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EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 17, Revision 2 (FEG.17Rev2): Pyrazine derivatives from chemical group 24

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 17, Revision 2 (FGE.17Rev2):

Pyrazine derivatives from chemical group 24¹

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate 21 flavouring substances in the Flavouring Group Evaluation 17, Revision 2, using the Procedure in Commission Regulation (EC) No 1565/2000. From the *in vitro* data available, genotoxic potential is indicated for the flavouring substances quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139]. Therefore, the Panel decided that the Procedure could not be applied to these two substances, so adequate genotoxicity data should be provided. For one substance [FL-no: 14.051] no intake data are available preventing it from being evaluated through the Procedure. The remaining 18 substances were evaluated through a stepwise approach that integrates information on structure-activity relationships, intake from current uses, toxicological threshold of concern, and available data on metabolism and toxicity. The Panel concluded that 17 substances [FL-no: 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.122, 14.127, 14.129, 14.148, and 14.161] do not give rise to safety concerns at their levels of dietary intake, estimated on the basis of the MSDI approach. For the remaining substance [FL-no: 14.052] additional toxicity data are required. Besides the safety assessment of these flavouring substances, the specifications for the materials of commerce have also been considered and for two substances information on specifications is lacking.

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1 On request from the Commission, Question No EFSA-Q- 2010-00006, adopted on 25 November 2010.

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to evaluate 21 flavouring substances in the Flavouring Group Evaluation 17, Revision 2 (FGE.17Rev2), using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. These 21 flavouring substances belong to chemical group 24 of Annex I of the Commission Regulation (EC) No 1565/2000.

The present Flavouring Group Evaluation (FGE) deals with 21 pyrazine derivatives. Three of these derivatives are quinoxalines.

Five of the 21 flavouring substances possess a chiral centre. For two of these substances the stereoisomeric composition has not been specified.

Seventeen of the flavouring substances are classified into structural class II and four are classified into structural class III.

Nineteen of the flavouring substances in the present group have been reported to occur naturally in a wide range of food items.

In its evaluation, the Panel as a default used the “Maximised Survey-derived Daily Intakes” (MSDIs) approach to estimate the *per capita* intakes of the flavouring substances in Europe. However, when the Panel examined the information provided by the European Flavouring 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 level reported by 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.

In the absence of more precise 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. In those cases where the mTAMDI approach indicated that the intake of a flavouring substance might exceed its corresponding threshold of concern, the Panel decided not to carry out a formal safety assessment using the Procedure. In these cases the Panel requires more precise data on use and use levels.

According to the default MSDI approach, the 18 flavouring substances in this group, which are evaluated through the Procedure and for which Industry has provided intake data, have intakes in Europe from 0.0024 to 0.12 microgram/*capita*/day which are below the threshold of concern value for both structural class II (540 microgram/person/day) and structural class III (90 microgram/person/day) substances.

For one candidate substance [FL-no: 14.051] no intake data are available preventing it from being evaluated through the Procedure.

No genotoxic potential at gene or chromosome level is indicated for the alkyl- and cycloalkyl-substituted pyrazines, which allow these substances to be evaluated through the Procedure. However, from the *in vitro* data available, genotoxic potential is indicated for the flavouring substance quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139]. Therefore, the Panel decided that the Procedure could not be applied to these two substances until adequate genotoxicity data become available.

Sixteen of the alkyl- and cycloalkyl-substituted pyrazines and one of the alkyl-substituted quinoxalines may be expected to be metabolised to innocuous products [FL-no: 14.051, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.127, 14.129, 14.148 and 14.161]. Regarding the remaining four substances, they cannot be anticipated to be metabolised to innocuous products. One sulphur-containing flavouring substance [FL-no: 14.122], may be converted to a reactive free thiol. It can also not be assumed that quinoxaline [FL-no: 14.147] and its derivative 2-methylquinoxaline [FL-no: 14.139] are metabolised to innocuous products. The fourth substance has a terminal double bond [FL-no: 14.052] (in conjugation with a heterocyclic aromatic ring), which may be epoxidated giving rise to reactive metabolites.

It was noted, that where toxicity data were available they were consistent with the conclusions in the present FGE.

It was considered that on the basis of the default MSDI approach 17 of the 18 flavouring substances evaluated through the Procedure would not give rise to safety concerns at the estimated levels of intake arising from their use as flavouring substances. For one flavouring substance [FL-no: 14.052] additional toxicity data are required.

When the estimated intakes, of the substances evaluated through the Procedure, were based on the mTAMDI approach they ranged from 190 to 400 microgram/person/day for the 16 flavouring substances from structural class II. Thus, the intakes for these 16 substances were below the threshold of concern for structural class II of 540 microgram/person/day. These substances are also expected to be metabolised to innocuous products. The estimated intake of the two flavouring substances [FL-no: 14.108 and 14.122] assigned to structural class III and evaluated through the Procedure are 190 and 270 microgram/person/day, respectively, which are above the threshold of concern for structural class III of 90 microgram/person/day. Therefore, for these substances more reliable exposure data are required. On the basis of such additional data, the flavouring substances should be re-evaluated using the Procedure. Subsequently, additional toxicity data might become necessary.

In order to determine whether the conclusion for the 18 flavouring substances, which have been evaluated using the Procedure, can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity for the materials of commerce have been provided for 16 of the 18 flavouring substances evaluated through the Procedure. Information on composition of mixture and/or chirality is missing for two these 18 substances. Thus, the final evaluation of the materials of commerce cannot be performed for these two substances [FL-no: 14.099 and 14.102], pending further information.

Thus, in conclusion, only 18 of the 21 candidate substances were evaluated through the Procedure, as two flavouring substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] could not be evaluated through the Procedure until adequate genotoxicity data become available and one substance [FL-no: 14.051] was presented with no intake data and therefore it could not be evaluated through the Procedure until adequate intake data and, if required, additional toxicological data become available.

For one substance [FL-no: 14.052] additional toxicity data are required. For two substances [FL-no: 14.099 and 14.102] the final evaluation of the materials of commerce cannot be performed pending further information on chirality and composition of mixture of structural isomers.

For the remaining 15 substances adequate specifications including complete purity criteria and identity for the materials of commerce have been provided and the Panel concluded that these candidate substances [FL-no: 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.108, 14.113, 14.122, 14.127, 14.129, 14.148, and 14.161] would present no safety concern at the level of intake estimated on the basis of the MSDI approach.

KEYWORDS

Flavourings, pyrazine, quinoxaline, food safety, FGE.17, FGE.50Rev1.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) lays down a Procedure for the establishment of a list of flavouring substances the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2008/163/EC (EC, 2009a). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999a). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

The FGE is revised to include substances for which data were submitted after the deadline as laid down in Commission Regulation (EC) No 622/2002 and to take into account additional information that has been made available since the previous Opinion on this FGE.

The Revision also includes newly notified substances belonging to the same chemical groups evaluated in this FGE.

After the completion of the evaluation programme the Union List of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996a).

HISTORY OF THE EVALUATION

In previous revisions of the present FGE, it was found that two of the candidate substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] showed possible genotoxic potential *in vitro*. Therefore, the Panel decided that the Procedure could not be applied to these two candidate substances nor for the structurally related 2,3-dimethylquinoxaline [FL-no: 14.108] until adequate genotoxicity data become available. This conclusion did also have implications for the structurally related 5-methyl quinoxaline [FL-no: 14.028], considered by the Panel in FGE.50, as the Procedure should not be applied until adequate genotoxicity data become available for this substance either (EFSA, 2008x).

FGE	Opinion adopted by EFSA	Link	No. of candidate substances
FGE.17	7 December 2005	http://www.efsa.eu.int/science/afc/afc_opinions/1291_en.html	18
FGE.17Rev1	30 January 2008	http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902101869.htm	20
FGE.17Rev2	23 November 2010		21

After finalisation of the previous version of this FGE (i.e. FGE.17, Revision 1), both *in vitro* and *in vivo* genotoxicity data have become available for the supporting substance, 5-methylquinoxaline [FL-no: 14.028] (Flavour Industry, 2009a). This substance is a candidate flavouring substance in FGE.50. The Panel explored the option of using the genotoxicity data submitted for 5-methylquinoxaline [FL-no: 14.028] to support the evaluation of the genotoxic potential of the candidate quinoxaline derivatives in FGE.17 for which in the previous version a concern for genotoxic potential could not be ruled out. (Flavour Industry, 2009a).

Furthermore, the present FGE.17Rev2 also include the evaluation of one new substance [FL-no: 14.051]. [FL-no: 14.051] is the one isomer of the mixture of the structural isomers of [FL-no: 14.077] (see Table 1 and Table 3, respectively). The Panel decided that the evaluation of [FL-no: 14.077] cannot cover the evaluation of [FL-no: 14.051] and accordingly the substance will be included in the present revision of FGE.17. No additional metabolism or toxicity data are available for this substance. Neither are European production figures available.

Finally, newly provided tonnage data for seven supporting substances, which were missing in previous revisions, have been included.

TERMS OF REFERENCE

The European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances in the Register prior to their authorisation and inclusion in a Union List according to Commission Regulation (EC) No 1565/2000 (EC, 2000a). In addition, the Commission requested EFSA to evaluate newly notified flavouring substances, where possible, before finalising the evaluation programme.

In addition, in letter of 1 April 2009 the Commission requested EFSA to carry out a re-evaluation of flavouring substances [FL-no: 14.028, 14.108 and 14.139] in accordance with Commission Regulation (EC) No 1565/2000:

“The European Commission requests the European Food Safety Authority to carry out a risk assessment on 5-methylquinoxaline ([FL-no: 14.028]), 2,3-dimethylquinoxaline ([FL-no: 14.108]) and 2-methylquinoxaline ([FL-no: 14.139]) in accordance with Commission Regulation (EC) No 1565/2000, if possible by the end of the evaluation programme, if not within nine month from finalisation of that programme”.

The deadline of the Terms of Reference was negotiated to 30 June 2010.

ASSESSMENT

1. Presentation of the Substances in Flavouring Group Evaluation 17, Revision 2

1.1. Description

The present revision of Flavouring Group Evaluation 17, FGE.17Rev2, using the procedure as referred to in the Commission Regulation EC No 1565/2000 (EC, 2000a) (The Procedure –shown in schematic form in Annex I of this FGE), deals with 21 pyrazine derivatives (candidate substances), which belong to chemical group 24 of Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000a).

The 21 candidate substances under consideration, as well as their chemical Register names, FLAVIS- (FL-), Chemical Abstract Service- (CAS-), Council of Europe- (CoE-) and Flavor and Extract Manufacturers Association- (FEMA-) numbers, structure and specifications, are listed in Table 1. All 21 candidate substances contain a pyrazine moiety. In 13 substances [FL-no: 14.051, 14.052, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.122, 14.127 and 14.129] only one heterocyclic ring is present. In five candidate substances a pyrazine ring is fused with either cyclopentane [FL-no: 14.099, 14.102, 14.113, and 14.161] or with cyclohexane [FL-no: 14.148]. All of these 18 substances have different ring substituents. In 15 of them, the substituents are simple alkyl chains or ketones. In the other three, the side chains are either a methoxy- or a thiomethyl- residue ([FL-no: 14.051 and 14.127] and [FL-no: 14.122], respectively). In the remaining three candidate substances the pyrazine ring is fused with benzene giving quinoxalines. In two of the quinoxalines

[FL-no: 14.108 and 14.139] the pyrazine ring also bears one or two methyl substituents; in the third no substituents are present (quinoxaline; [FL-no: 14.147]).

The 21 candidate substances are structurally related to 41 flavouring substances (supporting substances) evaluated at the 57th JECFA meeting in the group “Pyrazine Derivatives” (JECFA, 2002b). The name and structures of the 41 supporting substances are listed in Table 3, together with their evaluation status.

1.2. Stereoisomers

It is recognised that geometrical and optical isomers of substances may have different properties. Their flavour may be different, they may have different chemical properties resulting in possible variability in their absorption, distribution, metabolism, elimination and toxicity. Thus information must be provided on the configuration of the flavouring substance, i.e. whether it is one of the geometrical/optical isomers, or a defined mixture of stereoisomers. The available specifications of purity will be considered in order to determine whether the safety evaluation carried out for candidate substances for which stereoisomers may exist can be applied to the material of commerce. Flavouring substances with different configurations should have individual chemical names and codes (CAS number, FLAVIS number etc.).

Five of the 21 flavouring substances possess one or two chiral centres [FL-no: 14.099, 14.102, 14.113, 14.148 and 14.161]. Two of the substances [FL-no: 14.099 and 14.102] have been presented without specification of the stereoisomeric composition.

1.3. Natural Occurrence in Food

Nineteen out of the 21 candidate substances have been reported to occur in beef, chicken, cocoa, coffee, green tea, fruit juice, potato, pork, whisky, sherry, nuts, peanut, roasted sesame seed, malt, and wild rice. Quantitative data on the natural occurrence in food have been reported for seven of these substances (TNO, 2000).

These reports are:

- Isopropenylpyrazine [FL-no: 14.052]: 0.11 mg/kg in malt.
- 2-Acetyl-5-methylpyrazine [FL-no: 14.084]: up to 0.3 mg/kg in coffee.
- 2-Acetyl-6-methylpyrazine [FL-no: 14.087]: up to 0.55 mg/kg in coffee.
- 2-Butyl-3-methylpyrazine [FL-no: 14.091]: 1 mg/kg in cocoa.
- 2,5-Diethylpyrazine [FL-no: 14.097]: up to 0.1 mg/kg in coffee, and 0.02 mg/kg in peanut.
- Quinoxaline [FL-no: 14.147]: up to 0.1 mg/kg in malt.
- 6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine [FL-no: 14.161]: up to 5.1 mg/kg in coffee, and up to 0.0001 mg/kg in pork.

According to TNO two of the substances, 2,5 or 6-methoxy-3-ethylpyrazine and 2-isopropyl-3-methylthiopyrazine [FL-no: 14.051 and 14.122], have not been reported to occur naturally in any food items (TNO, 2000; TNO, 2009).

2. Specifications

Purity criteria for 20 of the 21 candidate substances have been provided by the Flavouring Industry (EFFA, 2003q; EFFA, 2006j; EFFA, 2007g). For one substance [FL-no: 14.051] no purity criteria has been provided.

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000 (EC, 2000a), this information is adequate for the 20 candidate substances, except that stereoisomeric information is needed for two substances [FL-no: 14.099 and 14.102]. Furthermore, according to Industry (EFFA, 2007e) [FL-no: 14.102] covers a mixture of 2,5-dimethyl-6,7-dihydro-5H-cyclopentapyrazine (60-100 %) & 3,5-dimethyl-6,7-dihydro-5H-cyclopentapyrazine (up to 40 %). The composition of this mixture has to be further specified (see Section 1.2 and Table 1). Specifications are lacking for [FL-no: 14.051].

3. Intake Data

Annual production volumes of the flavouring substances as surveyed by the Industry can be used to calculate the “Maximised Survey-derived Daily Intake” (MSDI) by assuming that the production figure only represents 60 % of the use in food due to underreporting and that 10 % of the total EU population are consumers (SCF, 1999a).

However, the Panel noted that due to year-to-year variability in production volumes, to uncertainties in the underreporting correction factor and to uncertainties in the percentage of consumers, the reliability of intake estimates on the basis of the MSDI approach is difficult to assess.

The Panel also noted that in contrast to the generally low *per capita* intake figures estimated on the basis of this MSDI approach, in some cases the regular consumption of products flavoured at use levels reported by the Flavour Industry in the submissions would result in much higher intakes. In such cases, the human exposure thresholds below which exposures are not considered to present a safety concern might be exceeded.

Considering that the MSDI model may underestimate the intake of flavouring substances by certain groups of consumers, the SCF recommended also taking into account the results of other intake assessments (SCF, 1999a).

One of the alternatives is the “Theoretical Added Maximum Daily Intake” (TAMDI) approach, which is calculated on the basis of standard portions and upper use levels (SCF, 1995) for flavourable beverages and foods in general, with exceptional levels for particular foods. This method is regarded as a conservative estimate of the actual intake by most consumers because it is based on the assumption that the consumer regularly eats and drinks several food products containing the same flavouring substance at the upper use level.

One option to modify the TAMDI approach is to base the calculation on normal rather than upper use levels of the flavouring substances. This modified approach is less conservative (e.g., it may underestimate the intake of consumers being loyal to products flavoured at the maximum use levels reported) (EC, 2000a). However, it is considered as a suitable tool to screen and prioritise the flavouring substances according to the need for refined intake data (EFSA, 2004a).

3.1. Estimated Daily *per Capita* Intake (MSDI Approach)

The intake estimation is based on the Maximised Survey-derived Daily Intake (MSDI) approach, which involves the acquisition of data on the amounts used in food as flavourings (SCF, 1999a). These data are derived from surveys on annual production volumes in Europe. These surveys were conducted in 1995 by the International Organization of the Flavour Industry, in which flavour manufacturers reported the total amount of each flavouring substance incorporated into food sold in the EU during

the previous year (IOFI, 1995). The intake approach does not consider the possible natural occurrence in food.

Average *per capita* intake (MSDI) is estimated on the assumption that the amount added to food is consumed by 10 % of the population⁴ (Eurostat, 1998). This is derived for candidate substances from estimates of annual volume of production provided by Industry and incorporates a correction factor of 0.6 to allow for incomplete reporting (60 %) in the Industry surveys (SCF, 1999a).

In the FGE.17Rev2 the total annual volume of production of 20 of the 21 candidate substances from use as flavouring substances in Europe has been reported to be approximately 6.2 kg (EFFA, 2003r; EFFA, 2006j; EFFA, 2007g). For one substance [FL-no: 14.051] no annual volumes of production in Europe have been submitted. For the 41 supporting substances the total annual volume of production is approximately 2700 kg. About 65 % of the total annual volume of production in Europe is accounted for by 2,3,5-trimethylpyrazine [FL-no: 14.019], 2-ethyl-3-methylpyrazine [FL-no: 14.006] and 3,(5- or 6-)-dimethyl-2-ethylpyrazine [FL-no: 14.100] (JECFA, 2002b).

On the basis of the annual volumes of production reported for the 20 candidate substances, the daily *per capita* intakes for each of these flavourings have been estimated (Table 2). Approximately 50 % of the total annual volume of production for the candidate substances (EFFA, 2003r) is accounted for by the candidate substances 2-Butyl-3-methylpyrazine [FL-no: 14.091], 2-methylquinoxaline [FL-no: 14.139] and quinoxaline [FL-no: 14.147]. The estimated daily *per capita* intakes of these three candidate substances from use as flavouring substances are in total 0.36 microgram, and below 0.07 microgram for each of the remaining candidate substances (Table 2).

3.2. Intake Estimated on the Basis of the Modified TAMDI (mTAMDI)

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995).

The assumption is that a person may consume a certain amount of flavourable foods and beverages per day.

For the 20 of the 21 candidate substances information on food categories and normal and maximum use levels^{5,6,7} were submitted by the Flavour Industry (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g). The 20 candidate substances are used in flavoured food products divided into the food categories, outlined in Annex III of the Commission Regulation (EC) No 1565/2000 (EC, 2000a), as shown in Table 3.1. For one substance [FL-no: 14.051] no use levels have been submitted. For the present calculation of mTAMDI, the reported normal use levels were used. In the case where different use levels were reported for different food categories the highest reported normal use level was used.

⁴ EU figure 375 millions. This figure relates to EU population at the time for which production data are available, and is consistent (comparable) with evaluations conducted prior to the enlargement of the EU. No production data are available for the enlarged EU.

⁵ "Normal use" is defined as the average of reported usages and "maximum use" is defined as the 95th percentile of reported usages (EFFA, 2002i).

⁶ The normal and maximum use levels in different food categories (EC, 2000) have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004e).

⁷ The use levels from food category 5 "Confectionery" have been inserted as default values for food category 14.2 "Alcoholic beverages" for substances for which no data have been given for food category 14.2 (EFFA, 2007a).

Table 3.1 Use of Candidate Substances

Food category	Description	Flavourings used
01.0	Dairy products, excluding products of category 2	All 20* substances
02.0	Fats and oils, and fat emulsions (type water-in-oil)	All 20* substances
03.0	Edible ices, including sherbet and sorbet	All 20* substances
04.1	Processed fruits	All 20* substances
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	None
05.0	Confectionery	All 20* substances
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	All 20* substances
07.0	Bakery wares	All 20* except [FL-no: 14.099 & 14.101]
08.0	Meat and meat products, including poultry and game	All 20* substances
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	All 20* substances
10.0	Eggs and egg products	All 20* substances
11.0	Sweeteners, including honey	All 20* substances
12.0	Salts, spices, soups, sauces, salads, protein products etc.	All 20* substances
13.0	Foodstuffs intended for particular nutritional uses	All 20* substances
14.1	Non-alcoholic ("soft") beverages, excl. dairy products	All 20* substances
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts	All 20* substances
15.0	Ready-to-eat savouries	All 20* substances
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 – 15	All 20* substances

* For one substance [FL-no: 14.051] no use levels are available

According to the Flavour Industry the normal use levels for the 20 candidate substances for which intake data are available are in the range of 0.1 - 2 mg/kg food, and the maximum use levels are in the range of 0.4 - 10 mg/kg (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g), Table II.1.2, Appendix II.

The mTAMDI values for the 16 candidate substances from structural class II (see Section 5) for which use levels are available range from 190 to 400 microgram/person/day. For the remaining four candidate substances from structural class III the mTAMDI range from 190 to 270 microgram/person/day.

For detailed information on use levels and intake estimations based on the mTAMDI approach, see section 6 and Annex II.

4. Absorption, Distribution, Metabolism and Elimination

A more detailed description of the metabolism of the candidate substances in this FGE is given in Annex III.

This group of flavouring substances consists of 21 candidate substances, all containing a pyrazine ring. In eight candidate substances the pyrazine ring is fused with either cyclopentane [FL-no: 14.099, 14.102, 14.113, and 14.161], cyclohexane [FL-no: 14.148] or benzene [FL-no: 14.108, 14.139 and 14.147]. All candidate substances, except one quinoxaline, have different substituents on the rings. In 16 of them, the substituents are simple alkyl chains or ketones. In other four, the substituents are either a methoxy- or a thiomethyl- residue ([FL-no: 14.127 and 14.051] and [FL-no: 14.122], respectively) or a terminal double bond (in conjugation with a heterocyclic aromatic ring), which may be epoxidated giving rise to reactive metabolites ([FL-no: 14.052]).

A group with 41 related supporting substances has been evaluated by JECFA (JECFA, 2002a).

Very little data on absorption, distribution and elimination of the candidate or supporting flavouring substances are available. The available data indicate that the weak basic heterocyclic substances, in this group may be well absorbed, mainly from the intestinal lumen, and may be rapidly excreted.

Limited information has been submitted to describe the metabolism of the pyrazines and alkyl-, aryl-, or alicyclic-substituted pyrazines in this group of flavouring substances. Almost all data available come from one paper (Hawksworth & Scheline, 1975) and a few review papers (Beedham, 1985; Beedham, 1988; Parkinson, 1996a). Additional information provided in other papers is supportive of the metabolic conversions that have been described, but of little quantitative relevance as they concern substances that are widely different from the candidate substances in this group and the supporting ones evaluated by the JECFA.

Pyrazines with a simple alkyl substituent may be expected to be oxidised at the side chain to give the corresponding carboxylic acid [FL-no: 14.097 and 14.101]. If such oxidations are not possible, e.g. due to steric hindrance, hydroxylation of the pyrazine ring may occur [FL-no: 14.091 and 14.129]. The bicyclic pyrazine derivatives with an additional substituent [FL-no: 14.099, 14.102, 14.108, 14.113, 14.139, 14.148 and 14.161] may be better substrates for ring hydroxylation, which seems to be carried out preferably by molybdenum hydroxylases. Five of the candidate substances bearing a ketone ring substituent [FL-no: 14.081, 14.083, 14.084, 14.086 and 14.087] may be reduced at the carbonyl in the side chain to give the corresponding alcohol. The two monocyclic pyrazine derivatives with a methoxy side chain [FL-no: 14.051 and 14.127] may also be expected to be metabolised via both ring hydroxylation and O-demethylation of the methoxy side chain. With the resulting products of any of these flavouring substances conjugation with glycine, sulphate or glucuronide may occur. In none of the studies N-oxidation or N-methylation, which would lead to the formation of bioactive metabolites, has been observed. This is in agreement with the reactive properties of the heterocyclic nitrogen in pyrazine moieties.

One candidate substance in this group, 2-isopropyl-3-methylthiopyrazine [FL-no: 14.122] is a thioether, which may be detoxified by formation of a sulphoxide and subsequently a sulphone, which are both stable and usually rapidly excreted. Alternatively, it may also be bioactivated via S-demethylation, resulting in the formation of a reactive free thiol. No data were provided to show that either route predominates. For that reason, it cannot be anticipated that this sulphur-containing pyrazine derivative is metabolised to innocuous products. Neither can it be assumed that quinoxaline [FL-no: 14.147] and its derivative 2-methylquinoxaline [FL-no: 14.139] are metabolised to innocuous products. One of the candidate substances, isopropenylpyrazine [FL-no: 14.052] has a terminal double-bond (in conjugation with a heterocyclic aromatic ring) and it may be anticipated to be epoxidated, thereby giving rise to reactive metabolites (as for alkenes with terminal double bonds). It can therefore not be concluded that isopropenylpyrazine can be metabolised to innocuous products.

Based on the available data, the other 17 substances in this group [FL-no: 14.051, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.127, 14.129, 14.148 and 14.161] may be expected to be metabolised to innocuous products.

5. Application of the Procedure for the Safety Evaluation of Flavouring Substances

The application of the Procedure is based on intakes estimated on the basis of the MSDI approach. Where the mTAMDI approach indicates that the intake of a flavouring substance might exceed its corresponding threshold of concern, a formal safety assessment is not carried out using the Procedure. In these cases the Panel requires more precise data on use and use levels. For comparison of the intake estimations based on the MSDI approach and the mTAMDI approach, see Section 6.

In previous versions of the present FGE, it was found that two of the candidate substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] showed possible genotoxic potential *in vitro*. Therefore, the Panel decided that the Procedure could not be applied to these two candidate substances nor for the structurally related 2,3-dimethylquinoxaline [FL-no: 14.108] until

adequate genotoxicity data become available. This conclusion did also have implications for the structurally related 5-methyl quinoxaline [FL-no: 14.028], considered by the Panel in FGE.50, as the Procedure should not be applied until adequate genotoxicity data become available for this substance either (EFSA, 2008x).

Additional genotoxicity data have now become available for the structurally related 5-methylquinoxaline [FL-no: 14.028] and in the present FGE these genotoxicity data submitted by the Industry (Flavour Industry, 2009a) have been evaluated. The available data indicate that there is no apparent structure-activity relationship for the genotoxicity of quinoxalines (Hashimoto et al., 1979): thus these compounds are to be considered individually. 2,3-Dimethylquinoxaline [FL-no: 14.108] is not genotoxic *in vitro* and can be evaluated through the Procedure; conversely, *in vitro* data indicate a genotoxic potential for quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139], for which no *in vivo* data are available. Therefore, for these two substances the Procedure cannot be applied until adequate genotoxicity data become available.

For one candidate substance, 2,5 or 6-methoxy-3-ethylpyrazine [FL-no: 14.051] no European production figures were available and consequently no European exposure estimates could be calculated. Accordingly, the safety in use could not be assessed using the Procedure for this substance.

For the safety evaluation of the 18 candidate substances which can be evaluated using the procedure, the Procedure as outlined in Annex I was applied, based on the MSDI approach. The stepwise evaluations of the substances are summarised in Table 2.

Step 1

According to the decision tree approach, presented by Cramer et al., 16 of the 18 candidate substances for which the Procedure could be applied are classified into structural class II. The two remaining substances, for which the Procedure could be applied, 2,3-dimethylquinoxaline [FL-no: 14.108] and 2-Isopropyl-3-methylthiopyrazine [FL-no: 14.122] are classified into structural class III (Cramer et al., 1978).

Step 2

Two of the 18 candidate substances cannot be anticipated to be metabolised to innocuous products: one sulphur-containing flavouring substance [FL-no: 14.122], which may be converted to a reactive free thiol and one substance with a terminal double bond [FL-no: 14.052] (in conjugation with a heterocyclic aromatic ring), which may be epoxidated giving rise to reactive metabolites.

Therefore these two substances are evaluated along the B-side of the Procedure scheme.

Based on the available data, 16 of the 18 substances in this group [FL-no: 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.127, 14.129, 14.148, and 14.161] may be expected to be metabolised to innocuous products. Therefore, these 16 flavouring substances are evaluated along the A-side of the Procedure scheme (Annex I).

Step A3

The 16 candidate substances evaluated along the A-side of the Procedure scheme are classified into structural class II. These substances have estimated daily *per capita* intakes from use as flavouring substances in the range from 0.0024 to 0.12 microgram. These intakes are below the threshold of concern of 540 and 90 microgram/person/day for structural class II and III, respectively.

Based on the results of the safety evaluation sequence of the Procedure, these 16 substances proceeding *via* the A-side of the Procedure scheme do not pose a safety concern when used as flavouring substances at the estimated levels of intake, based on the MSDI approach.

Step B3

The level of intake of the candidate substance isopropenylpyrazine [FL-no: 14.052] was estimated to be 0.012 microgram/*capita*/day, which is below the threshold of concern of 540 microgram/person/day for structural class II substances.

The level of intake of the candidate substance, 2-isopropyl-3-methyl thiopyrazine [FL-no: 14.122] was estimated to be 0.061 microgram/*capita*/day, which is below the threshold of concern of 90 microgram/person/day for structural class III substances.

Accordingly, these candidate substances proceed to step B4 of the Procedure.

Step B4

No valid toxicity study from which a NOAEL could be established was available for isopropenylpyrazine [FL-no: 14.052] or for any relevant supporting substance. Therefore, the Panel concluded that additional toxicity data are needed for this substance.

Ninety days oral feeding studies in rats are available for two supporting substances [FL-no: 14.031 and 14.034] structurally related to the candidate substance 2-isopropyl-3-methyl thiopyrazine [FL-no: 14.122]. Although the studies are not performed in accordance with modern guidelines (see Section 8.2), the studies have been considered adequate for deriving No Observed Adverse Effect Level (NOAEL) for each, which are 1.63 and 16.3 mg/kg body weight (bw) for [FL-no: 14.031 and 14.034], respectively. The MSDI of 0.061 microgram/*capita*/day corresponds to 0.001 microgram/kg bw/day at a body weight of 60 kg. Thus, a margin of safety of 1.6×10^6 using the NOAEL of 1.63 can be calculated. 2-Isopropyl-3-methyl thiopyrazine [FL-no: 14.122] is accordingly not expected to be of safety concern at its estimated level of intake.

Based on results of the safety evaluation sequence, 17 of the 18 candidate substances, for which the Procedure could be applied, are not expected to be of safety concern when used as flavouring substances at the estimated levels of intake, based on the MSDI approach.

6. Comparison of the Intake Estimations Based on the MSDI Approach and the mTAMDI Approach

For the candidate substance [FL-no: 14.051] no use levels were provided.

The estimated intakes for the 16 candidate substances in structural class II based on the mTAMDI range from 190 to 400 microgram/person/day, which is below the threshold of concern of 540 microgram/person/day for structural class II substances.

The estimated intakes of the four substances [FL-no: 14.108, 14.122, 14.139 and 14.147] assigned to structural class III, based on the mTAMDI range from 190 to 270 microgram/person/day, which are all above the threshold of concern for structural class III of 90 microgram/person/day.

For the candidate substances [FL-no: 14.108 and 14.122] further information is required. This would include more reliable intake data and then, if required, additional toxicological data.

For the candidate substances [FL-no: 14.139 and 14.147] additional genotoxicity data are required before they can be evaluated using the Procedure. Subsequently, more reliable intake data may be required.

For comparison of the intake estimates based on the MSDI approach and the mTAMDI approach see Table 6.1.

Table 6.1 Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI ($\mu\text{g}/\text{capita}/\text{day}$)	mTAMDI ($\mu\text{g}/\text{person}/\text{day}$)	Structural class	Threshold of concern ($\mu\text{g}/\text{person}/\text{day}$)
14.081	5-Acetyl-2,3-dimethylpyrazine	0.012	270	Class II	540
14.083	2-Acetyl-5-ethylpyrazine	0.012	270	Class II	540
14.084	2-Acetyl-5-methylpyrazine	0.0024	270	Class II	540
14.086	2-Acetyl-6-ethylpyrazine	0.0061	270	Class II	540
14.087	2-Acetyl-6-methylpyrazine	0.028	270	Class II	540
14.091	2-Butyl-3-methylpyrazine	0.12	270	Class II	540
14.097	2,5-Diethylpyrazine	0.024	270	Class II	540
14.099	6,7-Dihydro-5,7-dimethyl-5H-cyclopentapyrazine	0.032	190	Class II	540
14.101	2,5-Dimethyl-3-isopropylpyrazine	0.018	190	Class II	540
14.102	5,6-Dimethyldihydrocyclopentapyrazine	0.024	270	Class II	540
14.113	5-Ethyl-6,7-dihydro-5H-cyclopentapyrazine	0.012	270	Class II	540
14.127	2-Methoxy-3-propylpyrazine	0.061	270	Class II	540
14.129	2-Methyl-3-propylpyrazine	0.011	400	Class II	540
14.148	5,6,7,8-Tetrahydro-5-methylquinoxaline	0.0073	270	Class II	540
14.161	6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine	0.011	400	Class II	540
14.052	Isopropenylpyrazine	0.012	400	Class II	540
14.051	2,5 or 6-Methoxy-3-ethylpyrazine			Class II	540
14.108	2,3-Dimethylquinoxaline	0.049	190	Class III	90
14.122	2-Isopropyl-3-methylthiopyrazine	0.061	270	Class III	90
14.139	2-Methylquinoxaline	0.12	270	Class III	90
14.147	Quinoxaline	0.12	270	Class III	90

7. Considerations of Combined Intakes from Use as Flavouring Substances

Because of structural similarities of candidate and supporting substances, it can be anticipated that many of the flavourings are metabolised through the same metabolic pathways and that the metabolites may affect the same target organs. Further, in case of combined exposure to structurally related flavourings, the pathways could be overloaded. Therefore, combined intake should be considered. As flavourings not included in this FGE may also be metabolised through the same pathways, the combined intake estimates presented here are only preliminary. Currently, the combined intake estimates are only based on MSDI exposure estimates, although it is recognised that this may lead to underestimation of exposure. After completion of all FGEs, this issue should be readdressed.

The total estimated combined daily *per capita* intake of structurally related flavourings is estimated by summing the MSDI for individual substances.

As two of the candidate substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] show possible genotoxic potential *in vitro*, the substances are not taken through the Procedure. As no intake data are available for 2,5 or 6-methoxy-3-ethylpyrazine [FL-no: 14.051] the substance is not taken through the Procedure. These three substances are therefore not included in the calculation of the combined intake of the candidate substances evaluated in FGE.17Rev2.

On the basis of the reported annual production volumes in Europe (EFFA, 2003r), the combined estimated daily *per capita* intake as flavourings of the 16 candidate flavouring substances assigned to structural class II is 0.4 microgram, and of the two substances assigned to structural class III, 0.1 microgram. These values do not exceed the thresholds of concern for substances belonging to structural class II and III of 540 and 90 microgram/person/day, respectively.

The candidate substances are structurally related to 41 supporting substances evaluated by the JECFA at its 57th meeting (JECFA, 2002b). Based on reported production volumes, European *per capita* intakes (MSDI) could be estimated for the 41 supporting substances.

The total combined intake of the 16 candidate substances and 32 supporting substances from structural class II is approximately 300 microgram/*capita*/day, which is below the threshold of concern for a compound belonging to structural class II of 540 microgram/person/day. The total combined intake of the two candidate substances from structural class III, evaluated through the Procedure and nine

supporting substances from structural class III is approximately 37 microgram/*capita*/day, which is also below the threshold of concern for a compound belonging to structural class III of 90 microgram/person/day.

8. Toxicity

8.1. Acute Toxicity

Acute oral LD₅₀ values are available on 18 of the 41 supporting substances. The acute oral LD₅₀ in rats and mice range from 200 to more than 4000 mg/kg bw indicating a low level of oral toxicity in these species.

The acute toxicity data are summarised in Annex IV, Table IV.1

8.2. Subacute, Subchronic, Chronic and Carcinogenicity Studies

No data are available on subacute or subchronic toxicity for the candidate substances. Data on subchronic toxicity are available on 17 of the 41 supporting substances. Most of the studies were performed at single dose levels and all studies were performed in rats. NOAELs were in the range from 0.44 mg/kg bw to 55 mg/kg bw.

Toxicity studies on three supporting substances used for deriving the NOAELs used in the Procedure are briefly reported in the following.

Pyrazinyl methyl sulphide [FL-no: 14.034] and pyrazinylethanethiol [FL-no: 14.031]

The supporting substances pyrazinyl methyl sulphide [FL-no: 14.034] and pyrazinylethanethiol [FL-no: 14.031] were administered in the feed to male and female rats (16 animals/sex) for 13 weeks at doses of 1.63 mg/kg bw/day and 16.30 mg/kg bw/day, respectively. After 90 days, all animals were killed, subjected to detailed necropsy examinations, and liver and kidney weights were measured. A wide range of tissues and organs from each animal were preserved and histopathological examinations were performed on major organs and tissues. The authors stated that no major differences were observed between groups of treated and control animals, based on measurements of growth, food intake, haematological parameters, blood urea determinations, organ weights and gross and histopathologic examinations. However, no numeric data were reported. The only levels tested (1.63 and 16.30 mg/kg bw/day) have been taken as NOAELs for pyrazinyl methyl sulphide [FL-no: 14.034] and pyrazinylethanethiol [FL-no: 14.031], respectively (Posternak et al., 1975).

5-Methylquinoxaline [FL-no: 14.028]

The supporting substance 5-methylquinoxaline [FL-no: 14.028] was administered in the feed to male and female rats (16 animals/sex) for 90 days at a single dose of 17.1 mg/kg bw/day. After 7 weeks and after 13 weeks, 50 % of the animals were killed, and liver and kidney weights were measured and gross and histological examinations were carried out on a wide range of organs. The authors stated that no major differences were observed between groups of treated and control animals, based on measurements of growth, food intake, haematological parameters, blood urea determinations, organ weights and gross and histopathologic examinations. However, no numeric data were reported. The only level tested (17.1 mg/kg bw/day) has been taken as NOAEL for 5-methylquinoxaline [FL-no: 14.028] (Posternak et al., 1969)

No studies are available on chronic toxicity or on carcinogenicity for either candidate or supporting substances.

The repeated dose toxicity are summarised in Annex IV, Table IV.2.

8.3. Developmental / Reproductive Toxicity Studies

Data are available for four of the supporting substances 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine. 2,5-Dimethylpyrazine [FL-no: 14.020] has been reported to occur naturally in the urine of female mice at concentrations of 0.25 µg/l. A study of a mixture of 2,5-dimethylpyrazine with five naturally occurring ketones and esters was reported to delay puberty in juvenile female mice, but the compound responsible for this effect was not identified (Novotny et al., 1986). The effect of isomers of dimethylpyrazine on reproductive organs in male and female rats has been studied. (Yamada et al., 1993; Yamada et al., 1992; Yamada et al., 1994). The effects on reproductive organ parameters have been described after subcutaneous (s.c.) injection of 2,5-dimethylpyrazine [FL-no: 14.020] at doses > 30 mg/kg bw, which can be considered as the NOAEL. Also 2,6- methylpyrazine [FL-no: 14.021]) showed some effects, but to a lesser extent while, 2,3- dimethylpyrazine [FL-no: 14.050] showed no effect. However, the relevance of s.c. route of administration is limited, considering the use of the chemical as a flavouring substance. In addition, as recently reviewed, neither the mechanism(s) nor the relevance for the human reproductive system have been clarified (Koyama, 2004). In any case, the NOAEL of 30 mg/kg bw is orders of magnitude higher than the predicted level of exposure as a flavouring substance, according to the MSDI approach. A study on a fourth supporting substance, 2,3,5,6-tetramethylpyrazine [FL-no: 14.018], showed no adverse effects on several reproductive parameters after oral administration (Vollmuth et al., 1997)

Developmental/reproductive toxicity data are summarised in Annex IV, Table IV.3.

8.4. Genotoxicity Studies

Genotoxicity data were provided for three of the 20 candidate substances and for 11 of the 41 supporting substances. The three candidate substances are quinoxaline [FL-no: 14.147] and its derivatives 2-methylquinoxaline [FL-no: 14.139] and 2,3-dimethylquinoxaline [FL-no: 14.108]. After finalisation of the previous version of this FGE (i.e. FGE.17, Revision 1), both *in vitro* and *in vivo* genotoxicity data have become available for the supporting substance 5-methylquinoxaline [FL-no: 14.028]. This substance is a candidate flavouring substance in FGE.50. The Panel explored the option of using the genotoxicity data submitted for 5-methylquinoxaline [FL-no: 14.028] to support the evaluation of the genotoxic potential of the candidate quinoxaline derivatives in FGE.17.

Genotoxicity data on Candidate substances

In *in vitro* studies, quinoxaline [FL-no: 14.147], up to 10000 microgram/plate and 2,3-dimethylquinoxaline [FL-no: 14.108], up to 2500 microgram/plate, with and without metabolic activation, did not cause reverse mutation in various strains of *Salmonella typhimurium* (Table IV.4). Two studies on 2-methylquinoxaline [FL-no: 14.139] are available, one study with a positive, the other with negative result in the Ames test. However, quinoxaline [FL-no: 14.147] at 250 microgram/ml culture medium and with metabolic activation was found to induce TFT-mutants in the mouse lymphoma mutagenesis assay (L5178Y TK^{+/−} cells). This study was conducted in accordance with the OECD guideline 476 and therefore considered valid.

No adequate *in vivo* studies on genotoxicity of the candidate substances are available. A study of the potential of quinoxaline [FL-no: 14.147] to induce sperm head abnormalities (Topham, 1980) did not address a genetic endpoint and the Panel considered it could not be used for evaluation of genotoxicity of this substance.

Genotoxicity data on Supporting substances

Substituted pyrazines

In vitro, 2-methylpyrazine [FL-no: 14.027], ethylpyrazine [FL-no: 14.022], 2,3-dimethylpyrazine [FL-no: 14.050], 2,5-dimethylpyrazine [FL-no: 14.020], 2,6-dimethylpyrazine [FL-no: 14.021], 2,3-diethylpyrazine [FL-no: 14.005], 2,3,5-trimethylpyrazine [FL-no: 14.019], pyrazine [FL-no: 14.144] and (2, 5 or 6)-methoxy-3-methylpyrazine [FL-no: 14.025] were tested for their ability to cause reverse mutation in various strains of *S. typhimurium* and consistently revealed negative results with and without metabolic activation (Table IV.4).

In one of these studies, 2-methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, and pyrazine were also tested for their potential to cause genotoxicity in *Saccharomyces cerevisiae* and chromosomal aberrations in Chinese hamster ovary cells (Table IV.4) (Stich et al., 1980). This study has strong limitations for the following reasons. The positive results were observed only in a narrow range of exceedingly high and toxic concentrations. In the case of chromosome aberrations, the concentration used exceeded the maximum level (5 mg/ml) recommended by OECD. It has been shown (Galloway, 2000) that *in vitro* chromosome breaking can occur secondary to toxicity and/or changed physiological conditions (e.g., pH, osmolarity) with compounds not able to react with DNA and negative in the Ames test and *in vivo*. The *S.cerevisiae* D5 assay for induction of "aberrant colonies" is not routinely used and has not been validated at international level due to the uncertainty on the various effects involved. Thus, the positive results reported by Stich et al. (Stich et al., 1980) are considered of limited value and not relevant for hazard and risk assessment. Furthermore, pyrazine was found negative in a wide range of concentrations both in the *Salmonella* assay and in the mouse lymphoma TK assay (Fung et al., 1988).

Quinoxalines

5-Methylquinoxaline [FL-no: 14.028] was examined for its mutagenic potential in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, as well as in *Escherichia coli* strain WP2 uvrA. The study was conducted according to GLP and was in compliance with OECD Guideline 471. No evidence of mutagenicity was found with or without S9 metabolic activation at concentrations up to 5,000 µg/plate (Ogura & Wakamatsu, 2004).

5-Methylquinoxaline was examined for its potential to induce structural chromosome aberrations in mammalian cells. The study was conducted according to GLP and was in compliance with OECD Guideline 473. The test system used was a subculture of Chinese hamster lung-derived CHL/IU cells that were exposed to the test material at concentrations of 320, 480 and 720 µg/mL without S9 mix, and 72.0, 228 and 720 µg/mL with S9 mix. The percentage of "cell productivity" (the cell number was measured and expressed as relative growth rate compared to negative control) was reported as a parameter for cytotoxicity. The Panel considered that 5-methylquinoxaline was found to induce chromosomal aberrations in cultured mammalian cells in the presence of metabolic activation. Additionally, an increased frequency of polyploid cells up to 12.5 % of the middle dose compared to 0 % in the control was observed in the presence and absence of metabolic activation at concentrations which induced only low cytotoxicity (Ajimu & Kawaguchi, 2004a).

In vivo data are available for two structurally related substances only, (2, 5 or 6)-Methoxy-3-methylpyrazine [FL-no: 14.025 (mixture of three structural isomers)] and 5-methylquinoxaline [FL-no: 14.028].

5-Methylquinoxaline [FL-no: 14.028] was examined for its potential to induce micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow. The test material was administered daily (gavage) for two consecutive days to seven week old and six week old male SPF ICR (Crj:CD-1) mice at dosages of 125, 250 and 500 mg/kg/day (6 animals/dose). Microscopic examination of femoral bone marrow cells was conducted randomly from 5 animals. Two thousand polychromatic erythrocytes (PCE) per animal were analyzed microscopically (x1000), and the number of micronucleated polychromatic erythrocytes (MNCPE) was recorded. In order to evaluate the PCE/NCE ratio, the number of PCEs out of 200 total erythrocytes (PCEs plus NCEs) was recorded. The test was considered positive if the MNPCE frequencies in one or more treatment groups were significantly

higher than that in the negative control groups. No significant increase of micronucleated polychromatic erythrocyte frequency was observed in these treatment groups compared with the negative control group. The PCE/NCE ratio was not changed (Ajimu & Kawaguchi, 2004b). Based on the PCE/NCE ratio there is no indication that the substance reached the bone marrow, however, the Panel noted that the high dose was the maximum tolerated dose since clinical signs of toxicity have been observed after oral intake. Additionally, a doubling of this dose was lethal for two out of six animals in a preliminary test. Thus, the Panel considered it reasonable to assume that the substance was systemically available and reached the bone marrow.

For (2, 5 or 6)-methoxy-3-methylpyrazine [FL-no: 14.025], a test for Base mutation was performed in *Drosophila* with a concentration of 10 mmol/L (140 microgram/ml) in the solutions/emulsions fed to the flies, with no mutagenic effect (Table IV.5). Secondly, male and female NMRI mice were treated once orally with 87, 174 or 248 mg/kg bw, bone-marrow smears were prepared only at one sampling time (at 30 hours) after treatment. There was no increase in the frequency of micronuclei in polychromatic erythrocytes (Table IV.5). The PCE/NCE ratio was not reported and thus, it is not clear if the test substance reached the bone marrow. However from this study, there is no evidence of genotoxic potential.

Conclusion on Genotoxicity

The available data indicate that apparently there is no simple structure-activity relationship for the genotoxicity of quinoxalines, because the profile of genotoxic events *in vitro* differs for the various congeners (point mutations for [FL no: 14.139] and [FL-no: 14.147] vs chromosomal aberrations for [FL no: 14.028]). Therefore these compounds are to be evaluated based on substance-specific data for each individual quinoxaline derivative.

In vitro data indicate a genotoxic potential for quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139], for which no *in vivo* data are available. Therefore, for these two substances the Procedure cannot be applied until adequate genotoxicity data become available.

Conversely, 2,3-dimethylquinoxaline [FL-no: 14.108] is not considered genotoxic *in vitro* and hence can be evaluated through the Procedure (three negative bacterial reverse gene mutation assays which, although limited, consistently indicate lack of genotoxicity).

The Panel concluded that no genotoxic potential is indicated for 19 candidate substances, including 2,3-dimethylquinoxaline [FL-no: 14.108]. For these 19 substances, the available data do not preclude their evaluation through the Procedure.

Genotoxicity data are summarised in Annex IV, Table IV.4 and IV.5.

9. Conclusions

This group of flavouring substances consists of 21 candidate substances, all of which contain a pyrazine moiety. In 13 substances [FL-no: 14.051, 14.052, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.122, 14.127 and 14.129] only one heterocyclic ring is present. In five candidate substances a pyrazine ring is fused with either cyclopentane [FL-no: 14.099, 14.102, 14.113 and 14.161] or with cyclohexane [FL-no: 14.148]. All of these substances have different ring substituents. In 14 of them, the substituents are simple alkyl chains or ketones. In the other four, the side chains are either a methoxy- or a thiomethyl- residue ([FL-no: 14.051 and 14.127] and [FL-no: 14.122], respectively) or the substituent contains a terminal double-bond in conjugation with the heterocyclic aromatic ring ([FL-no: 14.052]). In the remaining three candidate substances the pyrazine ring is fused with benzene giving quinoxalines. In two of the quinoxalines [FL-no 14.139 and 14.108] the pyrazine ring also bears one or two methyl substituents; in the third no substituents are present (quinoxaline [FL-no: 14.147]).

Five of the 21 flavouring substances possess one or two chiral centres [FL-no: 14.099, 14.102, 14.113, 14.148 and 14.161]. In two of these cases [FL-no: 14.099 and 14.102], the stereoisomeric composition has not been specified.

Seventeen candidate substances are classified into structural class II. Four candidate substances [FL-no: 14.108, 14.122, 14.139 and 14.147] are classified into structural class III.

Nineteen of the flavouring substances in the present group have been reported to occur naturally in a wide range of food items.

According to the default MSDI approach, the 18 flavouring substances in this group, which are evaluated through the Procedure and for which Industry has provided intake data, have intakes in Europe from 0.0024 to 0.12 microgram/*capita*/day, which are below the threshold of concern value for both structural class II (540 microgram/person/day) and structural class III (90 microgram/person/day) substances.

On the basis of the reported annual production in Europe (MSDI approach), the combined intake of the candidate and supporting substances in the present FGE could be calculated. The total combined intake of the 16 candidate substances and 32 supporting substances from structural class II is approximately 300 microgram/*capita*/day which is below the threshold of concern for a compound belonging to structural class II of 540 microgram/person/day. The total combined intake of the two candidate substances from structural class III, evaluated through the Procedure and nine supporting substances from structural class III is approximately 37 microgram/*capita*/day which is also below the threshold of concern for a compound belonging to structural class III of 90 microgram/person/day.

In previous versions of the present FGE, the Panel concluded “No genotoxic potential at gene or chromosome level is indicated for the 17 alkyl- and cycloalkyl-substituted pyrazines, which allows these substances to be evaluated through the Procedure. However, from the *in vitro* data available, genotoxic potential is indicated for the candidate substances quinoxaline [FL-no: 14.147] and its derivative 2-methylquinoxaline [FL-no: 14.139]. Therefore, the Panel concluded that the Procedure could not be applied to these two candidate substances nor for the structurally related 2,3-dimethylquinoxaline [FL-no: 14.108] until adequate genotoxicity data become available”.

Data have become available for the structurally related substance, 5-methylquinoxaline [FL-no: 14.028], considered in FGE.50Rev1. However, these data indicate that apparently there is no simple structure-activity relationship for the genotoxicity of quinoxalines, because the profile of genotoxic events *in vitro* differs for the various congeners (point mutations for [FL no: [FL-no: 14.139] and [FL-no: 14.147] vs chromosomal aberrations for [FL no: [FL-no:14.028]). Therefore these compounds are to be evaluated based on substance-specific data for each individual quinoxaline derivative.

In vitro data indicate a genotoxic potential for quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139], for which no *in vivo* data are available. Therefore, for these two substances the Procedure cannot be applied until adequate genotoxicity data become available. Conversely 2,3-dimethylquinoxaline [FL-no: 14.108] is not considered genotoxic *in vitro* and hence can be evaluated through the Procedure

The Panel concluded that no genotoxic potential is indicated for 19 candidate substances, including 2,3-dimethylquinoxaline [FL-no: 14.108]. For these 19 substances, the available data do not preclude their evaluation through the Procedure.

Sixteen of the alkyl- and cycloalkyl-substituted pyrazines and one of the alkyl-substituted quinoxalines may be expected to be metabolised to innocuous products [FL-no: 14.051, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.127, 14.129, 14.148 and 14.161]. Regarding the remaining four substances, they cannot be anticipated to be metabolised to innocuous products. One sulphur-containing flavouring substance [FL-no: 14.122],

may be converted to a reactive free thiol. Neither can it be assumed that quinoxaline [FL-no: 14.147] and its derivative [FL-no: 14.139] are metabolised to innocuous products and one substance with a terminal double bond [FL-no: 14.052] (in conjugation with a heterocyclic aromatic ring), may be epoxidated giving rise to reactive metabolites.

Where toxicity data were available for substances evaluated using the Procedure, they were consistent with the conclusions in the present FGE.

It is considered that on the basis of the default MSDI approach, 17 of the 18 candidate substances evaluated through the Procedure would not give rise to safety concerns at the estimated levels of intake arising from their use as flavouring substances.

When the estimated intakes, of the substances evaluated through the Procedure, were based on the mTAMDI approach they ranged from 190 to 400 microgram/person/day for the 16 flavouring substances from structural class II. Thus, the intakes for these 16 substances were below the threshold of concern for structural class II of 540 microgram/person/day. These 16 substances are also expected to be metabolised to innocuous products.

Based on the mTAMDI approach, the estimated intake of the two flavouring substances [FL-no: 14.108 and 14.122] assigned to structural class III and evaluated through the Procedure are 190 and 270 microgram/person/day, respectively, which are above the threshold of concern for structural class III of 90 microgram/person/day. Therefore, for these substances more reliable exposure data are required. On the basis of such additional data, the flavouring substances should be re-evaluated using the Procedure. Subsequently, additional data might become necessary.

In order to determine whether the conclusion for the 18 candidate substances which have been evaluated using the Procedure can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity for the materials of commerce have been provided for 16 of the 18 flavouring substances evaluated through the Procedure. Information on composition of mixture and/or chirality is missing for two these 18 substances. Thus, the final evaluation of the materials of commerce cannot be performed for these two substances [FL-no: 14.099 and 14.102], pending further information.

Thus, in conclusion, only 18 of the 21 candidate substances were evaluated through the Procedure, as two flavouring substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] could not be evaluated through the Procedure until adequate genotoxicity data become available and one substance, 2,5 or 6-methoxy-3-ethylpyrazine [FL-no: 14.051], was presented with no intake data and therefore it could not be evaluated through the Procedure until adequate intake data and, if required, additional toxicological data become available.

For one candidate substance, isopropenylpyrazine [FL-no: 14.052] additional data are required as the substituent contains a terminal double-bond in conjugation with the heterocyclic aromatic ring, which may be epoxidated giving rise to reactive metabolites.

The final evaluation of the materials of commerce cannot be performed for two substances, 6,7-dihydro-5,7-dimethyl-5H-cyclopentapyrazine and 5,6-dimethyldihydrocyclopentapyrazine [FL-no: 14.099 and 14.102] pending further information on composition of mixture and/or chirality.

For the remaining 15 substances adequate specifications including complete purity criteria and identity for the materials of commerce have been provided and the Panel concluded that these candidate substances [FL-no: 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.108, 14.113, 14.122, 14.127, 14.129, 14.148, and 14.161] would present no safety concern at the level of intake estimated on the basis of the MSDI approach.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 17, REVISION 2

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 17, Revision 2

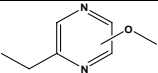
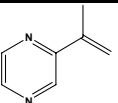
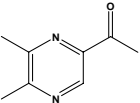
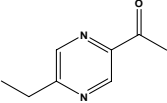
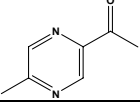
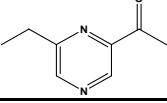
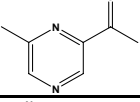
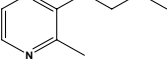
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
14.051	2,5 or 6-Methoxy-3-ethylpyrazine		3280 11329 68739-00-4	124.14				AV 7), BP 8), ID 9), MP 10), PF 11), RI 12), SG 13), SE 14), SW 15) Registname to be changed to mixture of 2-, 5- or 6-methoxy-3-ethylpyrazine
14.052	Isopropenylpyrazine		3296 11341 38713-41-6	Liquid C ₇ H ₈ N ₂ 120.16	Practically insoluble or insoluble Soluble	73-75 (23 hPa) NMR 99 %	1.480-1.486 0.964-0.968	
14.081	5-Acetyl-2,3-dimethylpyrazine		54300-10-6	Solid C ₈ H ₁₀ N ₂ O 150.18	Soluble Freely soluble	308 151 MS 95 %	n.a. n.a.	
14.083	2-Acetyl-5-ethylpyrazine		43108-58-3	Solid C ₈ H ₁₀ N ₂ O 150.18	Soluble Freely soluble	303 138 NMR 95 %	n.a. n.a.	
14.084	2-Acetyl-5-methylpyrazine		11297 22047-27-4	Solid C ₇ H ₈ N ₂ O 136.15	Soluble Freely soluble	80 (11 hPa) 56 MS 95 %	n.a. n.a.	
14.086	2-Acetyl-6-ethylpyrazine		11295 34413-34-8	Solid C ₈ H ₁₀ N ₂ O 150.18	Soluble Freely soluble	302 137 NMR 95 %	n.a. n.a.	
14.087	2-Acetyl-6-methylpyrazine		11298 22047-26-3	Solid C ₇ H ₈ N ₂ O 136.15	Soluble Freely soluble	280 33 MS 95 %	n.a. n.a.	
14.091	2-Butyl-3-methylpyrazine		15987-00-5	Solid C ₉ H ₁₄ N ₂ 150.22	Slightly soluble Freely soluble	84 (12 hPa) 100 MS 95 %	n.a. n.a.	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 17, Revision 2

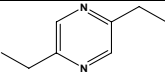
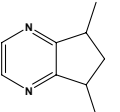
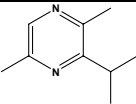
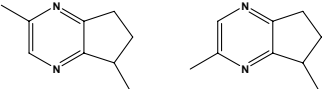
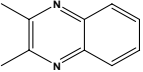
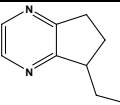
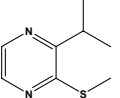
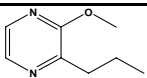
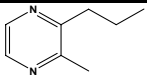
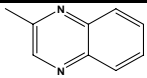
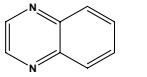
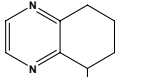
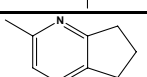
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
14.097	2,5-Diethylpyrazine		11306 13238-84-1	Solid C ₈ H ₁₂ N ₂ 136.20	Slightly soluble Freely soluble	187 88 MS 95 %	n.a. n.a.	
14.099	6,7-Dihydro-5,7-dimethyl-5H-cyclopentapyrazine 6)		41330-21-6	Solid C ₉ H ₁₂ N ₂ 148.21	Slightly soluble Freely soluble	84 (13 hPa) 113 MS 95 %	n.a. n.a.	Stereoisomeric composition to be specified.
14.101	2,5-Dimethyl-3-isopropylpyrazine		11318 40790-20-3	Solid C ₉ H ₁₄ N ₂ 150.22	Slightly soluble Freely soluble	276 97 NMR 95 %	n.a. n.a.	
14.102	5,6-Dimethyldihydrocyclopentapyrazine 6)		38917-61-2	Solid C ₉ H ₁₂ N ₂ 148.21	Slightly soluble Freely soluble	90 (16 hPa) 130 MS 95 %	n.a. n.a.	Change name to: 2,5-dimethyl-6,7-dihydro-5H-cyclopentapyrazine. Covers mix of 2,5-dimethyl-6,7-dihydro-5H-cyclopentapyrazine (60-100%) & 3,5-dimethyl-6,7-dihydro-5H-cyclopentapyrazine (up to 40 %). Composition of mixture and stereoisomeric composition to be more specified.
14.108	2,3-Dimethylquinoxaline		2379-55-7	Solid C ₁₀ H ₁₀ N ₂ 158.20	Slightly soluble Freely soluble	128 (2 hPa) 106 MS 95 %	n.a. n.a.	
14.113	5-Ethyl-6,7-dihydro-5H-cyclopentapyrazine		52517-53-0	Solid C ₉ H ₁₂ N ₂ 148.21	Slightly soluble Freely soluble	278 117 NMR 95 %	n.a. n.a.	Racemate.
14.122	2-Isopropyl-3-methylthiopyrazine		11342 67952-59-4	Solid C ₈ H ₁₂ N ₂ S 168.28	Slightly soluble Freely soluble	317 108 MS 95 %	n.a. n.a.	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 17, Revision 2

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
14.127	2-Methoxy-3-propylpyrazine		25680-57-3	Solid C ₈ H ₁₂ N ₂ O 152.20	Slightly soluble Freely soluble	271 111 MS 95 %	n.a. n.a.	
14.129	2-Methyl-3-propylpyrazine		15986-80-8	Liquid C ₈ H ₁₂ N ₂ 136.20	Practically insoluble or insoluble Freely soluble	190 MS 95 %	1.495-1.501 0.978-0.984	
14.139	2-Methylquinoxaline		7251-61-8	Solid C ₉ H ₈ N ₂ 144.18	Slightly soluble Freely soluble	245 132 MS 95 %	n.a. n.a.	
14.147	Quinoxaline		11365 91-19-0	Solid C ₈ H ₆ N ₂ 130.15	Slightly soluble Freely soluble	225 30 MS 95 %	n.a. n.a.	
14.148	5,6,7,8-Tetrahydro-5-methylquinoxaline		52517-54-1	Solid C ₉ H ₁₂ N ₂ 148.21	Slightly soluble Freely soluble	72 (4 hPa) 114 MS 95 %	n.a. n.a.	Racemate.
14.161	6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine		4702 11310 38917-61-2	Solid C ₉ H ₁₂ N ₂ 148.21	Practically insoluble or insoluble Freely soluble	91 (16 hPa) 110 MS 95 %	n.a. n.a.	Racemate.

- 1) Solubility in water, if not otherwise stated.
- 2) Solubility in 95 % ethanol, if not otherwise stated.
- 3) At 1013.25 hPa, if not otherwise stated.
- 4) At 20°C, if not otherwise stated.
- 5) At 25°C, if not otherwise stated.
- 6) Stereoisomeric composition not specified.
- 7) AV: Missing minimum assay value.
- 8) BP: Missing boiling point.
- 9) ID: Missing identification test.
- 10) MP: Missing melting point.
- 11) PF: Missing data on physical form.
- 12) RI: Missing refractive index.
- 13) SG: Missing specific gravity.
- 14) SE: Missing data on solubility in ethanol.

15) SW: Missing data on solubility.

TABLE 2: SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

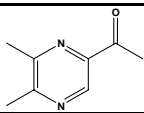
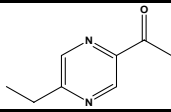
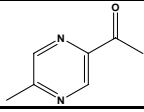
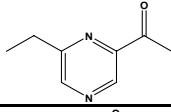
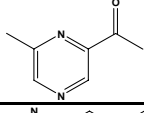
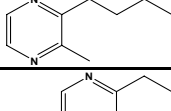
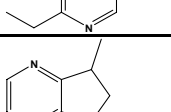
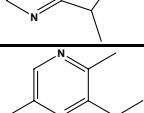
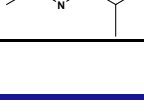
FL-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.081	5-Acetyl-2,3-dimethylpyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.083	2-Acetyl-5-ethylpyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.084	2-Acetyl-5-methylpyrazine		0.0024	Class II A3: Intake below threshold	4)	6)	
14.086	2-Acetyl-6-ethylpyrazine		0.0061	Class II A3: Intake below threshold	4)	6)	
14.087	2-Acetyl-6-methylpyrazine		0.028	Class II A3: Intake below threshold	4)	6)	
14.091	2-Butyl-3-methylpyrazine		0.12	Class II A3: Intake below threshold	4)	6)	
14.097	2,5-Diethylpyrazine		0.024	Class II A3: Intake below threshold	4)	6)	
14.099	6,7-Dihydro-5,7-dimethyl-5H-cyclopentapyrazine		0.032	Class II A3: Intake below threshold	4)	7)	
14.101	2,5-Dimethyl-3-isopropylpyrazine		0.018	Class II A3: Intake below threshold	4)	6)	

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

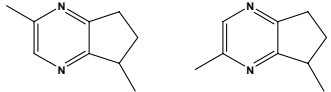
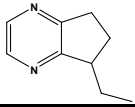
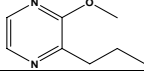
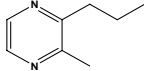
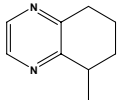
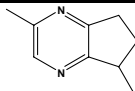
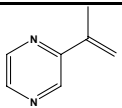
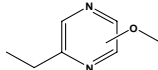
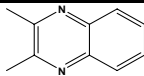
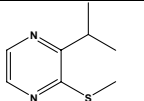
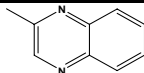
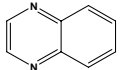
FL-no	EU Register name	Structural formula	MSDI 1 ($\mu\text{g}/\text{capita}/\text{day}$)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.102	5,6-Dimethyldihydrocyclopentapyrazine		0.024	Class II A3: Intake below threshold	4)	7)	
14.113	5-Ethyl-6,7-dihydro-5H-cyclopentapyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.127	2-Methoxy-3-propylpyrazine		0.061	Class II A3: Intake below threshold	4)	6)	
14.129	2-Methyl-3-propylpyrazine		0.011	Class II A3: Intake below threshold	4)	6)	
14.148	5,6,7,8-Tetrahydro-5-methylquinoxaline		0.0073	Class II A3: Intake below threshold	4)	6)	
14.161	6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine		0.011	Class II A3: Intake below threshold	4)	6)	
14.052	Isopropenylpyrazine		0.012	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
14.051	2,5 or 6-Methoxy-3-ethylpyrazine			Class II No evaluation			
14.108	2,3-Dimethylquinoxaline		0.049	Class III A3: Intake below threshold	4)	6)	
14.122	2-Isopropyl-3-methylthiopyrazine		0.061	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.139	2-Methylquinoxaline		0.12	Class III No evaluation			a)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI 1) ($\mu\text{g}/\text{capita}/\text{day}$)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.147	Quinoxaline		0.12	Class III No evaluation			a)

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = $\mu\text{g}/\text{capita}/\text{day}$.

2) Thresholds of concern: Class I = 1800, Class II = 540, Class III = 90 $\mu\text{g}/\text{person}/\text{day}$.

3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

4) No safety concern based on intake calculated by the MSDI approach of the named compound.

5) Data must be available on the substance or closely related substances to perform a safety evaluation.

6) No safety concern at estimated level of intake of the material of commerce meeting the specification of Table 1 (based on intake calculated by the MSDI approach).

7) Tentatively regarded as presenting no safety concern (based on intake calculated by the MSDI approach) pending further information on the purity of the material of commerce and/or information on stereoisomerism.

8) No conclusion can be drawn due to lack of information on the purity of the material of commerce.

a) Additional genotoxicity data required.

TABLE 3: SUPPORTING SUBSTANCES SUMMARY

Table 3: Supporting Substances Summary

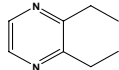
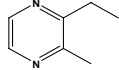
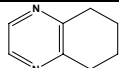
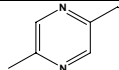
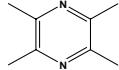
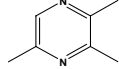
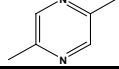
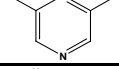
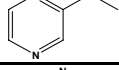
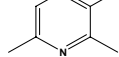
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
14.005	2,3-Diethylpyrazine		3136 534 15707-24-1	771 JECFA specification (JECFA, 2001c)	1.6	No safety concern a) Category B b)	
14.006	2-Ethyl-3-methylpyrazine		3155 548 15707-23-0	768 JECFA specification (JECFA, 2001c).	72	No safety concern a) Category B b)	
14.015	5,6,7,8-Tetrahydroquinoxaline		3321 721 34413-35-9	952 JECFA specification (JECFA, 2001c)	8	No safety concern a) Category B b)	
14.017	2-Ethyl-5-methylpyrazine		3154 728 13360-64-0	770 JECFA specification (JECFA, 2001c)	4.0	No safety concern a) Category B b)	
14.018	2,3,5,6-Tetramethylpyrazine		3237 734 1124-11-4	780 JECFA specification (JECFA, 2001c)	6.7	No safety concern a) Category B b)	
14.019	2,3,5-Trimethylpyrazine		3244 735 14667-55-1	774 JECFA specification (JECFA, 2001c)	100	No safety concern a) Category B b)	
14.020	2,5-Dimethylpyrazine		3272 2210 123-32-0	766 JECFA specification (JECFA, 2001c)	19	No safety concern a) Category B b)	
14.021	2,6-Dimethylpyrazine		3273 2211 108-50-9	767 JECFA specification (JECFA, 2001c)	1.3	No safety concern a) Category B b)	
14.022	Ethylpyrazine		3281 2213 13925-00-3	762 JECFA specification (JECFA, 2001c)	2.2	No safety concern a) Category B b)	
14.024	2-Ethyl-3,5-dimethylpyrazine		3150 2245 13925-07-0	776 JECFA specification (JECFA, 2001c)	1.2	No safety concern a)	

Table 3: Supporting Substances Summary

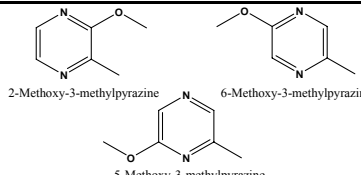
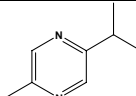

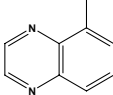
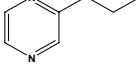
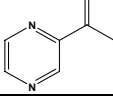
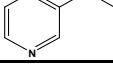
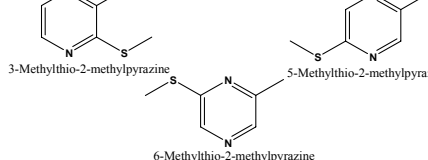
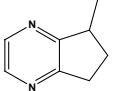
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1 (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
14.025	2,5 or 6-Methoxy-3-methylpyrazine	 <p>2-Methoxy-3-methylpyrazine 6-Methoxy-3-methylpyrazine 5-Methoxy-3-methylpyrazine</p>	3183 2266 63450-30-6	788 JECFA specification (JECFA, 2001c)	2.2	No safety concern a) Category B b)	JECFA evaluated (2,5 or 6)-methoxy-3-methylpyrazine (CASrn 2847-30-5, 2882-22-6, 2882-21-5). Register CASrn refers to methoxymethylpyrazine.
14.026	2-Isopropyl-5-methylpyrazine		3554 2268 13925-05-8	772 JECFA specification (JECFA, 2001c)	0.024	No safety concern a) Category B b)	
14.027	2-Methylpyrazine		3309 2270 109-08-0	761 JECFA specification (JECFA, 2001c)	17	No safety concern a) Category B b)	
14.028	5-Methylquinoxaline		3203 2271 13708-12-8	798 JECFA specification (JECFA, 2001c)	22	No safety concern a) Category B b)	
14.031	Pyrazineethanethiol		3230 2285 35250-53-4	795 JECFA specification (JECFA, 2001c)	0.13	No safety concern a) Category B b)	
14.032	Acetylpyrazine		3126 2286 22047-25-2	784 JECFA specification (JECFA, 2001c)	12	No safety concern a) Category B b)	
14.034	Pyrazinyl methyl sulfide		3231 2288 21948-70-9	796 JECFA specification (JECFA, 2001c)	0.0061	No safety concern a) Category B b)	
14.035	2-Methyl-3,5 or 6-methylthiopyrazine	 <p>3-Methylthio-2-methylpyrazine 5-Methylthio-2-methylpyrazine 6-Methylthio-2-methylpyrazine</p>	3208 2290 67952-65-2	797 JECFA specification (JECFA, 2001c)	6.3	No safety concern a) Category B b)	
14.037	6,7-Dihydro-5-methyl-5H-cyclopentapyrazine		3306 2314 23747-48-0	781 JECFA specification (JECFA, 2001c)	3.9	No safety concern a) Category B b)	

Table 3: Supporting Substances Summary

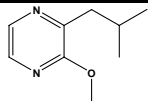
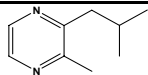
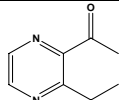

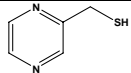

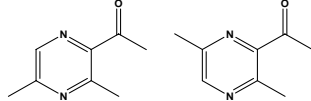
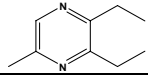
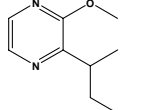
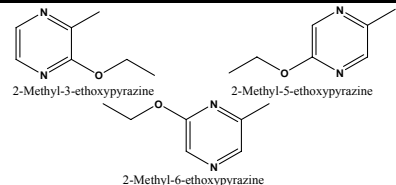
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1 (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
14.043	2-Isobutyl-3-methoxypyrazine		3132 11338 24683-00-9	792 JECFA specification (JECFA, 2001c)	1.6	No safety concern a)	
14.044	2-Isobutyl-3-methylpyrazine		3133 13925-06-9	773 JECFA specification (JECFA, 2001c)	0.037	No safety concern a)	
14.049	2-Acetyl-3-ethylpyrazine		3250 11293 32974-92-8	785 JECFA specification (JECFA, 2001c)	0.73	No safety concern a)	
14.050	2,3-Dimethylpyrazine		3271 11323 5910-89-4	765 JECFA specification (JECFA, 2001c)	14	No safety concern a)	
14.053	Mercaptomethylpyrazine		3299 11502 59021-02-2	794 JECFA specification (JECFA, 2001c)	0.012	No safety concern a)	
14.054	Methoxypyrazine		3302 11347 3149-28-8	787 JECFA specification (JECFA, 2001c)	3.0	No safety concern a)	
14.055	2-Acetyl-3,5-dimethylpyrazine		3327 11294 54300-08-2	786 JECFA specification (JECFA, 2001c)	0.97	No safety concern a)	
14.056	2,3-Diethyl-5-methylpyrazine		3336 11303 18138-04-0	777 JECFA specification (JECFA, 2001c)	0.11	No safety concern a)	
14.062	2-(sec-Butyl)-3-methoxypyrazine		3433 11300 24168-70-5	791 JECFA specification (JECFA, 2001c)	0.85	No safety concern a)	
14.067	2-Methyl-3,5 or 6-ethoxypyrazine		3569 11921 32737-14-7	793 JECFA specification (JECFA, 2001c)	0.055	No safety concern a)	JECFA evaluated methyl-3(or 5 or 6)-ethoxypyrazine (mixture of three substances with different CASrns for each substance). Register CASrns to be changed/deleted

Table 3: Supporting Substances Summary

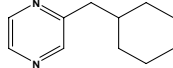
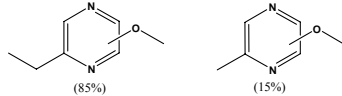
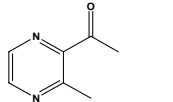
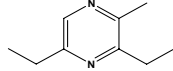
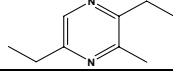
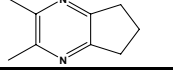
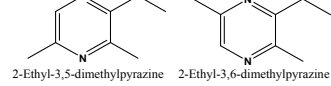
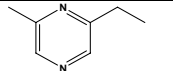
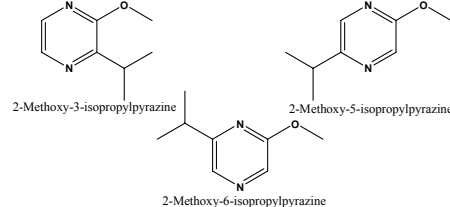
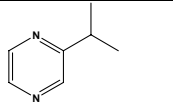
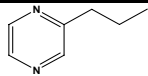

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1 (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
14.069	Cyclohexylmethylpyrazine		3631 28217-92-7	783 JECFA specification (JECFA, 2001c)	0.012	No safety concern a)	
14.077	2-Ethyl-(3,5 or 6)-methoxypyrazine (85%) and 2-Methyl-(3,5 or 6)-methoxypyrazine (13%)		3280 11329	789 JECFA specification (JECFA, 2001c)	1.3	No safety concern a)	
14.082	2-Acetyl-3-methylpyrazine		3964 11296 23787-80-6	950 JECFA specification (JECFA, 2001c)	0.1	No safety concern a)	
14.095	3,5-Diethyl-2-methylpyrazine		3916 11305 18138-05-1	779 JECFA specification (JECFA, 2001c)	0.012	No safety concern a)	
14.096	2,5-Diethyl-3-methylpyrazine		3915 11304 32736-91-7	778 JECFA specification (JECFA, 2001c)	0.012	No safety concern a)	
14.098	6,7-Dihydro-2,3-dimethyl-5H-cyclopentapyrazine		3917 11309 38917-62-3	782 JECFA specification (JECFA, 2001c)	0.012	No safety concern a)	
14.100	3,(5- or 6-)-Dimethyl-2-ethylpyrazine		3149 727 55031-15-7	775 JECFA specification (JECFA, 2001c)	38	No safety concern a)	JECFA evaluated 2-ethyl-3 (5 or 6)-dimethylpyrazine (CASrn 13360-65-1, 13925-07-0)
14.114	2-Ethyl-6-methylpyrazine		3919 11331 13925-03-6	769 JECFA specification (JECFA, 2001c)	0.37	No safety concern a)	
14.121	2-Isopropyl-(3,5 or 6)-methoxypyrazine		3358 11344 93905-03-4	790 JECFA specification (JECFA, 2001c)	0.0012	No safety concern a)	
14.123	Isopropylpyrazine		3940 11343 29460-90-0	764 JECFA specification (JECFA, 2001c)	0.12	No safety concern a)	

Table 3: Supporting Substances Summary

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1 (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
14.142	Propylpyrazine		3961 11362 18138-03-9	763 JECFA specification (JECFA, 2001c)	0.12	No safety concern a)	
14.144	Pyrazine		4015 11363 290-37-9	951 JECFA specification (JECFA, 2001c)	0.024	No safety concern a)	

1) EU MSDI: Amount added to food as flavouring substance in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

2) Category 1: Considered safe in use, Category 2: Temporarily considered safe in use, Category 3: Insufficient data to provide assurance of safety in use, Category 4: Not acceptable due to evidence of toxicity.

3) No safety concern at estimated levels of intake.

4) Category A: Flavouring substance, which may be used in foodstuffs, Category B: Flavouring substance which can be used provisionally in foodstuffs.

a) (JECFA, 2002b).

b) (CoE, 1992).

ND) No intake data reported.

ANNEX I: 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 (EC, 2000a), named the "Procedure", is shown in schematic form in Figure I.1. The Procedure is based on the Opinion of the Scientific Committee on Food expressed on 2 December 1999 (SCF, 1999a), 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; JECFA, 1996a; JECFA, 1997a; JECFA, 1999b).

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, III) for which thresholds of concern (human exposure thresholds) have been specified. Exposures below these thresholds 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 thresholds of concern for these structural classes of 1800, 540 or 90 microgram/person/day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996a).

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 products⁸ (Step 2)?
- do their exposures exceed the threshold of concern for the structural class (Step A3 and B3)?
- are the flavourings or their metabolites endogenous⁹ (Step A4)?
- does a NOAEL exist on the flavourings or on structurally related substances (Step 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.

⁸ "Innocuous metabolic products": Products that are known or readily predicted to be harmless to humans at the estimated intakes of the flavouring agent" (JECFA, 1997a).

⁹ "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, 1997a).

Procedure for Safety Evaluation of Chemically Defined Flavouring Substances

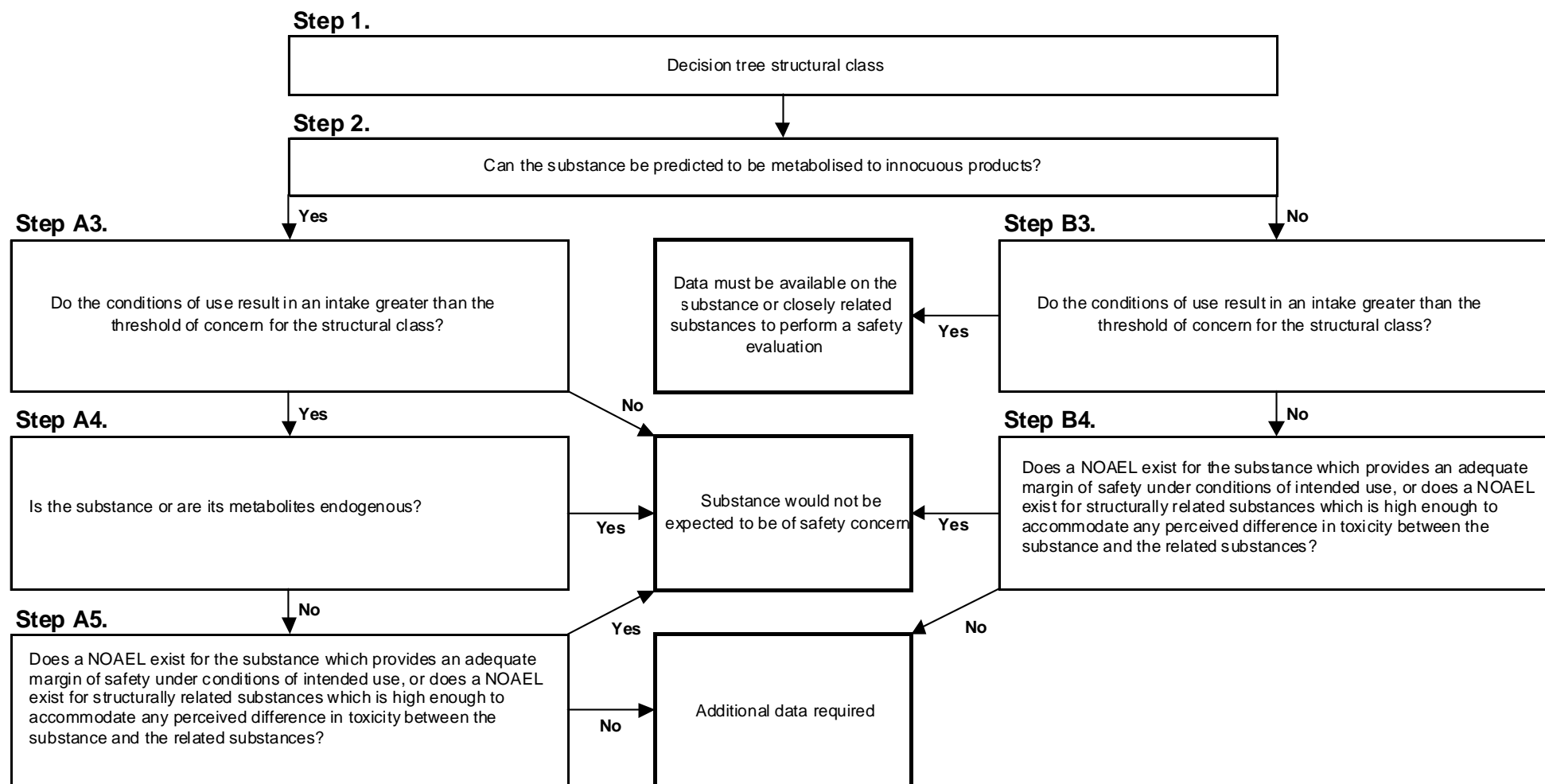


Figure I.1 Procedure for Safety Evaluation of Chemically Defined Flavouring Substances

ANNEX II: USE LEVELS / MTAMDI

II.1 NORMAL AND MAXIMUM USE LEVELS

For each of the 18 Food categories (Table II.1.1) in which the candidate substances are used, Flavour Industry reports a “normal use level” and a “maximum use level” (EC, 2000a). According to the Industry the “normal use” is defined as the average of reported usages and “maximum use” is defined as the 95th percentile of reported usages (EFFA, 2002i). The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004e).

Table II.1.1 Food categories according to Commission Regulation (EC) No 1565/2000 (EC, 2000a)

Food category	Description
01.0	Dairy products, excluding products of category 02.0
02.0	Fats and oils, and fat emulsions (type water-in-oil)
03.0	Edible ices, including sherbet and sorbet
04.1	Processed fruit
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds
05.0	Confectionery
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery
07.0	Bakery wares
08.0	Meat and meat products, including poultry and game
09.0	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.
13.0	Foodstuffs intended for particular nutritional uses
14.1	Non-alcoholic (“soft”) beverages, excl. dairy products
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts
15.0	Ready-to-eat savouries
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0 - 15.0

The “normal and maximum use levels” are provided by Industry for 20 of the 21 candidate substances in the present flavouring group (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g) (Table II.1.2).

Table II.1.2. Normal and Maximum use levels (mg/kg) for the candidate substances in FGE.17 (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g).

FL-no	Food Categories																	
	Normal use levels (mg/kg)																	
	Maximum use levels (mg/kg)																	
	01.0	02.0	03.0	04.1	04.2	05.0	06.0	07.0	08.0	09.0	10.0	11.0	12.0	13.0	14.1	14.2	15.0	16.0
14.052	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	2 10	0,2 1	0,2 1	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.081	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.083	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,5	0,1 0,5	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.084	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,5	0,1 0,5	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.086	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.087	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.091	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.097	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.099	0,4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	0,2 1	- -	0,1 0,4	0,1 0,4	- -	- -	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1

Table II.1.2. Normal and Maximum use levels (mg/kg) for the candidate substances in FGE.17 (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g).

FL-no	Food Categories																	
	Normal use levels (mg/kg)																	
	Maximum use levels (mg/kg)																	
	01.0	02.0	03.0	04.1	04.2	05.0	06.0	07.0	08.0	09.0	10.0	11.0	12.0	13.0	14.1	14.2	15.0	16.0
14.101	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	-	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.102	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.108	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	0,2 1	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.113	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.122	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.127	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.129	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	2 10	0,2 1	0,2 1	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.139	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.147	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.148	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	1 5	0,1 0,4	0,1 0,4	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.161	0,4 2	0,1 0,5	0,4 2	0,4 2	-	1 5	0,2 1	2 10	0,2 1	0,2 1	-	-	0,1 0,5	0,2 1	0,2 1	1 5	1 5	0,2 1

II.2 MTAMDI CALCULATIONS

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995). The assumption is that a person may consume the amount of flavourable foods and beverages listed in Table II.2.1. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table II.2.1 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 (EC, 2000a) and reported by the Flavour Industry in the following way (see Table II.2.2):

- Beverages (SCF, 1995) correspond to food category 14.1 (EC, 2000a)
- Foods (SCF, 1995) correspond to the food categories 1, 2, 3, 4.1, 4.2, 6, 7, 8, 9, 10, 13, and/or 16 (EC, 2000a)
- Exception a (SCF, 1995) corresponds to food category 5 and 11 (EC, 2000a)

- Exception b (SCF, 1995) corresponds to food category 15 (EC, 2000a)
- Exception c (SCF, 1995) corresponds to food category 14.2 (EC, 2000a)
- Exception d (SCF, 1995) corresponds to food category 12 (EC, 2000a)
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table II.2.2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 (EC, 2000a) into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

Food categories according to Commission Regulation 1565/2000		Distribution of the seven SCF food categories		
Key	Food category	Food	Beverages	Exceptions
01.0	Dairy products, excluding products of category 02.0	Food		
02.0	Fats and oils, and fat emulsions (type water-in-oil)	Food		
03.0	Edible ices, including sherbet and sorbet	Food		
04.1	Processed fruit	Food		
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Food		
05.0	Confectionery			Exception a
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Food		
07.0	Bakery wares	Food		
08.0	Meat and meat products, including poultry and game	Food		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	Food		
10.0	Eggs and egg products	Food		
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	Food		
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	Food		

The mTAMDI values (see Table II.2.3) are presented for 20 of the 21 substances in the present flavouring group, for which Industry has provided use and use levels (EFFA, 2003q; EFFA, 2006j; EFFA, 2007a; EFFA, 2007e; EFFA, 2007f; EFFA, 2007g). The mTAMDI values are only given for highest reported normal use levels.

Table II.2.3 Estimated intakes based on the mTAMDI approach

FL-no	EU Register name	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
14.081	5-Acetyl-2,3-dimethylpyrazine	270	Class II	540
14.083	2-Acetyl-5-ethylpyrazine	270	Class II	540
14.084	2-Acetyl-5-methylpyrazine	270	Class II	540
14.086	2-Acetyl-6-ethylpyrazine	270	Class II	540
14.087	2-Acetyl-6-methylpyrazine	270	Class II	540
14.091	2-Butyl-3-methylpyrazine	270	Class II	540
14.097	2,5-Diethylpyrazine	270	Class II	540
14.099	6,7-Dihydro-5,7-dimethyl-5H-cyclopentapyrazine	190	Class II	540
14.101	2,5-Dimethyl-3-isopropylpyrazine	190	Class II	540
14.102	5,6-Dimethyldihydrocyclopentapyrazine	270	Class II	540
14.113	5-Ethyl-6,7-dihydro-5H-cyclopentapyrazine	270	Class II	540
14.127	2-Methoxy-3-propylpyrazine	270	Class II	540
14.129	2-Methyl-3-propylpyrazine	400	Class II	540
14.148	5,6,7,8-Tetrahydro-5-methylquinoxaline	270	Class II	540
14.161	6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine	400	Class II	540
14.052	Isopropenylpyrazine	400	Class II	540
14.051	2,5 or 6-Methoxy-3-ethylpyrazine		Class II	540
14.108	2,3-Dimethylquinoxaline	190	Class III	90

Table II.2.3 Estimated intakes based on the mTAMDI approach

FL-no	EU Register name	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
14.122	2-Isopropyl-3-methylthiopyrazine	270	Class III	90
14.139	2-Methylquinoxaline	270	Class III	90
14.147	Quinoxaline	270	Class III	90

ANNEX III: METABOLISM

III.1. Introduction

This group of flavouring substances consists of 21 candidate substances, all of which contain a pyrazine ring. In 13 substances [FL-no: 14.051, 14.052, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.122, 14.127 and 14.129] only one heterocyclic ring is present. In five candidate substances a pyrazine ring is fused with either cyclopentane [FL-no: 14.099, 14.102, 14.113 and 14.161] or cyclohexane [FL-no: 14.148]. All these substances have different substituents. In 14 of them, the substituents are simple alkyl chains or ketones. In other three, the substituents are either a methoxy- or a thiomethyl- residue ([FL-no: 14.051 and 14.127] and [FL-no: 14.122], respectively) or an allyl residue [FL-no: 14.052].

In the remaining three candidate substances the pyrazine ring is fused with benzene giving quinoxalines. In two [FL-no 14.139 and 14.108] the pyrazine ring also bears one or two methyl substituents; in the third no substituents are present (= quinoxaline [FL-no 14.147]).

A group with 41 related supporting substances has been evaluated by JECFA (JECFA, 2002a).

III.2. Absorption, Distribution and Elimination

No pertinent absorption, distribution or elimination studies were found in the published or available unpublished literature for the candidate substances. Some information on supporting substances has been found, but the available information is still very limited.

In rats, orally administered substituted pyrazines are absorbed from the gastrointestinal tract and excreted (Hawksworth & Scheline, 1975). Approximately 90 – 100 % of a 100 mg/kg dose of 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine or 2-methoxypyrazine administered to male Wistar rats by stomach tube was excreted in the urine as polar metabolites within 24 hours. Also, 50 % of the orally administered dose of 100 mg 2,3-dimethylpyrazine/kg was recovered in the urine within 24 hours (Hawksworth & Scheline, 1975).

The supporting substance pyrazine [FL-no: 14.144] is a weak base with a $pK_a = 0.6$ (Damani & Crooks, 1982). At intestinal pH (pH = 5 - 7), absorption of weak amine bases such as pyrazine is optimal, because at these pHs such substances occur largely in the non-ionised state, which facilitates their absorption through the gastro-intestinal membranes (Hogben et al., 1959; Schranker et al., 1957). For the same reason it may be expected that the pyrazine derivatives in this group will be absorbed mainly after the passage through the stomach into the intestinal lumen.

Groups of five anaesthetised male Sprague-Dawley rats were administered a single intravenous bolus dose of 2, 5 or 10 mg tetramethylpyrazine/kg body weight (bw) through the femoral vein. Blood samples were withdrawn directly *via* heart puncture of the rat and collected from the same animals at 2.5, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes following administration to determine pharmacokinetic profiles in plasma. Distribution of tetramethylpyrazine was studied in various parts of the brain. It was determined that a two-compartment open model best described the plasma concentration-time curve for all the dose levels. The plasma distribution half-life ($t_{1/2, \text{alpha}}$) ranged from about 2.6 to 9.2 minutes and the elimination half-life ($t_{1/2, \text{beta}}$) ranged from 20 to 28 minutes for the 2 and 10 mg tetramethylpyrazine/kg bw doses, respectively. Area under the concentration curve (AUC) ranged from about 23 to 227 microg x min/ml and clearance (CL) ranged from 92 to 45 ml/min/kg for the 2 and 10 mg tetramethylpyrazine/kg bw doses, respectively. These

results indicate that rapid distribution, elimination and clearance occur in rats within the tested concentration range of 2 - 10 mg tetramethylpyrazine/kg bw. Fifteen minutes after intravenous administration of 10 mg tetramethylpyrazine/kg bw, the cerebral cortex concentration and plasma concentration of tetramethylpyrazine were 1.45 ± 0.09 microg/g and 6.14 ± 0.38 microg/ml, respectively. There were no significant differences in tetramethylpyrazine concentration among the various regions of the brain (Liang et al., 1999). The data on plasma clearance also indicate that the kinetics of tetramethylpyrazine may not be linear with the dose level.

An experimental design allowing simultaneous and continuous monitoring of tetramethylpyrazine concentrations in rat blood and brain was employed to study the distribution of an intravenously administered dose of 10 mg tetramethylpyrazine/kg bw. Microdialysis probes were inserted into the right jugular vein and striatum of four anaesthetised male Sprague-Dawley rats. Results indicate that both blood and brain pharmacokinetics of unbound tetramethylpyrazine fit best to a two-compartment model. The elimination half-life ($t_{1/2,\text{beta}}$) of tetramethylpyrazine in rat blood and brain were about 28 and 53 minutes and the AUCs were about 82 and 185 microg x min/ml, respectively (Tsai & Liang, 2001).

In conclusion:

Very few data on absorption, distribution and elimination of the candidate or supporting substances are available. The available data indicate that the (weak) basic heterocyclic substances in this group may be well absorbed, mainly from the intestinal lumen, and may be rapidly excreted.

III.3. Metabolism

III.3.1. Alkyl-, Alicyclic- and Alkylaryl-substituted Pyrazine Derivatives

For two candidate substances [FL-no: 14.147 and 14.108], some information on metabolism has been submitted. Supportive data have also been taken from general reviews on metabolism and from submitted studies, in which biotransformation of substances with remote resemblance to the candidate substances has been described.

In general, the biotransformation of the alkyl, alicyclic, and alkylaryl substituted pyrazine derivatives is expected to occur *via* oxidation of the side-chains (see Figure III.1). For example, methyl-substituted pyrazines are oxidised to yield the corresponding pyrazine-carboxylic acids, which may be excreted as glycine conjugates (Hawksworth & Scheline, 1975). An alternative pathway for substituted pyrazines and primary pathway for pyrazine involves hydroxylation of the pyrazine ring (Hawksworth & Scheline, 1975; Whitehouse et al., 1987; Yamamoto et al., 1987a; Yamamoto et al., 1987b). For example, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine are oxidised in rats almost exclusively *via* their aliphatic side-chains to carboxylic acid derivatives. Conversely, 2,3-dimethylpyrazine primarily undergoes ring hydroxylation, because side-chain oxidation is retarded (only 13 % of the administered dose oxidised) by the steric hindrance between the methyl groups (Hawksworth & Scheline, 1975).

At least 89 % of a 100 mg/kg oral dose of 2-methylpyrazine, 2,5-dimethylpyrazine, or 2,6-dimethylpyrazine was metabolised in the rat by side-chain oxidation to yield the corresponding pyrazine-2-carboxylic acid derivative. The acids were mainly excreted unconjugated, although 9 and 14 % of the administered doses were excreted as the corresponding glycine conjugates for 2-methylpyrazine and 2,5-dimethylpyrazine, respectively. No glycine conjugation was observed with 2-methylpyrazine-6-carboxylic acid. 2,3-Dimethylpyrazine was metabolised to 2-methyl-pyrazine-3-carboxylic acid (not conjugated with glycine) and 2,3-dimethyl-5-hydroxypyrazine (in total 50 % of the dose). No N-oxygenation products could be detected in the urine (Hawksworth & Scheline, 1975). Methyl side-chain oxidation to yield the

corresponding alcohols has also been demonstrated for quinoxaline derivatives (Turesky et al., 1988; Knize et al., 1989; Sjödin et al., 1989; Wallin et al., 1989).

Alicyclic-substituted pyrazines, such as candidate substances 6,7-dihydro-5,7-dimethyl-5H-cyclopentapyrazine [FL-no: 14.099], 5-ethyl-6,7-dihydro-5H-cyclopentapyrazine [FL-no: 14.113], 6,7-dihydro-2,5-dimethyl-5H-cyclopentapyrazine [FL-no: 14.161] and 5,6,7,8-tetrahydro-5-methylquinoxaline [FL-no: 14.148], are expected to undergo side-chain oxidation similar to that previously described for alkyl substituted pyrazines ($> C_1$), but no experimental evidence for this has been submitted. In addition, hydroxylation at various positions on the alicyclic ring is likely, based on general knowledge of metabolic conversion of alicyclic ring systems. The products of these oxidative metabolism reactions may be excreted unchanged or may be conjugated with glycine, glucuronic acid, or sulphate prior to excretion (Parkinson, 1996a).

Ring hydroxylation may be catalysed by molybdenum hydroxylases, e.g., xanthine oxidase and aldehyde oxidase, which are present in the cytosol of humans and other mammalian species, predominantly in the liver and small intestine. These enzymes catalyse ring hydroxylation of a wide range of endogenous and exogenous N-heterocycles bearing a substituent and/or a second fused ring. The molybdenum hydroxylases facilitate oxidation reactions involving nucleophilic attack by oxygen derived from water. Oxidation occurs at the most electropositive atom, which in N-heterocycles is generally the carbon adjacent to the ring nitrogen. The role of the molybdenum hydroxylases increases as the number of ring nitrogen atoms increase since each nitrogen activates the ring system toward nucleophilic attack. The oxidation action of the molybdenum hydroxylases is opposite from the microsomal monooxygenases (such as cytochrome-P450), which catalyse electrophilic attack by an oxygen atom derived from molecular oxygen (O_2) (Beedham, 1985; Beedham, 1988; Parkinson, 1996a). Although substituted monocyclic pyrazines may be substrates for the molybdenum hydroxylases when other pathways are unfavourable, as indicated by the results of (Hawksworth & Scheline, 1975), bicyclic heterocycles are the preferred substrates of these enzymes (Stubley et al., 1979; Beedham, 1985; Beedham, 2002).

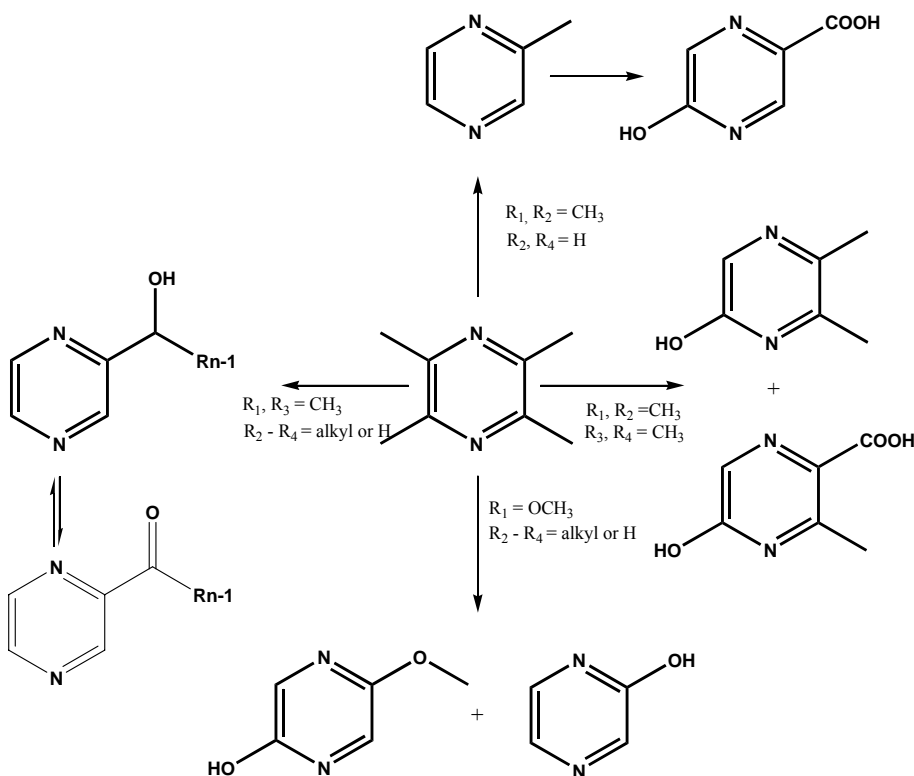


Figure III.1. Possible metabolic pathways of alkyl- and alkoxy-substituted pyrazine derivatives. Conjugates not shown. Excretion products are displayed in bold font.

III.3.2. N-oxidation and N-methylation

After *in vivo* administration of several methyl substituted pyrazine or pyridine derivatives to rats, no N-oxygenation products could be detected in the urine (Hawksworth & Scheline, 1975).

Generally, oxidation of ring nitrogen occurs in structures where the nitrogen atom has a nucleophilic character. N-oxidation of ring nitrogen has been reported in pyridine derivatives e.g. 3-acetyl pyridine is oxidised to give 3-acetyl pyridine N-oxide in *in vivo* and *in vitro* studies in rats and other laboratory animal species (Damani et al., 1980). The nucleophilicity of the ring nitrogen of pyrazine is much lower than in pyridine (pyrazine having a pK_a of 0.6 in comparison with pyridine having a pK_a of 5.17), suggesting that N-oxidation in pyrazine may not occur.

N-methylation of ring nitrogen in heterocyclic compounds has been reported, e.g. [2,6-¹⁴C]pyridine is methylated to N-methylpyridine in various mammals *in vivo* (D'Souza et al., 1980).

However, similarly as above, methylation of ring nitrogen occurs in structures where the nitrogen atom has nucleophilic character. N-methylation of pyrazine derivatives therefore is unlikely to occur, in contrast to the situation with pyridine derivatives.

Candidate substances

Quinoxaline [FL-no: 14.147]

Quinoxaline has been incubated in *in vitro* subcellular fractions to study the contribution of aldehyde oxidase (Mo-hydroxylase) and cytochrome P-450 in its metabolism. With aldehyde oxidase, formation of 2-hydroxyquinoxaline could be determined (K_m: 1.6 x 10⁻⁴ M) which could be further metabolised to 2,3-dihydroxyquinoxaline. With cytochrome P-450 only a trace of a phenolic reaction product was obtained (not further characterised). As with quinoxaline, with several other mono-aza and di-aza bicyclic heterocyclic compounds (quinolines, phthalazine, quinazolines, cinnoline), similar phenolic conversions were observed. Except with cinnoline, in all cases with aldehyde oxidase, but not with cytochrome P-450, the hydroxylation occurred at the carbon adjacent to an N atom. The study indicates that the conversions by aldehyde oxidase may be more efficient than those by cytochrome P-450 (Stubley et al., 1977).

Quinoxaline incubated *in vitro* with rabbit liver aldehyde oxidase is ring hydroxylated at a carbon atom adjacent to a ring nitrogen atom to yield 2-hydroxyquinoxaline and 2,3-dihydroxy quinoxaline. The apparent Michaelis-Menten constant of rabbit liver aldehyde oxidase for quinoxaline is K_m = 1.76 × 10⁻⁴ (at pH = 7 and 30°C). Incubation of quinoxaline with rat liver preparation yields qualitatively the same results as those using rabbit liver, but smaller amounts of the oxidation products are detected from rat liver incubations. Comparing the conversion of quinoxaline by rabbit and rat liver (10,000 g supernatants) during one hour incubations at 37°C, shows that the percentage conversion for rabbit is 6 and 5% and for rat 4.4 and 4.5% for 2-hydroxyquinoxaline and 2,3-dihydroxyquinoxaline, respectively (Stubley et al., 1979).

2,3-Dimethylquinoxaline [FL-no: 14.108]

Repeated administration of 2,3-dimethylquinoxaline [FL-no: 14.108] at a daily oral dose of 500 mg/kg bw for four days induces total hepatic cytochrome P-450 and cytochrome P-450-mediated biotransformations (aniline ring- hydroxylation, *p*-nitroanisole O-demethylation, aminopyrine N-demethylation, N-methylaniline N-demethylation) in female rat liver (Béraud et al., 1975).

III.3.3. Pyrazine Derivatives Containing an Oxygenated Functional Group in the Side-chain

Candidate substances with an oxygenated functional group in the side chain are [FL-no: 14.051, 14.081, 14.083, 14.084, 14.086, 14.087, and 14.127].

Rats were dosed with 100 mg/kg bw of 2-methoxypyrazine via gavage. Only 20 % of the urinary metabolites were identified as 2-hydroxypyrazine (i.e. the O-demethylated product). The other 80 % was one substance which was identified as a ring-hydroxylated 2-methoxypyrazine (Hawksworth & Scheline, 1975). Similar reactions can be expected to occur with candidate substance 2-methoxy-3-propylpyrazine [FL-no: 14.127] and 2,5 or 6-methoxy-3-ethylpyrazine [FL-no: 14.051].

Ring hydroxylation of the anti-tubercular agent, pyrazinamide, has been reported *in vitro* (Yamamoto et al., 1987b) and *in vivo* (Whitehouse et al., 1987; Yamamoto et al., 1987a) in both humans and rats. Within 12 hours after dosing male rats with 150 mg pyrazinamide/kg bw by gavage, 60 % of the dose was excreted as hydrolysis products via the urine (25 % as 5-hydroxypyrazine-2-carboxylic acid and 35 % as pyrazine-2-carboxylic acid). Parent compound and 5-hydroxypyrazinamide accounted for 14 and 3 % of the dose, respectively (Whitehouse et al., 1987). The same 5-hydroxylation products were detected in a urine sample of a man dosed with 12.5 mg pyrazinamide/kg bw. The hydroxylation of pyrazinamide and pyrazinoic acid *in vitro* to form 5-hydroxypyrazinamide and 5-hydroxypyrazine-2-carboxylic acid, respectively, occurred in the presence of xanthine oxidase-rich human liver cytosol (Yamamoto et al., 1987b).

It has been demonstrated that *in vitro* and *in vivo* 3-acetylpyridine can be reduced at the carbonyl group to give 1-(3-pyridyl)-ethanol (Damani et al., 1980). Based on this observation, it may be expected that the structurally related candidate substance acetylated pyrazines, such as 2-acetyl-5-methylpyrazine [FL-no: 14.084], 2-acetyl-6-methylpyrazine [FL-no: 14.087], 2-acetyl-5-ethylpyrazine [FL-no: 14.083], 2-acetyl-6-ethylpyrazine [FL-no: 14.086], and 5-acetyl-2,3-dimethylpyrazine [FL-no: 14.081] may also be metabolised by reduction of the ketone functional group resulting in the formation of the corresponding secondary alcohol. Subsequently, the terminal methyl group in the acetyl-chain may be oxidised to yield the corresponding alpha-hydroxy-carboxylic acid. Alternatively, the terminal carbon atom of the acetyl side chain may be completely removed, which results in the formation of the corresponding pyrazine-carboxylic acid. Similar conversions have been demonstrated for acetophenone, which can be metabolised to mandelic acid or benzoic acid (Sullivan et al., 1976).

Conclusion on metabolism of monocyclic or alicyclic- or aryl-bicyclic pyrazine derivatives with alkyl- or oxygenated functional group ring substituents:

Very little information has been submitted to describe the metabolism of the pyrazines and alkyl-, aryl- or alicyclic-substituted pyrazines in this group of flavouring substances. The available information shows that pyrazine with a simple alkyl substituent may be oxidised at the side chain to give the corresponding carboxylic acid [FL-no: 14.097 and 14.101]. If such oxidations are not possible, e.g. due to steric hindrance, hydroxylation of the pyrazine ring may also occur [FL-no: 14.091 and 14.129]. The bicyclic pyrazine derivatives with an additional alicyclic or aryl ring substituent [FL-no: 14.099, 14.102, 14.108, 14.113, 14.139, 14.147, 14.148, and 14.161] may be better substrates for ring hydroxylation which seems to be carried out preferably by molybdenum hydroxylases. The candidate substances bearing a ketone ring substituent [FL-no: 14.081, 14.083, 14.084, 14.086 and 14.087] may be reduced at the carbonyl in the side chain to give the corresponding alcohol. The two monocyclic pyrazine derivatives with a methoxy side chain [FL no: 14.051 and 14.127] may also be expected to be metabolised via both ring hydroxylation and O-demethylation of the methoxy side chain. With the resulting products of any of these flavouring substances conjugation with glycine, sulphate or glucuronide may occur. In addition, with some related substances N-oxidation or N-methylation have been observed, which may lead to biologically active metabolites. However, after *in vivo* administration of several methyl substituted pyrazine derivatives to rats, no N-oxidation products could be detected in the urine. Additionally, the nucleophilicity of the pyrazine ring

nitrogen is much lower than that of the pyridine ring nitrogen. It is concluded, therefore, that N-oxidation or N-methylation are unlikely to occur in the pyrazines.

III.3.4. Pyrazine Derivatives Containing a Thiol or Sulphide Functional Group in the Side-chain

The presence of sulphur in the side chain of pyrazines and alkylpyrazines provides a further metabolic option. The reactive lone pair of electrons on divalent sulphur in thiols and monosulphides permits rapid oxidation. Alkyl and aromatic sulphides, such as the candidate substance 2-isopropyl-3-methylthiopyrazine [FL-no: 14.122], can be oxidised to sulphoxides and then to sulphones (Hoodi & Damani, 1984; Nickson & Mitchell, 1994; Nickson et al., 1995). The oxidation to sulphoxides is catalysed by at least two enzyme systems, cytochrome P-450s and the flavin-containing monooxygenases (FMO) (Cashman & Williams, 1990; Cashman et al., 1990; Cashman et al., 1995a; Cashman et al., 1995b; Elfarra et al., 1995) and (Hoodi & Damani, 1984; Nnane & Damani, 1995; Rettie et al., 1990; Sadeque et al., 1992; Sadeque et al., 1995; Yoshihara & Tatsumi, 1990; Ziegler, 1980). The contribution of each system is highly dependent on molecular shape and nucleophilicity. For simple aliphatic, alicyclic and aromatic sulphides, oxidation is primarily catalysed by FMO and, to a lesser extent, by cytochrome P-450 (Hoodi & Damani, 1984). However, it is not clear which of these systems is of relevance for the one sulphur containing candidate substance [FL-no: 14.122] in this group of flavouring substances. The FMO enzymes are easily saturated, already at very low sulphide concentrations (Ziegler, 1980). However, quantitative data to assess the actual role of FMOs in thioether sulphoxidation *in vivo* were not provided (Ziegler, 1980) but the observation might indicate that their role could be limited at higher levels of exposure.

The final oxidation of the sulphoxide to the sulphone is an irreversible reaction (Damani, 1987; Williams et al., 1966). Essentially, all low molecular weight aliphatic and aromatic sulphones are metabolically stable. Hence, sulphoxides and sulphones are excreted in the urine of animals exposed to sulphides.

As described above, the major reactions by which simple sulphides can be metabolised involve oxidation of the S to give a sulphoxide, which can be further converted to a sulphone. Alternatively, sulphides can undergo oxidation of the carbon alpha to the -S-, resulting in the formation of an unstable hydroxyalkyl intermediate, which can be split to give an aldehyde and a free thiol. The aldehyde can be oxidised to its corresponding acid (Damani, 1987; Richardson et al., 1991). Similar reactions might occur with the candidate substance [FL-no: 14.122]. Thiols, such as the supporting substances 2-(mercaptomethyl) pyrazine and 2-pyrazinylethanethiol, are very reactive substances. *In vivo*, they become even more reactive mainly because most thiols exist in the ionised form at physiologic pH. Metabolic options for thiols include: oxidation to form unstable sulphenic acids (RSOH), which may be oxidised to sulphinic acid (RSO₂H) and sulphonic acid (RSO₃H); methylation to yield methyl sulphides, which then form sulphoxides and sulphones; reaction with physiologic thiols to form mixed disulphides and conjugation with glucuronic acid; or oxidation of the alpha-carbon, which results in desulphuration and the formation of an aldehyde (McBain & Menn, 1969; Dutton & Illing, 1972; Maiorino et al., 1989; Richardson et al., 1991).

Extensive discussions on the metabolism of sulphur-containing flavouring substances have been presented in FGE.08 and FGE.13.

Candidate substances

No pertinent metabolism studies were found in the published or available unpublished literature for the candidate substances.

III.4. Summary and Conclusions

This group of flavouring substances consists of 21 candidate substances, all of which contain a pyrazine moiety. In 13 substances [FL-no: 14.051, 14.052, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.101, 14.122, 14.127 and 14.129] only one heterocyclic ring is present. In five candidate substances a pyrazine ring is fused with either cyclopentane [FL-no: 14.099, 14.102, 14.113, and 14.161] or cyclohexane [FL-no: 14.148]. All these substances have different ring substituents patterns. In 14 of them, the substituents are simple alkyl chains or ketones. In the other three, the substituents are either a methoxy- or a thiomethyl-residue ([FL-no: 14.051 and 14.127] and [FL-no: 14.122], respectively) or an allyl residue [FL-no: 14.052].

In the remaining three candidate substances the pyrazine ring is fused with benzene giving quinoxalines. In two of the quinoxalines [FL-no 14.108 and 14.139] the pyrazine ring also bears one or two methyl substituents; in the third no substituents are present (quinoxaline; [FL-no 14.147]). A group with 41 related supporting substances has been evaluated by JECFA (JECFA, 2002a).

Very few data on absorption, distribution and elimination of the candidate or supporting flavouring substances are available. The available data indicate that the weak basic heterocyclic substances in this group may be well absorbed, mainly from the intestinal lumen, and may be rapidly excreted.

Limited information has been submitted to describe the metabolism of the pyrazines and alkyl-, aryl- or alicyclic-substituted pyrazines in this group of flavouring substances. Almost all data available come from one paper (Hawksworth & Scheline, 1975) and a few review papers (Beedham, 1985; Beedham, 1988; Parkinson, 1996a). Additional information provided in other papers is supportive of the metabolic conversions that have been described, but of little quantitative relevance as they concern substances that are widely different from the candidate substances in this group and the supporting ones evaluated by the JECFA.

Pyrazines with a simple alkyl substituent may be expected to be oxidised at the side chain to give the corresponding carboxylic acid [FL-no: 14.097, 14.101]. If such oxidations are not possible, e.g. due to steric hindrance, hydroxylation of the pyrazine ring may also occur [FL-no: 14.129 and 14.091]. The bicyclic pyrazine derivatives with an additional alicyclic or aryl ring substituent [FL-no: 14.099, 14.102, 14.108, 14.113, 14.139, 14.148, 14.147 and 14.161] may be better substrates for ring hydroxylation, which seems to be carried out preferably by molybdenum hydroxylases. The candidate substances bearing a ketone ring substituent [FL-no: 14.081, 14.083, 14.084, 14.086 and 14.087] may be reduced at the carbonyl in the side chain to give the corresponding alcohol. The two monocyclic pyrazine derivatives with a methoxy side chain [FL-no: 14.051 and 14.127] may also be expected to be metabolised via both ring hydroxylation and O-demethylation of the methoxy side chain. With the resulting products of any of these flavouring substances conjugation with glycine, sulphate or glucuronide may occur. In none of the studies N-oxidation or N-methylation, which would lead to the formation of bioactive metabolites, has been observed. This is in agreement with the reactive properties of the heterocyclic nitrogen in the pyrazine moieties of the various substances studied.

The one remaining candidate substance in this group [FL-no: 14.122] is a thioether, which may be detoxified by formation of a sulfoxide and subsequently a sulphone, which are both stable and usually rapidly excreted. Conversely, it may also be bioactivated via S-demethylation, resulting in the formation of a reactive free thiol. No data were provided to show that either route (sulphoxidation or S-demethylation) has prevalence over the other.

The limited amount of information on the metabolism of the substances in this flavouring group does not indicate that these substances will be metabolised to toxic products, except for the sulphur-containing flavouring substance [FL-no: 14.122], which may be converted to a reactive free thiol. For that reason, this sulphur-containing pyrazine derivative cannot be expected to be metabolised to innocuous products. Also

[FL-no: 14.052], having a terminal double bond, which may be epoxidated giving rise to reactive metabolites. cannot be expected to be metabolised to innocuous products.

Originally, it was considered that the results from the genotoxicity studies with quinoxaline and derivatives [FL-no: 14.108, 14.139 and 14.147] indicate that these three may be metabolised into substances that are reactive to DNA. However, new data have now become available for the structurally related substance, 5-methylquinoxaline [FL-no: 14.028], considered in FGE.50Rev1. Based on these data the Panel concluded that the *in vitro* genotoxicity alert could be ruled out for 5-methylquinoxaline [FL-no: 14.028] (FGE.50) as well as for the structurally related substance 2,3-dimethylquinoxaline [FL-no: 14.108] (FGE.17) but not for 2-methylquinoxaline [FL-no: 14.139] and quinoxaline itself [FL-no: 14.147].

Therefore, based on the available data, the following substances in this group [FL-no: 14.051, 14.081, 14.083, 14.084, 14.086, 14.087, 14.091, 14.097, 14.099, 14.101, 14.102, 14.108, 14.113, 14.127, 14.129, 14.148 and 14.161] may be expected to be metabolised to innocuous products.

ANNEX IV: TOXICITY

Oral acute toxicity data are available for none of the candidate substances of the present flavouring group evaluation from chemical group 24 but for 18 supporting substances evaluated by the JECFA at the 57th meeting. The supporting substances are listed in brackets.

Table IV.1: ACUTE TOXICITY

Chemical Name [FL-no]	Species	Sex	Route	LD ₅₀ (mg/kg bw)	Reference	Comments
(2-Methylpyrazine [14.027])	Rat	NR	Gavage	1800	(Moran et al., 1980)	
(2,3-Dimethylpyrazine [14.050])	Rat	NR	Gavage	613	(Moran et al., 1980)	
(2,5-Dimethylpyrazine [14.020])	Rat	NR	Gavage	1020	(Moran et al., 1980)	
(2,6-Dimethylpyrazine [14.121])	Rat	NR	Gavage	880	(Moran et al., 1980)	
(2-Ethyl-3-methylpyrazine [14.006])	Rat	NR	Gavage	600	(Moran et al., 1980)	
(2-Ethyl-5-methylpyrazine [14.017])	Rat	NR	Gavage	900	(Moran et al., 1980)	
(2,3,5-Trimethylpyrazine [14.019])	Rat	NR	Gavage	806	(Moran et al., 1980)	
(2-Ethyl-3, (5 or 6)-dimethylpyrazine [14.100])	Rat	NR	Gavage	456	(Moran et al., 1980)	
(2-Ethyl-3,5-dimethylpyrazine [14.024])	Rat	M, F	Gavage	504	(Posternak et al., 1975)	
(2,3,5,6-Tetramethylpyrazine [14.018])	Rat	NR	Gavage	1910	(Oser, 1969g)	
(2-Isobutyl-3-methoxypyrazine [14.043])	Mouse	NR	Gavage	2000	(Quest International, 1983a)	
	Rat	NR	Gavage	> 4000	(Roure Inc., 1974)	
(Acetylpyrazine [14.032])	Rat	M, F	Gavage	> 3000	(Posternak et al., 1975)	
(2-(sec-Butyl)-3-methoxypyrazine [14.062])	Mouse	NR	Gavage	2000	(Quest International, 1983b)	
(Cyclohexylmethylpyrazine [14.069])	Mouse	M, F	Gavage	2673	(Babish, 1978a)	
(Mercaptomethylpyrazine [14.053])	Rat	M, F	Gavage	2100	(Burdock & Ford, 1990b)	
(Pyrazinylethanethiol [14.031])	Rat	NR	Gavage	158	(Posternak et al., 1975)	
(Pyrazinyl methyl sulfide [14.034])	Rat	M, F	Gavage	2500	(Posternak et al., 1975)	
(2-Methyl-3,5 or 6-methylthiopyrazine [14.035])	Rat	NR	Gavage	1970	(Posternak et al., 1975)	

¹ Aspartic acid-fructose extracts were examined; 2,5-diethylpyrazine was 1 of 29 components identified in aspartic acid-fructose extract.

Subacute / Subchronic / Chronic / Carcinogenic toxicity data are available for none of the candidate substances of the present Flavouring Group Evaluation from chemical group 24 but for 17 supporting substances evaluated by the JECFA at the 57th meeting. The supporting substances are listed in brackets.

Table IV.2: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw/day)	Reference	Comments
(2-Ethyl-3-methylpyrazine [14.006])	Rat; M, F 32	Diet		90 days	M: 5.31 ¹ F: 5.22 ¹	(Posternak et al., 1969)	
(2-Ethyl-5-methylpyrazine [14.017])	Rat; M, F 30	Diet		90 days	M: 17 ¹ F: 18 ^{1,3}	(Oser, 1969d)	
(2,3-Diethylpyrazine [14.005])	Rat; M, F 32	Diet		90 days	1.75 ¹	(Posternak et al., 1969)	
(2,3,5-Trimethylpyrazine [14.019])	Rat; M, F 30	Diet		90 days	18 ¹	(Oser, 1969e)	
(2-Ethyl-3, (5 or 6)-dimethylpyrazine [14.100])	Rat; M, F 30	Diet		90 days	18 ¹	(Oser, 1969f)	
(2-Ethyl-3,5-dimethylpyrazine [14.024])	Rat; M, F 32	Diet		84 days	M: 12.7 ¹ F: 12.3 ¹	(Posternak et al., 1975)	
(2,3,5,6-Tetramethylpyrazine [14.018])	Rat; M, F 30	Diet		90 days	M: 50 ¹ F: 55 ³	(Oser, 1969c)	
(6,7-dihydro-5-Methyl-5H-cyclopentapyrazine [14.037])	Rat; M 10	Diet		90 days	50	(Wheldon et al., 1967)	This study can not be evaluated as a complete report could not be provided
(5,6,7,8-Tetrahydroquinoxaline [14.015])	Rat; M, F 30	Diet		90 days	M: 18.6 ¹ F: 19.3 ¹	(Oser, 1970d)	
(Acetylpyrazine [14.032])	Rat; M, F 32	Diet		91 days	M: 8.25 ¹ F: 8.15 ¹	(Posternak et al., 1975)	
(Methoxy-pyrazine [14.054])	Rat; M, F 10-16	Diet	20, 63, 200 mg/kg	91 days	20	(Osborne et al., 1981)	
((2,5 or 6)-Methoxy-3- methylpyrazine [14.025])	Rat; M, F 32	Diet		90 days	M: 45 ¹ F: 53 ¹	(Posternak et al., 1969)	70-80% 2-Methoxy-3- methylpyrazine, 20-30% (5 or 6)-Methoxy-3- methylpyrazine
(Cyclohexylmethylpyrazine [14.069])	Rat; M, F 30	Diet		90 days	M: 0.44 ¹ F: 0.47 ¹	(Babish, 1978b)	
(Pyrazinylethanethiol [14.031])	Rat; M, F 32	Diet		91 days	M: 16.56 ¹ F: 16.30 ¹	(Posternak et al., 1975)	
(Pyrazinyl methyl sulphide [14.034])	Rat; M, F 32	Diet		91 days	M: 1.66 ¹ F: 1.63 ¹	(Posternak et al., 1975)	This study was not performed in accordance with modern guidelines. No treatment related effects were observed in either haematological examination, on blood urea determinations or in histological examination of 25 organs or tissues. A complete clinical biochemical examination was not performed.
(2-Methyl-3,5 or 6-methylthiopyrazine [14.035])	Rat; M, F 32	Diet		91 days	4 ¹	(Posternak et al., 1975)	This study was not performed in accordance with modern guidelines. No treatment related effects were observed in either haematological examination, on blood urea determinations or in histological examination of 25 organs or tissues. A complete clinical biochemical examination was not performed.
(5-Methylquinoxaline [14.028])	Rat; M, F 32	Diet		90 days	17.1 ¹	(Posternak et al., 1969)	

¹ This study was performed at a single dose level.

² Decrease in food utilisation efficiency effects observed, but not accompanied by any evidence of pathology.

³ Growth rate and food utilisation efficiency effects were observed, but not accompanied by any evidence of pathology.

Developmental and reproductive toxicity data are available for none of the candidate substances of the present flavouring group evaluation from chemical group 24 but for four supporting substance evaluated by JECFA at the 57th meeting. Supporting substance listed in brackets.

Table IV.3: Developmental and Reproductive Toxicity Studies

Chemical Name [FL-no]	Study type Durations	Species/Sex No / group	Route	Dose levels	NOAEL (mg/kg bw/day), Including information of possible maternal toxicity	Reference	Comments
(2,3-Dimethylpyrazine [14.050])	Developmental toxicity 2 weeks	Rat; M 5 – 7	SC	0, 10, 30, 70, 100	100 ¹	(Yamada et al., 1993)	This study is considered of limited relevance, since the subcutaneous route of administration was used. ⁷
(2,5-Dimethylpyrazine [14.020])	Developmental toxicity : To 1 st oestrus; 1, 2 or 4 days	Rat; F 6 – 10	SC	100 ^{2,3}		(Yamada et al., 1992)	This study is considered of limited relevance, since the subcutaneous route of administration was used. ⁷
	Developmental toxicity : 2 weeks	Rat; M 5 – 7	SC	0, 10, 30, 70, 100	30 ¹	(Yamada et al., 1993)	This study is considered of limited relevance, since the subcutaneous route of administration was used. ⁷
	Developmental toxicity : 2 weeks	Rat; M 5	SC	0, 10, 30, 70, 100, 300	30 ⁶ 100 ⁵	(Yamada et al., 1994)	This study is considered of limited relevance, since the subcutaneous route of administration was used. ⁷
(2,6-Dimethylpyrazine [14.021])	Developmental toxicity : 2 weeks	Rat; M 5 - 7	SC	0, 10, 30, 70, 100	70 ¹	(Yamada et al., 1993)	This study is considered of limited relevance, since the subcutaneous route of administration was used. ⁷
(2,3,5,6-Tetramethylpyrazine [14.018])	Reproductive/ Developmental Toxicity ⁴	Rat; F 10	Gavage	25, 125, 250	Maternal: 25 Foetal: 250	(Vollmuth et al., 1997)	

¹Five to seven four-week old (juvenile) male Wistar rats/group were dosed subcutaneously once/day for two weeks.

²Subcutaneous injections of female Wistar rats beginning at the age of three weeks with 100 mg/kg bw once daily until the first oestrus.

³2,5-Dimethylpyrazine pretreatment of seven week old females twice per day for one, two and four days prior to oestradiol injection.

⁴Virgin rats administered 0, 25, 125, or 250 mg tetramethylpyrazine/kg body weight by gavage, seven days prior to and through cohabitation, gestation, delivery, and a four-day postparturition period.

⁵Five six-week old (mature) male Wistar rats/group were dosed subcutaneously once/day for two weeks at doses of 100 or 300 mg/kg bw.

⁶Five four-week old (juvenile) male Wistar rats/group were dosed subcutaneously once/day for two weeks at doses of 10, 30, 70, 100, or 300 mg/kg bw.

⁷It was concluded that the findings were of little relevance for the risk assessment of pyrazines as flavourings.

In vitro mutagenicity/genotoxicity data are available for three candidate substances of the present flavouring group evaluation from chemical group 24 and for 11 supporting substances evaluated by the JECFA at the 57th meeting. Supporting substances are listed in brackets.

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
(Pyrazine [14.144])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	64000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	64000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound within a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	10000 µg/ml	Negative ^{1,2}	(Fung et al., 1988)	Valid study in accordance with OECD guideline 471.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	60000 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	10000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mouse lymphoma mutagenesis assay	mouse lymphocytes L5178Y TK ^{+/+}	10000 µg/ml	Negative ¹	(Fung et al., 1988)	Study in accordance with former OECD guideline 476 (1983); colonies were not sized and results were not confirmed in a second study as requested by the OECD guideline in force. Therefore, chromosomal aberrations effects could not be ruled out.
(2-Methylpyrazine [14.027])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	94000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	94000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound of a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	67500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	40000 µg/ml 20000 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(Ethylpyrazine [14.022])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	67500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	5000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2,3-Dimethylpyrazine [14.050])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound within a large study, details are reported for positive compounds only.
(2,5-Dimethylpyrazine [14.020])	Ames test	<i>S. typhimurium</i>	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
		TA98; TA100; TA102			1989)	although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound of a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	200000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	135500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal aberration assay	Chinese hamster ovary cells	40000 µg/ml 20000 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2,6-Dimethylpyrazine [14.021])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	54000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	54000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	10800	Negative ⁴	(Lee et al., 1994a)	Well conducted study, valid although not in accordance with OECD guideline 471: two <i>S. typhimurium</i> strains only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	33800 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal aberration assay	Chinese hamster ovary cells	10000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2,3-Diethylpyrazine [14.005])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	109000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	109000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
(2,3,5-Trimethylpyrazine [14.019])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97735 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97735 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only.
((2,5 or 6)-Methoxy-3-methylpyrazine [14.025])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	3600 µg/plate	Negative ¹	(Wild et al., 1983)	Well conducted study, valid although not in accordance with OECD guideline 471: test concentrations not reported.
(Pyrzinyethanethiol [14.031])	Ames test	<i>S. typhimurium</i> TA97; TA98; TA100; TA1535	NR	Negative ¹	(Zeiger and Margolin, 2000)	Well conducted study, valid although not in accordance with OECD guideline 471: report does not give test concentrations, four test concentrations.
Quinoxaline [14.147]	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ³	(Beutin et al., 1981)	TA98 ; TA100: results presented in detail, without metabolic activation. TA1535,TA1537,TA1538: results incl. metabolic activation are mentioned in text (negative), but no data given. Not in accordance with OECD guideline 471.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	0.35 mmol	Negative ^{3,5}	(Aeschbacher et al., 1989)	0.35 mmol: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Modified Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538; G46; C3076; D3052 <i>E. coli</i> WP2; WP2uvrA	NR	Negative ³	(McMahon et al., 1979)	Review, of limited value (concentrations tested not reported).
	Ames test (plate incorporation method)	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	10000 µg/plate	Negative ³	(San, 1995)	Valid study in accordance with OECD guideline 471.
	DNA Polymerase deficiency assay	<i>E. coli</i>	NR	Negative ³	(Rosenkranz & Leifer, 1980)	Review, of limited value (concentrations tested not reported; without metabolic activation).

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
	SOS Chromosome test	<i>E. coli</i> PQ37	NR	Negative ¹	(Beutin et al., 1981)	
	Mouse lymphoma mutagenesis assay	L5178Y TK ⁺ - mouse lymphocytes	(with S9) 20 – 250 (without S9) 100 – 1500 microg/ml	Positive ⁶ Weakly Positive ³	(National Cancer Institute, 1998)	Valid study in accordance with OECD guideline 476.
2-Methylquinoxaline [14.139]	Ames test	<i>S. typhimurium</i> TA98; TA100	500 µg/plate	Positive ¹	(Hashimoto et al., 1979)	Well conducted study, valid although not in accordance with OECD guideline 471: two <i>S. typhimurium</i> strains only, highest dose but not individual doses reported. Positive only in TA98 and T100 with metabolic activation.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	0.007 - 700 µmol/plate (equal to 0.001 – 100 mg/plate)	Negative ^{1,7}	(Aeschbacher et al., 1989)	0.7 mmol: highest non-bactericidal dose. Well conducted study (valid), but not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
2,3-Dimethylquinoxaline [14.108]	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535;	2500 µg/plate	Negative ⁶	(Anderson and Styles, 1978)	Well conducted study, valid although not in accordance with OECD guideline 471 (with S9 metabolic activation only).
	Ames test	<i>S. typhimurium</i> TA100	NR	Negative ⁶	(Epler et al., 1978)	Review, no detailed information on test conditions incl. concentration. Authors pointed out the unanswered question whether the testing of negative compounds can sensibly be terminated (in 1978).
	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ¹	(Hashimoto et al., 1979)	Validity cannot be evaluated. Concentrations not reported. Results not reported in detail.
(5-Methylquinoxaline [14.028])	Reverse mutation	<i>S. typhimurium</i> TA 98, TA100, TA1535, TA1537 and <i>E. coli</i> WP2 uvrA	Up to 5000 microgram/plate	Negative ¹	(Ogura & Wakamatsu, 2004)	Valid. GLP-study in compliance with OECD 471 (except that no justification was provided for the use of duplicate instead of triplicate plating).
	Chromosomal aberration assay	Chinese hamster lung-derived CHL/IU cells	320, 480, 720 microgram/ml 72, 228, 720 microgram/ml	Negative ³ Positive ⁶	(Ajimu & Kawaguchi, 2004a)	Valid. GLP-study mainly in compliance with OECD 473 (duration of exposure not clearly reported). The authors noted in the discussion section that cytotoxicity was observed in the form of decreased cell viability and reproductive rate. However, it is not clear if one or two parameters for cytotoxicity were measured. The percentage of “cell productivity” (the cell number was measured and expressed as relative growth rate compared to negative control) was reported. According to the authors, there was a clear evidence of cytotoxicity in the form of decreased cell viability and reproductive rate at concentrations where chromosomal aberrations were observed. However, the results presented in tables demonstrate that 30 and 66 % of cell with chromosomal aberrations were induced at the limit of excessive cytotoxicity (54 and 46% of relative growth) in the preliminary test (in which 50 cells per slide were scored) at 180 and 360 µg/mL in the presence of S9, respectively. In the main test, the percentage of cells with chromosomal aberrations in the presence of S9 was 2.0, 2.5, 6.5 and 57.5 at 0, 72, 228 and 720 µg/mL, respectively, which was accompanied by 100, 90, 85 and 46% relative growth, respectively.

NR: Not reported.

¹ With and without S9 metabolic activation.

² Metabolic activation was provided with both rat and hamster liver S9 mix.

³ Without S9 metabolic activation.

⁴ Results were negative in TA100 with and without S9 metabolic activation; however, in TA98 the results were negative and positive with and without S9 metabolic activation, respectively.

- ⁵ Results were negative in TA100 with and without S9 metabolic activation. Weak results were noted in TA98 and TA102 with S9 metabolic activation. These changes may be related to the heat production products of the Maillard reaction in the presence of creatinine.
- ⁶ With S9 metabolic activation.
- ⁷ Weak results were noted in all strains with S9 metabolic activation. (*the number of revertants was increased up to 1.3-fold compared to control*). According to the authors (*Aeschbacher et al., 1989*), these changes may be related to the heat production products of the Maillard reaction in the presence of creatinine.

In vivo mutagenicity/genotoxicity data are available for one candidate substance of the present flavouring group evaluation from chemical group 24 and for one supporting substance evaluated by JECFA at the 57th meeting. The supporting substances are listed in brackets.

Table IV.5: GENOTOXICITY (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
(2,5 or 6)-Methoxy-3- methylpyrazine [14.025]	Basic test	<i>D. melangaster</i>		10 mM	Negative	(Wild et al., 1983)	Limited relevance for risk assessment as the test is not in a mammalian system and the test is not used routinely.
	Micronucleus assay	Mouse		87, 174, 248 mg/kg	Negative	(Wild et al., 1983)	Study design does not meet the criteria of current guidelines (PCE/NCE ratio was not reported, thus it is not clear if the test substance reached the bone marrow). Not in accordance with OECD guideline 474 (1983/1997).
Quinoxaline [14.147]	Sperm head abnormality test	Mouse	I.P	2500 mg/kg	Negative	(Topham, 1980)	Sperm head abnormality test does not make use of a genetic endpoint.
(5-Methylquinoxaline [14.028])	Micronucleus assay	Mouse	Gavage	125, 250, and 500 mg/kg/day	Negative	(Ajimu & Kawaguchi, 2004b)	Valid. GLP-study mainly in compliance with OECD 474 (only 5 male mice per group instead of 5 males and 5 females).

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ABBREVIATIONS

ADI	Acceptable Daily Intake
AUC	Area Under Curve
BW	Body weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids Chemical Abstract Service
CHO	Chinese hamster ovary (cells)
CL	Clearance
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EC	European Commission
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
FMO	Flavin-containing Monooxygenase
DLP	Dood Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavour Industry
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LD ₅₀	Lethal Dose, 50%; Median lethal dose
MNPCE	Micronucleated polychromatic erythrocytes
MS	Mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
No	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic erythrocytes

SC	Subcutaneous
SCE	Sister Chromatid Exchange
SCF	Scientific Committee on Food
SMART	Somatic Mutation and Recombination Test
TAMDI	Theoretical Added Maximum Daily Intake
UDS	Unscheduled DNA Synthesis
WHO	World Health Organisation