
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125			0.18	1.27	–	0.50			–	2				
02.0	Fats and oils and fat and oil emulsions (type water-in-oil)												0.2	1.1		
02.1	Fats and oils essentially free from water	15	–	0.5	0.10	0.31					0.00016	0.00016				
02.2	Fat emulsions mainly of type water-in-oil	15	–	0.5	0.10	0.31					0.00016	0.00016				
02.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	15	–	0.5	0.10	0.31					0.00016	0.00016				
02.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	50			0.17	0.17					0.00002	0.00002				
03.0	Edible ices, including sherbet and sorbet	50			0.14	0.25					0.00002	1	0.4	2	0.4	0.4
04.1.2	Processed fruit	125	–	0.5	0.002	0.003							0.3	1.5		
04.1.2.5	Jams, jellies, marmalades	30			0.17	0.18										
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter) and nuts and seeds	200			0.002	0.006					0.23	0.23				
05.0	Confectionery												0.4	2	0.4	0.4

CODEX code	Food categories[a],[b]	Standard portions[c],(g)	Occurrence level as added flavouring substance (mg/kg)													
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			**15.005[f]**		**15.018**		**15.029**		**15.030**		**15.032**		**15.130[d]**		**15.131[d]**	
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40			0.22	0.82					0.0005	0.005				
05.2	Confectionery, including hard and soft candy, nougats, etc., other than 05.1, 05.3 and 05.4	30			0.28	0.81	–	2			0.00051	1				
05.3	Chewing gum	3	–	0.5	1.26	2.06										
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35			0.55	0.80					0.49	0.49				
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery												0.2	1	0.4	0.4
06.3	Breakfast cereals, including rolled oats	30			0.38	0.39										
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pastas and noodles)	200			0.01	0.02	0.005	0.005	0.002	0.002	0.37	0.42				
06.5	Cereal and starch-based desserts (e.g. rice pudding, tapioca pudding)	200			0.04	0.75										
06.6	Batters (e.g. for breading or batters for fish or poultry)	30			0.01	0.02			–	10						
06.7	Pre-cooked or processed rice products, including rice cakes (Oriental type only)	200			0.002	0.006										
06.8	Soybean products (excluding soybean products of food category 12.9 and fermented soybean products of food category 12.10)	100			0.02	0.04										
07.0	Bakery wares												0.4	2	0.4	0.4

CODEX code	Food categories[a],[b]	Standard portions[c],(g)	Occurrence level as added flavouring substance (mg/kg)													
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			**15.005[f]**		**15.018**		**15.029**		**15.030**		**15.032**		**15.130[d]**		**15.131[d]**	
07.1	Bread and ordinary bakery wares	50			0.24[e]	0.21[e]	–	2			0.001	2.00				
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	80	–	0.5	0.53	1.51	0.01	0.01			0.00009	0.0005				
08.0	Meat and meat products, including poultry and game												0.1	0.4	0.1	0.1
08.2	Processed meat, poultry and game products in whole pieces or cuts	100			0.16	0.65	0.08	0.29	0.03	5.01	0.18	0.94				
08.3	Processed comminute meat, poultry and game products	100			0.16	0.65	0.08	0.29	0.03	5.01	0.18	0.94				
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms												0.1	0.4	0.1	0.1
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100			0.50	0.50			–	10	0.000017[e]	0.000004[e]				
09.3	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms	100			0.50	0.50			–	10	0.000017[e]	0.000004[e]				
09.4	Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms	100			0.50	0.50			–	10	0.000017[e]	0.000004 [e]				
10.0	Eggs and egg products															
10.2	Egg products	100			0.002	0.006										
10.3	Preserved eggs, including alkaline. salted and canned eggs	100			0.002	0.006										
10.4	Egg-based desserts (e.g. custard)	125			0.29	0.57										
11.0	Sweeteners, including honey															
11.1	Refined and raw sugar	10			0.003	0.004										

CODEX code	Food categories[a],[b]	Standard portions[c],(g)	Occurrence level as added flavouring substance (mg/kg)													
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			**15.005[f]**		**15.018**		**15.029**		**15.030**		**15.032**		**15.130[d]**		**15.131[d]**	
11.2	Brown sugar excluding products of food category 11.1	10			0.002	0.002										
11.3	Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1	30			0.03	0.08										
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30			0.13	0.18										
11.5	Honey	15			0.002	0.002										
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	1			0.02	0.02										
12.0	Salts, spices, soups, sauces, salads, protein products, etc.												0.2	1	1	1
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1			0.26	0.27	0.02	0.04	0.01	0.01	0.21	0.55				
12.3	Vinegars	15			0.002	0.006										
12.4	Mustards	15			0.22	0.88										
12.5	Soups and broths	200			0.01	0.03	–	0.50	–	10	0.13	0.49				
12.6	Sauces and like products	30			0.20	0.61					0.19[e]	0.10[e]				
12.7.1	Salads 120 g (e.g. macaroni salad, potato salad) excluding cocoa- and nut-based spreads of food categories	120			0.002	0.006										
12.7.2	Sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories	20			0.002	0.006										
12.9	Protein products	15			0.14	1.12					0.01	0.01				
12.10	Fermented soybean products	40			0.50	0.50										
13.0	Foodstuffs intended for particular nutritional uses												0.4	2		

CODEX code	Food categories[a],[b]	Standard portions[c],(g)	Occurrence level as added flavouring substance (mg/kg)													
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			**15.005[f]**		**15.018**		**15.029**		**15.030**		**15.032**		**15.130[d]**		**15.131[d]**	
13.3	Dietetic foods intended for special medical purposes (excluding food products of category 13.1)	200			0.23	0.75										
13.4	Dietetic formulae for slimming purposes and weight reduction	200			0.23	0.75										
13.5	Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1–13.4 and 13.6	200			0.23	0.75										
13.6	Food supplements	5			0.04	0.33					0.000003	0.00001				
14.1	Non-alcoholic ('soft') beverages	300			0.19	0.79	0.0003	0.0003			0.44	0.44	0.2	1	0.2	0.2
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts		–	0.1									0.2	1		
14.2.1	Beer and malt beverages	300			0.08	0.08					0.00008	0.00008				
14.2.2	Grape wines	150			0.13	0.13										
14.2.3	Mead	150			0.17	0.17										
14.2.4	Spirituous beverages	30			0.44	1.55										
15.0	Ready-to-eat savouries	30			0.12	0.48	0.06	1.61	0.02	5.27	0.20	0.53	1	5		
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0	300			0.02	0.05					0.19	0.49	0.2	1		

FL-No: FLAVIS number; '–': no value for normal or maximum use level was provided.

(a): Most of the categories reported are the sub-categories of Codex GSFA (General Standard for Food Additives, available at http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) used by the JECFA in the SPET technique (FAO/WHO, 2008).

(b): Only food categories for which use has been reported are included in the table.

(c): For Adults. In case of foods marketed as powder or as concentrates, occurrence levels must be reported for the reconstituted product, considering the instructions reported on the product label or one of the standard dilution factors established by the JECFA (FAO/WHO, 2008):- 1/25 for powder used to prepare water-based drinks such as coffee, containing no additional ingredients,- 1/10 for powder used to prepare water-based drinks containing additional ingredients such as sugars (ice tea, squashes, etc.),- 1/7 for powder used to prepare milk, soups and puddings,- 1/3 for condensed milk.

(d): Only use levels for 'main' food categories have been provided.

(e): The Panel noted that the normal and maximum use levels for this food category for [FL-no: 15.018 and 15.032] are not plausible. However, the lower value was provisionally used for the calculation of mTAMDI.

(f): For this substance only maximum use levels were provided. In the absence of normal use levels, the maximum use levels have been used in the calculation of mTAMDI for this substance.

C.2. mTAMDI calculation

The method for calculation of modified theoretical added maximum daily intake (mTAMDI) values is based on the approach used by the SCF up to 1995 (SCF, 1995). The assumption is that a person may consume the amount of flavourable foods and beverages listed in Table C.3. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table C.3: Estimated amount of flavourable foods, beverages and exceptions assumed to be consumed per person per day (SCF, 1995)

Class of product category	Intake estimate (g/day)
Beverages (non-alcoholic)	324.0
Foods	133.4
Exception a: Candy, confectionery	27.0
Exception b: Condiments, seasonings	20.0
Exception c: Alcoholic beverages	20.0
Exception d: Soups, savouries	20.0
Exception e: Others, e.g. chewing gum	e.g. 2.0 (chewing gum)

The mTAMDI calculations are based on the normal use levels reported by industry. The seven food categories used in the SCF TAMDI approach (SCF, 1995) correspond to the 18 food categories as outlined in Commission Regulation (EC) No 1565/2000 and reported by the Flavour Industry in the following way (see Table C.4):

- Beverages (SCF, 1995) correspond to food category 14.1
- Foods (SCF, 1995) correspond to the food categories 1, 2, 3, 4.1, 4.2, 6, 7, 8, 9, 10, 13, and/or 16
- Exception a (SCF, 1995) corresponds to food category 5 and 11
- Exception b (SCF, 1995) corresponds to food category 15
- Exception c (SCF, 1995) corresponds to food category 14.2
- Exception d (SCF, 1995) corresponds to food category 12
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table C.4: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 into the seven SCF food categories used for mTAMDI calculations (SCF, 1995)

Key	Food categories according to Commission Regulation 1565/2000	Distribution of the seven SCF food categories		
	Food category	**Foods**	**Beverages**	**Exceptions**
01.0	Dairy products, excluding products of category 02.0	Foods		
02.0	Fats and oils, and fat emulsions (type water-in-oil)	Foods		
03.0	Edible ices, including sherbet and sorbet	Foods		
04.1	Processed fruit	Foods		
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Foods		
05.0	Confectionery			Exception a
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Foods		
07.0	Bakery wares	Foods		
08.0	Meat and meat products, including poultry and game	Foods		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	Foods		
10.0	Eggs and egg products	Foods		
11.0	Sweeteners, including honey			Exception a
12.0	Salts, spices, soups, sauces, salads, protein products, etc.			Exception d
13.0	Foodstuffs intended for particular nutritional uses	Foods		
14.1	Non-alcoholic ('soft') beverages, excl. dairy products		Beverages	
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts			Exception c
15.0	Ready-to-eat savouries			Exception b
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0	Foods		

Table C.5: Estimated intakes based on the MSDI approach and the mTAMDI approach (DG SANCO, 2014; EFFA, 2010, 2012, 2018, 2022)

FL-no	EU Union List name	MSDI – EU (μg/*capita* per day)	mTAMDI (μg/person per day)	Structural class	TTC (μg/person per day)
15.004	5-Methyl-2-thiophenecarbaldehyde	0.73	n.a.	Class II	540
15.013	2-Isobutylthiazole	2.3	n.a.	Class II	540
15.014	5-(2-Hydroxyethyl)-4-methylthiazole	150	n.a.	Class II	540
15.015	4-Methyl-5-(2-acetoxyethyl)thiazole	8.6	n.a.	Class II	540
15.017	4,5-Dimethylthiazole	0.18	n.a.	Class II	540
15.019	2,4,5-Trimethylthiazole	0.61	n.a.	Class II	540
15.020	2-Acetylthiazole	9.7	n.a.	Class II	540
15.022	2-(sec-Butyl)thiazole	0.024	n.a.	Class II	540
15.026	2-Isopropyl-4-methylthiazole	19	n.a.	Class II	540
15.027	2-Propionylthiazole	0.056	n.a.	Class II	540
15.028	Thiazole	0.012	n.a.	Class II	540
15.033	2-Ethyl 4-methylthiazole	3.2	n.a.	Class II	540
15.035	4-Methylthiazole	0.097	n.a.	Class II	540
15.109	2,4,6-Trimethyldihydro-1,3,5(4H)-dithiazine	1.1	n.a.	Class II	540
15.113	5,6-Dihydro-2,4,6,tris(2-methylpropyl)-4H-1,3,5-dithiazine	2.4	n.a.	Class II	540
16.027	Thiamine hydrochloride	300	n.a.	Class II	540
15.001	2-Mercaptothiophene	0.012	0.02	Class III	90
15.002	2-Methyl-5-methoxythiazole	0.012	550	Class III	90
15.005	2,4-Dimethyl-5-vinylthiazole	0.012	70	Class III	90
15.008	2-Thienyl disulfide	0.061	15	Class III	90
15.011	5-Acetyl-2,4-dimethylthiazole	0.012	6.2	Class III	90
15.016	Benzothiazole	1.2	650	Class III	90
15.018	4-Methyl-5-vinylthiazole	5	171	Class III	90
15.021	2-Ethoxythiazole	0.012	4	Class III	90
15.029	2-(sec-Butyl)-4,5-dimethyl-3-thiazoline	0.012	12.4	Class III	90
15.030	4,5-Dimethyl-2-ethyl-3-thiazoline	0.012	4.6	Class III	90
15.032	4,5-Dimethyl-2-isobutyl-3-thiazoline	4.3	213	Class III	90
15.130	5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	0.012	157	Class III	90
15.131	5-Ethyl-4-methyl-2-(2-butyl)thiazoline	0.012	149	Class III	90

FL-No: FLAVIS number; MSDI: maximised survey-derived daily intake; mTAMDI: modified theoretical added maximum daily intake; n.a.: not available.

C.3. Natural occurrence

JECFA status (JECFA, 2002a, 2003)

JECFA reported that 18 flavouring substances have been detected as natural components in food and quantitative data have been reported for seven substances (former [FL-no: 15.028] and [FL-no: 15.013, 15.019, 15.016, 15.020, 15.035, 15.004]). The foods in which one or more of these flavouring substances can be found include lean meat, nuts, whole-grain cereals, fish, coffee, milk, beer, peanuts, popcorn, pork liver, shrimp, tomato, potato, grapes and apples.

Information provided by industry (EFFA, 2018)

The candidate chemicals [15.005, 15.018 and 15.060] have been reported to occur in foods (Nijssen et al., 2014). 2,4-Dimethyl-5-vinylthiazole is found in boiled beef. 4-Methyl-5- vinylthiazole is found in apple and grape brandy, cocoa, cognac, roasted hazelnut, garlic, passion fruit, grilled or roasted uncured pork, soursop and tequila. 2,4-Dimethyl-3-thiazoline is found in boiled beef and fried chicken.

Appendix D – Summary of Genotoxicity data considered in FGE.21Rev3

Table D.1: *In vitro* genotoxicity data considered in FGE.21Rev3 for subgroup B-III (EFSA CEF Panel, 2012)

Chemical Name[a] [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
Subgroup B-III						
2-Propylthiazolidine [15.099][b]	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 and 10 μg/ml: positive in TA100 (±S9); 100 μg/ml: positive in TA98 and TA100 (±S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).
2-Methylthiazolidine [15.090][b]	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 and 10 μg/ml: positive in TA100 (±S9); 100 μg/ml: positive in TA98 and TA100 (±S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).
(2-Ethylthiazolidine)	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 μg/ml: positive in TA100 (±S9) and TA98 (-S9); 10 μg/ml: positive in TA100 (±S9); 100 μg/ml: positive in TA98 and TA100 (±S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).
(2-Isopropylthiazolidine)	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 and 10 μg/ml: positive in TA100 (±S9); 100 μg/ml: positive in TA100 (±S9) and TA98 (-S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).
(2-Butylthiazolidine)	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 μg/ml: positive in TA100 (+S9); 10 μg/ml: positive in TA100 (±S9); 100 μg/ml: positive in TA100 (±S9) and TA98 (-S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).
(2-Isobutylthiazolidine)	Ames assay	S. Typhimurium TA98, TA100	1, 10, 100 μg/ml	1 μg/ml: positive in TA98 and TA100 (+S9); 10 μg/ml: positive in TA98 and TA100 (±S9); 100 μg/ml: positive in TA98 and TA100 (±S9)	Mihara and Shibamoto, 1980	The results were stated to be positive; however, the magnitude and a positive dose effect relationship could not be assessed (no numbers are given).

(a): Substances in brackets are structurally related substances for subgroup B-III in FGE.21. They have no FL-no and are not authorised for use as flavouring substances in EU. These substances have been studied for genotoxicity in the same publication as [FL-no: 15.090 and 15.099]. Since also for these structurally related substances genotoxicity was observed, they supported the concern for genotoxicity raised for [FL-no: 15.090 and 15.099] already in FGE.21 (EFSA, 2007).

(b): The use of 2-methylthiazolidine [FL-no: 15.090] and 2-propylthiazolidine [FL-no: 15.099] as flavouring substances in Europe was no longer of interest for Industry and no further data were provided. Therefore, these substances were not included in the Union List, i.e. Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC.

Appendix E – Summary of Genotoxicity data evaluated in FGE.76Rev2

Table E.1: Summary of *in vitro* Genotoxicity Data Submitted for [FL-no: 15.018 and 15.032] and evaluated in FGE.76Rev2

Chemical Name [FL-no]	Test System	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
4-Methyl-5-vinylthiazole [15.018]	Reverse bacterial mutation assay	S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102)	5–5000 μg/plate[a,b,c] 156.3–5000 μg/plate[b,c,d] 62.5–2000 μg/plate[b,d]	Negative	Covance, 2012a	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 471.
	Micronucleus assay	Human blood lymphocytes	70, 110 and 130 μg/mL[e] 600, 700 and 800 μg/mL[g] 700, 900 and 950 μg/mL[f]	Negative Negative Positive[f]	Covance, 2013a	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487. The given concentrations are those for the cultures that were scored for micronuclei.
	Micronucleus Assay with CREST staining	Mammalian TK6 cells	25, 150, 250 μg/mL[h] 100, 550, 650 μg/mL[i] 100, 300, 650 μg/mL[j] 100, 275, 300, 675 μg/mL[j]	Negative Negative Positive Positive	BioReliance, 2018a	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487. CREST analysis showed that 4-methyl-5-vinylthiazole induced MN mainly via a clastogenic mechanism. The given concentrations are those for the cultures that were scored for micronuclei.
4,5-Dimethyl-2-isobutyl-3-thiazoline [15.032]	Reverse bacterial mutation assay	Experiment 1: S. Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) Experiment 2: TA100 TA100 TA98, TA1535, TA1537 and TA102 Experiment 3: TA98, TA100, TA1535, TA1537 and TA102	5–5000 μg/plate[a,b,c] 78–5000 μg/plate[a,c] 156.3–5000 μg/plate[b,d] 156.3–5000 μg/plate[a,c],[b,d] 39–1250 μg/plate[b,d]	Negative	Covance, 2012b	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 471.

Chemical Name [FL-no]	Test System	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
	Micronucleus assay	Human blood lymphocytes	4, 8, 9 μg/mL[(e)] 10, 20, 60, 80 μg/mL[(f)] 40, 160, 210 μg/m[(g)]	Negative Positive[(f)] Positive[(g)]	Covance, 2013b	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487. The given concentrations are those for the cultures that were scored for micronuclei.
	Micronucleus assay	Mammalian TK6 cells	5, 15, 25 μg/mL[(h)] 5, 50, 100 μg/mL[(i)] 25, 125, 150 μg/mL[(j)]	Negative	BioReliance, 2018b	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 487. The given concentrations are those for the cultures that were scored for micronuclei.
	Micronucleus assay with FISH analysis	Human blood lymphocytes	129, 196, 228 μg/mL[(k)] 33.9, 70.9, 97.2 μg/mL[(e)] 222, 263, 272 μg/mL[(l)]	Positive	Charles River Laboratories, 2020	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487. FISH analysis indicates that 4,5-dimethyl-2-isobutyl-3-thiazoline induced MN via an aneugenic mechanism. The given concentrations are those for the cultures that were scored for micronuclei.

FL-No: FLAVIS number.
(a): plate incorporation method.
(b): with S9-mix.
(c): without S9-mix.
(d): pre-incubation method.
(e): 24-h treatment without S9-mix.
(f): 3 + 21-h treatment without S9-mix.
(g): 3 + 21-h treatment with S9-mix.
(h): 27-h treatment without S9-mix.
(i): 4 + 23-h treatment without S9-mix.
(j): 4 + 23-h treatment with S9-mix.
(k): 4 + 20-h treatment without S9-mix.
(l): 4 + 20-h treatment with S9-mix.

Table E.2: Summary of *in vivo* Genotoxicity Data for [FL-no: 15.018 and 15.032] evaluated in FGE.76Rev2

Chemical Name [FL-no]	Test System	Test Object	Route	Dose mg/kg bw per day	Result	Reference	Comments
4-Methyl-5-vinylthiazole [15.018]	Micronucleus assay (bone marrow)	Han Wistar Rat; M	gavage	45, 90 and 180 mg/kg bw per day[(a)]	Inconclusive (negative, but insufficient evidence of bone marrow exposure)	Covance, 2014a	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 474.
	Comet assay (liver and duodenum)	Han Wistar Rat; M	gavage		Negative		Reliable without restrictions. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.
4,5-Dimethyl-2-isobutyl-3-thiazoline [15.032]	Micronucleus assay (bone marrow)	Han Wistar Rat; M	gavage	87.5, 175 and 350 mg/kg bw per day[(a)]	Inconclusive (negative, but insufficient evidence of bone marrow exposure)	Covance, 2014b, 2015	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 474.
	Comet assay (liver and duodenum)	Han Wistar Rat; M	gavage		Negative		Reliable without restrictions. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.

FL-No: FLAVIS number; M: Male.
(a): Administered via gavage, 3 doses at times 0, 24 and 45 h with sacrifice and harvest at 48 h.

Appendix F – Summary of Toxicity data evaluated in FGE.76Rev2

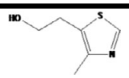
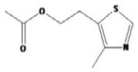
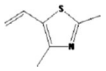
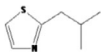
Table F.1: Summary of toxicity data evaluated by the Panel in FGE.76Rev2 and by JECFA at the 59th meeting (JECFA, 2002a, 2003)

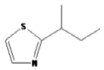
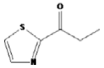
Chemical Name [FL-no]	Species; Sex No/group	Route	Doses (mg/kg bw per day)	Duration (days)	NOAEL (mg/kg bw per day)	Reference	Comments
2-(sec-butyl)-4,5-dimethyl-3-thiazoline [FL-no: 15.029]	Rat; M, F 15	Diet	0, 1.2	90	1.2	Food and Drug Research Laboratories, 1978	The study was performed with a single dose that produced no adverse effects. The study has several shortcomings, e.g. only one dose level, purity of test material is not specified and data on pathology and histopathology were not included in the study report.
2,4-dimethyl-5-vinylthiazole [FL-no: 15.005]	Rat; M, F 10–16	Diet	0, 0.92 (M) 0, 1.0 (F)	90	0.92	Posternak et al., 1969	The study was performed with a single dose that produced no adverse effects. The report is only a summary of the study. Poor study protocol and data reporting.

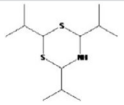
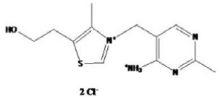
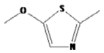
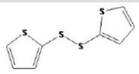
FL-No: FLAVIS number; M: Male; F: Female.

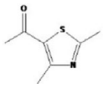
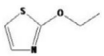
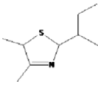
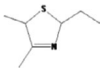
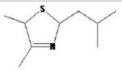
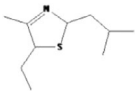
Appendix G – Summary of safety evaluations

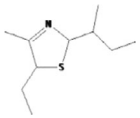
Table G.1: Summary of Safety Evaluation performed by JECFA and EFSA conclusions on flavouring substances in FGE.76Rev2

			JECFA conclusions	EFSA conclusions
FL-no JECFA-no	**EU Union List chemical name**	**Structural formula**	**Class[a] Evaluation procedure path [b],[c] Outcome on the named compound based on the MSDI approach**	**Procedural path if different from JECFA, Conclusion based on the MSDI[d] approach on the named compound and on the material of commerce**
15.014 1031	5-(2-Hydroxyethyl)-4-methylthiazole		Class II A3: Intake below threshold	
15.015 1054	4-Methyl-5-(2-acetoxyethyl) thiazole		Class II A3: Intake below threshold	
15.004 1050	5-Methyl-2-thiophenecarbaldehyde		Class II B3: Intake below threshold B4: Adequate NOAEL (290 mg/kg bw per day) exists	
15.005 1039	2,4-Dimethyl-5-vinylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	Class III
15.013 1034	2-Isobutylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.017 1035	4,5-Dimethylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.018 1038	4-Methyl-5-vinylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	Class III

FL-no JECFA-no	EU Union List chemical name	Structural formula	JECFA conclusions Class[a] Evaluation procedure path [b],[c] Outcome on the named compound based on the MSDI approach	EFSA conclusions Procedural path if different from JECFA, Conclusion based on the MSDI[d] approach on the named compound and on the material of commerce
15.019 1036	2,4,5-Trimethylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.020 1041	2-Acetylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (50 mg/kg bw per day) exists	
15.022 1033	2-(sec-Butyl)thiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.026 1037	2-Isopropyl-4-methylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.027 1042	2-Propionylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (50 mg/kg bw per day) exists	
15.028 1032	Thiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	No longer of interest for Industry for use as flavouring substance (DG SANCO, 2012) in Europe. No further data were submitted.
15.033 1044	2-Ethyl 4-methylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	

FL-no JECFA-no	EU Union List chemical name	Structural formula	JECFA conclusions Class[a] Evaluation procedure path [b],[c] Outcome on the named compound based on the MSDI approach	EFSA conclusions Procedural path if different from JECFA, Conclusion based on the MSDI[d] approach on the named compound and on the material of commerce
15.035 1043	4-Methylthiazole		Class II B3: Intake below threshold B4: Adequate NOAEL (0.92 mg/kg bw per day) exists	
15.109 1049	2,4,6-Trimethyldihydro-1,3,5(4H)-dithiazine		Class II B3: Intake below threshold B4: Adequate NOAEL (11 mg/kg bw per day) exists	Information on the stereochemical composition of the material of commerce is inadequate
15.113 1048	5,6-Dihydro-2,4,6,tris(2-methylpropyl)-4H-1,3,5-dithiazine		Class II B3: Intake below threshold B4: Adequate NOAEL (11 mg/kg bw per day) exists	Information on the stereochemical composition of the material of commerce is inadequate
16.027 1030	Thiamine hydrochloride		Class II A3: Intake above threshold A4: Not endogenous A5: Adequate NOAEL (36 mg/kg bw per day) exists	
15.001 1052	2-Mercaptothiophene		Class III B3: Intake below threshold B4: Adequate NOAEL (290 mg/kg bw per day) exists	
15.002 1057	2-Methyl-5-methoxythiazole		Class III B3: Intake below threshold B4: Adequate NOAEL (8.6 mg/kg bw per day) exists	
15.008 1053	2-Thienyl disulfide		Class III B3: Intake below threshold B4: Adequate NOAEL (290 mg/kg bw per day) exists	

			JECFA conclusions	EFSA conclusions
FL-no JECFA-no	EU Union List chemical name	Structural formula	Class[a] Evaluation procedure path [b],[c] Outcome on the named compound based on the MSDI approach	Procedural path if different from JECFA, Conclusion based on the MSDI[d] approach on the named compound and on the material of commerce
15.011 1055	5-Acetyl-2,4-dimethylthiazole		Class III B3: Intake below threshold B4: Adequate NOAEL (24 mg/kg bw per day) exists	
15.016 1040	Benzothiazole		Class III B3: Intake below threshold B4: Adequate NOAEL (5.1 mg/kg bw per day) exists	
15.021 1056	2-Ethoxythiazole		Class III B3: Intake below threshold B4: Adequate NOAEL (50 mg/kg bw per day) exists	
15.029 1059	2-(sec-Butyl)-4,5-dimethyl-3-thiazoline		Class III B3: Intake below threshold B4: Adequate NOAEL (1.2 mg/kg bw per day) exists	Genotoxicity data required
15.030 1058	4,5-Dimethyl-2-ethyl-3-thiazoline		Class III B3: Intake below threshold B4: Adequate NOAEL (1.2 mg/kg bw per day) exists	Genotoxicity data required
15.032 1045	4,5-Dimethyl-2-isobutyl-3-thiazoline		Class III B3: Intake below threshold B4: Adequate NOAEL (1.2 mg/kg bw per day) exists	
15.130 1761	5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline		Class III B3: Intake below threshold B4: Adequate NOAEL (1.2 mg/kg bw per day) exists	Genotoxicity data required

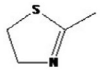
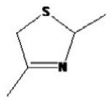
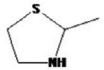
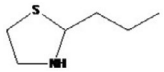
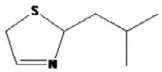
FL-no JECFA-no	EU Union List chemical name	Structural formula	JECFA conclusions Class[a] Evaluation procedure path [b],[c] Outcome on the named compound based on the MSDI approach	EFSA conclusions Procedural path if different from JECFA, Conclusion based on the MSDI[d] approach on the named compound and on the material of commerce
15.131 1762	5-Ethyl-4-methyl-2-(2-butyl)-thiazoline		Class III B3: Intake below threshold B4: Adequate NOAEL (1.2 mg/kg bw per day) exists	Genotoxicity data required

FL-No: FLAVIS number; FGE: Flavouring Group Evaluation; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; NOAEL: No observed adverse effect level; bw: body weight.
(a): Thresholds of concern: Class I = 1800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(b): JECFA, 2002a, 2003.
(c): JECFA, 2007, 2008a.
(d): EU MSDI: Amount added to food as flavouring in (kg/year) × 10^9/(0.1 × population in Europe (= 375 × 10^6) × 0.6 × 365) = μg/capita per day.

Appendix H – Summary of safety evaluations for structurally related substances from FGE.21Rev5

Only information related to the supporting substances relevant for the current evaluation is reported. Information on the full list of substances in FGE.21 can be retrieved in FGE.21Rev5 (EFSA CEF Panel, 2015).

Table H.1: Summary of Safety Evaluation Applying the Procedure for substances in FGE.21Rev5 (based on intakes calculated by the MSDI approach)

FL-no	Chemical name	Structural formula	MSDI[(a)] (μg/*capita* per day)	Class[(b)] Evaluation procedure path[(c)] Outcome on the named compound and on the material of commerce	EFSA comments
15.086	2-Methyl-2-thiazoline		0.012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists No safety concern based on intakes calculated by the MSDI approach	Concluded in FGE.21Rev4
15.060	2,4-Dimethyl-3-thiazoline		0.012	Class II No evaluation	Genotoxicity data required
15.090	2-Methylthiazolidine		0.024	Class II No evaluation	No longer of interest for Industry for use as flavouring substance in Europe (DG SANCO, 2012). No further data were submitted. Substance not included in the Union List[(d)]
15.099	2-Propylthiazolidine		0.012	Class II No evaluation	No longer of interest for Industry for use as flavouring substance in Europe (DG SANCO, 2012). No further data were submitted. Substance not included in the Union List[(d)]
15.119	2-Isobutyl-3-thiazoline		0.012	Class II No evaluation	Genotoxicity data required

FL-No: FLAVIS number; FGE: Flavouring Group Evaluation; MSDI: maximised survey-derived daily intake.
(a): EU MSDI: Amount added to food as flavour in (kg/year) × 10^9/(0.1 × population in Europe (= 375 × 10^6) × 0.6 × 365) = μg/capita per day.
(b): Thresholds of concern: Class I = 1800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC.