EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2013. Scientific Opinion on Flavouring Group Evaluation 24, Revision 2 (FGE.24Rev2)

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SCIENTIFIC OPINION

Scientific opinion on Flavouring Group Evaluation 24, Revision 2 (FGE.24Rev2):

Pyridine, pyrrole, indole and quinoline derivatives from chemical group 28[1]

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 24 flavouring substances in the Flavouring Group Evaluation 24, Revision 2, using the Procedure in Commission Regulation (EC) No 1565/2000. This revision was made required owing to the inclusion of the assessment of new toxicity data on one supporting substance, 2-acetylpyrrole [FL-no: 14.047], to support the re-evaluation of one candidate substance, 2-acetyl-5-methylpyrrole [FL-no: 14.085]. Nine of the original 33 candidate substances [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169], for which additional data were requested, are no longer supported by Industry for use as flavouring substances in Europe and will therefore not be considered any further. None of the 24 substances were considered to have genotoxic potential. These candidate substances were evaluated through a stepwise approach that integrates information on the structure-activity relationships, intake from current uses, toxicological threshold of concern, and available data on metabolism and toxicity. The Panel concluded that the 24 substances [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.110, 14.115, 14.116, 14.117, 14.118, 14.120, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150] do not give rise to safety concern at their levels of dietary intake, estimated on the basis of the MSDI approach. Besides the safety assessment of these flavouring substances, the specifications for the materials of commerce have also been considered. Adequate specifications including complete purity criteria and identity for the materials of commerce have been provided for all 24 candidate substances.

KEY WORDS

flavourings, pyridine, pyrrole, indole, quinoline, safety, FGE.24

[1] On request from the European Commission, Question No EFSA-Q-2013-00357, adopted on 25 October 2013.

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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 deliver a 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 24 flavouring substances in the Flavouring Group Evaluation 24, Revision 2 (FGE.24Rev2), using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. These 24 derivatives of pyridine, pyrrole, indole and quinoline belong to chemical group 28, Annex I of the Commission Regulation (EC) No 1565/2000.

The present revision of FGE.24, FGE.24Rev2, includes the assessment of new toxicity data on one supporting substance, 2-acetylpyrrole [FL-no: 14.047], to cover the re-evaluation of one candidate substance, 2-acetyl-5-methylpyrrole [FL-no: 14.085], for which additional data were required.

Since the publication of the previous version of FGE.24, FGE.24Rev1, nine of the original 33 candidate substances [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169], for which additional data were required, are no longer supported by Industry for use as flavouring substances in Europe and will therefore not be considered any further. This revision of FGE.24, FGE.24Rev2, therefore deals only with 24 candidate substances.

None of the 24 candidate substances can exist as geometrical or optical isomers.

Twenty-two of the candidate substances are classified into structural class II and two are classified into structural class III.

Twenty-one candidate 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 Intake" (MSDI) approach to estimate the per *capita* intakes of the flavouring substances in Europe. However, when the Panel examined the information provided by the European Flavour Industry on the use levels in various foods, it appeared obvious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavoured at the use level reported by the Industry, especially in those cases where the annual production values were reported to be small. In consequence, the Panel had reservations about the data on use and use levels provided and the intake estimates obtained by the MSDI approach.

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 to also 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 24 candidate substances in this group have intakes in Europe from 0.0012 to 0.73 µg/*capita*/day, which are all below the thresholds of concern for both structural class II (540 µg/person/day) and structural class III (90 µg/person/day) substances.

The genotoxicity data available for the candidate substances do not preclude their evaluation through the Procedure.

Two of the 24 candidate substances evaluated through the Procedure, ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120], are expected to be metabolised to innocuous products. For

the remaining 22 candidate substances it cannot be anticipated that they will be metabolised to innocuous products.

It was noted that where toxicity data were available they were consistent with the conclusions in the present flavouring group evaluation using the Procedure.

It was considered that, on the basis of the default MSDI approach, the 24 substances, to which the Procedure was applied would not give rise to safety concerns at the estimated levels of intake arising from their use as flavouring substances.

When the estimated intakes were based on the mTAMDI approach they are 400 µg/person/day for each of the 22 candidate substances from structural class II, which is below the threshold of concern for structural class II of 540 µg/person/day. For the two flavouring substances [FL-no: 14.088 and 14.131] assigned to structural class III, the estimated intakes based on the mTAMDI are 400 µg/person/day each. This is above the threshold of concern for structural class III of 90 µg/person/day. For the two candidate substances 1-acetylindole [FL-no: 14.088] and 2-methylindole [FL-no: 14.131], the intakes, estimated on the basis of the mTAMDI, exceed the threshold for the structural class to which the flavouring substance has been assigned. Therefore, for these two substances more reliable exposure data are required. On the basis of such additional data, these flavouring substances should be reconsidered along the steps of the Procedure. Following this procedure, additional toxicological data might become necessary.

In order to determine whether the conclusion for the 24 candidate substances could be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity tests for the materials of commerce have been provided for all 24 candidate substances.

Based on the available data, the Panel concluded that the 24 candidate substances [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.110, 14.115, 14.116, 14.117, 14.118, 14.120, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150] evaluated through the Procedure would present no safety concern at the estimated levels of intake based on the MSDI approach.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavourings is regulated under Regulation (EC) No 1334/2008 of the European Parliament and Council of 16 December 2008[4] on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union list of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

EFSA has evaluated 33 flavouring substances in the flavouring group evaluation 24 (FGE.24) and its revision. The last opinion was adopted on 27 September 2007. EFSA concluded in its opinion that for 2-acetyl-5-methylpyrrole [FL-no: 14.085] additional toxicological data are required.

EFSA has considered the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) evaluation of 26 pyridine, pyrrole and quinoline derivatives evaluated in the flavouring group evaluation 77 (FGE.77). The opinion was adopted on 31 January 2008. EFSA concluded in its opinion that for 2-acetylpyrrole [FL-no: 14.047] and 2-propionylpyrrole [FL-no: 14.068], No Observed Adverse Effect Levels (NOAELs) could not be derived as such or for structurally related substances. Accordingly, additional toxicological data are required for these substances.

The requested information on the representative material, 2-acetylpyrrole [FL-no: 14.047], has now been submitted by the European Flavour Association. This information is intended to cover the re-evaluation of this substance and of 2-propionylpyrrole [FL-no: 14.068] from FGE.77. In addition, it should cover the re-evaluation of 2-acetyl-5-methylpyrrole [FL-no: 14.085] from FGE.24.

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests European Food Safety Authority (EFSA) to carry out a safety assessment on the following three flavouring substances: 2-acetylpyrrole [FL-no: 14.047], 2-propionylpyrrole [FL-no: 14.068] and 2-acetyl-5-methylpyrrole [FL-no: 14.085] in accordance with Commission Regulation (EC) No 1565/2000.

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

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

[6] Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. Official Journal of the European Communities 19.7.2000, L 180, 8-16.

ASSESSMENT

1. History of the Evaluation of the Substances in the Present FGE

The first version of the Flavouring Group Evaluation 24 (FGE.24) dealt with 31 derivatives of pyridine, pyrrole, indole and quinoline.

The first revision of FGE.24 (FGE.24Rev1) included the assessment of two additional candidate substances [FL-no: 14.163 and 14.169]. No studies on toxicology or on metabolism for these two candidate substances were identified.

The data available gave rise to some concern regarding the genotoxic potential of the quinoline derivatives [FL-no: 14.002, 14.094 and 14.138], and accordingly the Panel concluded that the Procedure could not be applied to these three substances until adequate *in vivo* data become available. For seven candidate substances [FL-no: 13.100, 14.023, 14.085, 14.107, 14.145, 14.163 and 14.169], a No Observed Adverse Effect Level (NOAEL) could not be derived for the substance or a structurally related substance. Accordingly, additional data were required for these seven flavouring substances.

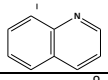
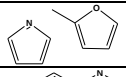
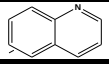
Since the publication of FGE.24Rev1, nine [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169] of the 33 candidate substances are no longer supported for use as flavouring substances in Europe by Industry (EFSA, 2011; DG SANCO, 2013) and will therefore not be considered any further. The nine substances are listed here below. Accordingly, FGE.24Rev2 only deals with 24 candidate substances.

The nine substances deleted:

FL-no	EU Register name
13.100	2-Acetyl-1-furfurylpyrrole
14.002	4-Methylquinoline
14.023	1-Methylpyrrole
14.094	4-Butylquinoline
14.107	2,5-Dimethylpyrrole
14.138	2-Methylquinoline
14.145	Pyrrole-2-carbaldehyde
14.163	1-Methylpyrrole-2-carboxaldehyde
14.169	1-Ethyl-2-pyrrolecarboxaldehyde

These nine substances will be excluded in the following text except in tables 1 and 7. Information in the text on these substances will only be kept if relevant for the remaining candidate substances.

As a consequence of this, the following supporting substances have been deleted from this revision:

FL-no JECFA no	EU Register name	Structural formula
- -	8-Methylquinoline	
13.134 1310	1-Furfurylpyrrole	
14.042 1302	6-Methylquinoline	

The table below gives information on publication date and links to the published versions.

FGE	Opinion adopted by EFSA	Link	No. of candidate substances
FGE.24	4 May 2006	http://www.efsa.europa.eu/EFSA/Scientific_Opinion/afc_op_ej372_fge	31

		24_op_en,0.pdf	
FGE.24Rev1	26 Sept. 2007	http://www.efsa.europa.eu/EFSA/ScientificPanels/AFC/efsa_locale-1178620753812_Opinions425.htm	33
FGE.24Rev2	25 October 2013		24

The present Revision of FGE.24, FGE.24Rev2, considers the re-evaluation of one candidate substance, 2-acetyl-5-methylpyrrole [FL-no: 14.085]. Additional information was provided to the Panel of a 90-day study on one supporting substance, 2-acetylpyrrole [FL-no: 14.047] which is structurally related to the candidate substance 2-acetyl-5-methylpyrrole [FL-no: 14.085]. A search in open literature was conducted for metabolism, genotoxicity and toxicity for 2-acetyl-5-methylpyrrole [FL-no: 14.085]. This search did not reveal any pertinent new information on the substance.

2. Presentation of the Substances in Flavouring Group Evaluation 24, Revision 2

2.1. Description

The present Flavouring Group Evaluation 24, Revision 2 (FGE24Rev2) using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000 (EC, 2000) (The Procedure – shown in schematic form in Appendix A), deals with 24 derivatives of pyridine, pyrrole and indole from chemical group 28, Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000).

The 24 flavouring substances have one heterocyclic N-atom in a five or six-membered ring structure. Twenty-two of the compounds are monocyclic, with either a five-membered ring (pyrroles) [FL-no: 14.085] or a six-membered ring (pyridines) [FL-no: 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.110, 14.115, 14.116, 14.117, 14.118, 14.120, 14.124, 14.125, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150]. The remaining two candidate substances are aromatic bicyclic structures consisting of one six- or one five-membered ring (indoles) [FL-no: 14.088 and 14.131]. The substances are substituted with simple alkyl, alcohol, ketone or ester moieties.

The 24 flavouring substances under consideration, as well as their chemical Register names, FLAVIS- (FL-), Chemical Abstract Service- (CAS-), Council of Europe- (CoE-) and Flavor and Extract Manufactures Association- (FEMA-) numbers, structure and specifications, are listed in Table 1.

A summary of the outcome of the safety evaluation of the candidate substances are listed in Table 7.

The hydrolysis products of the two esters [FL-no: 14.110 and 14.120] and the amide [FL-no: 14.088] contained in the present FGE as well as their evaluation status are listed in Table 8.

The 24 flavouring substances (candidate substances) are structurally closely related to 15 flavouring substances (supporting substances) evaluated at the 63rd JECFA meeting (JECFA, 2006) in the group of "Pyridine, pyrrole and quinoline derivatives". One other structurally related flavouring substance (indole-3-carbinol) is also included as supporting substance in this evaluation. The supporting substances are listed in Table 9.

SUMMARY OF SPECIFICATION DATA

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 24, Revision 2

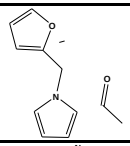
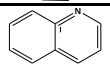
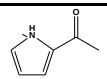
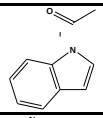
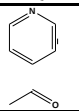
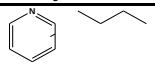
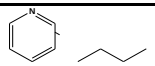
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
13.100	2-Acetyl-1-furfurylpyrrole		11941 13678-73-4	Solid $C_{11}H_{11}NO_2$ 189.21	Practically insoluble or insoluble Freely soluble	101 (0.04 hPa) 42 NMR 95 %	n.a. n.a.	No longer supported by Industry (EFSA, 2011).
14.002	4-Methylquinoline		488 491-35-0	Liquid $C_{10}H_9N$ 143.19	Slightly soluble Freely soluble	264 9 MS 95 %	1.615-1.621 1.083-1.089	No longer supported by Industry (EFSA, 2011).
14.023	1-Methylpyrrole		2217 96-54-8	Liquid C_5H_7N 81.12	Practically insoluble or insoluble Freely soluble	113 MS 99 %	1.482-1.490 0.902-0.914	No longer supported by Industry (EFSA, 2011).
14.085	2-Acetyl-5-methylpyrrole		6982-72-5	Solid C_7H_9NO 123.15	Practically insoluble or insoluble Freely soluble	240 88 MS 95 %	n.a. n.a.	
14.088	1-Acetylindole		576-15-8	Liquid $C_{10}H_9NO$ 159.19	Practically insoluble or insoluble Freely soluble	144 (13 hPa) MS 95 %	1.607-1.613 1.384-1.390	
14.089	4-Acetylpyridine		1122-54-9	Liquid C_7H_7NO 121.14	Practically insoluble or insoluble Freely soluble	212 MS 95 %	1.520-1.526 1.098-1.104	
14.092	2-Butylpyridine		5058-19-5	Liquid $C_9H_{13}N$ 135.21	Practically insoluble or insoluble Freely soluble	190 MS 95 %	1.487-1.493 0.911-0.917	
14.093	3-Butylpyridine		539-32-2	Liquid $C_9H_{13}N$ 135.21	Practically insoluble or insoluble Freely soluble	206 MS 95 %	1.492-1.498 0.909-0.915	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 24, Revision 2

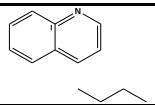
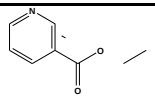
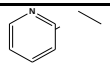
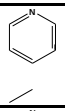
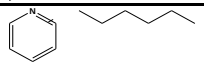
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
14.094	4-Butylquinoline		 74808-78-9	Solid $C_{13}H_{15}N$ 185.27	Practically insoluble or insoluble Freely soluble	328 30 NMR 95 %	n.a. n.a.	 No longer supported by Industry (EFSA, 2011).
14.103	2,3-Dimethylpyridine		 583-61-9	Liquid C_7H_9N 107.15	Slightly soluble Freely soluble	161 MS 95 %	1.503-1.509 0.943-0.949	
14.104 2151	2,4-Dimethylpyridine		4389 108-47-4	Liquid C_7H_9N 107.15	Slightly soluble Freely soluble	156 MS 95 %	1.496-1.502 0.929-0.935	
14.105	3,4-Dimethylpyridine		 583-58-4	Liquid C_7H_9N 107.15	Slightly soluble Freely soluble	176 MS 95 %	1.507-1.513 0.954-0.960	
14.106	3,5-Dimethylpyridine		 11382 591-22-0	Liquid C_7H_9N 107.15	Slightly soluble Freely soluble	170 MS 95 %	1.500-1.506 0.939-0.945	
14.107	2,5-Dimethylpyrrole		 11383 625-84-3	Liquid C_6H_9N 95.14	Practically insoluble or insoluble Freely soluble	168 MS 95 %	1.500-1.506 0.929-0.935	 No longer supported by Industry (EFSA, 2011).
14.110	Ethyl nicotinate		 614-18-6	Liquid $C_8H_9NO_2$ 151.16	Slightly soluble Freely soluble	224 9 MS 95 %	1.498-1.504 1.105-1.111	
14.115	2-Ethylpyridine		 11767 100-71-0	Liquid C_7H_9N 107.15	Practically insoluble or insoluble Freely soluble	149 MS 95 %	1.494-1.500 0.927-0.937	
14.116	4-Ethylpyridine		 11387 536-75-4	Liquid C_7H_9N 107.15	Practically insoluble or insoluble Freely soluble	166 MS 95 %	1.496-1.502 0.939-0.945	
14.117	2-Hexylpyridine		 1129-69-7	Liquid $C_{11}H_{17}N$ 163.26	Practically insoluble or insoluble	111 (20 hPa) MS	1.480-1.490 0.892-0.902	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 24, Revision 2

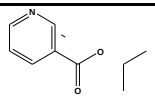
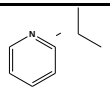
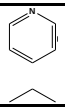
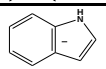
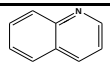
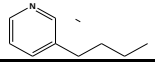
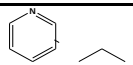
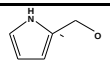
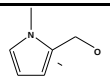
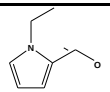
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
					Freely soluble	95 %		
14.118	2-Hydroxypyridine		142-08-5	Solid C_5H_5NO 95.10	Slightly soluble Freely soluble	281 107 MS 95 %	n.a. n.a.	
14.120	Isopropyl nicotinate		553-60-6	Liquid $C_9H_{11}NO_2$ 165.19	Slightly soluble Freely soluble	110 (9 hPa) MS 95 %	1.498-1.504 1.061-1.067	
14.124	2-Isopropylpyridine		11400 644-98-4	Liquid $C_8H_{11}N$ 121.18	Practically insoluble or insoluble Freely soluble	156 MS 95 %	1.488-1.494 0.931-0.937	
14.125	4-Isopropylpyridine		696-30-0	Liquid $C_8H_{11}N$ 121.18	Practically insoluble or insoluble Freely soluble	180 MS 95 %	1.492-1.498 0.923-0.929	
14.131	2-Methylindole		95-20-5	Solid C_9H_9N 131.18	Practically insoluble or insoluble Freely soluble	273 60 MS 95 %	n.a. n.a.	
14.134	2-Methylpyridine		11415 109-06-8	Liquid C_6H_7N 93.13	Practically insoluble or insoluble Freely soluble	128 MS 95 %	1.494-1.500 0.941-0.947	
14.135	3-Methylpyridine		11801 108-99-6	Liquid C_6H_7N 93.13	Practically insoluble or insoluble Freely soluble	143 MS 95 %	1.496-1.502 0.953-0.959	
14.136	4-Methylpyridine		11416 108-89-4	Liquid C_6H_7N 93.13	Practically insoluble or insoluble Freely soluble	142 MS 95 %	1.500-1.506 0.951-0.957	
14.138	2-Methylquinoline		11358 91-63-4	Liquid $C_{10}H_9N$ 143.19	Slightly soluble Freely soluble	246 MS 95 %	1.608-1.614 1.055-1.061	No longer supported by Industry (EFSA, 2011).
14.140	3-Pentylpyridine			Liquid $C_{10}H_{15}N$	Practically insoluble or	100 (12 hPa)	1.486-1.492 0.905-0.911	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 24, 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
			1802-20-6	149.24	insoluble Freely soluble	MS 95 %		
14.143	3-Propylpyridine		11419 4673-31-8	Liquid $C_8H_{11}N$ 121.18	Practically insoluble or insoluble Freely soluble	181 MS 95 %	1.493-1.499 0.922-0.928	
14.145	Pyrrole-2-carbaldehyde		11393 1003-29-8	Solid C_5H_5NO 95.10	Sparingly soluble Freely soluble	218 46 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2013).
14.150	2,4,6-Trimethylpyridine		108-75-8	Liquid $C_8H_{11}N$ 121.18	Practically insoluble or insoluble Freely soluble	168 MS 95 %	1.493-1.499 0.910-0.916	
14.163 2152	1-Methylpyrrole-2-carboxaldehyde		4332 1192-58-1	Liquid C_6H_7NO 109.13	Very slightly soluble Freely soluble	192-194 IR NMR MS 98 %	1.558-1.564 1.012-1.018	No longer supported by Industry (DG SANCO, 2013).
14.169 2150	1-Ethyl-2-pyrrolecarboxaldehyde		4317 2167-14-8	Liquid C_7H_9NO 123.15	Slightly soluble Freely soluble	205 NMR 99 %	1.541-1.547 1.033-1.039	No longer supported by Industry (DG SANCO, 2013).

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.

2.2. Stereoisomers

None of the 24 candidate substances can exist as geometrical or optical isomers.

2.3. Natural Occurrence in Food

Twenty-one out of 24 flavouring substances have been reported to occur in one or more of the following food items: tea, coffee, cocoa, fruits, almond, nuts, bread, asparagus, cheese, milk powder, pork, chicken, beef, shrimps, beer, whisky, wine, rum, malt, popcorn and peppermint oil. Quantitative data on the natural occurrence in food have been reported for 12 of the 21 candidate substances (Table 2)

Table 2: Candidate Substances Reported to Occur in Food (TNO, 2000)

FL-no:	Name:	Quantitative data reported
14.085	2-Acetyl-5-methylpyrrole	0.3 mg/kg in coffee, 0.02 mg/kg in malt
14.103	2,3-Dimethylpyridine	0.00008 mg/kg in pork, 2 mg/kg in tea
14.104	2,4-Dimethylpyridine	Trace amounts in chicken, up to 0.00005 mg/kg in pork, 0.002 mg/kg in shrimps
14.105	3,4-Dimethylpyridine	Up to 0.0002 mg/kg in whisky
14.106	3,5-Dimethylpyridine	Up to 0.15 mg/kg in tea
14.110	Ethyl nicotinate	Up to 0.06 mg/kg in apricot, up to 0.05 mg/kg in arctic bramble, up to 1.4 mg/kg in beer, up to 0.3 mg/kg in papaya
14.115	2-Ethylpyridine	Trace amounts in asparagus, 0.0001 mg/kg in pork
14.116	4-Ethylpyridine	0.0005 mg/kg in pork
14.134	2-Methylpyridine	0.08 mg/kg in cheese, 0.0005 mg/kg in chicken, 0.0003 mg/kg in pork, up to 0.02 mg/kg in shrimps, 0.002 mg/kg in whisky
14.135	3-Methylpyridine	0.0008 mg/kg in beer, 1.3 mg/kg in coffee and up to 0.0006 mg/kg in whisky
14.136	4-Methylpyridine	0.0001 mg/kg in pork, trace amounts in shrimps
14.150	2,4,6-Trimethylpyridine	Up to 0.15 mg/kg in tea

Three of the 24 substances have not been reported to occur naturally in any food items according to TNO (TNO, 2000) (Table 3).

Table 3: Candidate Substances Not Reported to Occur in Food (TNO, 2000)

FL-no:	Name:
14.088	1-Acetylindole
14.120	Isopropyl nicotinate
14.125	4-Isopropylpyridine

3. Specifications

Purity criteria for the 24 candidate substances have been provided by the Flavouring Industry (EFFA, 2004b) (Table 1).

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000 (EC, 2000), the information is adequate for all of the candidate substances (see Section 2.2 and Table 1).

4. 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, 1999).

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, 1999).

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, 2000). However, it is considered as a suitable tool to screen and prioritise the flavouring substances according to the need for refined intake data (EFSA, 2004).

4.1. 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, 1999). 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 (IOFI), 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 population[7] (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, 1999).

[7] 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.

In the present Flavouring Group Evaluation 24, Revision 2 (FGE.24Rev2), the total annual production volume of the 24 candidate substances from use as flavouring substances in Europe was reported to be 12 kg (EFFA, 2004c; Flavour Industry, 2005). For the 15 supporting substances the total annual volume of production in Europe is approximately 980 kg (JECFA, 2006).

On the basis of the annual volumes of production reported for the 24 candidate substances, the daily *per capita* intakes for each of these flavourings have been estimated (Table 6 and 7). More than 50 % (7.7 kg) of the total annual volume of production for the candidate substances (EFFA, 2004c) is accounted for by 4-methylpyridine [FL-no: 14.136] and 2-methylpyridine [FL-no: 14.134]. The estimated daily *per capita* intakes of these candidate substances from use as flavouring substances are 0.73 and 0.21 µg, respectively. The daily *per capita* intakes for each of the remaining substances are less than 0.15 µg (Table 6 and Table 7).

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

The method for calculation of the modified 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 present evaluation of the 24 candidate substances, information on food categories and normal and maximum use levels[8,9,10] were submitted by the Flavour Industry (EFFA, 2004b; EFFA, 2007; Flavour Industry, 2005). The 24 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, 2000), as shown in Table 4. For the present calculation of mTAMDI, the reported normal use levels were used. In cases where different use levels were reported for different food categories the highest reported normal use level was used.

Table 4: Use of Candidate Substances in Various Food Categories for 24 Candidate Substances for which Data on Use have been provided

[8] Normal use" is defined as the average of reported usages and "maximum use" is defined as the 95th percentile ``of reported usages (EFFA, 2002).

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

[10] 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, ``2007).

Food category	Description	Flavourings used
01.0	Dairy products, excluding products of category 2	All 24
02.0	Fats and oils, and fat emulsions (type water-in-oil)	All 24
03.0	Edible ices, including sherbet and sorbet	All 24
04.1	Processed fruits	All 24
04.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes), and nuts and seeds	None
05.0	Confectionery	All 24
06.0	Cereals and cereal products, including flours and starches from roots and tubers, pulses and legumes, excluding bakery	All 24
07.0	Bakery wares	All 24
08.0	Meat and meat products, including poultry and game	All 24
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	All 24 except [FL-no: 14.115]
10.0	Eggs and egg products	Only [FL-no: 14.115]
11.0	Sweeteners, including honey	None
12.0	Salts, spices, soups, sauces, salads, protein products etc.	All 24
13.0	Foodstuffs intended for particular nutritional uses	All 24
14.1	Non-alcoholic ("soft") beverages, excluding dairy products	All 24
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	All 24
15.0	Ready-to-eat savouries	All 24
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 – 15	All 24

According to the Flavour Industry the normal use levels for the 24 candidate substances are in the range of 0.1 - 2 mg/kg food, and the maximum use levels are in the range of 0.5 - 10 mg/kg (EFFA, 2004b; EFFA, 2007; Flavour Industry, 2005).

The mTAMDI value is 400 µg/person/day for each of the 22 candidate substances from structural class II and for the two candidate substances from structural class III. For detailed information on use levels and intake estimations based on the mTAMDI approach, see Section 7 and Appendix B.

5. Absorption, Distribution, Metabolism and Elimination

The 24 candidate substances are divided into four subgroups with more closely related structural properties within the subgroups as shown in Table 5 (The substances previously allocated to subgroup 2 and 6 are no longer supported by Industry (See Section 1)).

Table 5: Candidate Substances Divided into Groups of Related Chemical Structures

Subgroup	Candidate substance	FL-no	Chemical group
1	2-Acetyl-5-methylpyrrole	14.085	Pyrroles
2	The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry		N-substituted pyrroles
3	2-Methylindole	14.131	Indoles
	1-Acetylindole	14.088	
4	2-Methylpyridine	14.134	Pyridines with alkyl, hydroxyl or acetyl groups
	3-Methylpyridine	14.135	
	4-Methylpyridine	14.136	
	2-Ethylpyridine	14.115	
	4-Ethylpyridine	14.116	
	3-Propylpyridine	14.143	
	2-Isopropylpyridine	14.124	
	4-Isopropylpyridine	14.125	

	2-Butylpyridine	14.092	
	3-Butylpyridine	14.093	
	3-Pentylpyridine	14.140	
	2-Hexylpyridine	14.117	
	2,3-Dimethylpyridine	14.103	
	2,4-Dimethylpyridine	14.104	
	3,4-Dimethylpyridine	14.105	
	3,5-Dimethylpyridine	14.106	
	2,4,6-Trimethylpyridine	14.150	
	2-Hydroxypyridine	14.118	
	4-Acetylpyridine	14.089	
5	Ethyl nicotinate	14.110	Pyridines with ester groups
	Isopropyl nicotinate	14.120	
6	The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry		Quinolines

Subgroup 1: The group contains one pyrrol derivative, 2-acetyl-5-methylpyrrole [FL-no: 14.085]. Further oxidation of the acetyl group is expected. According to (Damani and Crooks, 1982) pyrroles are likely substrates for enzymatic oxidation at N1.

Subgroup 3: The group contains two indole derivatives, 1-acetylindole [FL-no: 14.088] (an amide) and 2-methylindole [FL-no: 14.131]. No data are available on the hydrolysis of 1-acetylindole [FL-no: 14.088] or structurally related substances. Amides are known to be hydrolysed but less rapidly than esters. A rapid hydrolysis of 1-acetylindole in the gastro-intestinal tract is not anticipated although hydrolysis by amidases in the tissues might be expected. The candidate substance 2-methylindole [FL-no: 14.131] and indole, the hydrolysis product of 1-acetylindole [FL-no: 14.088], will be metabolised through two main pathways. Oxidation of the methyl group in 2-methylindole and in the ring structure is anticipated, resulting in introduction of hydroxyl groups which are conjugated with glucuronic acid or sulphate. Epoxidation of the double bonds on the pyrrole ring is also expected, leading to oxindole compounds which can be further hydroxylated and conjugated with glucuronic acid or sulphate. The supporting substance 3-methylindole is anticipated to be metabolised to the reactive metabolite 3-methyleneindolenine which is conjugated with glutathione. Since a methyleneindolenine is not expected to be formed from 2-methylindole, conjugation with glutathione is unlikely for this substance.

Subgroup 4: The group contains 19 pyridine derivatives substituted with alkyl, hydroxyl or acetyl groups. Two main metabolism pathways can be predicted for the alkyl substituted pyridines. The main pathway is hydroxylation of the alkyl groups, with additional oxidation to the corresponding carboxyl compounds. The carboxyl metabolites are excreted in the urine as such or conjugated with glycine. Alternatively the candidate pyridines may be oxidised on the nitrogen atom, and excreted in the urine without conjugation. Hydroxylation of the pyridine ring through epoxide intermediates cannot be excluded, although it has not been shown to be a major pathway of the supporting substances 3-ethylpyridine, 2,3-dimethylpyridine and 2,6-dimethylpyridine However, this is probably dependent on the positions of the substitutions. From the metabolism studies on the supporting substance 3-acetylpyridine, the major metbolic pathway for 4-acetylpyridine [FL-no: 14.089] is expected to be via reduction of the keto group followed by conjugation by glucoronic acid and excretion via the urine. To a minor extend, N-oxidation or N-methylation may take place. 2-Hydroxypyridine [FL-no: 14.118] is expected to be excreted as such or as conjugate.

Subgroup 5: The group contains two esters of nicotinic acid, ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120], which are expected to be hydrolysed *in vivo* to nicotinic acid and ethanol, and to nicotinic acid and isopropanol, respectively.

The two candidate substances [FL-no: 14.110 and 14.120] (subgroup 5) in this flavouring group evaluation are expected to be metabolised to innocuous products. For the remaining 22 candidate substances [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.115, 14.116, 14.117, 14.118, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150], the Panel would expect metabolites such as *N*-oxides or epoxides, which cannot be anticipated to be innocuous products.

A more detailed description of the metabolism is given in Appendix C.

6. 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 7.

For the safety evaluation of the 24 candidate substances the Procedure as outlined in Appendix A was applied, based on the MSDI approach. The stepwise evaluations of the 24 substances are summarised in Table 7.

Step 1

Twenty-two of the 24 candidate substances for which the Procedure could be applied are classified into structural class II and two candidate substances into structural class III [FL-no: 14.088 and 14.131] according to the decision tree approach by Cramer et al. (1978).

Step 2

Ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120] are expected to be hydrolysed *in vivo* to nicotinic acid and ethanol, and to nicotinic acid and isopropanol, respectively. Nicotinic acid is a vitamin and accordingly the evaluation of the two candidate substances proceeds via the A-side of the Procedure.

For the remaining 22 candidate substances [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.115, 14.116, 14.117, 14.118, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150] of this flavouring group, metabolites such as N-oxides or epoxides are expected. Therefore, for these 22 candidate substances it cannot be anticipated that they will be metabolised to innocuous products and accordingly they proceed via the B-side of the Procedure.

Step A3

The two candidate substances [FL-no: 14.110 and 14.129] from subgroup 5, evaluated along the A-side of the Procedure, are both classified in structural class II. The substances have estimated daily *per capita* intakes from use as flavouring substances of 0.013 and 0.0012 µg. These intakes are below the threshold of concern of 540 µg/person/day for structural class II and accordingly ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120] are not expected to be of safety concern at the estimated level of intake. Tolerable upper intake levels for nicotinic acid and nicotinamide are 10 and 900 mg/person/day, respectively (SCF, 2002).

Step B3

The levels of intake of the remaining 20 candidate substances classified into structural class II were estimated to be between 0.0012 and 0.73 µg/*capita*/day, which are below the threshold of concern of 540 µg/person/day. The levels of intake of the candidate substances, evaluated through the Procedure,

classified into structural class III were estimated to be 0.0012 µg/*capita*/day each, which is below the threshold of concern of 90 µg/person/day. Therefore, these 22 candidate substances, evaluated via the B-side, proceed to step B4.

Step B4

Subgroup 1:

For the candidate substance 2-acetyl-5-methylpyrrole [FL-no: 14.085], a NOAEL of 48 mg/kg bw per day for the supporting substance 2-acetylpyrrole [FL-no: 14.047] is derived. The estimated daily *per capita* intake of 0.0012 µg for 2-acetyl-5-methylpyrrole [FL-no: 14.085] corresponds to 0.02 ng/kg bw/day at a body weight of 60 kg. Thus, a margin of safety of 2.4×10^9 can be calculated. 2-Acetyl-5-methylpyrrole is accordingly not expected to be of safety concern at the estimated level of intake.

Subgroup 3:

In an oral 37 weeks feeding study in rats on indole-3-carbinole, a substance structurally related to the two indole derivatives in this FGE, a NOAEL of 50 mg/kg body weight (bw)/day could be derived. The combined estimated daily *per capita* intake of 0.0024 µg for 1-acetylindole [FL-no: 14.088] and 2-methylindole [FL-no: 14.131] corresponds to 0.04 ng/kg bw/day at a body weight of 60 kg. Thus, a margin of safety of 1.3×10^9 can be calculated. 1-Acetylindole [FL-no: 14.088] and 2-methylindole [FL-no: 14.131] are accordingly not expected to be of safety concern at the estimated level of intake.

Subgroup 4:

A 90 days oral feeding study in rats is available for the supporting substance 2-acetylpyridine [FL-no: 14.038]. The NOAEL derived is 37 mg/kg bw/day. The MSDI values for the 19 pyridine derivatives in this FGE are between 0.012 and 0.21 µg/*capita*/day. The combined estimated daily *per capita* intake of these 19 derivatives is 1.5 µg corresponding to 0.025 µg/kg bw/day. Thus, a margin of safety of 1.5×10^6 can be calculated using the NOAEL of 37 mg/kg bw/day. The 19 pyridine derivatives in this flavouring group are accordingly not expected to be of safety concern at the estimated level of intake.

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

The mTAMDI values are 400 µg/person/day for each of the 24 candidate substances. Twenty-two candidate substances are from structural class II and two are from structural class III. These intake estimates are above the threshold of concern for the structural class III substances of 90 µg/person/day. For comparison of the MSDI- and mTAMDI-values see Table 6.

Thus, for two candidate substances [FL-no: 14.088 and 14.131], further information is required. This would include more reliable intake data, and if required, additional toxicological data.

Table 6: Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI (µg/*capita*/day)	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
14.110	Ethyl nicotinate	0.013	400	Class II	540
14.120	Isopropyl nicotinate	0.0012	400	Class II	540
14.085	2-Acetyl-5-methylpyrrole	0.0012	400	Class II	540
14.089	4-Acetylpyridine	0.0073	400	Class II	540
14.092	2-Butylpyridine	0.012	400	Class II	540
14.093	3-Butylpyridine	0.061	400	Class II	540
14.103	2,3-Dimethylpyridine	0.037	400	Class II	540
14.104	2,4-Dimethylpyridine	0.024	400	Class II	540
14.105	3,4-Dimethylpyridine	0.13	400	Class II	540
14.106	3,5-Dimethylpyridine	0.073	400	Class II	540
14.115	2-Ethylpyridine	0.027	400	Class II	540

14.116	4-Ethylpyridine	0.027	400	Class II	540
14.117	2-Hexylpyridine	0.012	400	Class II	540
14.118	2-Hydroxypyridine	0.024	400	Class II	540
14.124	2-Isopropylpyridine	0.021	400	Class II	540
14.125	4-Isopropylpyridine	0.012	400	Class II	540
14.134	2-Methylpyridine	0.21	400	Class II	540
14.135	3-Methylpyridine	0.027	400	Class II	540
14.136	4-Methylpyridine	0.73	400	Class II	540
14.140	3-Pentylpyridine	0.0012	400	Class II	540
14.143	3-Propylpyridine	0.0012	400	Class II	540
14.150	2,4,6-Trimethylpyridine	0.012	400	Class II	540
14.088	1-Acetylindole	0.0012	400	Class III	90
14.131	2-Methylindole	0.0012	400	Class III	90

8. 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.

On the basis of the reported annual production volumes in Europe (EFFA, 2004c; Flavour Industry, 2005), the combined estimated daily *per capita* intake as flavourings of the 22 candidate substances assigned to structural class II is 1.5 µg, which does not exceed the threshold of concern for a compound belonging to structural class II of 540 µg/person/day. The combined daily *per capita* intake as flavourings of the two candidate substances assigned to structural class III and evaluated using the Procedure is 0.0024 µg, which does not exceed the threshold of concern for a compound belonging to structural class III of 90 µg/person/day.

The 24 candidate substances, to which the Procedure has been applied, are structurally related to 15 supporting substances evaluated by JECFA at its 63rd meeting (JECFA, 2006). Based on reported production volumes, European *per capita* intakes (MSDI) could be estimated for all 15 supporting substances. The total combined intakes of the candidate and supporting substances are approximately 90 and 0.0085 µg/*capita*/day for structural class II and III, respectively, which do not exceed the thresholds of concern for a compound belonging to structural class II of 540 µg/person/day and structural class III of 90 µg/person/day.

9. Toxicity

9.1. Acute Toxicity

Data are available for four candidate substances and for eight structurally related substances of which seven have been evaluated by the JECFA (JECFA, 2006) (supporting substances). The structurally related 2,5-dimethylpyrrole [former FL-no: 15.107] is no longer supported by Industry (See Section 1). The LD_{50} values range from 60 to 3500 mg/kg body weight (bw).

The acute toxicity data are summarised in Table 10.

9.2. Subacute, Subchronic, Chronic and Carcinogenicity Studies

No data are available on subacute or subchronic oral toxicity on the candidate substances. Data on subacute or subchronic oral toxicity are available for three supporting substances, indole [FL-no: 14.007], 2-acetylpyridine [FL-no: 14.038], 2-acetylpyrrole [FL-no: 14.047], and the structurally

related indole-3-carbinol. For the supporting substance [FL-no: 14.007], there are data on chronic toxicity and carcinogenicity.

Indole-3-carbinol (subgroup 3)

In two studies, indole-3-carbinole was administered in the feed to male rats (8 animals/group) for 29 or 37 weeks at doses of 0 and 50 mg/kg bw/day. All tissues and gross lesions were subjected to histological examination and body and liver weights were measured. No differences between the groups of treated and control animals were detected. The only dose of 50 mg/kg bw has been taken as a NOAEL (Tanaka et al., 1990; Tanaka et al., 1992).

2-Acetylpyridine [FL-no: 14.038] (subgroup 4)

2-Acetylpyridine was administered to male and female rats (10 animals/sex/group) by gavage for 91 days at doses of 0, 37, 110, 330 and 1000 mg/kg bw/day. Urine analysis, haematological and histological examinations were performed. Liver enlargement was observed in the two highest dosed groups. Slight anaemia was observed in females dosed with 110 mg/kg bw and in males dosed with 330 mg/kg bw. The NOAEL derived was 37 mg/kg bw/day (Til and van der Meulen, 1971).

2-Acetylpyrrole [FL-no: 14.047] (subgroup 1)

2-Acetylpyrrole was administered to male and female rats (10 animals/sex/group) by gavage for 91 days at doses of 0 (dietary control), 1.050, 2.100 and 4.200 mg/kg feed daily in an OECD Guideline 408 study. However, the test material was not stable in the diet, and part of it was probably not more detected by the extraction method employed due to complexation with metal ions in the feed. Bauter (2012) calculated that over the course of the study the animals received concentrations of 35 - 40 % of the target intake level on average. Therefore, values for exposure levels based on the measured intake are proportionally lower. Based on this analysis of the test diets, the mean dietary concentrations were calculated to be 367, 754 and 1705 mg/kg feed. These dietary concentrations correspond to average daily intakes of 24, 48 and 107 mg/kg bw for males and 28, 56 and 121 mg/kg bw for females, respectively, over 90 days. Haematological and histological examinations were performed. Female rats of the high intake groups displayed minimal to moderate dark bilateral thyroid glands. Microscopic changes of note were slight thyroid hypertrophy/hyperplasia among 4/10 and 10/10 high intake group males and females, respectively (Bauter, 2012). However, statistically significantly decreased total white blood cell counts, erythrocyte counts, haemoglobin concentrations, haematocrit, absolute lymphocyte counts, absolute monocyte counts and absolute basophil counts and increased red cell distribution width were reported in the high intake group females on day 86. These parameters are outside of historical control levels although the variation are low in magnitude. The thyroid effects at the exposure level are of concern, as well as haematological changes in the high dose females. Therefore, the NOAEL for 2-acetylpyrrole is derived from the middle dose 48 mg/kg bw/day in males and 56 mg/kg bw/day in females. The NOAEL value of 48 mg/kg bw per day was used in calculating the margin of safety. The fact that this value is based on calculated lower exposure levels rather than the higher levels of 2-acetylpyrrole added to the diet results in a NOAEL level which is on the safe side because the effects observed at and ascribed to the lower exposure level, in fact may be due to a higher exposure if the loss of 2-acetylpyrrole is less than measured.

Repeated dose toxicity data are summarised in Table 11.

9.3. Developmental / Reproductive Toxicity Studies

There are no data available on developmental or reproductive toxicity for the candidate or the supporting substances.

9.4. Genotoxicity Studies

Genotoxicity data were provided for seven of the 24 candidate substances. In *in vitro* studies on the candidate substances 2-methylindole [FL-no: 14.131], 2-methylpyridine [FL-no: 14.134], 3-methylpyridine [FL-no: 14.135], 4-methylpyridine [FL-no: 14.136], 2,4-dimethylpyridine [FL-no: 14.104], 3,5-dimethylpyridine [FL-no: 14.106] and 4-acetylpyridine [FL-no: 14.089] in doses up to 10000 µg/plate, with and without metabolic activation, did not cause reverse mutations in various strains of *S. typhimurium* (Table 12).

Studies on induction of aneuploidy in *S. cerevisiae* D61.M are available on the three candidate substances 2-methylpyridine [FL-no: 14.134], 2,4-dimethylpyridine [FL-no: 14.104] and 4-acetylpyridine [FL-no: 14.089] gave positive results. The positive results were obtained at high doses inhibiting the growth of the yeast. Furthermore, fungal systems for measuring aneuploidy have little relevance compared to the mammalian system.

No *in vivo* studies on genotoxicity of the candidate substances were available.

Genotoxicity tests are available for the eight supporting substances [FL-no: 14.004, 14.007, 14.038, 14.039, 14.041, 14.047, 14.061 and 14.065]. 2-Acetylpyrrole [FL-no: 14.047] (methyl 2-pyrrolyl ketone) was positive in TA98 without metabolic activation at the two highest concentrations. Negative results were obtained at the lowest concentration as well as with metabolic activation. This study is considered of limited relevance. Pyrrole [FL-no: 14.041], indole [FL-no: 14.007], 3-methylindole [FL-no: 14.004] (skatole), 3-ethylpyridine [FL-no: 14.061] and 2-acetylpyridine [FL-no: 14.038] gave negative results in bacterial mutation assays.

Studies on induction of aneuploidy in *S. cerevisiae* D61.M are available on three supporting substances, 2,6-dimethylpyridine [FL-no: 14.065], 2-acetylpyridine [FL-no: 14.038] and 3-acetylpyridine [FL-no: 14.039], which gave positive results. However, as for the three candidate substances, the positive results were obtained at high doses inhibiting the growth of the yeast. Furthermore, fungal systems for measuring aneuploidy have little relevance compared to the mammalian system.

In vivo data are available for one supporting substance.

3-Methylindole (skatole) [FL-no: 14.004] was reported negative in the micronucleus test in mice. The validity of this study, however, cannot be evaluated, as only an abstract is available.

Positive results were obtained for some candidate and supporting substances in the Rec, DNA breaking, CHO and DNA synthesis assays. These results are, however, not considered valid (Table 12).

Conclusion on genotoxicity

The genotoxicity data available for the candidate substances do not preclude their evaluation through the Procedure.

Data on genotoxicity are summarised in Table 12 and 13.

CONCLUSIONS

The present Flavouring Group Evaluation deals with 24 pyridine, pyrrole, indole and quinoline derivatives. Since the publication of the previous version of FGE.24, FGE.24Rev1, nine of the original 33 candidate substances [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169], for which additional data were required, are no longer supported by Industry for use as flavouring substances in Europe and will therefore not be considered any further. This revision of FGE.24, FGE.24Rev2, therefore only deals with 24 candidate substances.

Further, the present revision of FGE.24, FGE.24Rev2, includes the assessment of new toxicity data on one supporting substance, 2-acetylpyrrole [FL-no: 14.047], to cover the re-evaluation of one candidate substance, 2-acetyl-5-methylpyrrole [FL-no: 14.085], for which additional data were required.

None of the 24 candidate substances can exist as geometrical or optical isomers.

Twenty-two of the candidate substances are classified into structural class II and two are classified into structural class III.

Twenty-one of the candidate 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 24 candidate substances in this group have intakes in Europe from 0.0012 to 0.73 µg/*capita*/day, which are all below the thresholds of concern for both structural class II (540 µg/person/day) and structural class III (90 µg/person/day) substances.

On the basis of the reported annual production volumes in Europe (MSDI approach), the total combined intakes of the candidate and supporting substances are approximately 90 and 0.0085 µg/*capita*/day for structural class II and III, respectively, which do not exceed the thresholds of concern for a compound belonging to structural class II of 540 µg/person/day and structural class III of 90 µg/person/day, respectively.

The genotoxicity data available for the candidate substances do not preclude their evaluation through the Procedure.

Two of the 24 candidate substances evaluated through the Procedure, ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120], are expected to be metabolised to innocuous products. For the remaining 22 candidate substances it cannot be anticipated that they will be metabolised to innocuous products.

It was noted that where toxicity data were available they were consistent with the conclusions in the present flavouring group evaluation using the Procedure.

It was considered that, on the basis of the default MSDI approach, the 24 substances, to which the Procedure was applied, would not give rise to safety concerns at the estimated levels of intake arising from their use as flavouring substances.

When the estimated intakes were based on the mTAMDI approach they were 400 µg/person/day for each of the 22 candidate substances from structural class II, which is below the threshold of concern for structural class II of 540 µg/person/day. For the two flavouring substances [FL-no: 14.088 and 14.131] assigned to structural class III, the estimated intakes based on the mTAMDI are 400 µg/person/day each. This is above the threshold of concern for structural class III of 90 µg/person/day. For the two candidate substances 1-acetylindole [FL-no: 14.088] and 2-methylindole [FL-no: 14.131], the intakes, estimated on the basis of the mTAMDI, exceed the threshold for the structural class to which the flavouring substance has been assigned. Therefore, for these substances more reliable exposure data are required. On the basis of such additional data, these flavouring substances should be reconsidered along the steps of the Procedure. Following this procedure additional toxicological data might become necessary.

In order to determine whether the conclusion for the 24 candidate substances could be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity tests for the materials of commerce have been provided for all 24 candidate substances.

Based on the available data, the Panel concluded that the 24 candidate substances [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.110, 14.115, 14.116, 14.117, 14.118, 14.120, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150] evaluated through the Procedure would present no safety concern at the estimated levels of intake based on the MSDI approach.

SUMMARY OF SAFETY EVALUATION

Table 7: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

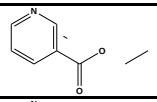
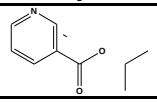
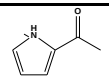
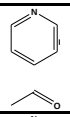
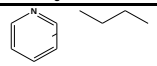
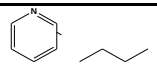
FL-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.110	Ethyl nicotinate		0.013	Class II A3: Intake below threshold	4)	6)	
14.120	Isopropyl nicotinate		0.0012	Class II A3: Intake below threshold	4)	6)	
14.023	1-Methylpyrrole		0.3	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		a)
14.085	2-Acetyl-5-methylpyrrole		0.0012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.089	4-Acetylpyridine		0.0073	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.092	2-Butylpyridine		0.012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.093	3-Butylpyridine		0.061	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.103	2,3-Dimethylpyridine		0.037	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.104 2151	2,4-Dimethylpyridine		0.024	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.105	3,4-Dimethylpyridine		0.13	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.106	3,5-Dimethylpyridine		0.073	Class II B3: Intake below threshold,	4)	6)	

Table 7: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

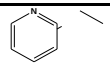
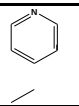
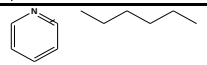
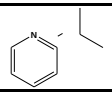
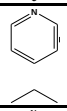
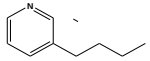
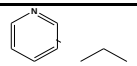
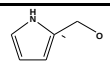
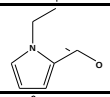
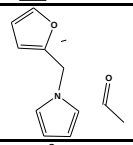
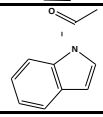
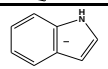
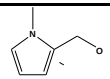
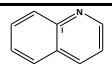
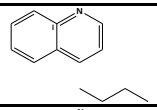
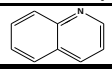
FL-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
				B4: Adequate NOAEL exists			
14.107	2,5-Dimethylpyrrole		0.061	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		a)
14.115	2-Ethylpyridine		0.027	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.116	4-Ethylpyridine		0.027	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.117	2-Hexylpyridine		0.012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.118	2-Hydroxypyridine		0.024	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.124	2-Isopropylpyridine		0.021	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.125	4-Isopropylpyridine		0.012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.134	2-Methylpyridine		0.21	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.135	3-Methylpyridine		0.027	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.136	4-Methylpyridine		0.73	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.140	3-Pentylpyridine		0.0012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.143	3-Propylpyridine		0.0012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	

Table 7: 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
14.145	Pyrrole-2-carbaldehyde		0.12	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		b)
14.150	2,4,6-Trimethylpyridine		0.012	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.169 2150	1-Ethyl-2-pyrrolecarboxaldehyde		0.12	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		b)
13.100	2-Acetyl-1-furfurylpyrrole		0.091	Class III B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		a)
14.088	1-Acetylindole		0.0012	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.131	2-Methylindole		0.0012	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.163 2152	1-Methylpyrrole-2-carboxaldehyde		0.0024	Class III B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		b)
14.002	4-Methylquinoline		0.12	Class III No evaluation			a)
14.094	4-Butylquinoline		0.0012	Class III No evaluation			a)
14.138	2-Methylquinoline		0.012	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) = µg/capita/day.

2) Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.

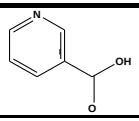
3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

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 the estimated level of intake of the material of commerce meeting the specification requirement (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) No longer supported by Industry (EFSA, 2011).
b) No longer supported by Industry (DG SANCO, 2013).

EVALUATION STATUS OF HYDROLYSIS PRODUCTS OF CANDIDATE ESTERS

Table 8: Evaluation Status of Hydrolysis Products of Candidate Esters

FL-no	EU Register name JECFA no	Structural formula	SCF status 1) JECFA status 2) CoE status 3) EFSA status	Structural class 4) Procedure path (JECFA) 5)	Comments
	Nicotinic acid		Not evaluated as flavouring substance		Not in EU-Register.
02.078	Ethanol 41		Category 1 a) No safety concern b)	No evaluation	At the forty-sixth JECFA meeting (JECFA, 1997), the Committee concluded that ethanol posed no safety concern at its current level of intake when ethyl esters are used as flavouring agents.
02.079	Isopropanol 277		Category 1 a) No safety concern c)	Class I A3: Intake above threshold, A4: Endogenous	
08.002	Acetic acid 81		Category 1 a) No safety concern d) Category A e)	Class I A3: Intake above threshold, A4: Endogenous	
14.007	Indole 1301		No safety concern f) Category A e)	Class I A3: Intake below threshold	

1) 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.

2) No safety concern at estimated levels of intake.

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

4) Threshold of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.

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

a) (SCF, 1995).

b) (JECFA, 1997).

c) (JECFA, 2000).

d) (JECFA, 1999).

e) (CoE, 1992).

f) (JECFA, 2005b).

SUPPORTING SUBSTANCES SUMMARY

Table 9: Supporting Substances Summary

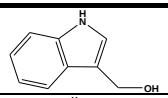
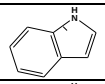
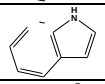
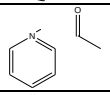
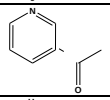
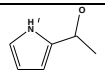
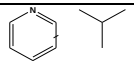
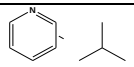
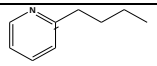
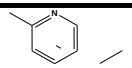
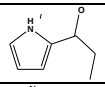
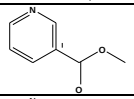
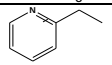
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (μg/*capita*/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
	Indole-3-carbinol		700-06-1				Not in EU-Register.
14.004	3-Methylindole		3019 493 83-34-1	1304 JECFA specification (JECFA, 2005a).	2.4	No safety concern a) Category B b)	
14.007	Indole		2593 560 120-72-9	1301 JECFA specification (JECFA, 2005a).	26	No safety concern a) Category A b)	
14.038	2-Acetylpyridine		3251 2315 1122-62-9	1309 JECFA specification (JECFA, 2005a).	50	No safety concern a) Category B b)	
14.039	3-Acetylpyridine		3424 2316 350-03-8	1316 JECFA specification (JECFA, 2005a).	23	No safety concern a) Category B b)	
14.041	Pyrrole		3386 2318 109-97-7	1314 JECFA specification (JECFA, 2005a).	0.11	No safety concern a) Category B b)	
14.047	2-Acetylpyrrole		3202 11721 1072-83-9	1307 JECFA specification (JECFA, 2005a).	3.3	No safety concern a)	
14.058	2-Isobutylpyridine		3370 11395 6304-24-1	1311 JECFA specification (JECFA, 2005a).	0.0061	No safety concern a)	
14.059	3-Isobutylpyridine		3371 11396 14159-61-6	1312 JECFA specification (JECFA, 2005a).	0.049	No safety concern a)	
14.060	2-Pentylpyridine		3383 11412 2294-76-0	1313 JECFA specification (JECFA, 2005a).	0.061	No safety concern a)	
14.061	3-Ethylpyridine		3394 11386 536-78-7	1315 JECFA specification (JECFA, 2005a).	9.3	No safety concern a)	
14.065	2,6-Dimethylpyridine		3540 11381 108-48-5	1317 JECFA specification (JECFA, 2005a).	0.26	No safety concern a)	

Table 9: 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
14.066	5-Ethyl-2-methylpyridine		3546 11385 104-90-5	1318 JECFA specification (JECFA, 2005a).	0.12	No safety concern a)	
14.068	2-Propionylpyrrole		3614 11942 1073-26-3	1319 JECFA specification (JECFA, 2005a).	0.012	No safety concern a)	
14.071	Methyl nicotinate		3709 93-60-7	1320 JECFA specification (JECFA, 2005a).	0.49	No safety concern a)	
14.164	2-Propylpyridine		 622-39-9	1322 JECFA specification (JECFA, 2005a).	0.61	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, 2005b).

b) (CoE, 1992).

TOXICITY DATA

Table 10: Acute Toxicity

Chemical Name [FL-no]*	Species	Sex	Route	LD_{50} (mg/kg bw)	Reference	Comments
(2,5-Dimethylpyrrole [14.107], no longer supported)	Rat	M, F	Oral	59	(Burdock and Ford, 1990)	
(Indole [14.007])	Rat	M	Oral	1000	(Smyth et al., 1962)	
(3-Methylindole [14.004])	Rat	NR	Oral	3450	(McGee Laboratories Inc., 1974)	
(2-Propionylpyrrole [14.068])	Mouse	M, F	Oral	1620	(Moran et al., 1980)	
2-Methylpyridine [14.134]	Rat	M	Oral	> 950[1]	(Dow Chemical Company, 1976)	
	Rat	NR	Oral	400 – 800	(Eastman Kodak Co., 1980)	
	Rat	NR	Oral	1410	(Smyth et al., 1951)	
	Rat	NR	Oral	> 500 and < 1000[2]	(Taylor and Olson, 1964)	
	Rat	M, F	Oral	810	(Birch, 1972a)	
	Mouse	NR	Oral	400 – 800	(Eastman Kodak Co., 1980)	
3-Methylpyridine [14.135]	Rat	M, F	Oral	710	(Birch, 1972b)	
	Rat	M	Oral	630	(Dow Chemical Company, 1983)	
4-Methylpyridine [14.136]	Rat	F	Oral	841	(Pullin et al., 1973)	
	Rat	M, F	Oral	700	(Birch, 1972c)	
	Rat	NR	Oral	1290	(Goe, 1984)	
(5-Ethyl-2-methylpyridine [14.066])	Rat	NR	Oral	1540	(Smyth et al., 1951)	
	Rat	NR	Oral	368	(Izmerov et al., 1982)	
	Rat	M	Oral	1.30 ml/kg (1195)	(Myers and Ballantyne, 1997)	
	Mouse	NR	Oral	282	(Izmerov et al., 1982)	
2,4,6-Trimethylpyridine [14.150]	Rat	M	Oral	>1000	(Dow Chemical Company, 1985)	
(2-Acetylpyridine [14.038])	Rat	NR	Oral	2280	(Posternak et al., 1975)	
	Rat	M, F	Oral	2.0 ml/kg (2160)	(Spanjers and Til, 1968)	
(3-Acetylpyridine [14.039])	Rat	M, F	Oral	M: 0.052 ml/kg (57) F: 0.046 ml/kg (51)	(Costello et al., 1992)	
(Methyl nicotinate [14.071])	Mouse	NR	Oral	2800[3]	(Pellmont, 1977)	

* Supporting substances are listed in brackets.

1 LD_{50} not determined. Animals were dosed once and monitored for 28 days following dose administration. At 950 mg/kg bw, 4/10 animals were found dead indicating that the LD_{50} for 2-methylpyridine is slightly greater than 950 mg/kg bw.

2 LD_{50} not determined. At 500 mg/kg bw, 2/2 animals survived, while at 1000 mg/kg bw 2/2 animals died.

3 Administered as a 16 % solution in 5 % gum Arabic.

Table 11: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]*	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw/day)	Reference	Comments
(Indole [14.007])	Rat; M 6	Diet	0, 0.25, 0.50 and 0.75 % (~ 0, 138, 275 and 413 mg/kg bw/day)	21 days	< 125	(Martinez and Roe, 1972)	Old study with limited quality. Reduced body weight at the lowest dose tested, and reduced food intake at the two highest doses. No effect on liver weight.
	Rat; M, F 25 (20 F, 5 M)	Diet	100 mg/kg bw/day first 460 days, then 200 mg/kg bw/day	590 days	No NOAEL derived	(Kaiser, 1953)	Dose increased in the end of the study, and adverse effects at the lowest dose tested, shown by reduced weight, and erythrocyte count, and increased leukocyte count.
(Indole-3-carbinol[1])	Rat; F 20	Diet	0 and 2000 ppm (0 and 100 mg/kg bw/day)	25 weeks	No NOAEL derived	(Stoner et al., 2002)	Non-GLP study of reasonable quality. Indole-3-carbinol significantly increased liver foci development compared to control and reduced body weight gain.
	Rat; F 10	Gavage	5, 25, 50, 100 and 200 mg/kg bw/day	6 weeks	100	(Grubbs et al., 1995)	Published non-GLP study of limited quality. 200 mg/kg bw gave a 20 % increase in liver weight. Additionally 100 days (14 weeks) study with 100 mg/kg bw indole-3-carbinol increased liver weight by 33 %. Experimental details and results insufficiently reported.
	Rat; M 8	Diet	0 and 1000 ppm (0 and 50 mg/kg bw/day)	37 weeks	50	(Tanaka et al., 1992)	Study of neoplastic lesions in oral cavity, especially the tongue. Histological examination performed on all major tissues. No effect on body weight, liver weight or neoplasm in tongue or liver. Reasonable quality.
			0 and 1000 ppm (0 and 50 mg/kg bw/day) for seven weeks	29 weeks	50	(Tanaka et al., 1990)	Published non-GLP study of reasonable quality. Histological examination. Study on liver carcinogenesis. No effect of indole-3-carbinol.
(2-Acetylpyrrole [14.047])	Rat; M, F 32	Diet	0, 87 (M) and 86 (F) mg/kg bw/day	91 days	M: 87.46[2] F: 86.31[2]	(Posternak et al., 1975)	Haematological examination. Histological examination and weight of liver and kidney. Slightly reduction in body weight gain in males. Poorly reported study and it can therefore not be used in the Procedure.
	Rat; M, F 10	Diet	0, 68, 133 and 263 mg/kg bw for males 0, 79, 155 and 298 mg/kg bw for females	91 days	M: 48 F: 56	(Bauter, 2012)	OECD Guideline study (408). Test material was not stable in the diet. Bauter calculated that over the course of the study the animals received only 35 - 40 %. The mean dietary concentrations were calculated to be 367, 754 and 1705 mg/kg feed. These dietary concentrations correspond to average daily intakes of 24, 48 and 107 mg/kg bw for males and 28, 56 and 121 mg/kg bw for females, respectively, over 90 days.
(2-Acetylpyridine [14.038])	Rat; M, F 32	Diet	0, 3.13 (M) and 3.06 (F) mg/kg	91 days	M: 3.13[2] F: 3.06[2]	(Posternak et al., 1975)	Haematological examination. Histological examination and weight of liver and kidney. No

Table 11: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]*	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw/day)	Reference	Comments
			bw/day				significant effect. Poorly reported study and it can therefore not be used in the Procedure.
	Rat; M, F 20	Gavage	0, 37, 110, 330 and 1000 mg/kg bw/day six days a week	91 days	37	(Til and van der Meulen, 1971)	Old study of reasonable quality. Urine, haematological and histological examination. Liver enlargement in the two highest dosage groups. Slight anaemia in females from 110 mg/kg bw and in males from 330 mg/kg bw.

* Supporting substances are listed in brackets.

1 Major metabolite of 3-methylindole (skatole).

2 Study performed with either a single dose level or multiple dose levels that produced no adverse effect.

3 Study performed with a single dose level that produced no adverse effect. This study was conducted to determine if 6-methylquinoline induced lesions or tumours similar to structurally-related substances. 6-Methylquinoline did not induce a statistically significant quantity of lesions or tumours in the rat when administered via the diet at 0.05 %.

Developmental and reproductive toxicity data are not available neither for the candidate substances of the present flavouring group evaluation from chemical group 28, nor for the supporting substance evaluated by JECFA at the 63rd meeting.

Table 12: Genotoxicity (*In Vitro*)

Chemical Name [FL-no]*	Test System	Test Object	Concentration	Result	Reference	Comments
(Pyrrole [14.041])	Ames assay (modified pre-incubation method)	*S. typhimurium* TA98; TA100; TA102	1.4 mmol/plate (93926 µg/plate)	Negative[1]	(Aeschbacher et al., 1989)	
	Ames assay (pre-incubation method)	*S. typhimurium* TA100; TA1535; TA1537	3 µmol/plate (201 µg/plate)	Negative[1]	(Florin et al., 1980)	
	Ames assay (pre-incubation method)	*S. typhimurium* TA98	30 µmol/plate (2013 µg/plate)	Negative[1]		
	Ames assay (plate incorporation method)	*S. typhimurium* TA98; TA100	Not reported	Negative[3]	(Lee et al., 1994)	
	Rec assay	*B. subtilis* H17 (rec+), M45 (rec-)	4 and 20 mg/disk	Positive[3]	(Kim et al., 1987)	Poor predictive value for mutagenicity. Limited value.
	Unscheduled DNA synthesis	Rat hepatocytes	Not reported	Negative	(Williams, 1984)	
(Indole [14.007])	Ames assay (pre-incubation method)	*S. typhimurium* TA100	20 µg/plate	Negative[2]	(Ochiai et al., 1986)	
	Ames assay	*S. typhimurium* TM677	4 mM (469 µg/ml)	Negative[3]	(Kaden et al., 1979)	
	Ames assay (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1538	2500 µg/plate	Negative[1]	(Anderson and Styles, 1978)	
	Ames assay	*S. typhimurium* TA98; TA100	500 nmol/plate (59 µg/plate)	Negative[2]	(Vance et al., 1986)	
	Ames assay (pre-incubation method)	*S. typhimurium* TA100; TA1535; TA1537	3 µmol/plate (351 µg/plate)	Negative[1]	(Florin et al., 1980)	
		S. typhimurium TA98	30 µmol/plate (3515 µg/plate)	Negative[3]		
		S. typhimurium TA97; TA102	1000 µg/plate	Negative[1]	(Fujita et al., 1994)	
		S. typhimurium TA98; TA100 *E. coli* WP2uvrA/ pKM101	0.4 µmol/plate (47 µg/plate)	Negative[1]	(Sasagawa and Matsushima, 1991)	
		S. typhimurium TA100	500 µg/plate	Negative[2]	(Hashizume et al., 1991)	
2-Methylindole [14.131]	Ames assay (pre-incubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1538	4, 20, 100, 500 and 2500 µg/plate	Negative[1]	(Anderson and Styles, 1978)	The study is considered valid.
		S. typhimurium TA98; TA100; TA1535; TA1537	3 µmol/plate (394 µg/plate)	Negative[1]	(Florin et al., 1980)	Single dose study.
	Ames assay	*S. typhimurium* TA98	3 nmol - 30 µmol/plate (12 doses) (3935 µg/plate)	Negative[1]	(Curvall et al., 1982)	The study is considered valid.
		S. typhimurium TM677	2 mM (262 µg/ml)	Negative	(Kaden et al., 1979)	Single dose study.
(3-Methylindole [14.004])	Ames assay (pre-incubation method)	*S. typhimurium* TA100; TA1535; TA1537	3 µmol/plate (394 µg/plate)	Negative[1]	(Florin et al., 1980)	
		S. typhimurium	30 µmol/plate	Negative[1]	(Florin et al., 1980)	

Table 12: Genotoxicity (*In Vitro*)

Chemical Name [FL-no]*	Test System	Test Object	Concentration	Result	Reference	Comments
		TA98	(3935 µg/plate)			
	Ames assay	*S. typhimurium* TA98; TA100	Not reported	Negative[3]	(Kim et al., 1989)	
	Ames assay (pre-incubation method)	*S. typhimurium* TA98; TA100 *E. coli* WP2uvrA/ pKM101	0.4 µmol/plate (52 µg/plate)	Negative[1]	(Sasagawa and Matsushima, 1991)	
		S. typhimurium TA100	100 µg/plate	Negative[2]	(Ochiai et al., 1986)	
		S. typhimurium TA100	Up to 3.33 mM (437 µg/ml)	Negative[3]	(Reddy et al., 2002)	
	Mutation assay (paper-disk method)	*E. coli* Sd-4-73	0.025 ml/disk	Negative	(Szybalski, 1958)	
	Chromosomal aberration assay	Chinese hamster ovary cells	1.3, 1.4, 1.5 mM (+ S9) 1.4, 1.5, 1.6 mM (- S9)	Positive[1]	(Reddy et al., 2002)	Aberrations were only detected at cytotoxic concentrations that showed marked inhibition of DNA synthesis.
	Alkaline elution assay	Rat primary hepatocytes (unindiced and PB/β-NF induced)	0.5, 0.6, 0.7, 0.8, 0.9 and 1 mM	Negative	(Reddy et al., 2002)	The study is considered valid.
	DNA modification assay	Isolated human genomic DNA	25 and 500 µM (66 µg/ml)	Positive[3] Negative[2]	(Laws et al., 2001)	Assay demonstrating inhibition of PCR amplification and spots demonstrated by postlabeling. Limited predictive value.
	DNA Single strand break assay	Bovine kidney cells	10 µM - 1 mM (131.2 µg/ml)	Positive	(Kim et al., 1989)	Abstract only. Validity cannot be evaluated.
(2-Acetylpyrrole [14.047])	Ames assay (plate incorporation method)	*S. typhimurium* TA98	4, 20 and 100 µmol/plate (10913 µg/plate)	Negative[3] Positive[2]	(Lee et al., 1994)	Positive without S9 only at the two highest concentrations. High concentrations. Technically acceptable, but of limited relevance due to high concentrations.
		S. typhimurium TA100	100 µmol/plate (10913 µg/plate)	Negative[1]		
	Ames assay	*S. typhimurium* TA98; TA100	Up to 200 µg/plate	Negative[1]	(Wang et al., 1994)	
2-Methylpyridine [14.134]	Ames assay (pre-incubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	3 µmol/plate (279 µg/plate)	Negative[1]	(Florin et al., 1980)	Single dose study.
	Ames assay (modified pre-incubation method)	*S. typhimurium* TA98; TA100; TA102	10 nmol - 1 mmol/plate (6 doses) (93 µg/ml)	Negative[1]	(Aeschbacher et al., 1989)	The study is considered valid.
	Ames assay (plate incorporation method)	*S. typhimurium* TA97; TA98; TA100; TA102	Up to 5000 µg/plate (6 doses)	Negative[1]	(Claxton et al., 1987)	Individual dose levels not reported. The study is considered valid.
		S. typhimurium TA98; TA100; TA1535; TA1537	50, 160, 500, 1600 and 5000 nl/plate (4722 µg/plate)	Negative[1]	(Vleminckx et al., 1993a)	The study is considered valid.
	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.5 - 0.74% (6 doses) (6988 µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded.

Table 12: Genotoxicity (*In Vitro*)

Chemical Name [FL-no]*	Test System	Test Object	Concentration	Result	Reference	Comments
						Limited relevance.
	HGPRT Gene mutation assay	Chinese hamster V79 lung cells	4.5, 4.75, 5, 5.25 and 5.5 µl/ml (5194 µg/ml)	Negative[2]	(Vleminckx et al., 1993b)	The study is considered valid.
	Alkaline elution assay	Chinese hamster V79 lung cells	2, 3, 4, 5 and 6 µl/ml (5666 µg/ml)	Negative[2]	(Schriewer et al., 1993)	The study is considered valid.
3-Methylpyridine [14.135]	Ames assay (modified pre-incubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	85, 280, 840 and 8540 µg/plate	Negative	(Haworth et al., 1983)	The study is considered valid.
	Ames assay (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	50, 160, 500, 1600 and 5000 nl/plate (4785 µg/plate)	Negative[1]	(Vleminckx et al., 1993a)	The study is considered valid.
	Mutagenicity assay	*E. coli* WP2 uvrA	5 - 10 mg/plate (5000 – 10,000 µg/plate)	Negative	(Pai et al., 1978)	Single dose study. Very few experimental details. The validity cannot be evaluated.
	HGPRT Gene mutation assay	Chinese hamster V79 lung cells	3, 3.25, 3.5, 3.75 and 4 µl/ml (3828 µg/ml)	Negative[2]	(Vleminckx et al., 1993b)	The study is considered valid.
	Alkaline elution assay	Chinese hamster V79 lung cells	2, 3, 4 and 5 µl/ml (4785 µg/ml)	Negative[2]	(Schriewer et al., 1993)	The study is considered valid.
4-Methylpyridine [14.136]	Ames assay (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	50, 160, 500, 1600 and 5000 nl/plate (4779 µg/plate)	Negative[1]	(Vleminckx et al., 1993a)	The study is considered valid.
	HGPRT Gene mutation assay	Chinese hamster V79 lung cells	3.75, 4, 4.25 and 4.5 µl/ml (4301 µg/ml)	Negative[2]	(Vleminckx et al., 1993b)	The study is considered valid.
	Alkaline elution assay	Chinese hamster V79 lung cells	3.75, 4, 4.25 and 4.5 µl/ml (4301 µg/ml)	Negative[2]	(Schriewer et al., 1993)	The study is considered valid.
(3-Ethylpyridine [14.061])	Ames assay (pre-incubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	3 µmol/plate (321µg/plate)	Negative[1]	(Florin et al., 1980)	Single dose study.
2,4-Dimethylpyridine [14.104]	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.4 - 0.60% (6 doses) (5551µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded. Limited relevance.
(2,6-Dimethylpyridine [14.065])	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.5 - 0.60% (4 doses) (5551 µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded. Limited relevance.
3,5-Dimethylpyridine [14.106]	Ames assay (pre-incubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	3 µmol/plate (321 µg/plate)	Negative[1]	(Florin et al., 1980)	Single dose study.
(2-Acetylpyridine [14.038])	Ames assay (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	100 - 10,000 µg/plate	Negative	(Longfellow, 1997)	Very short summary. The results cannot be validated. High doses.
	Mouse lymphoma assay	Mouse lymphocytes L5178Y tk+/–	2500 - 4500 µg/ml (-S9) 1000 - 4000 µg/ml(+S9)	Positive[1]		Very short summary. The results cannot be validated.
	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.5 - 0.87%(4 doses) (9396 µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded. Limited relevance.
(3-Acetylpyridine [14.039])	Mutation	*E. coli*	10000 µg/plate	Negative	(Pai et al., 1978)	Single dose study. Very few

Table 12: Genotoxicity (*In Vitro*)

Chemical Name [FL-no]*	Test System	Test Object	Concentration	Result	Reference	Comments
		WP2uvrA				experimental details. The validity cannot be evaluated.
	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.5 - 1.11% (5 doses) (1223 µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded. Limited relevance.
4-Acetylpyridine [14.089]	Ames assay (pre-incubation method)	*S. typhimurium* TA97; TA98; TA100; TA102; TA104; TA1535; TA1537; TA1538	5, 100, 300, 100, 3000 and 10,000 µg/plate	Negative[1]	(Zeiger et al., 1992)	The study is considered valid.
	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	0.5 - 1.19% (5 doses) (13,114 µg/ml)	Positive	(Zimmermann et al., 1986)	Very high doses. The effect is considered thresholded. Limited relevance.
	Mitotic aneuploidy assay	*S. cerevisiae* D61.M	Up to 11 mg/ml	Positive	(Whittaker et al., 1989)	Purity 88 %. Very high doses. The effect is considered thresholded. Limited relevance.

* Supporting substances are listed in brackets.
[1] With and without metabolic activation.
[2] Without metabolic activation.
[3] With metabolic activation.

Table 13: Genotoxicity (*In Vivo*)

Chemical Name	Test System	Test Object	Route	Dose	Result	Reference	Comments
(3-Methylindole [14.004])*	*In vivo* Micronucleus test	Mouse	Oral	1000 mg/kg day	Negative	(Reddy et al., 2003)	Abstract only. The validity cannot be evaluated.

* Supporting substance.

REFERENCES

Aeschbacher HU, Wolleb U, Loliger J, Spadone JC and Liardon R, 1989. Contribution of coffee aroma constituents to the mutagenicity of coffee. Food and Chemical Toxicology 27(4), 227-232.

Anderson D and Styles JA, 1978. An evaluation of 6 short-term tests for detecting organic chemical carcinogens. Appendix 2. The bacterial mutation test. British Journal of Cancer 37, 924-930.

Bauter MR, 2012. Draft Report. 2-Acetylpyrrole: A 90-day dietary study in rats. Product Safety Labs. Study no. 33327. Marts 2, 2012. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Birch MD, 1972a. Initial submission: Toxicological investigation of: 0.4 mole fraction 2-methylpyridine with cover letter dated 08/17/92. Monsanto Co. EPA Doc. 88-920007591, microfiche no. OTS0545782. Date 04/19/72. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Birch MD, 1972b. Initial submission: Toxicological investigation of: 0.4 mole fraction 3-methylpyridine - LOT: QT 195729 (final report) with cover letter dated 11/26/91. Monsanto Co. EPA Doc. 88-92000037, microfiche no. OTS0534823. Date 4/19/72. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Birch MD, 1972c. Initial submission: 4-Methylpyridine - Neat - LOT: QET 195729 (final report) with cover letter dated 11/26/91. Monsanto Co. EPA Doc. 88-920000385, microfiche no. OTS0534837. Date 4/19/72. Unpublished data submitted by EFFA to FLAVIS Secretariat.

Burdock GA and Ford RA, 1990. Acute oral toxicity (LD50) study in the rat with 2,5-dimethylpyrole. Acute Toxicity Data. Journal of the American College of Toxicology Part B, 1, 6.

Claxton LD, Dearfield KL, Spanggord RJ, Riccio ES and Mortelmans K, 1987. Comparative mutagenicity of halogenated pyridines in the *Salmonella typhimurium*/mammalian microsome test. Mutation Research 176, 185-198.

CoE, 1992. Flavouring substances and natural sources of flavourings. 4th Ed. vol. I. Chemically defined flavouring substances. Council of Europe, partial agreement in the social and public health field. Strasbourg.

Costello AC, Myers RC, Manderfield CE and Osimitz TG, 1992. Acute oral toxicity determination of methyl pyridyl ketone in the rat. Journal of the American College of Toxicology 11(6), 681.

Cowan DA, Damani LA and Gorrod JW, 1978. Metabolic n-oxidation of 3-substituted pyridines: Identification of products by mass spectrometry. Biomedical Mass Spectrometry 5(9), 551-556.

Cramer GM, Ford RA and Hall RL, 1978. Estimation of toxic hazard - a decision tree approach. Food and Cosmetics Toxicology 16(3), 255-276.

Curvall M, Florin I and Jansson T, 1982. Mutagenicity of some indoles and related compounds in the Ames test. Toxicology 23, 1-10.

Damani LA and Crooks PA, 1982. Oxidative metabolism of heterocyclic ring systems. In: Jakoby WB, Bend JR and Caldwe J (Ed.). Metabolic Basis of Detoxication. 2nd Ed. Academic Press, New York, pp. 69-89.

Damani LA, Bryan JB, Cowan DA and Gorrod JW, 1980. The origin of l-(3-pyridyl-N-oxide)ethanol as a metabolite of 3-acetylpyridine. Xenobiotica 10(7/8), 645-653.

DG SANCO (Directorate General for Health and Consumer Affairs), 2013. Information from DG SANCO 14/05 2013, concerning a list of 18 non-supported substances. FLAVIS.2.26.

Dow Chemical Company, 1976. Acute oral toxicity of alpha-picoline in rats with cover letter to EPA 080983. EPA Doc. 40-8341086, microfiche no. OTS0507474. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Dow Chemical Company, 1983. Initial submission: 3-methylpyridine: Acute toxicological properties and industrial handling hazards with cover letter dated 042192. EPA Doc. 88-920002220, microfiche no. OTS0539237. Date 7/21/83. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Dow Chemical Company, 1985. Initial submission: 2,4,6-trimethylpyridine: acute toxicological properties in male rats (final report) with cover letter dated 05/15/92. EPA Doc. 88-920003187, microfiche no. OTS0539775. Date 2/26/85. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Durrer A, Wernly-Chung GN, Boss G and Testa B, 1992. Enzymic hydrolysis of nicotinate esters: Comparison between plasma and liver catalysis. Xenobiotica 22(3), 273-282.

Eastman Kodak Company, 1980. Initial submission: Toxicity report: m-Ethylphenol with cover letter dated 09/2/892. EPA Doc. 88-920009161, microfiche no. OTS0546443. Date 11/26/80. Unpublished data submitted by EFFA to FLAVIS Secretariat.

EC (European Commission), 2000. Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. Official Journal of the European Communities 19.7.2000, L 180, 8-16.

EFFA (European Flavour and Fragrance Association), 2002. Letter from EFFA to Dr. Joern Gry, Danish Veterinary and Food Administration. Dated 31 October 2002. Re.: Second group of questions. FLAVIS/8.26.

EFFA (European Flavour and Fragrance Association), 2004a. Intake - Collection and collation of usage data for flavouring substances. Letter from Dan Dils, EFFA to Torben Hallas-Møller, EFSA. May 31, 2004.

EFFA (European Flavour and Fragrance Association), 2004b. Submission 2004-2. Flavouring group evaluation of 34 flavouring substances (candidate chemicals) of the chemical group 28 (annex I of 1565/2000/EC) structurally related to pyridine, pyrrole and quinoline derivatives [FEMA 2004-1] used as flavouring substances. 1 June 2004. Unpublished report submitted by EFFA to FLAVIS Secretariat. FLAVIS/8.36.

EFFA (European Flavour and Fragrance Association), 2004c. Submission 2004-2. Flavouring group evaluation of 34 flavouring substances (candidate chemicals) of the chemical group 28 (annex I of 1565/2000/EC) structurally related to pyridine, pyrrole and quinoline derivatives [FEMA 2004-1] used as flavouring substances. 1 June 2004. FLAVIS/8.36. European inquiry on volume of use. IOFI, International Organization of the Flavor Industry, 1995. Private communication to FEMA. Unpublished report submitted by EFFA to FLAVIS Secretariat.

EFFA (European Flavour and Fragrance Association), 2007. E-mail from Jan Demyttenaere, EFFA to FLAVIS Secretariat, National Food Institute, Technical University of Denmark. Dated 8 February 2007. RE: FLAVIS submissions - use levels for Category 14.2 - Alcoholic beverages. FLAVIS/8.70.

EFSA (European Food Safety Authority), 2004. Minutes of the 7th Plenary Meeting of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, Held in Brussels on 12-13 July 2004. Adopted on September 2004.

EFSA, 2011. List of substances for which the Commission withdraw its request to EFSA for an opinion. FLAVIS/2.23Rev1.

Eurostat, 1998. Total population. Cited in Eurostat, 2004. The EU population, Total population.

Flavour Industry, 2005. Unpublished information submitted by Flavour Industry to DG SANCO and forwarded to EFSA. A-24.

Florin I, Rutberg L, Curvall M and Enzell CR, 1980. Screening of tobacco smoke constituents for mutagenicity using the Ames' test. Toxicology 18, 219-232.

Fujita H, Aoki N and Sasaki M, 1994. Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102 (IX*). Annual Report of Tokyo Metropolitan Research Laboratory of Public Health 45, 191-199. (In Japanese)

Gillam EMJ, Notley LM, Cai H, De Voss JJ and Guengerich FP, 2000. Oxidation of indole by cytochrome P450 enzymes. Biochemistry 39(45), 13817-13824.

Goe GL, 1984. Initial submission: Letter from Reilly Tar & Chem. Corp. to US EPA regarding production, process, safety & handling, and toxicity of 4-methylpyridine. Reilly Tar & Chemical Corp. EPA Doc. FYIOTS-0794-0986, microfiche no. OTS0000986. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Gorrod JW and Damani LA, 1980. The metabolic N-oxidation of 3-substituted pyridine in various animal species in vivo. Eur. J. Drug Metab. Pharm. 5(1), 53-57.

Grubbs CJ, Steele VE, Casebolt T, Juliana MM, Eto I, Whitaker LM, Dragnev KH, Kelloff GJ and Lubet RL, 1995. Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol. Anticancer Research 15, 709-716.

Hashizume T, Santo H, Tsujisawa H, Kosaka K, Ozawa T, Yamashita M and Kinae N, 1991. Mutagenic activities of tryptophan metabolites before and after nitrite treatment. Food and Chemical Toxicology 29(12), 839-844.

Hawksworth G and Scheline RR, 1975. Metabolism in the rat of some pyrazine derivatives having flavour importance in foods. Xenobiotica 5(7), 389-399.

Haworth S, Lawlor T, Mortelmans K, Speck W and Zeiger E, 1983. Salmonella mutagenicity test results for 250 chemicals. Environmental Mutagenesis 5(Suppl. 1), 3-142.

IOFI (International Organization of the Flavor Industry), 1995. European inquiry on volume of use. IOFI, International Organization of the Flavor Industry, 1995.

Izmerov NF, Sanotsky IV and Sidorov KK, 1982. Toxicometric Parameters of Industrial Toxic Chemicals Under Single Exposure. Moscow, Centre of International Project, GKNT, 1982. Cited in RTECS. Chemical name: hexanal, 2-ethyl-. Section Toxicity data.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1995. Evaluation of certain food additives and contaminants. Forty-fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. 14-23 February 1995. WHO Technical Report Series, no. 859. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1996. Toxicological evaluation of certain food additives. Forty-fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives and contaminants. WHO Food Additives Series: 35. IPCS, WHO, Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1997. Evaluation of certain food additives and contaminants. Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, 6-15 February 1996. WHO Technical Report Series, no. 868. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1999. Evaluation of certain food additives and contaminants. Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. Rome, 17-26 June 1997. WHO Technical Report Series, no. 884. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2000. Evaluation of certain food additives. Fifty-first Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, 9-18 June 1998. WHO Technical Report Series, no. 891. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2005a. Compendium of food additive specifications. Addendum 12. Joint FAO/WHO Expert Committee of Food Additives 63rd session. Rome, 8-17 June 2004. FAO Food and Nutrition paper 52 Add. 12.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2005b. Evaluation of certain food additives. Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, no. 928. Geneva, 8-17 June 2004.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2006. Safety evaluation of certain food additives and contaminants. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives, WHO Food Additives Series: 54. IPCS, WHO, Geneva.

Kaden DA, Hites RA and Thilly WG, 1979. Mutagenicity of soot and associated polycyclic aromatic hydrocarbons to Salmonella typhimurium. Cancer Research 39, 4152-4159.

Kaiser K, 1953. [Investigation on the carcinogenic activity of indol in rats]. Z. Krebsforsch. 59(4), 488-495. (In German)

Kim E-H, Shinohara K, Murakami H and Omura H, 1987. Pyrrole compounds as food mutagens. Journal of the Faculty of Agriculture, Kyushu University 31, 279-285.

Kim HY, Hincks JR, Huie JM, Yost GS and Coulombe Jr RA, 1989. Deuterated 3-methylindole is less genotoxic than 3-methylindole. Toxicologist 9(1), 154.

King LJ, Parke DV and Williams RT, 1966. The metabolism of (2-^{14}C) indolo in the rat. Biochemical Journal 98, 266-277.

LaVoie EJ, Shigematsu A, Adams EA and Hoffmann D, 1983. Comparative studies on the metabolism of quinoline and isoquinoline: Molecular basis for differences in mutagenic and carcinogenic activities. Toxicology Letters 18(Suppl. 1), 141.

Laws GM, Skopek TR, Reddy MV, Storer RD and Glaab WE, 2001. Detection of DNA adducts using a quantitative long PCR technique and the fluorogenic 5' nuclease assay (TaqMan). Mutation Research 484, 3-18.

Lee H, Bian SS and Chen YL, 1994. Genotoxicity of 1,3-dithiane and 1,4-dithiane in the CHO/SCE assay and the Salmonella/microsomal test. Mutation Research 321, 213-218.

Lehninger AL, 1982. Principles of biochemistry. Worth Publisers, Inc., New York.

Longfellow D, 1997. Mutagenicity studies. Benzothiazole. Short-term test program sponsored by the Division of Cancer Etiology, National Cancer Institute.

Martinez OB and Roe DA, 1972. Interrelationships of sulfate and glucuronide conjugation in indole-fed rats. Journal of Nutrition 102(3), 365-374.

McGee Laboratories Inc., 1974. Acute oral LD50 (rats). Dermal toxicity (rabbits - 5 gm/kg body weight dose). Skatole. Laboratories Inc. October, 29. Unpublished report submitted by EFFA to FLAVIS Secretariat.

McKee RH, Phillips RD, Lerman SA, Slesinski RS, Rogers-Back AM, Curren RD and Putman DL, 1987. The genotoxic potential of isophorone. Environmental Mutagenesis 9(8), 71.

McKennis H, Turnbull LB and Bowman ER, 1964. Additional routes in the metabolism of 3-acetylpyridine. Journal of Biological Chemistry 239(4), 1215-1220.

Miller ON, Hamilton JG and Goldsmith GA, 1960. Investigation of the mechanism of action of nicotinic acid on serum lipid levels in man. American Journal of Clinical Nutrition 8, 480-490.

Moran EJ, Easterday DD and Oser BL, 1980. Acute oral toxicity of selected flavor chemicals. Drug and Chemical Toxicology 3(3), 249-258.

Myers RC and Ballantyne B, 1997. Comparative acute toxicity and primary irritancy of various classes of amines. Toxic Subst. Mech. 16(2), 151-193.

Nguyen PL, Saint-Jalm Y, Duterte-Catella H, Truhaut R and Claude JR, 1988. Biotransformations of gamma-picoline in the rat. Archives of Toxicology (Suppl. 12), 308-312.

Novello NJ, 1927. The fate of certain heterocyclic ring compounds in the animal body. Journal of Biological Chemistry 74(1), 33-36.

Ochiai M, WakabaYashi K, Sugimura T and Nagao M, 1986. Mutagenicities of indole and 30 derivatives after nitrite treatment. Mutation Research 172, 189-197.

Pai V, Bloomfield SF, Jones J and Gorrod JW, 1978. Mutagenicity testing of nitrogenous compounds and their N-oxidised products using Trp+ reversion in E. coli. In: Gorrod JW (Ed.). Biological Oxidation of Nitrogen in organic compounds, Elsevier North Holland Biomedical Press, New York, pp. 375-382.

Parkinson A, 1996. Biotransformation of xenobiotics. In: Klaassen, C.D. (Ed.). Casarret and Doull's Toxicology: The Basic Science of Poisons. 5th Ed. McGraw-Hill, New York, pp.113-186.

Pellmont B, 1977. Acute oral toxicology of methylnicotinate. August 18. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Posner HS, Mitoma C and Udenfriend S, 1961. Enzymatic hydroxylation of aromatic compounds. II. Further studies of the properties of microsomal hydroxylating system. Archives of Biochemistry and Biophysics 94(2), 269-279.

Posternak JM, Dufour JJ, Rogg C and Vodoz CA, 1975. Summaries of toxicological data. Toxicological tests on flavouring matters. II. Pyrazines and other compounds. Food and Cosmetics Toxicology 13, 487-490.

Pullin TG, Edwards HN and Schwebel RL, 1973. Acute toxicological properties of gamma picoline (4-methyl pyridine) with cover letter. Dow Chemical Co. EPA Doc. 878214752, microfiche no. OTS0206650. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Reddy MV, Storer RD, Laws GM, Armstrong MJ, Barnum JE, Gara JP, McKnight CG, Skopek TR, Sina JF, DeLuca JG and Galloway SM, 2002. Genotoxicity of naturally occurring indole compounds: Correlation between covalent DNA binding and other genotoxicity tests. Environmental and Molecular Mutagenesis 40(1), 1-17.

Reddy MV, Laws GM, Armstrong MJ, Galloway SM and Storer RD, 2003. *In vivo* genotoxicity of 3-methylindole and melatonin: Formation of DNA adducts but not micronuclei. Proceedings of the American Association for Cancer Research 44, 291-292.

Reigh G, McMahon H, Ishizaki M, Ohara T, Shimane K, Esumi Y, Green C, Tyson C and Ninomiya S-I, 1996. Cytochrome P450 species involved in the metabolism of quinoline. Carcinogenesis 17(9), 1989-1996.

Ruangyuttikarn W, Skiles GL and Yost GS, 1992. Identification of a cysteinyl adduct oxidized 3-methylindole from goat lung and human liver microsomal proteins. Chemical Research in Toxicology 5, 713-719.

Sasagawa C and Matsushima T, 1991. Mutagen formation on nitrile treatment of indole compounds derived from indole-glucosinolate. Mutation Research 250(1/2), 169-174.

SCF (Scientific Committee for Food), 1995. Scientific Committee for Food. First annual report on chemically defined flavouring substances. May 1995, 2nd draft prepared by the SCF Working Group on Flavouring Substances (Submitted by the SCF Secretariat, 17 May 1995). CS/FLAV/FL/140-Rev2. Annex 6 to Document III/5611/95, European Commission, Directorate-General III, Industry.

SCF (Scientific Committee for Food), 1999. Opinion on a programme for the evaluation of flavouring substances (expressed on 2 December 1999). Scientific Committee on Food. SCF/CS/FLAV/TASK/11 Final 6/12/1999. Annex I to the minutes of the 119th Plenary meeting. European Commission, Health & Consumer Protection Directorate-General.

SCF (Scientific Committee for Food), 2000. Scientific Committee on Food. Guidelines of the Scientific Committee on Food for the development of tolerable upper intake levels for vitamins and minerals. (Adopted on 19 October 2000). Scientific Committee on Food SCF/CS/NUT/UPPLEV/11 Final. 28 November, 2000. European Commission, Health & Consumer Protection Directorate-General.

SCF (Scientific Committee for Food), 2002. Opinion of the Scientific Committee for Food on the Tolerable Upper Intake Level of Nicotinic Acid and Nicotinamide, expressed on 17 April 2002. SCF/CS/NUT/UPPLEV/39 Final, 6th May 2002. European Commission, Health & Consumer Protection Directorate-General.

Scharping CE, Duke CC, Holder GM and Larden D, 1993. The hepatic metabolism of two methylquinolines. Carcinogenesis 14(5), 1041-1047.

Schriewer L, Vleminckx C, Rigaux G, Ottogali M and Lakhanisky T, 1993. Evaluation of the genotoxic potential of pyridine and methylated pyridines. Pyridine, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine. C. DNA single-stranded breaks measurement in V79 cells. Reilly Industries. Institute of Hygiene and Epidemiology. Vleminckx, C. Study no. IHE-TOX-1003. EPA Doc 86-930000182, microfiche no. OTS0538163. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Shigenaga MK, Kim BH, Caldera-Munoz P, Cairns T, Jacob III P, Trevor AJ and Castagnoli Jr N, 1989. Liver and lung microsomal metabolism of the tobacco alkaloid beta-nicotyrine. Chemical Research in Toxicology 2, 282-287.

Skiles GL and Yost GS, 1992. Stable-isotope mechanistic studies on the oxidation of 3-methylindole. Toxicologist 12(1), 289.

Skiles GL, Appleton ML, Smith DJ, Carlson JR and Yost GS, 1991. Isolation of a mercapturate adduct produced subsequent to glutathione conjugation of bioactivated 3-methylindole. Toxicology and Applied Pharmacology 108, 531-537.

Skordos KW, Laycock JD and Yost GS, 1998. Thioether adducts of a new imine reactive intermediate of the pneumotoxin 3-methylindole. Chemical Research in Toxicology 11(11), 1326-1331.

Smith DJ, Skiles GL, Appleton ML, Carlson JR and Yost GS, 1993. Identification of goat and mouse urinary metabolites of the pneumotoxin, 3-methylindole. Xenobiotica 23(9), 1025-1044.

Smyth Jr HF, Carpenter CP and Weil CS, 1951. Range finding toxicity data: List IV. Archives of Industrial Hygiene and Occupational Medicine 4, 119-122.

Smyth Jr HF, Carpenter CP, Weil CS, Pozzani UC and Striegel JA, 1962. Range-finding toxicity data: List VI. American Industrial Hygiene Association Journal 23, 95-107.

Spanjers MT and Til HP, 1968. Acute oral toxicity of acetylpyridine in rats. Centraal Instituut Voor Voedingsonderzioek, Netherlands. Report no. R2794. December, 1968. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Steiner A, Mayer JM and Testa B, 1992. Nicotinate esters: Their binding to and hydrolysis by human serum albumin. Journal of Pharmacy and Pharmacology 44(9), 745-749.

Stoner G, Casto B, Ralston S, Roebuck B, Pereira C and Bailey G, 2002. Development of a multi-organ rat model for evaluating chemopreventive agents: efficacy of indole-3-carbinol. Carcinogenesis 23 (2), 265-272.

Szybalski W, 1958. Special microbiological systems. II. Observations on chemical mutagenesis in microorganisms. Annals of the New York Academy of Sciences 76, 475-489.

Tada M, Takahashi K and Kawazoe Y, 1982. Metabolites of quinoline, a hepatocarcinogen, in a subcellular microsomal system. Chemical and Pharmaceutical Bulletin 30(10), 3834-3837.

Tanaka T, Mori Y, Morishita Y, Hara A, Ohno T, Kojima T and Mori H, 1990. Inhibitory effect of sinigrin and indole-3-carbinol on diethylnitrosamine-induced hepatocarcinogenesis in male ACI/N rats. Carcinogenesis 11(8), 1403-1406.

Tanaka T, Kojima T, Morishita Y and Mori H, 1992. Inhibitory effects of the natural products indole-3-carbinol and sinigrin during initiation and promotion phases of 4-nitroquinoline 1-oxide induced rat tongue carcinogenesis. Jpn. J. Cancer Res. 83, 835-842.

Taylor ML and Olson KJ, 1964. Results of range finding toxicological tests on 2-picoline. Dow Chemical Co. EPA Doc 878214756, microfiche no. OTS0206650. Date 010864. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Thornton-Manning JR, Nichols WK, Manning BW, Skiles GL and Yost GS, 1993. Metabolism and bioactivation of 3-methylindole by Clara cells, alveolar macrophages and subcellular fractions from rabbit lungs. Toxicology and Applied Pharmacology 122, 182-190.

Til HP and van der Meulen HC, 1971. Subchronic (90-day) toxicity study with 2-acetylpyridine in albino rats. Centraal Instituut Voor Voedingsonderzioek, Netherlands. Report no. R3373. February, 1971. Unpublished report submitted by EFFA to FLAVIS Secretariat.

TNO (Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek), 2000. VCF Volatile Compounds in Food. Nijssen LM, van Ingen-Visscher CA and Donders JJH (Eds.). Database. Zeist, The Netherlands. TNO Triskelion, 1963-2000.

Vance WA, Okamoto HS and Wang YY, 1986. Structure-activity relationships of nitro and methyl-nitro derivatives of indoline, indole, indazole and benzimidazole in *Salmonella typhimurium*. Mutation Research 173 (3), 169-176.

Vleminckx C, Ottogali M, Schriewer L, Rigaux G and Lakhanisky T, 1993a. Evaluation of the genotoxic potential of pyridine and methylated pyridines. Pyridine, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine. A. Salmonella/microsome test. Reilly Industries with cover letter dated 03/29/93. Study no. IHE-TOX-1003. Date 3/29/93. EPA Doc. 86-930000180, microfiche no. OTS0538163. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Vleminckx C, Rigaux G, Schriewer L, Ottogali M and Lakhanisky T, 1993b. Evaluation of the genotoxic potential of pyridine and methylated pyridines. Pyridine, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine. B. HGPRTgene mutation assay in V79 cells. Reilly Industries. Study no. IHE-TOX-1003. EPA Doc. 86-930000181, microfiche no. OTS0538163. Unpublished report submitted by EFFA to FLAVIS Secretariat.

Wang CJ, Lin YL and Lin JK, 1994. Mutagenicity and cytotoxicity of nitropyrrole compounds derived from the reaction of 2-acetyl pyrrole with nitrite. Food and Chemical Toxicology 32(9), 839-844.

Whittaker SG, Zimmermann FK, Dicus B, Piegorsch WW, Fogel S and Resnick MA, 1989. Detection of induced mitotic chromosome loss in *Saccharomyces cerevisiae* - an interlaboratory study. Mutation Research 224(1), 31-78.

Williams GM, 1984. DNA damage and repair tests for the detection of genotoxic agents. Food Additives and Contaminants 1(2), 173-178.

Yost GS, Kuntz DJ and McGill LD, 1990. Organ-selective switching of 3-methylindole toxicity by glutathione depletion. Toxicology and Applied Pharmacology 103, 40-51.

Zeiger E, Anderson B, Haworth S, Lawlor T and Mortelmans K, 1992. Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. Environmental and Molecular Mutagenesis 19(21), 2-141.

Zimmermann FK, Henning JH, Schell I and Oehler M, 1986. Genetic and anti-tubulin effects induced by Pyridine derivatives. Mutation Research 163, 23-31.

APPENDIX A: PROCEDURE FOR THE SAFETY EVALUATION

The approach for a safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000 (EC, 2000), 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, 1999), which is derived from the evaluation Procedure developed by the Joint FAO/WHO Expert Committee on Food Additives at its 44th, 46th and 49th meetings (JECFA, 1995; JECFA, 1996; JECFA, 1997; JECFA, 1999).

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 µg/person/day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996).

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

- can the flavourings be predicted to be metabolised to innocuous products[11] (Step 2)?
- do their exposures exceed the threshold of concern for the structural class (Step A3 and B3)?
- are the flavourings or their metabolites endogenous[12] (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.

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

[12] *"Endogenous substances": Intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included (JECFA, 1997).*

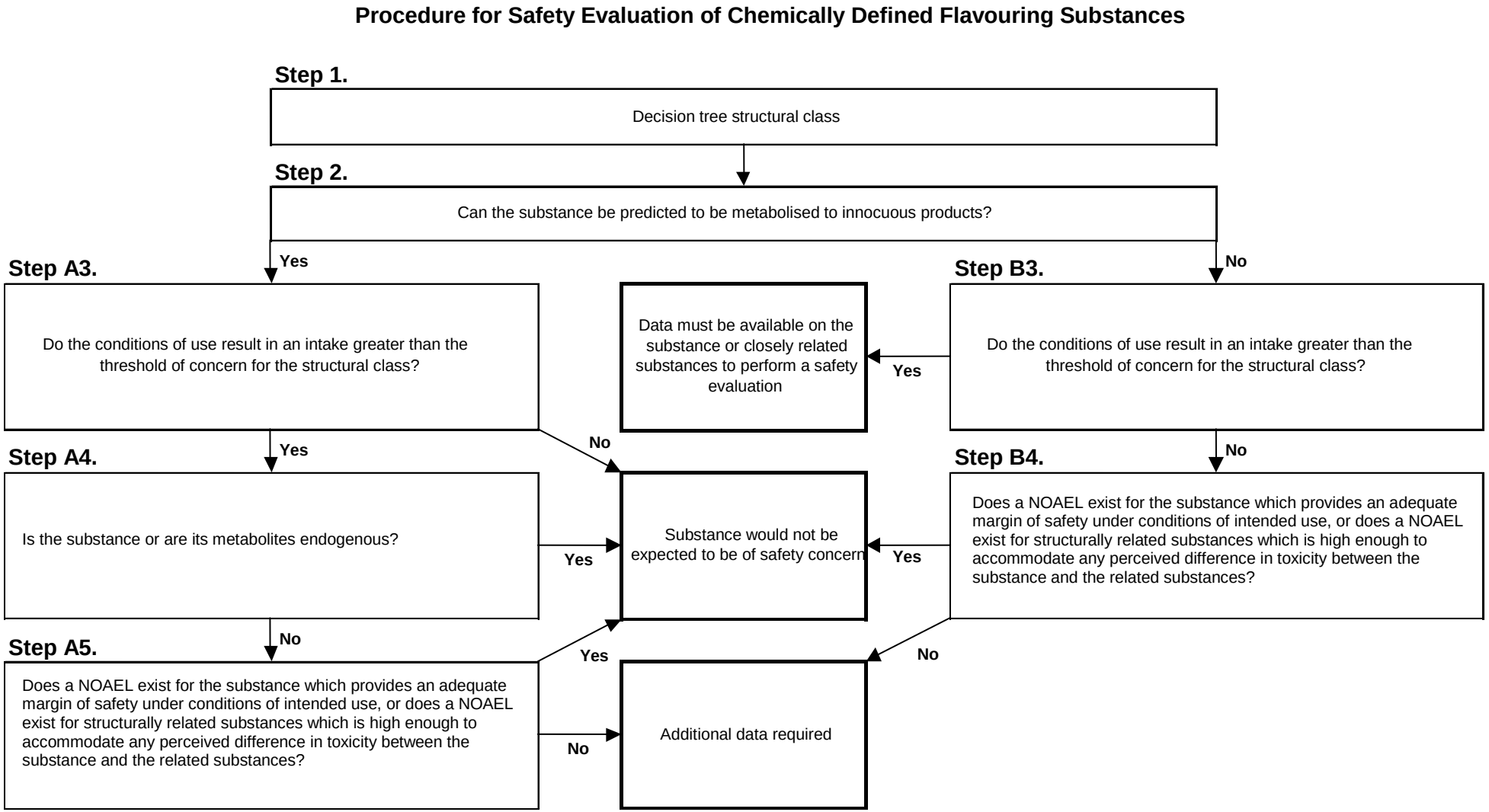


Figure I.1 *Procedure for Safety Evaluation of Chemically Defined Flavouring Substances*

APPENDIX B: 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, 2000). 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, 2002). The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004a).

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

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)
V 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 the 24 candidate substances in the present flavouring group (Table II.1.2). The table also contains "normal and maximum use levels" for the nine substances no longer supported by Industry for use as flavouring substances in Europe: [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169] (DG SANCO, 2013; EFSA, 2011).

Table II.1.2 Normal and Maximum use levels (mg/kg) for the candidate substances in FGE.24 (EFFA, 2004b; EFFA, 2007; Flavour Industry, 2005).

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
13.100	0,4 2	0,2 1	0,4 2	0,3 1,5	- -	0,4 2	0,2 1	0,4 2	0,1 0,4	0,1 0,4	- -	- -	0,2 1	0,4 2	0,2 1	0,4 2	1 5	0,2 1
14.002	0,4 2	0,2 1	0,4 2	0,3 1,5	- -	0,4 2	0,2 1	0,4 2	0,1 0,4	0,1 0,4	- -	- -	0,2 1	0,4 2	0,2 1	0,4 2	1 5	0,2 1
14.023	4 2	0,1 0,5	0,4 2	0,4 2	- -	1 5	2 1	2 10	0,2 1	0,2 1	- -	- -	0,3 1,5	0,2 1	0,2 1	1 5	1 5	0,2 1
14.085	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
14.088	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
14.089	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
14.092	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
14.093	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
14.094	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
14.103	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

Table II.1.2 Normal and Maximum use levels (mg/kg) for the candidate substances in FGE.24 (EFFA, 2004b; EFFA, 2007; Flavour Industry, 2005).

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
14.104	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.105	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.106	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.107	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.110	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.115	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	-	0,2	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	-	1	-	0,5	1	1	5	5	1
14.116	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.117	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.118	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.120	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.124	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.125	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.131	0,4	0,2	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	1	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.134	0,4	0,2	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	1	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.135	0,4	0,2	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	1	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.136	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.138	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.140	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.143	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.145	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.150	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.163	0,4	0,1	0,4	0,4	-	1	0,2	2	0,2	0,2	-	-	0,1	0,2	0,2	1	1	0,2
	2	0,5	2	2	-	5	1	10	1	1	-	-	0,5	1	1	5	5	1
14.169	0,6	-	0,6	-	-	1,2	0,6	1,2	-	-	-	-	-	-	0,3	1,2	-	0,6
	1,8	-	1,8	-	-	3,6	1,8	3,6	-	-	-	-	-	-	0,9	3,6	-	1,8

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, 2000) 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, 2000)
- 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, 2000)
- Exception a (SCF, 1995) corresponds to food category 5 and 11 (EC, 2000)
- Exception b (SCF, 1995) corresponds to food category 15 (EC, 2000)
- Exception c (SCF, 1995) corresponds to food category 14.2 (EC, 2000)
- Exception d (SCF, 1995) corresponds to food category 12 (EC, 2000)
- 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, 2000) 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 each of the 24 flavouring substances in the present flavouring group, for which Industry has provided use and use levels (EFFA, 2004b; Flavour Industry, 2005; EFFA, 2007). The mTAMDI values are only given for the highest reported normal use levels (See Table II.2.3). The table also contains the mTAMDI values for the nine substances no longer supported by Industry for use as flavouring substances in Europe: [FL-no: 13.100, 14.002, 14.023, 14.094, 14.107, 14.138, 14.145, 14.163 and 14.169] (DG SANCO, 2013; EFSA, 2011).

TableII.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)**
14.110	Ethyl nicotinate	400	Class II	540
14.120	Isopropyl nicotinate	400	Class II	540
14.023	1-Methylpyrrole	670	Class II	540
14.085	2-Acetyl-5-methylpyrrole	400	Class II	540

14.089	4-Acetylpyridine	400	Class II	540
14.092	2-Butylpyridine	400	Class II	540
14.093	3-Butylpyridine	400	Class II	540
14.103	2,3-Dimethylpyridine	400	Class II	540
14.104	2,4-Dimethylpyridine	400	Class II	540
14.105	3,4-Dimethylpyridine	400	Class II	540
14.106	3,5-Dimethylpyridine	400	Class II	540
14.107	2,5-Dimethylpyrrole	400	Class II	540
14.115	2-Ethylpyridine	400	Class II	540
14.116	4-Ethylpyridine	400	Class II	540
14.117	2-Hexylpyridine	400	Class II	540
14.118	2-Hydroxypyridine	400	Class II	540
14.124	2-Isopropylpyridine	400	Class II	540
14.125	4-Isopropylpyridine	400	Class II	540
14.134	2-Methylpyridine	400	Class II	540
14.135	3-Methylpyridine	400	Class II	540
14.136	4-Methylpyridine	400	Class II	540
14.140	3-Pentylpyridine	400	Class II	540
14.143	3-Propylpyridine	400	Class II	540
14.145	Pyrrole-2-carbaldehyde	400	Class II	540
14.150	2,4,6-Trimethylpyridine	400	Class II	540
14.169	1-Ethyl-2-pyrrolecarboxaldehyde	310	Class II	540
13.100	2-Acetyl-1-furfurylpyrrole	160	Class III	90
14.088	1-Acetylindole	400	Class III	90
14.131	2-Methylindole	400	Class III	90
14.163	1-Methylpyrrole-2-carboxaldehyde	400	Class III	90
14.002	4-Methylquinoline	160	Class III	90
14.094	4-Butylquinoline	400	Class III	90
14.138	2-Methylquinoline	400	Class III	90

APPENDIX C: METABOLISM

III.1. Introduction

This flavouring group consists of 24 candidate substances, all of which have one heterocyclic N-atom in a five- or six-membered ring structure. Twenty-two of the compounds are monocyclic, with either a five-membered ring (pyrroles) [FL-no: 14.085] or a six-membered ring (pyridines) [FL-no: 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.110, 14.115, 14.116, 14.117, 14.118, 14.120, 14.124, 14.125, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150]. The remaining two candidate substances are aromatic bicyclic structures consisting of a six- and a five-membered ring (indoles) [FL-no: 14.088 and 14.131]. The substances are substituted with simple alkyl, alcohol, ketone or ester moities.

The candidate substances are divided into four subgroups with similar structural properties as shown in Table III.1 (The substances previously allocated to subgroups 2 and 6 are no longer supported by Industry (See Section 1)).

A group with 15 related supporting substances has been evaluated by the JECFA (JECFA, 2006).

Table III.1 Candidate substances divided into groups of related chemical structures.

Subgroup	Candidate substance	FL-no	Chemical group
1	2-Acetyl-5-methylpyrrole	14.085	
2	The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry		N-substituted pyrroles
3	2-Methylindole	14.131	Indoles
	1-Acetylindole	14.088	
4	2-Methylpyridine	14.134	Pyridines with alkyl, hydroxyl or acetyl groups
	3-Methylpyridine	14.135	
	4-Methylpyridine	14.136	
	2-Ethylpyridine	14.115	
	4-Ethylpyridine	14.116	
	3-Propylpyridine	14.143	
	2-Isopropylpyridine	14.124	
	4-Isopropylpyridine	14.125	
	2-Butylpyridine	14.092	
	3-Butylpyridine	14.093	
	3-Pentylpyridine	14.140	
	2-Hexylpyridine	14.117	
	2,3-Dimethylpyridine	14.103	
	2,4-Dimethylpyridine	14.104	
	3,4-Dimethylpyridine	14.105	
	3,5-Dimethylpyridine	14.106	
	2,4,6-Trimethylpyridine	14.150	
	2-Hydroxypyridine	14.118	
	4-Acetylpyridine	14.089	
5	Ethyl nicotinate	14.110	Pyridines with ester groups
	Isopropyl nicotinate	14.120	
6	The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry		Quinolines

III.2. Absorption, Distribution and Elimination

Nicotinic acid resulting from the hydrolysis of isopropyl nicotinate [FL-no: 14.120] and ethyl nicotinate [FL-no: 14.110] is absorbed and excreted in humans given an oral dosage of 3000 mg as shown by 88 % recovery in urine (time not reported) (Miller et al., 1960).

Rabbits given subcutaneous injections of 500 mg of pyrrole on alternate days exhibited a rapid increase in the urinary elimination of urea nitrogen (40 to 50 % of the pyrrole dose) over the first 24 hours (Novello, 1927).

The excretion of indole in rats is fairly rapid with 75 % of the dosage excreted after 24 hours, and more than 80 % of the radioactivity recovered from the pooled 48-hour urine of female albino Wistar rats given a single oral dose of 64 to 80 mg/kg [2-^{14}C]-indole. Urine, faeces and expired air contained 80.6, 11.1, and 2.4 %, respectively, of the radioactivity (King et al., 1966).

Twenty-four male Holtzman rats were administered diets supplemented with 0, 0.25, 0.50 or 0.75 % (corresponding to 0, 150, 300 and 450 mg/kg bw/day) indole for three weeks. A fifth group of six rats receiving 0.75 % indole was supplemented with 0.25 % methionine in the diet. During week 3, all groups (including controls) received a single dose of [2-^{14}C]-indole via stomach tube. Urine collected for 72 hours after the single dose administration revealed that 32, 47, 49, 51 and 62 % of the radioactive dose were recovered from the 0 (control), 0.25, 0.50, 0.75 % indole groups, and the 0.75 % indole plus 0.25 % methionine groups, respectively, demonstrating a slower elimination of indole from the body than reported by King et al., 1966 (Martinez and Roe, 1972).

Mice and rats given single intraperitoneal injections of 400 mg/kg of [^{14}C]-3-methylindole excreted 69.4 % and 66.2 % of the total radioactivity, respectively, after 48 hours (Skiles et al., 1991).

In male rats administered a dosage of 100 mg/kg bw of 2,3-dimethylpyridine [FL-no: 14.103] by gavage, 50 % of the administered dosage were excreted in the urine after 24 hours (Hawksworth and Scheline, 1975). Ninety-six per cent of a 100 mg/kg bw dose of 2-methylpyridine administered via gavage to male albino Wistar rats was excreted within 24 hours (Hawksworth and Scheline, 1975). More than 50 % of a 300 mg/kg bw dose of 4-methylpyridine, administered to Wistar rats (3/sex) via gavage was excreted in the urine after 24 hours (Nguyen et al., 1988).

Studies with indoles and 2-methyl- and 2,3-dimethyl pyridine indicate that the candidate indoles and pyridines are absorbed from the gastrointestinal tract and fairly rapidly excreted in the urine. No data on absorption and excretion are available for the candidate pyrroles and quinolines.

III.3. Biotransformation

Ester and amide hydrolysis

Two studies on the hydrolysis of the candidate substances ethyl nicotinate [FL-no: 14.110] and isopropyl nicotinate [FL-no: 14.120] are reported.

Several nicotinates were studied *in vitro* for their binding to and hydrolysis by human serum albumin. After 90 minutes of dialysis, 4.1 and 0 % of ethyl nicotinate and isopropyl nicotinate, respectively, were hydrolysed in human serum albumin as compared to 0.1 and 0 % in buffer. The percentage bound by human serum albumin was 35.6 and 34.1 % for ethyl nicotinate and isopropyl nicotinate, respectively (Steiner et al., 1992).

In a study of the effect of substrate on the rate of carboxyesterase-catalysed hydrolysis, the steady state kinetic constants for a series of nicotinates were determined. Purified hog liver carboxylesterase and human plasma containing carboxyesterase were incubated with ethyl nicotinate and isopropyl

nicotinate. The maximal velocity (Vmax) for ester hydrolysis in purified hog liver carboxyesterase and human plasma is 18.4 mmol/min/mg protein and 2.16 µmol/min/mg protein, respectively, for ethyl nicotinate and 97.8 mmol/min/mg protein and 0.20 µmol/min/mg protein, respectively, for isopropyl nicotinate (Durrer et al., 1992).

No study on the hydrolysis of the amide bond in the candidate substance 1-acetylindole [FL-no: 14.088] was reported. Amides are hydrolysed by the same enzymes as the esters, although the hydrolysis of amides generally occurs more slowly (Parkinson 1996, Chapter 6 Casarett & Doull's).

The hydrolysis of ethyl nicotinate and isopropyl nicotinate in human serum is expected to be relatively slow. However, purified hog liver carboxylesterase hydrolyses the same compounds rapidly, which might result from the different enzyme concentrations used. A rapid hydrolysis of 1-acetylindole in the Gastro-intestinal (GI) tract is not anticipated although hydrolysis by amidases in the tissues might be expected.

Metabolism of pyrroles (subgroup 1)

Pyrrole is an electron-rich compound, electron density being high at all positions of the ring but greatest at C-2 and C-5. It is very reactive toward electrophilic attack at C-2 and resistant to nucleophiles. Pyrrole is a likely substrate for electrophilic enzymatic oxidation at N-1 and hydroxylation at C-2 and C-5 (Damani and Crooks, 1982).

No metabolism studies were found for the candidate substance 2-acetyl-5-methylpyrrole [FL-no: 14.085].

Metabolism of indoles (subgroup 3)

No metabolism studies were found for the candidate substances 2-methylindole and 1-acetylindole [FL-no: 14.131 and 14.088]. However, for the supporting compounds indole [FL-no: 14.007] and 3-methylindole (skatole) [FL-no: 14.004] several metabolic studies are available.

The main metabolite of indole in various species is 3-hydroxylindole (indoxyl) and with the 2-oxygenated and 5-hydroxylated metabolites oxindole and 5-hydroxyoxindole as minor metabolites (Figure III.2) (Damani and Crooks, 1982).

Ring hydroxylation on both rings of indole compounds has been well documented (Figures III.1 and III.2), and indole is very reactive toward electrophilic attack at C-3. Following oral dosing of three albino Wistar rats with single doses of 64 to 74 mg/kg bw of [2-^{14}C]-indole, 63 % of the dose was detected in 48-hour urine pools as 3-hydroxyindole (indoxyl), as sulphate conjugate (49.6 %) and glucuronide conjugate (13.2 %). In two of the rats, other metabolites identified in the urine included 3.5 % 5-hydroxyoxindole, 1.4 % indole-2-one and 5.8 % indole-2,3-dione (isatin). Analysis of the faecal excretions showed 0.14, 0.40, and 0.64 % radiolabel as indole, 3-hydroxyindole sulphate and total 3-hydroxyindole metabolites, respectively (King et al., 1966). In a parallel study, 48-hour bile samples of two female albino Wistar rats given 49 to 63 mg/kg bw [2-^{14}C]-indole orally, contained 0.56, 0.80, and 0.82 % of the radiolabel as 5-hydroxyoxindole, 3-hydroxyindole sulphate and total 3-hydroxyindole metabolites, respectively, as minor metabolites (King et al., 1966).

Twenty-four male Holtzman rats were administered diets supplemented with 0, 0.25, 0.50 or 0.75 % (corresponding to 0, 150, 300 and 450 mg/kg bw/day) indole for three weeks. A fifth group of six rats receiving 0.75 % indole was supplemented with 0.25 % methionine in the diet. During week 3, all groups (including controls) received a single dose of [2-^{14}C]-indole *via* stomach tube. Urine collected for 72 hours after the single dose administration revealed the two main radioactive metabolites as 3-indolyl sulphate and 3-indolyl glucuronidate. The amount excreted as glucuronidate was increasing with increasing dose of administered indole (Martinez and Roe, 1972)

3-Hydroxyindole was the primary metabolite isolated when indole was incubated with freshly prepared rabbit liver microsomes (Posner et al., 1961). 3-Hydroxyindole may further be oxidised to indigo (2-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,2-dihydro-3*H*-indol-3-one) (Posner et al., 1961). Incubation of indole with rat liver microsomes in the presence of glucose-6-phosphate, nicotinamide, and NADPH for one hour demonstrated the formation of ring oxidised metabolites including indigo, indirubin (3-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,3-dihydro-2*H*-indol-2-one) and oxindole (1,3-dihydro-2*H*-indol-2-one) (King et al., 1966). When indole was incubated with recombinant human CYP450 enzymes, 2A6, 2C19, and 2E1 co-expressed in *Escherichia coli* with NADPH-P450 reductase, several metabolites was detected. Oxindole was detected as the major metabolite, while indole-2,3-dione, 6-hydroxyindole, dioxindole, indigo and indirubin was detected in less amounts. 3-Hydroxyindole was observed as a transient product (Gillam et al., 2000).

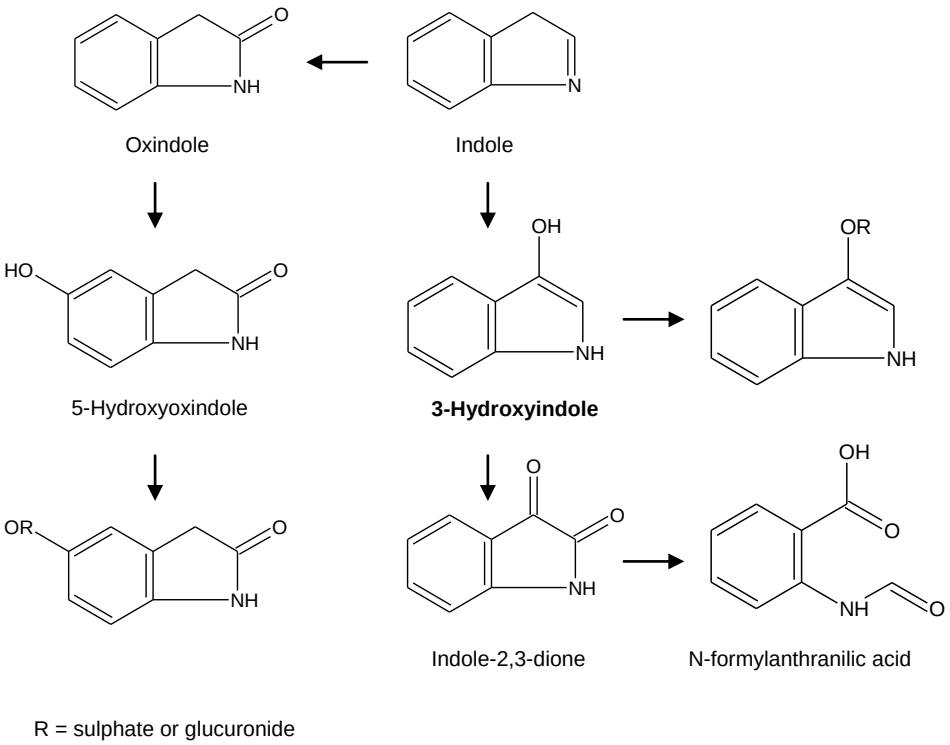


Figure III.1. *The metabolism of indole in rats (King et al., 1966). Main metabolism product is in bold.*

Mice and rats given single intraperitoneal injections of 400 mg/kg of [*methyl*-^{14}C]-3-methylindole excreted 2.6 and 7.3 %, respectively, as the mercapturic acid conjugate of 3-methylindole, 3-[(*N*-acetylcystein-Syl)methyl]indole (Skiles et al., 1991). The mercapturic acid conjugate is likely formed *via* a reactive 3-methylene iminium ion that may be generated either directly *via* CYP450 mediated oxidation of the methyl substituent or indirectly *via* ready dehydration of indole-3-carbinol (Skiles and Yost, 1992).

At least six metabolites were isolated from the urine 36 hours after male Swiss-Webster mice received a 400 mg/kg dose of ring labelled [*ring*-UL-^{14}*C*]-3-methylindole by intraperitoneal injection (Figure III.2). Three primary pathways were characterised. In one, side chain oxidation yields indole-3-carbinol. Indole-3-carbinol is then oxidised to the corresponding carboxylic acid. Alternatively, 3-methylindole can be converted to the reactive substance 3-methyleneindolenine, which subsequently is conjugated with glutathione to yield 3-[(*N*-acetylcystein-*S*-yl)methyl]indole. In the third pathway, the 2,3-alkene is epoxidised to yield 3-methyloxindole or 3-hydroxy-3-methylindolenine intermediates. These metabolites are conjugated with glucuronic acid or sulphate, followed by excretion in the urine or are further oxidised to yield a series of dihydroxy-3-methyloxindole metabolites that are also

conjugated and excreted. The second pathway through epoxidation of 3-methylindole predominates (Smith et al., 1993). The existence of a cytochrome P450-dependent 2,3-epoxide of 3-methylindole was indirectly confirmed using stable isotope techniques and mass spectrometry. After incubation of goat lung microsomes with [2-^{2}H]-3-methylindole, metabolically formed 3-methyloxindole was produced by NIH shift of the deuterium from position 2 to position 3, a process which is consistent with epoxide ring opening (Skordos et al., 1998).

Incubation of 3-methylindole (0.5 mM) with rabbit Clara cells and alveolar macrophages in combination with either *N*-acetylcystein (NAC) or glutathione (GSH) yielded four metabolites, two metabolites derived from side chain oxidation were indole-3-carbinol and the 3-(glutathione-*S*-yl)methylindole or 3-(*N*-acetylcysteine-*S*-yl)-3-methylindole from incubation with GSH or NAC, respectively. The latter two were the major microsomal metabolites. Two other metabolites, presumably derived from epoxidation, were 3-methyloxindole and the metabolites from NAC and GSH incubation respectively, 2-(*N*-acetylcystein-*S*-yl)-3-hydroxy-3-methylindoline and 2-(glutathione-*S*-yl)-3-hydroxy-3-methylindoline. Incubation with excess glutathione (4 mM) produced the corresponding glutathione conjugates instead of the mercapturic acid conjugates (Thornton-Manning et al., 1993). Human liver microsomal protein fraction was incubated with labelled [^{14}C]-3-methylindole in the presence of NADPH. Hydrolysis of the isolated protein fraction indicated the presence of a cysteine conjugate at the 3 position of 3-methylindole. The authors suggested that a reactive 3-methyleneindolenine intermediate reacts with the cysteine thiol groups of target proteins (Ruangyuttikarn et al., 1992).

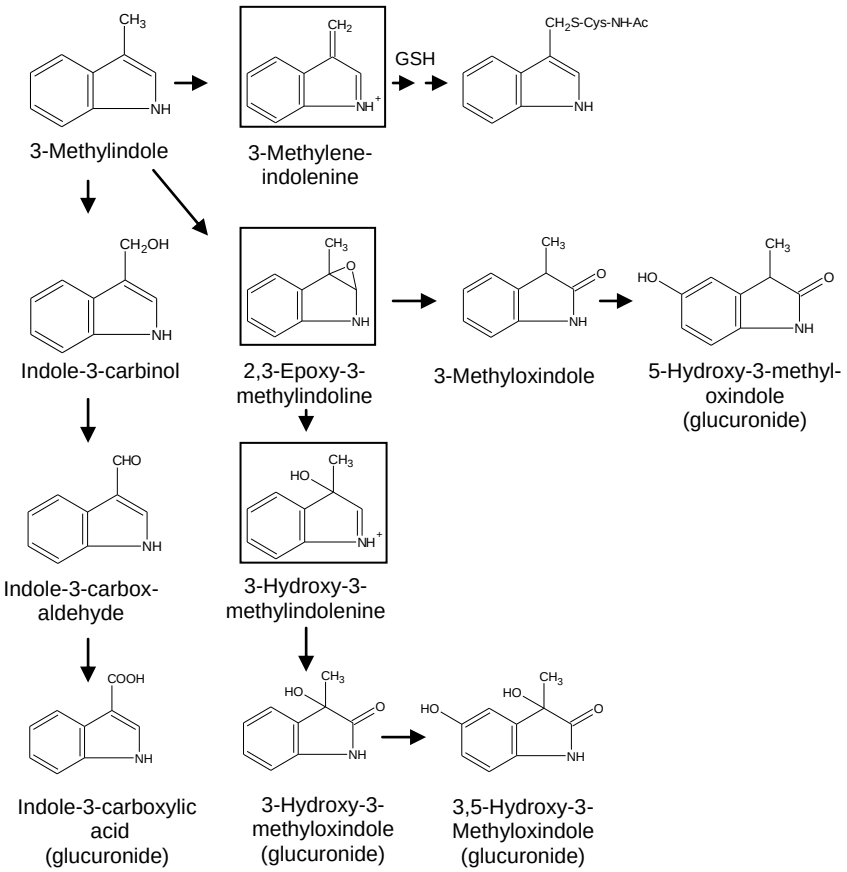


Figure III.2. *Metabolism of 3-methylindole in mice and goat (Skiles et al., 1991; Smith et al., 1993). Main metabolism product is in bold. Suggested intermediates are in frame.*

The major pathways of metabolism of 3-methylindole are oxygenation of the double bond in position 2 and 3 (epoxidation product), dehydrogenation of the methyl group and hydroxylation of the methyl

group and the ring structure, leading to metabolites mainly conjugated to glucuronic acid, sulphate and glutathione (Figure III.2).

Mice administered intraperitoneally 0 - 6 mmol/kg bw of *L*-buthionine-(*S*,*R*)sulphoximine (BSO), a specific inhibitor of GSH synthesis, three hours prior to treatment with 75 mg/kg bw of [*methyl*^{14}C]-3-methylindole, showed a shift in toxicity from the pulmonary tissue to renal tissue. The reduction in glutathione level was high in the liver, with 88 % reduction, while the effect in the kidneys and lungs was less dramatic with only 38 % and 32 % reduction, respectively. Inhibition with BSO results in the lowest level of gluthatione in the kidneys, an organ which will also be affected by reduced detoxification by the liver. This shows the importance of glutathione in the detoxification of 3-methylindole and its metabolites (Yost et al., 1990).

Metabolism of pyridines

Electron density calculations show that electrophilic attack in pyridine is favoured at C-3, whereas nucleophilic attack occurs preferentially at C-2. The pyridyl nitrogen is a likely target for electrophilic enzymatic oxidation because of its relatively high electron negativity and its unshared pairs of electrons (Damani and Crooks, 1982).

Metabolism of pyridines substituted with alkyl groups (subgroup 4)

There are metabolism studies for four of the candidate pyridines substituted with alkyl groups, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine and 2,3-dimethylpyridine [FL-no: 14.134, 14.135, 14.136 and 14.103]. For the rest of the thirteen candidate pyridines substituted with alkyl groups no metabolism studies were found (see Table III.1). There are also metabolism studies for the supporting substances 3-ethylpyridine [FL-no: 14.061], 3-acetylpyridine [FL-no: 14.039] and 2,6-dimethylpyridine [FL-no: 14.065], as well as the structurally related 3-hydroxypyridine.

Ninety-six per cent of a 100 mg/kg bw dose of 2-methylpyridine [FL-no: 14.134] administered *via* gavage to male albino Wistar rats was excreted as the glycine conjugate, pyridine-2-carboxylic acid (picolinic acid) within 24 hours. No *N*-oxidation of 2-methylpyridine was observed with a detection limit of the metabolite at 1 % of the dose (Hawksworth and Scheline, 1975).

Intraperitoneal administration of 40 mg/kg bw of 3-methylpyridine [FL-no: 14.135] to mice, hamsters, rats, guinea pigs, or rabbits resulted in the excretion of 6.4, 0.3, 4.0, 0.7 and 0.1 %, respectively, in the urine as the corresponding *N*-oxide within 24 hours (Gorrod and Damani, 1980). 3-Methylpyridine was converted to the corresponding *N*-oxide by fortified hepatic microsomal preparations from hamster, guinea-pig, rabbit, rat and mouse, and by pulmonary microsomal preparations from guinea-pig and rabbit (Cowan et al., 1978).

More than 50 % of a 300 mg/kg bw dose of 4-methylpyridine [FL-no: 14.136], administered to Wistar rats (3/sex) *via* gavage, was excreted in the urine, principally as pyridine-4-carboxylic acid (50 %) or its glycine conjugate (5 %) after 24 hours. Minor metabolites included approximately 5 % unchanged 4-methylpyridine from expired air and urine and 1.5 % 4-methylpyridine-*N*-oxide in urine. Two metabolic pathways for 4-methylpyridine were confirmed in this study:

1) *N*-oxidation leading to the formation of *N*-oxide and

2) methyl oxidation leading to the formation of pyridine-4-carboxylic acid (isonicotinic acid) excreted mainly unchanged and partly as the glycine conjugate, isonicotinuric acid (Nguyen et al., 1988).

The supporting substance 3-ethylpyridine [FL-no: 14.061] was converted to the corresponding *N*-oxide by fortified hepatic microsomal preparations from hamster, guinea-pig, rabbit, rat and mouse, and by pulmonary microsomal preparations from guinea-pig and rabbit (Cowan et al., 1978).

Three metabolites were detected in rat urine administered a dosage of 100 mg/kg bw of 2,3-dimethylpyridine [FL-no: 14.103] by gavage corresponding to 50 % of the administered dosage after 24 hours. The main metabolite was a result of oxidation at the methyl group at the C-3 position, 2-methylpyridine-3-carboxylic acid (30 % of dose), which was excreted in the urine. In addition, 2,3-dimethylpyridine was ring hydroxylated at position C-5 and C-6 resulting in the metabolites 2,3-dimethyl-5-hydroxypyridine and 2,3-dimethyl-6-hydroxypyridine (7 % and 13 % of the dosage). The metabolites were excreted in the urine as such and not as glycine conjugate. No urinary *N*-oxidation products were found (with a detection limit of 1 % of the administered compound) (Hawksworth and Scheline, 1975).

For the supporting substance 2,6-dimethylpyridine [FL-no: 14.065], one study on metabolism was found. Ninety per cent of a 100 mg/kg bw dose of 2,6-dimethylpyridine administered *via* gavage to male albino Wistar rats was excreted as the glycine conjugate of the 6-methyl-2-carboxylic acid (Hawksworth and Scheline, 1975).

The metabolism of 3-acetylpyridine [FL-no: 14.039] was investigated in dogs after oral (500 mg/kg bw every day for eight days) and intraperitoneal (50 mg/kg bw, single dosage) administration. The metabolites isolated from urine were 1-(3-pyridyl)ethanol, *N*-methylated 1-(3-pyridyl)ethanol and (3-pyridyl)-1,2-ethanediol, which was oxidised to nicotinic acid (McKee et al., 1987) (Figure III.3). 3-Acetylpyridine was converted to the corresponding *N*-oxide by fortified hepatic microsomal preparations from hamster, guinea-pig, rabbit, rat and mouse, and by pulmonary microsomal preparations from guinea-pig and rabbit (Cowan et al., 1978). Similar *in vitro* results were obtained when 3-acetylpyridine was incubated with hepatic supernatant fraction and microsomal fractions from rats, guinea pigs, rabbits, hamster and mice, where they identified *N*-oxide metabolites of both 1-(3-pyridyl)ethanol and 3-acetylpyridine itself. 1-(3-pyridyl-*N*-oxide)ethanol is produced by further reduction of 3-acetylpyridine-*N*-oxide, and not from 1-(3-pyridyl)ethanol (Damani et al., 1980).

In contrast to 3-acetylpyridine, no *N*-oxidation products of 3-hydroxypyridine were found after incubation with liver microsomes from hamster, guinea-pig, rabbit, rat and mouse. (Cowan et al., 1978).

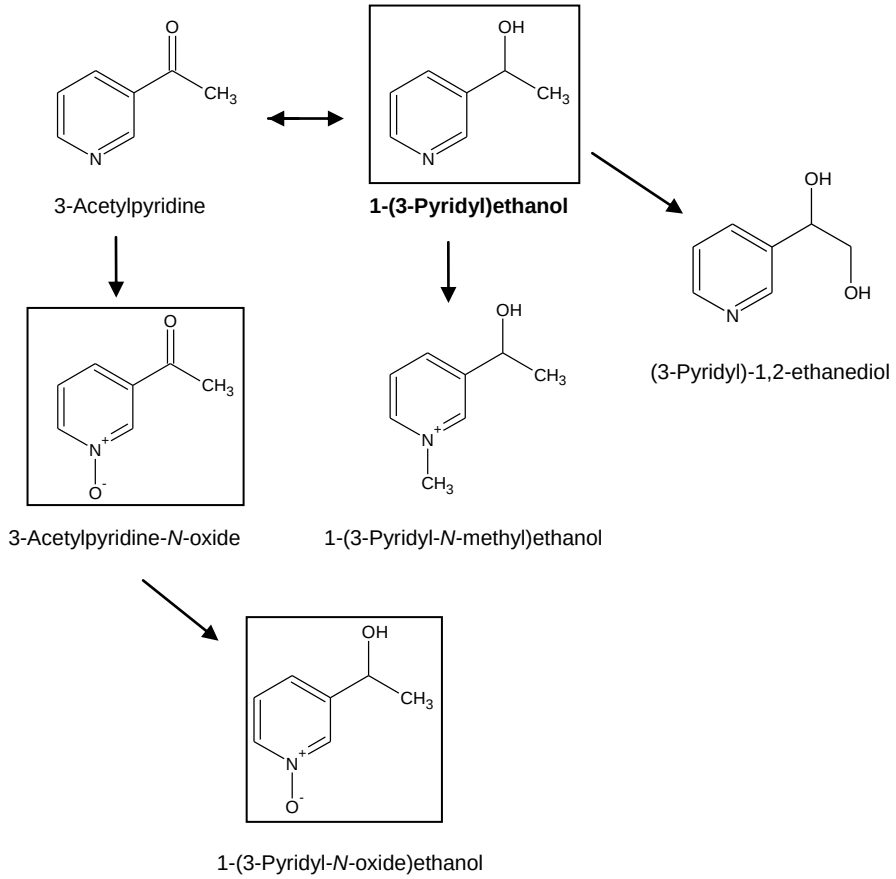


Figure III.3. *Metabolism of 3-acetylpyridine in urine from dogs (McKennis et al., 1964) and with microsomal preparation from liver of rats, guinea-pigs, rabbits, hamster and mice (in frame)(Damani et al., 1980). Main metabolism product in vivo is in bold.*

Metabolism of pyridines substituted with ester groups (subgroup 5)

No metabolism studies were found for the candidate pyridine substances ethyl nicotinate and isopropyl nicotinate [FL-no: 14.110 and 14.120].

However, there are metabolism studies on the hydrolysis product nicotinic acid from hydrolysis of ethyl nicotinate and isopropyl nicotinate. No *N*-oxidation products of nicotinic acid were found after incubation with liver microsomes from hamster, guinea-pig, rabbit, rat and mouse (Cowan et al., 1978). Nicotinic acid is a vitamine metabolite of the nicotinates (Lehninger, 1982). Nicotinic acid reacts with 5-phosphoribosyl-1-pyrophosphate to form the nicotinic acid mononucleotide, which then condenses with ATP to form the nicotinic acid analogue of nicotinamide adenine dinucleotide (NAD), which is subsequently converted to NAD by a reaction with glutamine and ATP. For adults a tolerable upper intake level for nicotinic acid of 10 mg/day was established by the Scientific Committee on Food in 2000 (SCF, 2000).

III.4. Summary on metabolism

Ethyl nicotinate and isopropyl nicotinate [FL-no: 14.110 and 14.120] are expected to be hydrolysed *in vivo* to nicotinic acid and ethanol, and nicotinic acid and isopropanol, respectively. No data are available on the hydrolysis of 1-acetylindole [FL-no: 14.088] or supporting substances. Amides are

known to be hydrolysed but less rapidly than esters from carboxylic acids. A rapid hydrolysis of 1-acetylindole is not anticipated due to steric hindrance.

Subgroup 1: The candidate substance with alkyl substitutions in these positions, 2-acetyl-5-methylpyrrole [FL-no: 14.085], is more likely hydroxylated on the alkyl group. Further oxidation of the acetyl group on 2-acetyl-5-methylpyrrole is also expected. According to (Damani and Crooks, 1982) pyrroles are likely substrates for elecrophilic enzymatic oxidation at N-1.

Subgroup 3: The candidate substance 2-methylindole [FL-no: 14.131] and indole, the hydrolysis product of 1-acetylindole [FL-no: 14.088], will be metabolised through two main pathways. Oxidation of the methyl group in 2-methylindole and in the ring structure, resulting in addition of hydroxyl groups which is followed by conjugation to glucuronic acid and sulphate. Secondary epoxidation of the double bonds on the pyrrole ring is expected, leading to oxindole compounds which can be further oxidised by addition of hydroxyl groups and conjugated with glucuronic acid and sulphate. The supporting substance 3-methylindole is metabolised to the reactive metabolite 3-methyleneindolenine which is conjugated to GSH. Since a methyleneindolenine is not formed from 2-methylindole, conjugation to GSH seems less likely for this substance. The possible metabolic intermediates from 2-methylindole are probably less reactive than the intermediates formed from 3-methylindole, although epoxide intermediates are likely to be formed.

Subgroup 4: Two main metabolism pathways can be predicted for the alkyl substituted pyridines. The main pathway is hydroxylation of the alkyl groups, with additional oxidation to the corresponding carboxyl compounds. The carboxyl metabolites are excreted in the urine as such or in conjugation with glycine. Alternatively the candidate pyridines may be oxidised on the nitrogen atom, and excreted in the urine without conjugation. Hydroxylation of the pyridine ring through epoxide intermediates cannot be excluded, although not shown to be a major pathway of the supporting compound. However, this is probably dependent on the positions of the substitutions. From the metabolism studies on the supporting substance 3-acetylpyridine, the major metbolic pathway for 4-acetylpyridine [FL-no: 14.089] is expected to be via reduction of the keto group followed by conjugation by glucoronic acid and excretion via the urine. To a minor extend, *N*-oxidation or *N*-methylation may take place. 2-Hydroxypyridine [FL-no: 14.118] is expected to be excreted as such or as conjugate.

Subgroup 5: Nicotinic acid is a vitamin metabolite of the candidate substances ethyl nicotinate and isopropyl nicotinate. Nicotinic acid reacts with 5-phosphoribosyl-1-pyrophosphate to form the nicotinic acid mononucleotide, which then condenses with ATP to form the nicotinic acid analogue of NAD, which is subsequently converted to NAD by a reaction with glutamine and ATP.

Two of the candidate substances [FL-no: 14.110 and 14.120] in this group are expected to be metabolised to innocuous products. For the remaining 22 candidate substances of this flavouring group [FL-no: 14.085, 14.088, 14.089, 14.092, 14.093, 14.103, 14.104, 14.105, 14.106, 14.115, 14.116, 14.117, 14.118, 14.124, 14.125, 14.131, 14.134, 14.135, 14.136, 14.140, 14.143 and 14.150] the Panel would expect metabolites as *N*-oxides, epoxides or iminium intermediates. For the 22 candidate substances it cannot be anticipated that they will be metabolised to innocuous products or excluded that they might be converted to toxic metabolites or intermediates.

ABBREVIATIONS

ATP	Adenosine Triphosphate
BSO	Buthionine-sulphoximine
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)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EC	European Commission
EFFA	European Flavour and Fragrance Association
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)
GLP	Good Laboratory Practice
GSH	Glutathione
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
MS	Mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NAC	N-acetylcystein
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
No	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
OECD	Organisation for Economic Co-operation and Developmen
SCF	Scientific Committee on Food

TAMDI	Theoretical Added Maximum Daily Intake
WHO	World Health Organisation