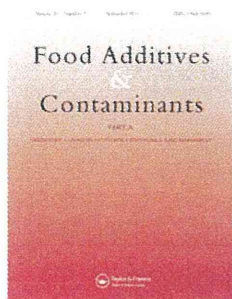


- 2) 厚生省薬務局 : 単回及び反復投与毒性試験ガイドラインの改正について。薬務局新医薬品課長・審査課長通知。薬新薬第 88 号, 平成 5 年 8 月 10 日。  
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## The Japan Flavour and Fragrance Materials Association's (JFFMA) safety assessment of acetal food flavouring substances uniquely used in Japan

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## REVIEW

### The Japan Flavour and Fragrance Materials Association's (JFFMA) safety assessment of acetal food flavouring substances uniquely used in Japan

Hiroyuki Okamura<sup>a</sup>, Hajime Abe<sup>b</sup>, Yasuko Hasegawa-Baba<sup>b</sup>, Kenji Saito<sup>a</sup>, Fumiko Sekiya<sup>a</sup>, Shim-Mo Hayashi<sup>a</sup>, Yoshiharu Mirokuji<sup>a</sup>, Shinpei Maruyama<sup>a</sup>, Atsushi Ono<sup>c</sup>, Madoka Nakajima<sup>c</sup>, Masakuni Degawa<sup>f</sup>, Shogo Ozawa<sup>g</sup>, Makoto Shibutani<sup>b\*</sup> and Tamio Maitani<sup>d</sup>

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Using the procedure devised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), we performed safety evaluations on five acetal flavouring substances uniquely used in Japan: acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal. As no genotoxicity study data were available in the literature, all five substances had no chemical structural alerts predicting genotoxicity. Using Cramer's classification, acetoin dimethyl acetal and hexanal dibutyl acetal were categorised as class I, and acetaldehyde 2,3-butanediol acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal as class III. The estimated daily intakes for all five substances were within the range of 1.45–6.53 µg/person/day using the method of maximised survey-derived intake based on the annual production data in Japan from 2001, 2005, 2008 and 2010, and 156–720 µg/person/day using the single-portion exposure technique (SPET), based on the average use levels in standard portion sizes of flavoured foods. The daily intakes of the two class I substances were below the threshold of toxicological concern (TTC) – 1800 µg/person/day. The daily intakes of the three class III substances exceeded the TTC (90 µg/person/day). Two of these, acetaldehyde 2,3-butanediol acetal and hexanal glyceryl acetal, were expected to be metabolised into endogenous products after ingestion. For 4-methyl-2-pentanone propyleneglycol acetal, one of its metabolites was not expected to be metabolised into endogenous products. However, its daily intake level, based on the estimated intake calculated by the SPET method, was about 1/15 000th of the no observed effect level. It was thus concluded that all five substances raised no safety concerns when used for flavouring foods at the currently estimated intake levels. While no information on *in vitro* and *in vivo* toxicity for all five substances was available, their metabolites were judged as raising no safety concerns at the current levels of intake.

**Keywords:** acetals; Cramer's decision tree; food flavours; Joint FAO/WHO Expert Committee on Food Additives (JECFA); safety; threshold of toxicological concern (TTC)

## Introduction

### Flavouring substances in Japan

A wide variety of flavouring substances have been developed to improve the taste and aroma of foodstuffs but which have no nutritional properties. This wide variety enables the food industry to meet its needs to mimic the flavours of foods. According to the annual usage survey by the Japan Flavour and Fragrance Materials Association (JFFMA), approximately 3230 flavouring substances have been used commercially in Japan (Someya 2012). Among them, 129 substances are currently approved as designated additives by the Ministry of Health, Labour and Welfare, Japan. The remaining substances have been classified into 18 chemical structural groups and approved for use in Japan under the compliance of the Food Sanitation Act without a safety evaluation.

### Safety evaluation procedures

In 1996, JECFA adopted a novel safety evaluation procedure for flavouring substances (WHO 1996). Using this procedure, the safety of many flavouring substances can be efficiently evaluated using the safety evaluation data for substances that have similar chemical structures or metabolic pathways, even if the substances under investigation have no safety data. Further evaluation protocols similar to the JECFA procedure are accepted for use in the United States and the European Union. In addition, the results of safety evaluations by JECFA, the Flavour and Extract Manufacturers Association (FEMA) Expert Panel, and EFSA are now widely used in more than 70 countries (Someya 2012; Konishi et al. 2014). Since 2003, the Food Safety Commission of Japan (FSCJ) has been performing safety evaluations on

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individually selected flavouring substances. Its conclusions are consistent with those obtained using the group evaluation by JECFA and, as of May 2014, 51 substances have already been designated as food additives in Japan based on these results (Someya 2012).

Therefore, of approximately 3230 flavouring substances used in Japan, about 2400 have been judged to be safe under limited conditions of use for flavouring by JECFA, FEMA, EFSA and FSCJ. However, this leaves about 800 flavouring substances whose safety has not been evaluated by any governmental or international organisation. Thus, in 2009, JFFMA established a Safety Re-evaluation Committee, comprised of expert scientists in the fields of toxicology and food chemistry as well as representative members of JFFMA, to evaluate substances uniquely used in Japan using the JECFA evaluation procedure (see Figure S1 in the Supplemental data online), with the order of priority being determined by the annual volume of production.

Using the JECFA evaluation procedure, the present study evaluates the safety of five acetal flavouring substances uniquely used in Japan: acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal. These substances have unique odour characteristics and are used with other flavouring substances to improve the taste of processed foods such as candy, chewing gum and soft drinks.

### Collecting information for the evaluation procedure

#### Chemical information of five flavouring substances and their hydrolysis products

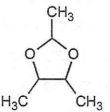

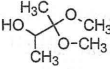

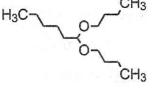
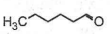
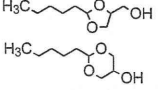
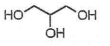
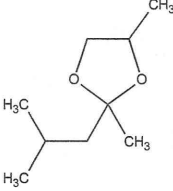
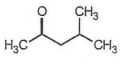
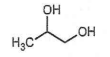
The CAS No., molecular weight and chemical structure for the five flavouring substances, acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal, are listed in Table 1. These substances have not been reported to occur naturally, and thus all five substances are expected to be ingested only from foods containing them as flavouring agents (JFFMA 2003, 2006, 2011).

In general, aliphatic acetals undergo hydrolysis to their components – aldehydes and alcohols (Knoefel 1934; Morgareidge 1962), and the hydrolysis products of five acetal flavouring substances are acetaldehyde, 2,3-butanediol, acetoin, methanol, hexanal, butyl alcohol, glycerol, 4-methyl-2-pentanone and propylene glycol (Table 1).

#### Prediction of genotoxic potential by chemical structure and in silico models

We evaluated the genotoxic potential of each acetal by analysing the biological potential of its chemical

Table 1. Chemical information on five acetal flavouring substances uniquely used in Japan.

| Flavouring substances   | Hydrolysed products   |
|---|---|
| Name/CAS No./molecular weight   | Name/CAS No./molecular weight   |
| Chemical structure  | Chemical structure  |
| Acetaldehyde 2,3-butanediol acetal/3299-32-9/116.16                                 | Acetaldehyde/75-07-0/44.05  |
|    | <br>2,3-butanediol/513-85-9/90.12    |
| Acetoin dimethyl acetal/2371-17-7/134.17  | Acetoin/513-86-0/88.11  |
|    | <br>Methanol/67-56-1/32.04           |
| Hexanal dibutyl acetal/93892-07-0/230.39  | Hexanal/66-25-1/100.16  |
|  | <br>Butyl alcohol/71-36-3/74.12    |
| Hexanal glyceryl acetal/4379-20-8, 4740-88-9/174.24                                 | Hexanal/66-25-1/100.16  |
|  | <br>Glycerol/56-81-5/92.09         |
| 4-Methyl-2-pentanone propyleneglycol acetal/25186-44-1/158.24                       | 4-Methyl-2-pentanone/108-10-1/100.16  |
|  | <br>Propylene glycol/57-55-6/76.09 |
|   |                                    |

structure. While the JECFA decision tree approaches do not cover genotoxicity in its evaluation procedure (WHO 1996), the potential of genotoxicity is now evaluated before the decision tree steps by incorporating structural alerts and other available genotoxicity information from structurally related chemicals in JECFA (WHO 1998; 1999a, 1999b) and FSCJ

(2005a, 2005b). In the present study, following the JECFA procedures, we examined structural alerts to chemical substructure groups, as is generally done for chemicals lacking genotoxicity information (Ashby & Tennant 1988, 1991; Tennant et al. 1990). While genotoxicity study data were not available for any of the five substances, there were no structural alerts on their chemical structures. We also examined the genotoxic potential of the five substances using structure-activity relationship (SAR) analyses (Hansch 1969) using *in silico* prediction software that has been applied to the regulatory evaluation of flavouring substances (Cronin et al. 2003; Ono et al. 2012). We used three SAR software programs with different algorithms for analysis: DEREK (version 10.0.2; Lhasa Ltd, Leeds, UK), MultiCASE (version 1.90; Multicase Inc., Cleveland, OH, USA), and ADMEWORKS (version 4.0; Fujitsu Kyusyu Systems Ltd, Fukuoka, Japan). All the software programs used in the study calculated that no substances except acetaldehyde 2,3-butanediol acetal had the potential for genotoxicity. Regarding acetaldehyde 2,3-butanediol acetal, MultiCASE analysis gave a positive result for genotoxicity, but the other two programs gave negative results. Furthermore, alcohols and aldehydes or ketones, which are metabolites of acetals, have no genotoxic potential except for acetaldehyde (WHO 1999a, 1999b). In addition, the genotoxicity information of structurally related substances was used for evaluation. Based on these results, we decided that the JECFA evaluation procedure could be applied to these five acetal flavouring substances.

#### Estimating the daily intake of flavouring substances by the MSDI and SPET methods

According to the annual production data from 2001, 2005, 2008 and 2010 in Japan, the total annual estimated volumes of acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal were 2.02–5.51, 0.10–23.00, 5.85–11.97, 3.78–6.72 and 0–24.80 kg, respectively (JFFMA 2003, 2006, 2011; unpublished information, 2008). Based on these data, the daily intake of each substance was calculated using the maximised survey-derived intake (MSDI; Young et al. 2006) and single-portion exposure technique (SPET; WHO 2008) procedures.

The development of the MSDI procedure was based on disappearance data from periodic surveys of ingredient manufacturers using the volume of ingredients produced during the survey year (Young et al. 2006). Because the production volume changes every year, the maximum production volume of each substance was selected from the annual production data obtained to avoid any underestimation of the intakes. The resultant daily intakes per person of each of the five flavouring substances were estimated to be between 1.45 and 6.53  $\mu\text{g}$  (Table 2).

Using the SPET method, the estimated daily intakes of acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal were calculated as 720, 200, 156, 300 and 320  $\mu\text{g}$ , respectively, based on the average use levels in standard portion sizes of flavoured foods (Table 2).

Table 2. Estimated daily intake data of five acetal flavouring substances uniquely used in Japan.

| Substance                                   | Annual volume of production<br>(kg year <sup>-1</sup> ) |       |       |      | Estimated daily intake<br>( $\mu\text{g}/\text{person}/\text{day}$ ) |                            | Name and code of the food group(s) showing<br>the highest use level <sup>c</sup> |
|---|---|-------|-------|------|--|----------------------------|--|
|   | 2001  | 2005  | 2008  | 2010 | MSDI<br>value <sup>a</sup>   | SPET<br>value <sup>b</sup> |  |
| Acetaldehyde 2,3-butanediol acetal          | 5.51  | 2.02  | –     | 2.51 | 1.45   | 720                        | 14.1 Non-alcoholic 'soft' beverages (includes fruit juices, coffee, tea)         |
| Acetoin dimethyl acetal                     | 0.10  | 23.00 | –     | –    | 6.06   | 200                        | 01.2 Fermented and renneted milk products (plain)                                |
| Hexanal dibutyl acetal                      | 5.85  | 11.97 | –     | 8.17 | 3.15   | 156                        | 05.2 Confectionery (hard and soft candy)   |
| Hexanal glyceryl acetal                     | 6.72  | 3.78  | –     | 3.82 | 1.77   | 300                        | 14.1 Non-alcoholic 'soft' beverages (includes fruit juices, coffee, tea)         |
| 4-Methyl-2-pentanone propyleneglycol acetal | 0   | 0     | 24.80 | –    | 6.53   | 320                        | 07.2 Fine bakery goods (sweet, salty, savoury)                                   |

Notes: MSDI, maximised survey-derived intake; SPET, single-portion exposure technique.

<sup>a</sup>Calculated by the MSDI method based on the maximum usage volume from the annual production data of 2001, 2005, 2008 and 2010 in Japan.

MSDI value ( $\mu\text{g}/\text{person}/\text{day}$ ) = annual volume of production (kg)  $\times 10^9$  ( $\mu\text{g kg}^{-1}$ )/population of consumers  $\times 0.8 \times 365$  days. The population of consumers was assumed to be  $13 \times 10^8$ .

<sup>b</sup>Calculated by the SPET method based on the average use levels in standard portion sizes of flavoured foods ( $\mu\text{g}/\text{person}/\text{day}$ ).

<sup>c</sup>Name and code of the food group(s) as listed in Codex General Standard for Food Additives, Codex Stan 192-1995 (FAO 2015) (see [www.codexalimentarius.net/gsfaonline/docs/CXS\\_192e.pdf](http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf)).

Because the daily intakes estimated by the SPET method were higher than those by the MSDI method, it was decided to conduct a safety evaluation using the values calculated by the SPET method.

#### *Estimation of absorption, metabolism and excretion*

There was no information on the five acetals regarding absorption, metabolism and excretion from the JECFA assessment reports. Therefore, the components or structurally related substances of these five acetals were examined to estimate their metabolic fate. Among these structurally related acetals, 1,2,3-*tris*[(1'-ethoxy)ethoxy] propane was easily hydrolysed into acetaldehyde and glycerol (De Simone 1976). Potassium 2-(1'-ethoxy)ethoxypropanoate, an acetal of lactic acid, was completely hydrolysed into lactic acid, acetaldehyde and ethanol by incubation in gastric fluids (Moreno et al. 1984). 1,1-Dimethoxyethane, acetal and related acetals were hydrolysed within 1–5 h in simulated gastric fluid and, to a lesser extent, in simulated intestinal fluid (Morgareidge 1962). An ingested suspension of 1,1-dimethoxyethane, acetal and other aliphatic acetals was rapidly hydrolysed within the stomach (Knoefel 1934). In a study examining the synthesis of valproic acid (2-propylpentanoic acid) by incubation of dimethyl, diethyl and diisopropyl acetals of 2-propylpentanal with an S10 fraction of rat liver homogenates or microsomal fraction, synthesis was inhibited by SKF-525A, a cytochrome P450 (CYP) inhibitor (Vicchio & Callery 1989). Synthesis was also inhibited by removal of nicotine adenine amide dinucleotide phosphoric hydrase or oxygen. These results were in accordance with the responses mediated by CYP. 2-Propyl-1-pentanol was detected as a major metabolite after incubation with the microsomal fraction after removing enzymes from the soluble fraction. Moreover, the incubation of deuterated diethyl acetal of 2-propylpentanal indicated the oxidation of ether methylene as a metabolism pathway of acyclic acetal. These results showed that acetal-related substances were hydrolysed into aldehydes and alcohols.

Linear saturated acyclic alcohols, aldehydes and carboxylic acids, metabolites of acetals, are absorbed from the gastrointestinal tract (Dawson & Hullin 1954; von Oettingen 1960; Brabec 1993; Katz & Guest 1994; Lington & Bevan 1994). While most of these are endogenous biogenic substances making it difficult for their plasma half-life to be measured (Lington & Bevan 1994), these acetal metabolites are known to be further metabolised through  $\beta$ -oxidation and tricarboxylic acid pathways into innocuous products.

Aliphatic ketones are excreted into urine as glucuronic acid conjugates through reduction to secondary alcohols (Neubreuer 1901; Leibman 1971; Bosron & Li 1980;

Felsted & Bachur 1980). In the case of short-chain aliphatic ketones, it is possible that they are excreted directly into expired air or undergo  $\omega$ -oxidation or  $\omega$ -1-oxidation (Haggard et al. 1945; Scopinaro et al. 1947; Saito 1975; Brown et al. 1987; Schwartz 1989), metabolised into the corresponding carboxylic acids, and then metabolised into innocuous substances through the tricarboxylic acid pathway. Although the metabolism of short-chain aliphatic ketones through  $\omega$ -oxidation and  $\omega$ -1-oxidation are competitive with long-chain aliphatic ketones when they exist at high concentrations (Dietz et al. 1981; Topping et al. 1994), such competition does not occur at the intake levels when used as a flavouring substance.

Orally administered acetoin is rapidly absorbed from the gastrointestinal tract in rats (Gabriel et al. 1972). In humans, aliphatic acyclic  $\alpha$ -diketones are metabolised into ketocarboxylic acids through  $\alpha$ -hydroxylation and oxidation of the terminal methyl group, when they exist at low concentrations (WHO 1999b).  $\alpha$ -Ketocarboxylic acids are metabolised into simple aliphatic carboxylic acids and carbon dioxide through oxidative decarboxylation and further into innocuous products through  $\beta$ -oxidation and the tricarboxylic acid cycle (WHO 1999b). In rats, rabbits and dogs, aliphatic acyclic  $\alpha$ -diketones are reduced to diols and further conjugated with glucuronic acid to excrete into urine when they are exposed to animals at high concentrations (Westerfeld & Berg 1943; Gabriel et al. 1972; Otsuka et al. 1996).

#### **Safety evaluation of the five substances according to the JECFA evaluation procedure**

##### *Decision tree approach*

Details of the JECFA evaluation procedure are described in WHO (1996). A summary of the decision tree approach for this procedure is shown in Figures S1 and S2 in the Supplemental data online.

*Step 1:* In the first step, each flavouring substance was subjected to classification into three chemical structure classes: I, II, or III, according to Cramer's decision tree, as shown in Figure S2 (Cramer et al. 1976). Substances in class I have a simple chemical structure with known efficient metabolic pathways that would suggest a low oral toxicity. Substances in class II are less innocuous than class I, but do not contain structural features suggestive of having toxicity like substances in class III. Substances in class III have a chemical structure that may cause significant toxicity.

According to the results of the Cramer decision tree (Figure S2; Cramer et al. 1976), two substances, acetoin dimethyl acetal and hexanal dibutyl acetal, were assigned to structural class I, and the remaining three substances, acetaldehyde 2,3-butanediol acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal, to

Table 3. Safety evaluation of acetal flavouring substances according to the JECFA evaluation procedure – Step 1 Cramer's decision tree approach.

| Substance                                   | Cramer's decision tree<br>(N = no, Y = yes)                      | Chemical structural class |
|---|--|---------------------------|
| Acetaldehyde 2,3-butanediol acetal          | Q1N, Q2N, Q3N, Q5N, Q6N, Q7Y, Q8N, Q10N, Q11N, Q12N, Q22N, Q33N  | III                       |
| Acetoin dimethyl acetal                     | Q1N, Q2N, Q3N, Q5N, Q6N, Q7N, Q16N, Q17N, Q19Y, Q20Y, Q21N, Q18N | I                         |
| Hexanal dibutyl acetal                      | Q1N, Q2N, Q3N, Q5N, Q6N, Q7N, Q16N, Q17N, Q19Y, Q20Y, Q21N, Q18N | I                         |
| Hexanal glyceryl acetal                     | Q1N, Q2N, Q3N, Q5N, Q6N, Q7Y, Q8N, Q10N, Q11N, Q12N, Q22N, Q33N  | III                       |
| 4-Methyl-2-pentanone propyleneglycol acetal | Q1N, Q2N, Q3N, Q5N, Q6N, Q7Y, Q8N, Q10N, Q11N, Q12N, Q22N, Q33N  | III                       |

structural class III (Table 3). In addition, hydrolysed products of aliphatic acetals, i.e., alcohols and aldehydes or ketones, which are components of these substances, also belong to structural class I.

*Step 2:* In the second step, the metabolic fate of each substance was predicted by chemical structure and applied using a decision-tree approach consisting of two branches: one for substances that are metabolised into innocuous products, and the other for substances that are not. This second step showed that all five substances were easily hydrolysed into alcohols and aldehydes or ketones by carboxylesterases or esterases, and the resultant substances are metabolised into innocuous products as mentioned above (WHO 1999b) (Table 4). Then the evaluation of all substances proceeded to the A-side of the decision tree (Figure S1, Step A3).

*Step A3:* In this step, the estimated daily intake of each substance was compared with the threshold level of human exposure, according to Cramer's structural classes (Cramer et al. 1976), in which the estimated threshold levels are 1800, 540, and 90 µg/person/day for structural classes I, II, and III, respectively (Munro et al. 1996, 1999).

As mentioned above, the estimated daily intakes of the five acetal flavouring substances as calculated by the SPET method were higher than those by the MSDI method. The daily intake of two class I substances were within the range of 156–200 µg kg<sup>-1</sup> day<sup>-1</sup> (Table 4). These intake values were below the threshold of toxicological concern (TTC) applied to structural class I substances. However, the daily intake level of three class III substances were within the range of 300–720 µg kg<sup>-1</sup> day<sup>-1</sup>, values greater than the TTC. Therefore, the evaluation of these three substances proceeds to step A4.

*Step A4:* None of the three structural class III substances is a normal constituent of the body. It is expected that acetaldehyde 2,3-butanediol acetal is hydrolysed into acetaldehyde and 2,3-butanediol, and hexanal glyceryl acetal into hexanal and glycerol. These metabolites are normal constituents of the body (Table 4). On the other hand, it is expected that 4-methyl-2-pentanone propyleneglycol acetal is hydrolysed into 4-methyl-2-pentanone and propyleneglycol. Propyleneglycol is expected to be further metabolised into endogenous substances; however, 4-methyl-2-pentanone is not. Therefore, the evaluation of this substance proceeds to step A5.

*Step A5:* A no-observed-effect-level (NOEL) of 50 mg kg<sup>-1</sup> day<sup>-1</sup> was reported in a 13-week study using Sprague–Dawley rats for 4-methyl-2-pentanone (Microbiological Associates Inc. 1986), a metabolite of 4-methyl-2-pentanone propyleneglycol acetal that is not a normal constituent of the body. This NOEL provides a safety margin of > 15 000 for the intake of 4-methyl-2-pentanone calculated by the daily intake level of 4-

Table 4. Safety evaluation of acetal flavouring substances according to the JECFA evaluation procedure – Steps 2, A3, A4 and A5.

| Structure class | Substance                                   | Step 2 | Step A3 <sup>a</sup>        | Step A4          | Step A5          | Decision   |
|-----------------|---|--------|-----------------------------|------------------|------------------|--|
| Class I         | Acetoin dimethyl acetal                     | Yes    | No (MSDI: 6.56, SPET: 200)  | –                | –                | Substance would not be expected to be a safety concern |
|                 | Hexanal dibutyl acetal                      | Yes    | No (MSDI: 3.42, SPET: 156)  | –                | –                |  |
| Class III       | Acetaldehyde 2,3-butanediol acetal          | Yes    | Yes (MSDI: 1.57, SPET: 720) | Yes <sup>b</sup> | –                |  |
|                 | Hexanal glyceryl acetal                     | Yes    | Yes (MSDI: 1.92, SPET: 300) | Yes <sup>b</sup> | –                |  |
|                 | 4-Methyl-2-pentanone propyleneglycol acetal | Yes    | Yes (MSDI: 7.08, SPET: 320) | No               | Yes <sup>c</sup> |  |

Notes: <sup>a</sup>Estimated threshold levels are 1800 and 90 µg/person/day for structural classes I and III, respectively.

<sup>b</sup>Hydrolysed products of acetaldehyde 2,3-butanediol acetal and hexanal glyceryl acetal are normal constituents of the body.

<sup>c</sup>A no observed effect level (NOEL) of 4-methyl-2-pentanone, a metabolite of 4-methyl-2-pentanone propyleneglycol acetal is 50 mg kg<sup>-1</sup> day<sup>-1</sup>.

methyl-2-pentanone propyleneglycol acetal using the SPET method (Table 4).

#### **Consideration of the combined intakes from use as flavouring agents**

Because JECFA considers that evaluating the combined intake is not necessary if the highest MSDI value is less than  $20 \mu\text{g day}^{-1}$  (WHO 2011), we decided to omit the evaluation of combined intakes for all five substances.

#### **Assessing toxicological information for hydrolysed products of the five flavouring substances**

As no information on *in vitro* and *in vivo* toxicity for all five substances was available, instead their metabolites were examined using the data tabulated by JECFA (WHO 1998; 1999a, 1999b; FSCJ 2005a, 2005b).

#### **Subacute toxicity**

The subacute toxicity by oral administration of acetaldehyde, acetoin, hexanal, butyl alcohol and methyl isobutyl ketone (MIBK; also known as 4-methyl 2-pentanone) in rats has been reported in several studies (Table 5). There are no subacute toxicity data by oral administration of 2,3-butanediol, methanol, glycerol and propylene glycol.

In a study of Wistar rats (24 per group) given drinking water containing acetaldehyde for 11 weeks, liver cell fatty degeneration was detected at  $500 \text{ mg kg}^{-1} \text{ day}^{-1}$  (Matysiak-Budnik et al. 1996). In a study of Wistar rats (10 per group) given drinking water containing acetaldehyde for 4 weeks, an increase in relative kidney weight and thickening of the limiting ridge of the forestomach were noted at  $625 \text{ mg kg}^{-1} \text{ day}^{-1}$  (Til et al. 1986).

In a study of CFE rats (15 per group) given drinking water containing acetoin for 13 weeks, the body weight gain was reduced at  $12\ 000 \text{ mg kg}^{-1}$  ( $1300 \text{ mg kg}^{-1} \text{ day}^{-1}$ ) (Gaunt et al. 1972). Low values in erythrocyte counts and haemoglobin concentration were also noted at  $1300 \text{ mg kg}^{-1} \text{ day}^{-1}$ . However, haematocrit values were not influenced by acetoin treatment. There were also no toxicologically relevant effects on the clinical signs, urinalysis, blood chemistry or histopathological examination.

Another study of Sprague–Dawley rats (10 per group) given drinking water containing hexanal for 4 weeks, there were no toxicologically relevant effects in the treated groups (Komsta et al. 1988).

A study of Sprague–Dawley rats (30 per group) orally administered butyl alcohol for 13 weeks showed increased incidences of ataxia or hypokinesia in both sexes at  $500 \text{ mg kg}^{-1} \text{ day}^{-1}$  from weeks 7 to 13 (USEPA 1987).

In a study of Sprague–Dawley rats (30 rats per group) orally administered MIBK for 13 weeks, high values for

haemoglobin concentration and haematocrit and low values for lymphocytes at  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$  were observed. Blood biochemically, a high level of serum alanine aminotransferase was observed at  $\geq 250 \text{ mg kg}^{-1} \text{ day}^{-1}$ . At  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$ , there were high levels of serum alkaline phosphatase and total protein, high levels for total cholesterol, and low values for the albumin/globulin ratio, high values for blood urea nitrogen and serum potassium and low values for serum glucose. In addition, increases in kidney and liver weights were observed at doses of  $\geq 250$  and of  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$ , respectively. Increases in urinary protein and ketones were observed at  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$ . Histopathologically, an increased incidence of mild nephropathy was observed at  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$  (Microbiological Associates Inc. 1986). In a study of a group of five HLA, Wistar rats given drinking water containing MIBK for 120 days revealed increases in kidney weights at  $1040 \text{ mg kg}^{-1} \text{ day}^{-1}$  (Carnegie-Mellon Institute of Research 1977a, 1977b).

#### **Long-term toxicity/carcinogenicity**

No information was available on chronic toxicity of the components or structurally related substances of these acetals. Regarding carcinogenicity, there are several sets of data on acetaldehyde. Groups of Sprague–Dawley rats were given drinking water containing acetaldehyde for 104 weeks (Soffritti et al. 2002). The total incidence of malignant tumours increased in both sexes at a concentration of  $2500 \text{ mg l}^{-1}$ . However, in their evaluation report of acetaldehyde, FSCJ determined that the study results by Soffritti et al. (2002) could only be used as reference data, because the tumours were sporadically observed without showing dose-related increases or any apparent target organs (FSCJ 2005a).

In an inhalation carcinogenicity study of acetaldehyde targeting the nasal mucosa in rats, cancer developed at doses of 750, 1500 and  $3000 \text{ mg kg}^{-1}$  (the dose was reduced from 3000 to  $1000 \text{ mg kg}^{-1}$  after 11 months),  $6 \text{ h day}^{-1}$ , 5 days  $\text{week}^{-1}$  for the maximum period of 27 months. It also developed when targeting the larynx in hamsters at a dose of  $2500 \text{ mg kg}^{-1}$  (the dose was later reduced to  $1650 \text{ mg kg}^{-1}$ ),  $7 \text{ h day}^{-1}$ , 5 days  $\text{week}^{-1}$  for 52 weeks (IARC 1999). However, FSCJ (2005a) has judged that these inhalation study data are irrelevant for oral exposure to food additives.

#### **Genotoxicity**

Several *in vivo* and *in vitro* genotoxicity studies of acetaldehyde, acetoin, hexanal, butyl alcohol and 4-methyl 2-pentanone, which are components of acetals, have been reported (Obe & Ristow 1977; Florin et al. 1980; Garst et al. 1983; He & Lambert 1985; Marnett et al. 1985;



Table 5. Summary of *in vivo* oral exposure studies for acetaldehyde, acetoin, hexanal, butanol and 4-methyl-2-pentanone.a

| Substance                       | Animals used                 | Exposure method<br>(experimental period) | Dose  | Toxicological finding  | Reference   |
|---------------------------------|------------------------------|--|---|--|---|
| Acetaldehyde                    | Wistar rat (male)            | Drinking water<br>(11 weeks)             | 0, 120, 500 mg kg <sup>-1</sup> day <sup>-1</sup>                 | Pathology data: fatty degeneration of hepatocytes<br>(500 mg kg <sup>-1</sup> day <sup>-1</sup> )  | Matysiak-Budnik et al. (1996)                           |
|                                 | Wistar rat (male/<br>female) | Drinking water<br>(4 weeks)              | 0, 25, 125, 625 mg<br>kg <sup>-1</sup> day <sup>-1</sup>          | Organ weight: increase in relative kidney weight<br>(625 mg kg <sup>-1</sup> day <sup>-1</sup> ); Pathology data: thickening<br>of the limiting ridge of the forestomach<br>(625 mg kg <sup>-1</sup> day <sup>-1</sup> )   | Til et al. (1986)                                       |
|                                 | SD rat (male/female)         | Drinking water<br>(104 weeks)            | 0, 50, 250, 500, 1500,<br>2500 mg l <sup>-1</sup>                 | No toxicologically relevant carcinogenicity data   | Soffritti et al. (2002) and<br>FSCJ (2005a)             |
| Acetoin                         | CFE rat (male/female)        | Drinking water<br>(13 weeks)             | 0, 85, 330, 1300 mg<br>kg <sup>-1</sup> day <sup>-1</sup>         | Decrease in body weight gain (1300 mg kg <sup>-1</sup> day <sup>-1</sup> );<br>haematology: low values in erythrocyte and<br>haemoglobin concentration (1300 mg kg <sup>-1</sup> day <sup>-1</sup> )   | Gaunt et al. (1972)                                     |
| Hexanal                         | SD rat (male/female)         | Drinking water<br>(4 weeks)              | 0, 0.1, 1.2, 12.6,<br>124.7 mg kg <sup>-1</sup> day <sup>-1</sup> | No toxicological findings  | Komsta et al. (1988)                                    |
| Butyl alcohol                   | SD rat (male/female)         | Oral administration<br>(13 weeks)        | 0, 30, 125, 500 mg<br>kg <sup>-1</sup> day <sup>-1</sup>          | Behavioural test: increase of ataxia or hypokinesia<br>(500 mg kg <sup>-1</sup> day <sup>-1</sup> )  | USEPA (1987)  |
| 4-Methyl-2-<br>pentanone (MIBK) | SD rat (male/female)         | Oral administration<br>(13 weeks)        | 0, 50, 250, 1000 mg<br>kg <sup>-1</sup> day <sup>-1</sup>         | Haematology: high values in haemoglobin<br>concentration and haematocrit (1000 mg<br>kg <sup>-1</sup> day <sup>-1</sup> ); blood biochemistry: high levels of<br>serum alanine aminotransferase (250 and<br>1000 mg kg <sup>-1</sup> day <sup>-1</sup> in females), serum alkaline<br>phosphatase and total protein (1000 mg kg <sup>-1</sup><br>in females), blood urea nitrogen and serum<br>potassium (1000 mg kg <sup>-1</sup> day <sup>-1</sup> in males), and<br>total cholesterol (1000 mg kg <sup>-1</sup> in both sexes),<br>low level of albumin/globulin ratio and serum<br>glucose (1000 mg kg <sup>-1</sup> day <sup>-1</sup> in males); organ<br>weights: increase in kidney weight (250 and<br>1000 mg kg <sup>-1</sup> day <sup>-1</sup> in both sexes); increase in<br>liver weight (1000 mg kg <sup>-1</sup> day <sup>-1</sup> in both sexes);<br>pathology data: increased incidence of mild<br>nephropathy (1000 mg kg <sup>-1</sup> day <sup>-1</sup> in males) | Microbiological Associates<br>Inc. (1986)               |
|                                 | HLA Wistar rat<br>(female)   | Drinking water<br>(120 days)             | 0, 1040 mg kg <sup>-1</sup> day <sup>-1</sup>                     | Organ weight: increase in relative kidney weight<br>(1040 mg kg <sup>-1</sup> day <sup>-1</sup> )  | Carnegie-Mellon Institute of<br>Research (1977a, 1977b) |

Note: <sup>a</sup>There were no short-term/subchronic toxicological reports of 2,3-butanediol, methanol, glycerol and propylene glycol.

Norppa et al. 1985; Kim et al. 1987; Wangenheim & Bolcsfoldi 1988; Brooks et al. 1988; O'Donoghue et al. 1988; Brambilla et al. 1989; Furnus et al. 1990; Helander & Lindahl-Kiessling 1991; Dillon et al. 1992; Zeiger et al. 1992; Kapp et al. 1993; Müller et al. 1993; Martelli et al. 1994; WHO 1998; FSCJ 2005b). The results of these studies are summarised in Table 6.

The Ames test for acetaldehyde showed negative results (Dillon et al. 1992), but mutagenicity in a mouse lymphoma thymidine kinase test with and without metabolic activation revealed positive results (Wangenheim & Bolcsfoldi 1988). The chromosomal aberration test for acetaldehyde using human lymphocytes showed negative results, but positive results using lymphocytes from a patient suffering from Fanconi anaemia (Obe et al. 1979). Acetaldehyde increased the frequency of DNA strand breakage in mice by inhalation exposure (IARC 1999), as well as that of sister chromatid exchange in cultured human lymphocytes (He & Lambert 1985; Norppa et al. 1985) and in mice by intraperitoneal administration (IARC 1999). Acetaldehyde also increased the frequency of micronucleus formation in mice by intraperitoneal administration (FSCJ 2005a). On the other hand, negative results were detected in micronucleus tests using germ cells by intraperitoneal administration (IARC 1999). DNA adduct formation in humans was detected in granulocytes and lymphocytes in the blood caused by acetaldehyde through alcohol intake (Fang & Vaca 1997; Brooks & Zakhari 2014).

Positive results were reported in *in vitro* forward- or reverse-mutation assays of acetoin, hexanal and butyl alcohol (Garst et al. 1983; Brambilla et al. 1989; WHO 1998), but negative results were detected in other mutation tests. 4-Methyl 2-pentanone showed no genotoxicity in either *in vitro* or *in vivo* assays (WHO 1999a, 1999b).

While acetaldehyde is considered to be genotoxic, JECFA concluded that it does not raise any safety concerns at the current levels of intake because acetaldehyde can be predicted to undergo complete metabolism to endogenous products via the fatty acid and tricarboxylic acid pathways (WHO 1998). With regard to acetoin, hexanal and butyl alcohol that showed some positive results in genotoxicity studies, these hydrolysed products belong to structural class I, according to the results of the Cramer decision tree. Therefore, it does not raise any safety concerns at the current levels of intake because hydrolysed products can be predicted to undergo complete metabolism to endogenous products.

#### Evaluation of acetals based on JECFA's procedure

This review has presented the key scientific data relevant to the safety evaluation of acetals for use as food flavours using the JECFA evaluation procedure. Regarding the prediction of genotoxicity, four of the

five substances had no structural alerts so it was considered that these substances had no genotoxic potential based on *in silico* software analyses. While genotoxicity study data are not available for all five acetals of concern, it is considered that these substances can be rapidly hydrolysed into alcohols and aldehydes or ketones. Moreover, structurally acetal-related substances are also reported to be hydrolysed into aldehydes and alcohols. In contrast, according to the Cramer's decision tree (Cramer et al. 1976), two of the five substances, acetoin dimethyl acetal and hexanal dibutyl acetal, were categorised as class I and the other three substances, acetaldehyde 2,3-butanediol acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal, were categorised as class III. The estimated daily intakes for each of these substances were between 1.45 and 6.53  $\mu\text{g}/\text{person}/\text{day}$  or between 156 and 720  $\mu\text{g}/\text{person}/\text{day}$ , using the MSDI or SPET method, respectively. The daily intake of the two class I substances were below the toxicological threshold of concern of 1800  $\mu\text{g}/\text{person}/\text{day}$ . Conversely, the daily intake of the three class III substances exceeded the TTC of 90  $\mu\text{g}/\text{person}/\text{day}$ . Two of them, acetaldehyde 2,3-butanediol acetal and hexanal glyceryl acetal, were immediately metabolised after ingestion and excreted as endogenous products. Ingested 4-methyl-2-pentanone propyleneglycol acetal is hydrolysed into 4-methyl-2-pentanone and propyleneglycol. Among them, propyleneglycol will be metabolised into endogenous products (WHO 2002). However, 4-methyl-2-pentanone is not endogenously metabolised with a NOEL of 50  $\text{mg kg}^{-1} \text{day}^{-1}$ , which is more than 15 000 times the daily intake level of 4-methyl-2-pentanone propyleneglycol acetal determined by the SPET method. Thus, it was concluded that all five substances raise no safety concerns when used for flavouring foods at the currently estimated intake levels.

#### Evaluation of toxicity data of acetal metabolites

The subacute toxicity studies of acetal metabolites revealed no major toxicity with these compounds. Available studies on the genotoxicity of acetal components also provided negative results except for some positive results with acetaldehyde. Acetaldehyde was also reported to be carcinogenic by high-dose inhalation exposure in rodents.

JECFA has previously concluded that acetaldehyde does not raise any safety concerns at the current levels of intake because ingested acetaldehyde will undergo complete metabolism to endogenous products and its level do not exceed the physiological range (WHO 1998). On the other hand, there is genetic-epidemiological evidence showing that humans deficient in the oxidation of acetaldehyde to acetate have a substantially increased