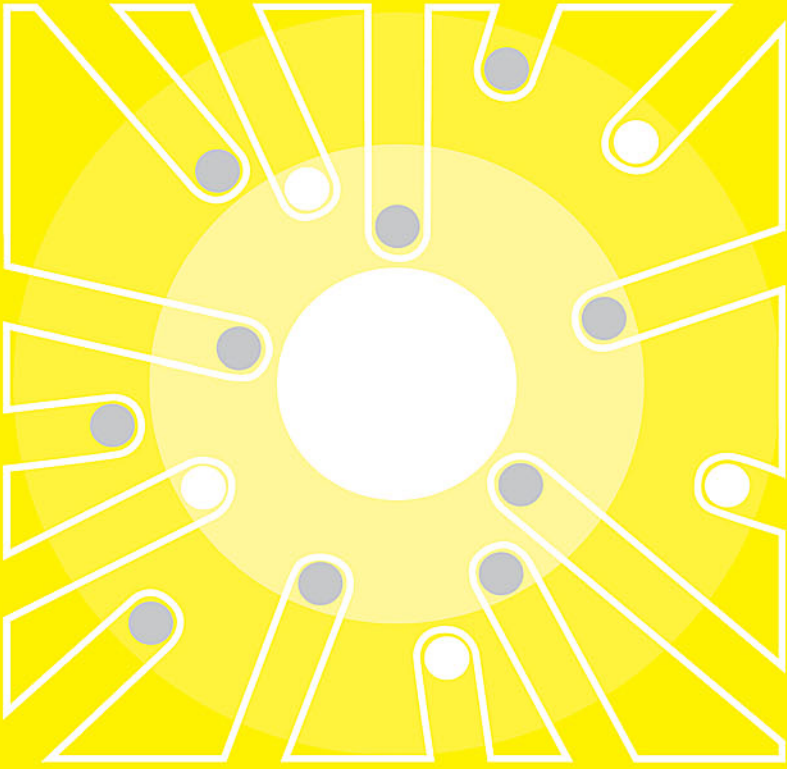
WHO FOOD ADDITIVES SERIES: 68

Safety evaluation of certain food additives and contaminants

Prepared by the
Seventy-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

Food and Agriculture Organization of the United Nations

World Health Organization



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CONTENTS

PREFACE

The monographs contained in this volume were prepared at the seventy-seventh meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at FAO headquarters in Rome, Italy, on 4–13 June 2013. These monographs summarize the data on selected food additives reviewed by the Committee.

The seventy-seventh report of JECFA has been published by WHO as WHO Technical Report No. 983. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and dietary exposure and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in this volume are based on working papers that were prepared by WHO experts. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in WHO concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: WHO Joint Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SPECIFIC FOOD ADDITIVES

ADVANTAME

First draft prepared by

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1. EXPLANATION

Advantame (*N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl) propyl]-L-α-aspartyl]-L-phenylalanine-1-methyl ester, monohydrate, Chemical Abstracts Service No. 714229-20-6) is an *N*-substituted (aspartic acid portion) derivative of aspartame that is structurally similar to another *N*-substituted aspartame derivative, neotame (Figure 1).

Advantame has not previously been evaluated by the Committee. Although it was submitted at the seventy-sixth meeting (Annex 1, reference *211*) for consideration as a flavouring agent, the Committee decided that it would be inappropriate to evaluate this substance as a flavouring agent because it is a high-intensity sweetener, and evaluation as a food additive had been requested by the Forty-fourth Session of the Codex Committee on Food Additives (FAO/WHO, 2012a).

1.1 Chemical and technical considerations

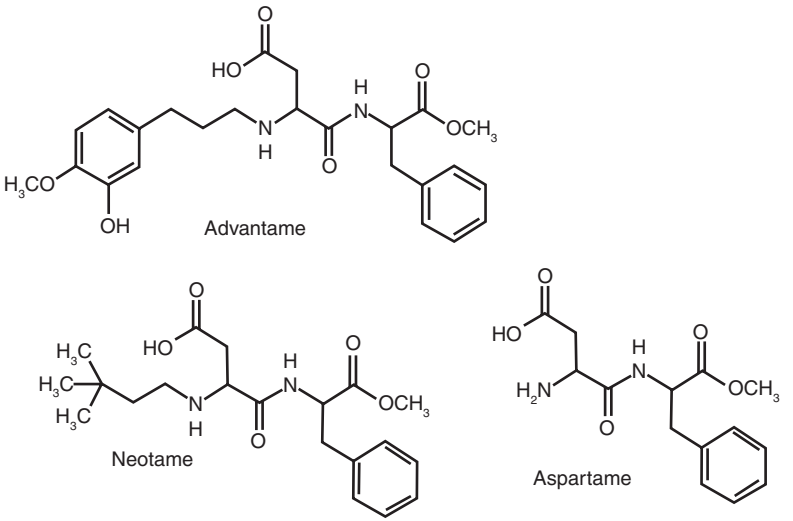
Advantame appears as a white to yellow powder that is very slightly soluble in water and sparingly soluble in ethanol. It is used as a high-intensity, non-nutritive sweetener in tabletop sweeteners and in a wide variety of foods, and it has been demonstrated to be approximately 100 times sweeter than aspartame and approximately 20 000–37 000 times sweeter than sucrose (Amino et al., 2006; Ajinomoto, 2012).

Advantame is manufactured by *N*-alkylation of the aspartic acid portion of aspartame (L-α-aspartyl-L-phenylalanine methylester), with 3-(3-hydroxy-4-methoxyphenyl) propionaldehyde produced by selective catalytic hydrogenation from 3-hydroxy-4-methoxycinnamaldehyde. Methanol and ethyl acetate are used as reaction solvents and recrystallization solvents in the preparation of advantame.

The final advantame product has a purity of not less than 97.0% on an anhydrous basis. Specifications of not more than 1.0% and 1.5% were set for the advantame acid (*N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl) propyl]-L-α-aspartyl]-L-phenylalanine) and other related substances, respectively. In addition, the limits for residual solvents, residues on ignition and lead were specified.

Advantame slowly degrades under acidic conditions and at high temperature under baking conditions. The main degradation product is the advantame acid. In contrast to aspartame, advantame does not form the diketopiperazine derivative, as there is no free amino group to start the internal reaction of cyclization.

Figure 1. Chemical structures of advantame, neotame and aspartame



2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

The absorption, distribution, metabolism and excretion of advantame have been extensively studied in mice, rats, rabbits, dogs and humans. After oral administration, advantame acid (demethylated advantame) is rapidly formed in the gastrointestinal tract as a result of the hydrolysis of the methyl ester group of the parent compound. Advantame (primarily in the form of metabolites) is mainly excreted in the faeces in rats, dogs and humans, with urinary excretion representing a minor route. Advantame acid is generally the predominant metabolite found in plasma, urine and faeces of all species studied. Based on measured urinary concentrations of radioactivity following oral dosing and the proportion of radioactivity present in faeces following intravenous dosing, the bioavailability of advantame (predominantly in the form of advantame acid) is estimated to be approximately 10% in rats and dogs. Although humans are exposed to significantly lower doses than experimental animals, bioavailability in humans, based on measured urinary metabolites, was estimated to be at least 6%.

(a) In vitro studies

[^{14}C]Advantame (50 µg/ml) and its primary metabolite, [^{14}C]advantame acid (25 µg/ml), were incubated in simulated gastric fluid and simulated intestinal fluid (USP, 1995) at 37 °C for up to 120 minutes to mimic conditions in the human digestive tract. Samples were collected 0, 1, 5, 15, 30, 60 and 120 minutes after incubation and analysed by high-performance liquid chromatography (HPLC) and liquid scintillation counting (LSC).

Advantame was found to be stable in the simulated gastric fluid, both with and without pepsin, for the entire incubation period, with only approximately 3.5% hydrolysis to advantame acid. Incubation of advantame in simulated intestinal fluid containing pancreatin resulted in rapid and almost complete hydrolysis to advantame acid within 5 minutes, whereas only a very limited amount of hydrolysis (about 3.4%) occurred in intestinal fluid without added pancreatin. Advantame acid was very stable in both simulated gastric fluid and simulated intestinal fluid, with greater than 98.0% remaining in all cases after the 120-minute incubation period (HLS, 2002).

(b) In vivo studies

(i) Mice

Advantame was administered in the diet at a concentration of 0, 5000, 15 000 or 50 000 mg/kg to groups of Charles River CD-1 mice (40–44 days old, 40 of each sex per group) for a period of 13 weeks. The dietary concentrations of advantame resulted in doses of 0, 734, 2129 and 7444 mg/kg body weight (bw) per day for males and 0, 892, 2593 and 9317 mg/kg bw per day for females for the 0, 5000, 15 000 and 50 000 mg/kg diet groups, respectively. Blood samples were collected for analyses of advantame and advantame acid during weeks 1, 6 and 12 (six sampling times per collection period).

The extent of systemic exposure to advantame, based on maximum plasma concentration (C_{max}) and area under the plasma concentration–time curve at 24 hours (AUC_{24}) values, generally increased with dose, but was not consistently proportional (Table 1). At the highest dose, the observed AUC_{24} values were 30–66% less than what would be predicted based on linearity. There was no significant trend observed with respect to increasing concentrations of advantame or advantame acid over time. Advantame acid concentrations were significantly greater (3.6- to 23-fold) than advantame concentrations at all time points (Table 2), suggesting extensive metabolism of advantame (Baldrey, Flack & Carr, 2002).

(ii) Rats

[^{14}C]Advantame (measured purity 99.9%; specific activity 2.6 MBq/mg) was administered either by gavage (5 or 150 mg/kg bw in 1% [weight per volume (w/v)] aqueous sodium carboxymethyl cellulose) or by intravenous injection (5 mg/kg bw in isotonic saline) as a single dose to male and female Han Wistar rats (7–10 weeks of age; four of each sex per dose). Urine and faeces were collected 0–6, 6–12, 12–24, 24–48, 48–72 and 72–96 hours after dosing for LSC, and urine and faeces were analysed for metabolites by HPLC with radiodetection.

Following oral dosing, very low amounts of radioactivity (<2% of the total dose) were detected in the urine within 96 hours. In comparison, 23.9–25.8% of the dose administered by intravenous injection was excreted in the urine within 96 hours.

The faeces represented the major route of excretion for radioactivity, with approximately 95–98% of the oral dose being excreted within 96 hours; 55–75% of the low dose was excreted within the first 12–24 hours, and between 84% and 97%

Table 1. Toxicokinetics of advantame in the 13-week mouse study

Dietary concentration (mg/kg)	Mean C_{max} (ng/ml)						Mean AUC_{24} (ng·h/ml)					
	Week 1		Week 6		Week 12		Week 1		Week 6		Week 12	
	M	F	M	F	M	F	M	F	M	F	M	F
5 000	106	135	75.9	65.1	176	167	1 258	1 824	751	849	1 807	2 294
15 000	267	342	167	319	169	1 514	4 149	4 844	2 340	3 351	2 517	9 065
50 000	615	1 253	217	342	628	635	7 812	12 431	3 825	6 196	6 268	9 125

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration; F, female; M, male

Table 2. Toxicokinetics of advantame acid in the 13-week mouse study

Dietary concentration (mg/kg)	Mean C_{max} (ng/ml)						Mean AUC_{24} (ng·h/ml)					
	Week 1		Week 6		Week 12		Week 1		Week 6		Week 12	
	M	F	M	F	M	F	M	F	M	F	M	F
5 000	670	711	442	783	338	697	12 207	13 811	8 602	12 704	6 518	10 953
15 000	1 172	2 327	1 292	2 358	1 159	1 946	25 591	42 433	26 624	46 231	23 133	36 826
50 000	4 907	7 659	5 617	8 416	5 818	6 655	90 548	136 701	95 680	141 734	97 421	138 790

AUC_{24}, area under the plasma–concentration time curve after 24 hours; C_{max}, maximum plasma concentration; F, female; M, male

of the high dose was excreted within the first 6–24 hours. The faeces were also the major route of excretion after the 5 mg/kg bw intravenous dose, with 72.5% of the administered dose excreted within 96 hours. A comparison of the percentage of radioactivity excreted in the urine following the low and high oral doses with the percentage excreted in the urine following the intravenous dose indicated that approximately 4–8% of the oral doses was absorbed.

No advantame parent compound was detected in urine or faeces following oral dosing (advantame was detected in urine of female rats only following intravenous dosing). Advantame acid accounted for approximately 20–30% of total urinary radioactivity at the low dose and approximately 50% at the high dose (0.3–0.6% of the total administered dose). Other minor metabolites detected in urine after oral dosing included HU-1 (3(3-hydroxy-4-methoxyphenyl)-1-propylamine) and HF-1 (demethylated advantame acid; *N*-(3-(3-hydroxy-4-methoxyphenyl)) propyl-L-aspartic acid). The main metabolite in urine following intravenous dosing was advantame acid (approximately 94% of total urinary radioactivity in males and females). At the low oral dose and following intravenous dosing, three faecal metabolites were detected (advantame acid; RF-1, demethylated advantame acid; and RF-2, unidentified metabolite), whereas only advantame acid was detected at the high oral dose (Table 3) (Ubukata, Nakayama & Mihara, 2011).

[^{14}C]Advantame (purity 99.9%; radiochemical purity >98%) was administered to male and female Han Wistar rats (7–10 weeks of age) by oral gavage (30 of each sex) as a single dose of 5 or 150 mg/kg bw or intravenously (33 of each sex) at a dose of 5 mg/kg bw. Blood samples were taken from subgroups of six rats (three of each sex) from the oral dose groups at 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 12 and 24 hours after dosing, with identical sampling for the intravenously dosed animals (plus one additional time point at 0.1 hour). Control animals (three per dose group) received equivalent volumes of dosing solvent controls (carboxymethyl cellulose [1% (w/v)] for gavage, isotonic saline for intravenous). The plasma concentration of radioactivity was measured using LSC, whereas the plasma concentrations of advantame and the major metabolite, advantame acid, were measured using HPLC equipped with a radiodetector and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Analysis was performed to determine various pharmacokinetic end-points, which are presented in Tables 4 and 5.

Note: The original analytical extraction procedure was reported to result in the partial re-esterification of advantame acid to advantame; experimental data indicated that a maximum of 5% of advantame acid might be converted back to advantame. All values for advantame acid are expressed in terms of “advantame acid equivalents”, which represent the total of free, re-esterified advantame and conjugated advantame acid present in the rat plasma samples. This LC-MS/MS underestimation was corrected in the rat chronic bioassay (see below).

The time to reach the maximum plasma radioactivity concentrations (T_{max}) following the oral doses was rapid, occurring at 15 minutes post-dosing in rats administered advantame at 5 mg/kg bw, whereas male and female rats dosed with 150 mg/kg bw had T_{max} values of 45 minutes and 30 minutes, respectively. Mean C_{max} and AUC values increased approximately proportionally to the oral

Table 3. Mean total proportion of the administered dose of advantame excreted as metabolites in the faeces of rats

Dose (mg/kg bw)	Sex	Metabolites excreted (% of dose administered)		
		Advantame acid	RF-1[a]	RF-2
5 (oral)	Male	29.1	40.9	11.5
	Female	28.8	41.1	11.9
150 (oral)	Male	86.2	nd	nd
	Female[b]	88.1	nd	nd
5 (intravenous)	Male	20.7	26.9	8.1
	Female	26.2	27.6	8.3

bw, body weight; nd, not detected

[a] Demethylated advantame acid.

[b] Only data from 48- to 72-hour collection period were analysed.

Table 4. Pharmacokinetic parameters for total radioactivity in rats orally dosed with ^{14}C-labelled advantame

Parameter	5 mg/kg bw		150 mg/kg bw	
	Males	Females	Males	Females
C_{max} (ng eq/g)	105	136	2 366	4 066
T_{max} (h)	0.25	0.25	0.75	0.50
AUC_t (ng eq·h/g)	313	348	7 760	9 510
AUC (ng eq·h/g)	334	382	8 010	10 100
$t_{½}$ (h)	7.2	8.1	6.0	7.3

AUC, area under the 24-hour mean plasma concentration–time curve; AUC_t, area under the 24-hour mean plasma concentration–time curve at the final quantifiable time point; C_{max}, maximum plasma concentration; eq, equivalents; $t_{½}$, half-life; T_{max}, time to reach maximum plasma concentration

Table 5. Pharmacokinetic parameters for advantame acid equivalents in rats orally dosed with ^{14}C-labelled advantame

Parameter	5 mg/kg bw		150 mg/kg bw	
	Males	Females	Males	Females
C_{max} (ng eq/g)	7.6	8.1	226.7	412.9
T_{max} (h)	0.75	0.25	1.0	0.50
AUC_t (ng eq·h/g)	13.1	23.2	875	1010
AUC (ng eq·h/g)	14.3	25.1	877	1020
$t_{½}$ (h)	1.9	2.1	3.1	3.6

AUC, area under the 24-hour mean plasma concentration–time curve; AUC_t, area under the 24-hour mean plasma concentration–time curve at the final quantifiable time point; C_{max}, maximum plasma concentration; eq, equivalents; $t_{½}$, half-life; T_{max}, time to reach maximum plasma concentration

dose, with the oral bioavailability of radioactivity calculated to be less than 10% of the administered dose. The half-life of radioactivity following oral dosing was on average 7.1 hours, with no significant difference between the two dose groups.

Advantame was rapidly and completely hydrolysed to advantame acid equivalents, with peak plasma concentrations measured at the earliest sampling time point (0.1 hour) after intravenous administration of 5 mg/kg bw (results not shown).

In comparison, the time to reach C_{max} (T_{max}) for advantame acid equivalents was 45 and 60 minutes for males dosed with 5 or 150 mg/kg bw, with slightly shorter times observed for the female rats (15 and 30 minutes, respectively). The advantame acid equivalents present in the plasma accounted for approximately 6% and 11% of the 5 and 150 mg/kg bw oral doses, respectively. The plasma half-life of advantame acid equivalents was on average 2 and 3.4 hours at the 5 and 150 mg/kg bw oral doses, respectively (Aikens, Kane & Houchen, 2004; Ubukata, Nakayama & Mihara, 2011).

[^{14}C]Advantame (radiochemical purity 98.3%) was administered to 21 male Lister Hooded (Crl:LISBR) rats (196–217 g; 6–7 weeks of age) as a single gavage dose of 5 mg/kg bw in 1% (w/v) carboxymethyl cellulose. Three rats were sacrificed at 0.25, 1, 2, 6, 12, 24 and 48 hours after dosing, and a range of organs and tissues was analysed for radioactivity by LSC.

The majority of radioactivity was detected in the contents of the gastrointestinal tract (approximately 70% of the administered dose over the first 6 hours), with the tissues of the digestive tract (stomach, small and large intestine, caecum) having the next highest levels. Radioactivity rapidly declined in all tissues, with the majority excreted in the faeces within 24 hours. Based on the level of radioactivity in organs and tissues other than the gastrointestinal tract and its contents (89.2–97.1% at 6 hours), the level of gastrointestinal absorption was estimated to be 3–11% of the administered dose, with no indication of tissue-specific accumulation (Aikens et al., 2002). In a similar study conducted with five pregnant Hans Wistar rats, the tissue distribution of radioactivity analysed by whole-body autoradiography demonstrated that up to 12 hours following a single 5 mg/kg bw oral dose of advantame, no radioactivity was detected in the placenta or fetus (Aikens, Kane & Houchen, 2004).

Groups of male and female Wistar (Han) rats (36–44 days of age; nine of each sex per group) were fed, ad libitum, diets containing advantame (purity 99.5%; batch no. 001227) at a concentration of 0, 1500, 5000, 15 000 or 50 000 mg/kg, equal to doses of 0, 118, 415, 1231 and 4227 mg/kg bw per day for males and 0, 146, 481, 1487 and 5109 mg/kg bw per day for females, respectively, for 13 weeks. Blood samples were collected for analysis of advantame and advantame acid during weeks 1 and 13 (samples taken at 6 times over a 24-hour period).

Systemic exposure to advantame and advantame acid during weeks 1 and 13, based on calculated C_{max} and AUC_{24} values, generally increased with increasing concentration over the range 1500–50 000 mg/kg diet. However, exposure increased by less than the proportionate dose increment; in general, there

was statistically significant evidence of non-linearity ($P < 0.001$). At the highest concentration (50 000 mg/kg diet), the C_{max} and AUC_{24} values in male and female rats were approximately 40–92% lower than those values predicted from a linear relationship. While systemic exposure of rats to advantame acid was higher during week 13 than during week 1 in the 1500 mg/kg diet group, exposure indices were generally similar for the other groups (i.e. no statistically significant evidence for a time-related difference) (Chase, 2004).

As part of the in utero carcinogenicity study (see section 2.2.3), groups of HsdBrl Han:Wist (Han Wistar) rats (12 of each sex per dose) were fed, ad libitum, diets containing advantame (purity ≥99.2%; lot no. 010212) at a concentration of 0, 2000, 10 000 or 50 000 mg/kg for 104 weeks. Dietary concentrations were equal to doses of 0, 97, 488 and 2621 mg/kg bw per day for males and 0, 125, 630 and 3454 mg/kg bw per day for females, respectively. Blood was sampled during weeks 14, 26, 52 and 104 for toxicokinetic analysis (blood sampled 6 times over a 24-hour period and analysed for advantame and advantame acid).

Systemic exposure to advantame and advantame acid, based on C_{max} and AUC_{24} values, during weeks 14, 26, 52 and 104 generally did not increase proportionally to increasing dose (Tables 6 and 7). At the highest dose, the C_{max} and AUC_{24} values for both advantame and advantame acid were 64–98% lower than what would be predicted from a linear relationship. There was no consistent sex- or time-related difference in AUC_{24} values for either advantame or advantame acid. The systemic exposure to advantame acid was significantly greater than exposure to advantame at all sampling time points, at all doses and in both sexes.

(iii) Rabbits

As part of a preliminary developmental toxicity study (see section 2.2.5), advantame (purity >99%) in 1% (w/v) methyl cellulose was administered by gavage to groups of pregnant New Zealand White rabbits (six per dose) at a dose of 0, 500, 1000 or 2000 mg/kg bw per day from day 6 to day 28 of gestation. Rabbits were approximately 18–22 weeks old and 3.59–4.62 kg at the beginning of the study. Blood was sampled from all animals on days 6 and 27 of gestation beginning 30 minutes after dosing and then at various intervals up to 24 hours, for analysis of advantame and advantame acid by LC-MS/MS.

Maximum mean plasma concentrations of advantame and advantame acid (Tables 8 and 9) were generally observed approximately 0.5–1 hour after dosing, indicating that absorption was rapid. Average metabolite ratios (AUC_{24} for advantame acid / AUC_{24} for advantame) over the course of the experiment were approximately 30, indicating extensive conversion of advantame to advantame acid. As noted by the study authors, based on the extraction method used for the LC-MS/MS analysis, it is likely that there was an overestimation of the amount of advantame and an underestimation of the amount of advantame acid at many of the time points due to re-esterification (up to 5%). Therefore, it is likely that the average metabolic ratio would be greater than 30.

While systemic exposure to both advantame and advantame acid was evident over the study duration, it was less than proportional to the dose at both time

Table 6. Toxicokinetics of advantame in the 104-week rat study

Dietary concentration (mg/kg)	Mean C_{max} (ng/ml)								Mean AUC_{24} (ng·h/ml)							
	Week 14		Week 26		Week 52		Week 104		Week 14		Week 26		Week 52		Week 104	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2 000	46.8	86.6	135	299	61.2	45.3	29.7	33.2	595	813	1 020	1 560	661	509	426	490
10 000	20.8	56.0	341	166	68.5	105	118	70.5	393	840	1 880	1 630	731	1 180	1 140	981
50 000	97.5	92.8	99.7	154	61.1	242	194	332	1 660	1 720	1 520	1 730	934	1 900	1 510	3 490

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration; F, female; M, male

Table 7. Toxicokinetics of advantame acid in the 104-week rat study

Dietary concentration (mg/kg)	Mean C_{max} (ng/ml)								Mean AUC_{24} (ng·h/ml)							
	Week 14		Week 26		Week 52		Week 104		Week 14		Week 26		Week 52		Week 104	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2 000	142	245	128	216	84.4	89.8	130	64.2	2 310	3 010	2 140	2 770	1 450	1 720	1 570	1 110
10 000	537	378	661	658	448	468	321	197	6 950	7 030	10 500	8 530	6 380	6 530	5 270	3 910
50 000	1 710	2 910	2 500	4 450	959	3 310	513	1 880	22 800	44 700	25 100	59 800	14 400	42 600	7 460	25 700

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration

Table 8. Toxicokinetics of advantame in the preliminary rabbit developmental study

Dose (mg/kg bw per day)	C_{max} (ng/ml)		AUC_{24} (ng·h/ml)	
	Day 6	Day 27	Day 6	Day 27
500	944	847	4 982	3 906
1000	714	1 178	3 737	11 594
2000	1 070	2 980	8 382	22 367

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration

Table 9. Toxicokinetics of advantame acid in the preliminary rabbit developmental study

Dose (mg/kg bw per day)	C_{max} (ng/ml)		AUC_{24} (ng·h/ml)	
	Day 6	Day 27	Day 6	Day 27
500	10 513	14 338	151 896	147 414
1000	13 694	21 382	117 112	268 999
2000	18 923	30 400	274 417	486 206

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration

points. After repeated dosing (day 27), there was some evidence of accumulation at the higher doses (1000 and 2000 mg/kg bw per day), but not at the lowest dose (Baldrey et al., 2002).

(iv) Dogs

Advantame (purity 99.9%), labelled with ^{14}C in the 3-hydroxy-4-methoxyphenyl moiety, was administered as a single oral dose of 5 mg/kg bw (1% [w/v] carboxymethyl cellulose vehicle) to male Beagle dogs (5–7 months of age; 9.0–10.0 kg). Single animals were sacrificed 6, 72, 144 and 288 hours after dosing, with blood samples collected 1, 2, 4, 6, 8, 12 and 24 hours after dosing and at subsequent 24-hour intervals up to sacrifice (last three sacrifice times). Feed was withheld 18 hours prior to and 4 hours after dosing. Radioactivity was quantified by LSC.

Peak concentrations of radioactivity in the plasma generally occurred 6–8 hours after dosing, with plasma containing the highest concentration of radioactivity detected at all sample times. The highest tissue/organ concentrations of radioactivity were detected in the large intestinal wall and the bile, with the amount of radioactivity in the bile representing only 0.1% of the dose. Radioactivity in plasma declined relatively slowly, with a mean terminal half-life of 71 hours (range 56–95 hours). The rate of decline of radioactivity observed in tissues was similar to the rate of decline observed in plasma. This, combined with relatively constant tissue to plasma ratios, suggests that radioactivity measured in most tissues was probably due to perfusion by circulating plasma (Aikens et al., 2002).

[^{14}C]Advantame (radiochemical purity >98%; batch no. AJO/139/TW1) was administered to three Beagle dogs of each sex (6 months of age; 9–11.3 kg) as a single gavage dose of 5 or 150 mg/kg bw (both in 1% [w/v] carboxymethyl cellulose) or a single intravenous dose of 5 mg/kg bw (in isotonic saline). Dosing was provided sequentially, beginning with the lowest oral dose, with a 4-week washout period between the different doses. Dogs were fasted prior to dosing, with feed and water made available ad libitum 4 hours after dosing. Blood samples were collected immediately prior to dosing and then at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hours after dosing. Urine and faeces were collected separately at 0–6, 6–12, 12–24, 24–48, 48–72, 72–96 and 96–120 hours after dosing. Radioactivity was analysed by LSC, whereas the concentrations of advantame and advantame acid were measured by LC-MS/MS and HPLC.

Following oral dosing, gastrointestinal absorption of radioactivity was relatively slow, with C_{max} values obtained between 6 and 8 hours after dosing (T_{max}). This contrasts with a T_{max} of 0.5–1 hour for advantame acid. Although there was a dose-related increase in plasma radioactivity and advantame acid following oral dosing, it was less than proportional to the dose increment. The bioavailability of radioactivity was approximately 14–15% and 8–9% at the low and high oral doses, respectively, based on comparisons of AUC values for oral and intravenous dosing.

No advantame was detected in plasma samples from the majority of dogs administered the 5 mg/kg bw oral dose (detection limit 0.5 ng/ml), whereas advantame and advantame acid were quantifiable in all dogs administered the high oral dose. Advantame was estimated to account for only approximately 0.001–0.002% of total plasma radioactivity, whereas advantame acid accounted for on average 0.34% of total plasma radioactivity. Based on the original analytical methodology, advantame and advantame acid were collectively shown to account for on average 79% of plasma radioactivity. This difference was attributed to the possible presence of conjugated metabolites of advantame acid, which would have been hydrolysed during extraction of the samples (Aikens et al., 2005). The majority of radioactivity after oral dosing was excreted in faeces within 24 hours after dosing (82–89%), with relatively low levels detected in urine (3.7–7.4%). Overall, urine, faeces and cage washings accounted for approximately 94% of the administered oral doses. The major metabolites detected in faeces were advantame acid (70–78% of the oral dose) and HF-1 (*N*-(3-(3-hydroxy-4-methoxyphenyl))propyl-L-aspartic acid) (1–4% of the oral dose), whereas advantame acid accounted for approximately 50% of the urinary radioactivity (3% of the oral dose). Other metabolites detected in urine included HU-1 (3-(3-hydroxy-4-methoxyphenyl)-1-propylamine) (approximately 1.5% of the oral dose) and an unidentified metabolite named D3 (0.5–1.0% of the oral dose). As D3 was detected only in the urine of dogs, it was not further characterized.

The half-life of plasma total radioactivity following oral dosing was 74–85 hours, which the study authors attributed to the slow elimination of a possible sulfate conjugate of advantame acid. However, as there were no sulfate conjugates of advantame acid detected in the urine or faeces, enterohepatic recirculation is also a possibility. The half-life of advantame acid in plasma was on average 5.4 hours (Aikens et al., 2005; Ubukata, Nakayama & Mihara, 2011).

Advantame (purity >97%; batch nos 010319 and 010626) was fed to groups of four Beagle dogs of each sex at a dietary concentration of 0, 2000, 10 000 or 50 000 mg/kg for 52 weeks. An additional group of two dogs of each sex was assigned to the control, intermediate-dose and high-dose groups and allowed to recover for 6 weeks following treatment. The average achieved doses over 52 weeks of treatment were 0, 83, 421 and 2058 mg/kg bw per day for males and 0, 82, 406 and 2416 mg/kg bw per day for females at 0, 2000, 10 000 and 50 000 mg/kg, respectively. Dogs were approximately 24 weeks of age at the beginning of the study, with body weights ranging from 6.4 to 10.0 kg. Blood was sampled for residue analysis during weeks 1, 13, 27 and 52 at 0, 0.5, 1, 3, 6, 12 and 24 hours after the diet was offered.

C_{max} and AUC_{24} values were always significantly higher for advantame acid than for advantame (Tables 10 and 11), indicating higher systemic exposure to the metabolite than to the parent compound. Based on the C_{max} and AUC_{24} values, systemic exposure to advantame and advantame acid was increased in a less than dose-proportionate manner, with females consistently showing higher values. There was no consistent evidence of bioaccumulation over the study duration. Metabolic ratios (AUC_{24} for advantame acid / AUC_{24} for advantame) ranged from 70 to 1324, demonstrating extensive conversion of advantame to advantame acid.

It was noted that by using the modified analytical method, a previously unidentified late-eluting peak was observed during the analysis of advantame acid. Based on retention times and fragmentation patterns using mass spectrometry, it was concluded by the study authors that the unidentified peak was likely to be a sulfate conjugate of advantame acid (Otabe, Fujieda & Masuyama, 2011a).

(v) Humans

Advantame (purity not stated; batch no. 010228), at 0.1, 0.25 or 0.5 mg/kg bw, was provided as single oral doses in 150 ml of water to groups of eight male volunteers (average age 26.1 years; mean body weight 77.6 kg), with blood samples collected for analysis at regular intervals (every 15–30 minutes for the first 6 hours and then at longer time periods) after dosing for up to 168 hours. Subjects were fasted for 11 hours prior to dosing and 2 hours following dosing; meals were then provided at three time periods throughout the day.

Plasma concentrations of advantame were below the limit of quantification (0.5 ng/ml) for all samples from the 0.1 mg/kg bw dose group and in all but nine samples from the 0.25 mg/kg bw dose group (not detected in three of eight participants). Advantame was detected consistently only in plasma samples from the highest dose group, but only up to the 1-hour collection time point (below detection limits after 1 hour post-dosing). Conversely, advantame acid was detected in the plasma of all dose groups, with a maximum plasma concentration reached in 1.4–1.6 hours after dosing. C_{max} and AUC values for advantame acid increased approximately proportionally to dose, with the elimination rate seemingly decreasing with increasing dose. The C_{max} of advantame acid was 11.8, 24.5 and 46.8 ng/ml in the 0.1, 0.25 and 0.5 mg/kg bw dose groups, respectively. Advantame acid was undetected in any samples collected 12, 36 or 48 hours after dosing for the 0.1, 0.25 and 0.5 mg/kg bw dose groups, respectively. The half-life of advantame acid

Table 10. Toxicokinetics of advantame in the 52-week dog study[a]

Dietary concentration (mg/kg)	C_{max} (ng/ml)								AUC_{24} (ng·h/ml)							
	Day 1		Week 13		Week 27		Week 52		Day 1		Week 13		Week 27		Week 52	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2 000	4.68	5.85	4.13	7.67	2.13	4.51	1.27	2.59	23.5	31.3	24.2	28.3	12.8	16.1	6.93	7.01
10 000	30.0	35.3	19.6	14.3	10.6	15.7	6.32	7.00	170	145	73.4	61.2	47.9	70.9	37.8	37.3
50 000	109	122	69.4	151	37.9	123	23.8	56.0	581	756	353	755	233	730	148	388

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration; F, female; M, male

[a] Mean values for $n = 4$ (2000 mg/kg diet) or $n = 6$ (10 000 and 50 000 mg/kg diet).

Table 11. Toxicokinetics of advantame acid in the 52-week dog study[a]

Dietary concentration (mg/kg)	C_{max} (ng/ml)								AUC_{24} (ng·h/ml)							
	Day 1		Week 13		Week 27		Week 52		Day 1		Week 13		Week 27		Week 52	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2 000	520	691	699	682	595	834	350	544	5 350	8 010	8 750	10 100	6 440	10 000	4 470	8 240
10 000	2 610	2 960	2 020	1 580	1 260	1 670	1 240	1 200	28 400	30 300	22 300	22 000	15 600	18 600	17 900	13 700
50 000	5 560	6 620	3 510	10 300	1 990	3 940	2 200	3 270	42 100	51 400	46 900	130 000	27 000	44 200	27 500	39 500

AUC_{24}, area under the plasma concentration–time curve after 24 hours; C_{max}, maximum plasma concentration; F, female; M, male

[a] Mean values for $n = 4$ (2000 mg/kg diet) or $n = 6$ (10 000 and 50 000 mg/kg diet).

was estimated to be 3.6 hours for the lowest dose group. It was noted by the authors that progressively longer half-life estimates for the higher dose groups were not directly comparable due to measurements being taken at different time periods (Warrington, 2004).

[^{14}C]Advantame (purity >98%; specific activity 0.11 MBq/mg) was administered to six fasted male volunteers (mean age 49.5 years; mean body weight 80.4 kg) as a single oral dose of approximately 18.75 mg (0.23 mg/kg bw) in 150 ml of sterile water. Blood was sampled prior to dosing and at timed intervals up to 168 hours post-dosing (initially every 10–15 minutes and then at progressively longer durations). Food was provided 4 and 10 hours after dosing. All samples of urine and faeces were collected 12 hours prior to dosing and during predetermined intervals up to 168 hours post-dosing. The blood, plasma, urine and faeces were analysed for radioactivity by LSC. Plasma was analysed for advantame and advantame acid by LC-MS/MS. Radioactive metabolites were separated from urine and faeces by HPLC with radiodetection, with additional confirmation of compounds present in the urine and the faeces determined using tandem mass spectrometry with multiple reaction monitoring.

On average, 6.2% of the radioactivity was excreted in the urine, with 60% of this excretion occurring within the first 24 hours. A mean of 89.5% of radioactivity was excreted in the faeces, with the majority excreted for most subjects within the first 72 hours. The mean amount of administered radioactivity recovered was 95.7%, with essentially all excretion of radioactivity complete by 120 hours after dosing. The maximum mean concentration of radioactivity in plasma occurred 1.25 hours after dosing, whereas the mean half-life for elimination of radioactivity was 3.9 hours. By 24 hours after dosing, plasma radioactivity was below the limit of quantification for five of the six subjects.

Advantame was below the limit of quantification (0.5 ng/ml) in plasma at all sampling times for three of the six subjects and quantifiable at only two sampling times for each of the remaining three subjects. In contrast, advantame acid was detectable in plasma samples from all subjects within 15 minutes after dosing, reaching a maximum value (mean 22.7 ng/ml) by 1.75 hours after dosing. Concentrations of advantame acid in plasma rapidly declined, with an average half-life of 5.7 hours. Comparison of total plasma radioactivity with concentrations of advantame acid indicated that 84.6–92.0% of plasma radioactivity could be accounted for by advantame acid.

No advantame was detected in any urine sample. Advantame acid was the predominant metabolite detected and represented a mean total of 2.3% of the dose. Two other metabolites were also detected in urine: HU-1 (3-(3-hydroxy-4-methoxyphenyl)-1-propylamine), which represented a mean total of 1.9% of the dose, and HF-1 (*N*-(3-(3-hydroxy-4-methoxyphenyl)) propyl-L-aspartic acid), which represented a mean total of 1.0% of the dose. Treatment of urine samples with β-glucuronidase and/or sulfatase enzymes yielded no additional advantame, implying that glucuronide or sulfate conjugates of advantame were not present. Co-chromatographic analysis (thin-layer chromatography with reversed-phase HPLC) of human, dog and rat urine demonstrated that the three major metabolites identified in human urine were also detected in urine from rats and dogs following oral dosing with advantame.

As with urine, no advantame was detected in any faecal samples. The two major metabolites that accounted for the majority of faecal radioactivity were identified as advantame acid (mean 52% of the dose) and HF-1 (mean 30% of the dose). HU-1 was not detected in faeces (Ubukata, Nakayama & Mihara, 2011).

In a follow-up study, a group of 12 volunteers (6 males, 6 females; average age 31.8 years) received oral doses of 10 mg of advantame (purity >99%) 3 times/day with meals for 4 consecutive weeks. Doses were provided in cellulose capsules and were equal to 0.375–0.5 mg/kg bw per day. Subjects were fasted overnight prior to dosing. Blood samples were collected immediately prior to dosing on days 8, 15, 22 and 29 and analysed for the presence of advantame and advantame acid.

Advantame was below the limit of quantification (0.5 ng/ml) in all blood samples. Although variable, plasma concentrations of advantame acid appeared to be at steady state after 8 days, with no evidence of accumulation or a significant difference between males and females. Mean plasma levels of advantame acid were 9.7, 10.3, 11.8 and 11.9 ng/ml on days 8, 15, 22 and 29, respectively (Warrington et al., 2011).

A similar study to Warrington (2004) (see above) was performed with volunteers with Type 2 diabetes. Inclusion criteria for participants were 31–70 years of age, diagnosed with relatively stable diabetes at least 1 year prior to enrolment and acceptable blood glucose control. Thirty-six subjects (18 of each sex; average age 61.4 years; average body weight 82.9 kg) ingested 10 mg of advantame (purity >99%) in cellulose capsules 3 times/day with meals for 12 weeks. Blood samples were collected for analysis of advantame and advantame acid prior to the start of the experiment and on dosing days 8, 29, 57 and 85. Daily doses ranged from 0.375 to 0.5 mg/kg bw per day.

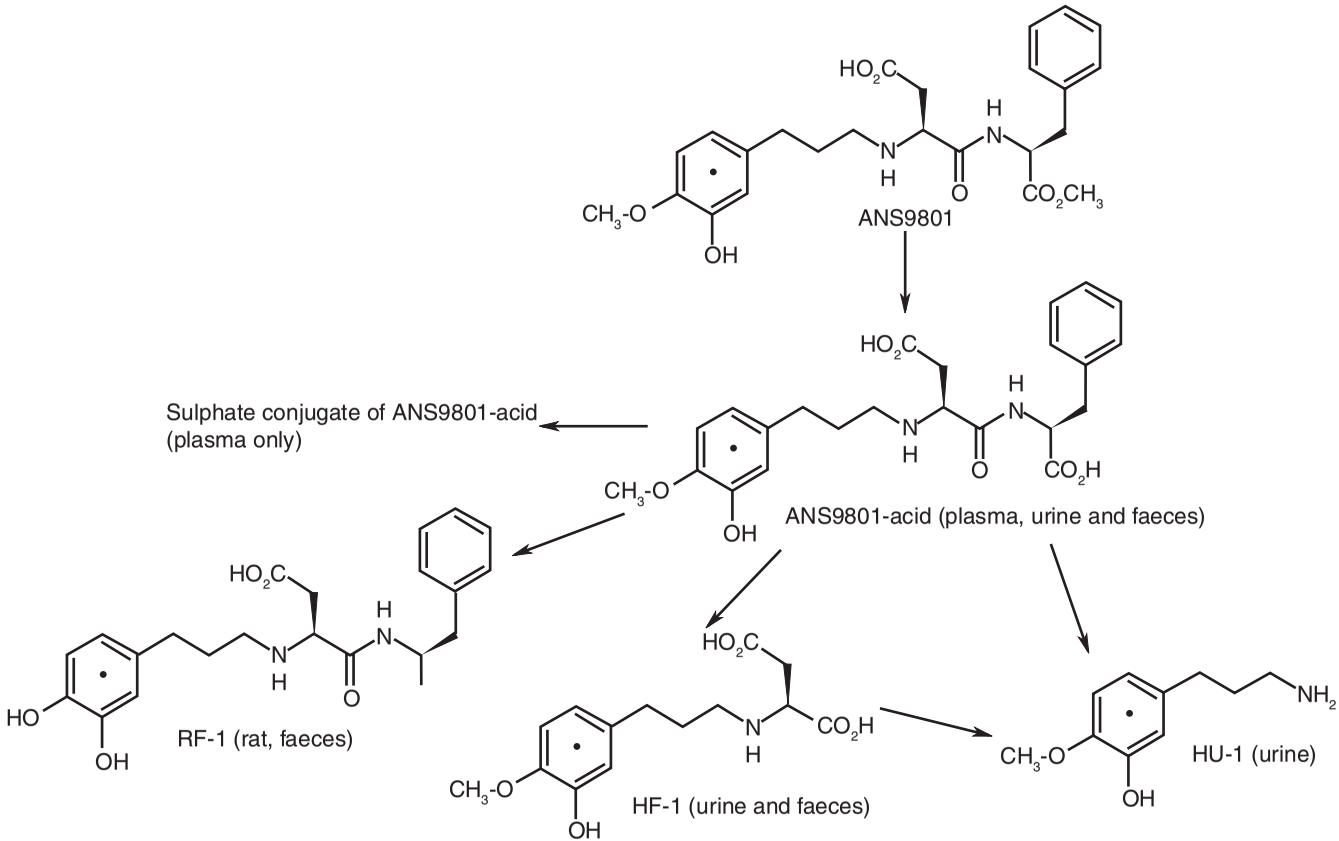
Advantame was not detected in any test subject (detection limit 1.0 ng/ml) throughout the study. Plasma concentrations of advantame acid were highly variable between subjects, with mean concentrations of 16.2, 14.6, 12.5 and 13.6 ng/ml at the 8-, 29-, 57- and 85-day sampling points, respectively. The findings suggested that steady state had been achieved over the dosing period and that there was no evidence of bioaccumulation (Pirage, 2006).

Based on the analysis of metabolites, the metabolic pathway shown in Figure 2 was proposed for advantame.

2.1.2 Effects on enzymes and other biochemical parameters

The in vitro binding of ^{14}C-labelled advantame and of ^{14}C-labelled advantame acid (purity >99%) to proteins in human, dog and rat plasma was investigated. Pooled human plasma samples were incubated with various concentrations of advantame (10, 100, 500 or 1000 ng/ml) or advantame acid (10, 100, 1000 or 5000 ng/ml) for 10 minutes at 37 °C. Similarly, pooled samples of dog plasma were mixed with advantame at 20, 200, 2000 or 20 000 ng/ml or with advantame acid at 100, 1000, 10 000 or 25 000 ng/ml, whereas rat plasma was mixed with

Figure 2. Proposed metabolic pathway for advantame (ANS9801) (Ubukata, Nakayama & Mihara, 2011)



advantame acid only at 10, 100, 1000 or 10 000 ng/ml. At the end of the incubation period, samples were subjected to centrifugal ultrafiltration to separate bound from unbound fractions, and the radioactivity was quantified by LSC.

On average, 81.3–92.4% of advantame was bound to human plasma proteins, whereas 96.2–96.9% of advantame acid was bound. Similarly, the extent of advantame acid binding to rat plasma protein ranged from 90.5% to 91.8%. In dog plasma samples, lower percentages of binding were observed: 63.1–64.9% for advantame and 61.6–71.3% for advantame acid. There was no concentration effect noted for any binding or evidence of saturation (Aikens & Hickson, 2004).

2.2 Toxicological studies

The results of the toxicokinetics analysis conducted as part of the study protocols of the experimental animal and human clinical studies are discussed in section 2.1.1 above.

The non-neoplastic effects and no-effect levels from the critical toxicity studies discussed below are summarized in Table 12.

2.2.1 Acute toxicity

(a) Rats

Groups of five male and five female Wistar (Han) rats (5–7 weeks of age) were administered advantame (purity 100.1%; lot no. 000825) by gavage at a single dose of 5000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. The study was conducted according to good laboratory practice (GLP) requirements (Organisation for Economic Co-operation and Development [OECD], United Kingdom, European Commission, United States Food and Drug Administration [USFDA] and Japan) and test guidelines (European Economic Community, OECD Test Guideline 423, USFDA). Animals were observed for clinical signs twice daily over a 15-day period after treatment, and body weights were recorded on days 7 and 14 after treatment. Animals were sacrificed and examined for gross pathological changes on day 15 after treatment.

There were no deaths during the study. One female rat exhibited increased salivation immediately after dosing, whereas all rats, both male and female, had white-coloured faeces on day 2 after treatment. All animals appeared fully recovered from treatment by day 3. Body weight gains were unremarkable, and there were no treatment-related macroscopic abnormalities. The acute oral median lethal dose (LD_{50}) in Wistar rats was in excess of 5000 mg/kg bw (Blanchard & Clemson, 2001).

2.2.2 Short-term studies of toxicity

(a) Mice

In a 13-week range-finding study, groups of CD-1 mice (20 of each sex per group; 40–44 days of age) were given, ad libitum, diets containing advantame (purity

Table 12. Overview of NOAEL values from the toxicity studies conducted with advantame

Species, study duration, study type	Route	Dose (male/female, mg/kg bw per day)	NOAEL (mg/kg bw per day)	Treatment-related effects *[comments]*	Reference
Rat (single dose)	Oral gavage	5000/5000	N/A	All animals: white-coloured faeces One female: increased salivation *[all animals recovered by day 3; no macroscopic abnormalities]* LD_{50} > 5000 mg/kg bw	Blanchard & Clemson (2001)
Mouse 13 weeks (range finding)	Dietary	0, 734/892, 2129/2593 or 7444/9317	7444	High dose: reduced body weight gain and feed conversion efficiency *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Chase (2002a)
Rat 4 weeks (range finding)	Dietary	0, 174/187, 1739/1920, 3672/3739 or 6126/6490	6126	All doses: discoloured faeces *[incidence increased in two highest dose groups]*; green/purple/blue discoloured cage lining High dose: decreased body weight gain and feed conversion efficiency; pale contents of lower gastrointestinal tract in females *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Chase (2002b)
Rat 13 weeks	Dietary	0, 118/146, 415/481, 1231/1487 or 4227/5109	4227	All except low dose: pink/green discoloured cage lining High-middle and high doses: pale faeces; decreased plasma urea *[attributed to decreased dietary protein; adaptive response not toxicologically significant]* High dose: pale contents of caecum and colon *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Chase (2004)
Dog 4 weeks	Dietary	0, 232/254, 737/743 or 2385/2488	2385	Middle and high doses: reduced thymus weight *[no correlating histopathology/haematology; not toxicologically significant]* High dose: pale faeces, reduced body weight gain and feed conversion efficiency *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Barker (2002)

Table 12 (continued)

Species, study duration, study type	Route	Dose (male/female, mg/kg bw per day)	NOAEL (mg/kg bw per day)	Treatment-related effects *[comments]*	Reference
Dog 13 weeks	Dietary	0, 205/229, 667/703 or 2230/2416	2230	All doses: loose/liquid faeces High dose: pale faeces, reduced body weight gain *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*; reduced relative thymus weight *[with corroborating macropathology and histopathology but within historical control data; not toxicologically significant]*	Powell & Scott (2005)
Dog 52 weeks	Dietary	0, 82.5/81.9, 420.9/406.2 or 2058/2139	2058	Middle and high doses: pale faeces *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Powell, Lelasseux & Crome (2005); Otabe, Fujieda & Masuyama (2011a)
Mouse 104 weeks Carcinogenicity	Dietary	0, 213/272, 1057/1343 or 5693/7351	5693	High dose: decreased body weight gain and feed conversion efficiency *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Horne (2006)
Rat 104 weeks Combined toxicity and carcinogenicity	Dietary (in utero prior to selection for main study)	0, 97/125, 488/630 or 2621/3454	2621	Middle and high doses: pale/dark green faeces; green/pink/purple discoloured cage lining *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]* High dose: consistently lower plasma urea concentrations *[attributed to decreased dietary protein; adaptive response not toxicologically significant]* High dose: pale and swollen anus; decreased body weight gain and feed conversion efficiency; green content in gastrointestinal tract (females killed in extremis) *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Horne (2005); Otabe, Fujieda & Masuyama (2011b)

Rat Two-generation reproductive toxicity	Dietary	F_0 prior to mating: 0, 164/184, 833/907 or 4410/4776 F_0 females gestation/ lactation: 0, 163/320, 795/1575 or 4136/8192 F_1 prior to mating: 0, 204/229, 1036/1139 or 5431/5920 F_1 females gestation/ lactation: 0, 167/316, 865/1592 or 4457/8447	Highest dietary concentration (equal to doses in the range of 4000–6000 during period prior to mating and gestation and over 8000 during lactation)	F_0 and F_1 middle and high doses: pale faeces; green/purple discoloured cage lining F_0 and F_1 high dose: higher feed consumption and decreased feed conversion efficiency *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Willoughby (2004); Otabe, Fujieda & Masuyama (2011d)
Rat Days 0–20 of gestation (embryo-fetal developmental toxicity)	Dietary	0, 465, 1418 or 4828	Maternal and developmental: 4828	All doses: pale faeces; green/purple/blue discoloured cage lining *[highest incidence in high-dose group; attributed to high concentration of non-caloric substance in diet; not toxicologically significant]* High dose: reduced body weight and body weight gain; reduced feed consumption on days 0–2 only *[attributed to high concentration of non-caloric substance in diet; not toxicologically significant]*	Willoughby (2002); Otabe, Fujieda & Masuyama (2011e)

Table 12 (continued)

Species, study duration, study type	Route	Dose (male/ female, mg/kg bw per day)	NOAEL (mg/kg bw per day)	Treatment-related effects *[comments]*	Reference
Rabbit Days 6–28 of gestation (embryo-fetal developmental toxicity; preliminary range finding)	Oral gavage	0, 500, 1000 or 2000	N/A	All doses: green/purple/pink discoloured cage lining; gaseous dark contents of caecum Low dose: dark/green bladder contents Middle dose: green/purple urine Middle and high doses: pale faeces; green staining of body surfaces High dose: dark/green bladder contents *[attributed to high concentration of non-caloric substance in diet; not considered adverse]*	Fulcher, Renaut & Bottomly (2002)
Rabbit Days 6–28 of gestation (embryo-fetal developmental toxicity)	Oral gavage	0, 500, 1000 or 2000	Maternal: 500 Developmental: 2000	All doses: green/purple/pink discoloured cage lining; green/purple/ blue/pink urine; green staining of body surfaces; green bladder content Middle dose: 1 dam sacrificed in extremis, with loss of body weight, collapsed posture, loss of coordination/locomotion, vocalization; kidneys green, green content of gastrointestinal tract High dose: 5 dams sacrificed in extremis, with loss of body weight, low feed consumption, low faecal output, dark content of gastrointestinal tract; 1 dam aborted litter, with loss of body weight, low feed consumption, dark content of caecum *[similarity of clinical signs and necropsy findings of mortalities in the mid- and high-dose dams considered adverse treatment-related effect]* **Maternal LOAEL = 1000 mg/kg bw per day**	Fulcher, Renaut & Bottomly (2003); Otabe, Fujieda & Masuyama (2011e)

bw, body weight; LD_{50}, median lethal dose; LOAEL, lowest-observed-adverse-effect level; N/A, not applicable; NOAEL, no-observed-adverse-effect level

99.5%; batch no. 001227) at a concentration of 0, 5000, 15 000 or 50 000 mg/kg, equal to doses of 0, 734, 2129 and 7444 mg/kg bw per day for males and 0, 892, 2593 and 9317 mg/kg bw per day for females, respectively. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Satellite groups of 40 mice of each sex were dosed similarly for toxicokinetic analysis. Animals were examined throughout the study period, and body weight and feed consumption were monitored weekly. Blood samples were collected for toxicokinetic analyses during weeks 1, 6 and 12. After sacrifice, a macroscopic examination of all animals was undertaken, as well as measurement of organ weights (except thyroid). This study was designed as a preliminary dose range–finding study for a 2-year carcinogenicity study (see section 2.2.3 (a)) and therefore did not include analyses of haematology, clinical chemistry or urine analysis parameters. In addition, no histopathology or ophthalmoscopy was performed.

There were no treatment-related mortalities or clinical signs. Mean body weight gain over the 13 weeks of treatment was approximately 10% lower in both sexes of the 50 000 mg/kg diet group compared with the control (males: 95%, 99% and 89%; females: 90%, 105% and 89% of the control at 5000, 15 000 and 50 000 mg/kg diet, respectively), but the difference was not statistically significant. There was no difference in feed consumption between treated and control groups. Feed conversion efficiency varied over time and between doses and overall was lower at 50 000 mg/kg diet compared with the control (males: 93%, 97% and 83%; females: 94%, 111% and 89% of the control at 5000, 15 000 and 50 000 mg/kg diet, respectively). The effect observed on body weight gain and feed conversion efficiency at the highest dose is attributed to the high concentration of test material in the diet rather than a direct toxicological effect.

There was no treatment-related effect on absolute or relative organ weights; however, the relative kidney weights of females treated in the high-dose group were slightly higher (8%) than for controls. This finding was not considered toxicologically relevant because of the small magnitude of the inter-group difference, which was within the normal background range for the laboratory. In males, there was an increase in the incidence of congestion of the lungs across all treated groups relative to the controls (5%, 15%, 32% and 30% at 0, 5000, 15 000 and 50 000 mg/kg diet, respectively); however, in the absence of a similar trend in females (40%, 15%, 15% and 40%, respectively), a dose–response relationship or statistical significance, this finding was not considered treatment related. There were no other remarkable gross pathological findings.

The administration of advantame in the diet at concentrations up to 50 000 mg/kg was well tolerated, and 50 000 mg/kg diet was considered a suitable high concentration for the subsequent carcinogenicity study in mice. The no-observed-adverse-effect level (NOAEL) following 13 weeks of dietary exposure to advantame was 50 000 mg/kg diet (equal to 7444 mg/kg bw per day), the highest dietary concentration tested (Chase, 2002a).

(b) Rats

In a 4-week range-finding study, groups of Wistar (Han) rats (10 of each sex per group; 38–42 days of age) were given, ad libitum, diets containing advantame

(purity 99.2%; batch no. 000530) at a concentration of 0, 1500, 15 000, 30 000 or 50 000 mg/kg, equal to doses of 0, 174, 1739, 3672 and 6126 mg/kg bw per day for males and 0, 187, 1920, 3739 and 6490 mg/kg bw per day for females, respectively. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Satellite groups of nine rats of each sex were dosed similarly for toxicokinetic analysis. Animals were examined throughout the study period, and body weight and feed consumption were monitored weekly. Ophthalmoscopic examinations were conducted prior to treatment and during week 4. Blood and urine samples were collected for haematological, clinical chemistry and urine analyses during week 4. During weeks 1 and 4, blood samples were collected for toxicokinetic analyses. Macroscopic examination of all animals was undertaken, as well as measurement of organ weights. Histopathological examinations were performed on a standard suite of tissues in control and high-dose animals, as well as any tissues classified as abnormal. In addition, the kidneys of all treated females were subject to histopathological examination due to the observation of an increased incidence of corticomedullary mineralization in the kidneys of the high-dose females.

No treatment-related mortality or clinical signs of toxicity were observed. Occurrences of green, purple or blue staining of the cage lining were noted in some rats from day 15 onward, which was stated by the author to be associated with contact of faeces with the lining. The occurrence of discoloured faeces was increased in treated groups compared with controls. The incidence was generally higher in the 30 000 and 50 000 mg/kg diet groups. These findings are considered to be associated with the high concentration of advantame and/or its metabolites in faeces; although they are treatment related, they are not considered toxicologically relevant. In high-dose animals, body weight gains and feed conversion efficiency were approximately 15% lower than in controls (82–85% and 81–86% of controls, respectively). There was no clear effect of treatment on feed consumption. The observed effect on body weight gain and feed conversion efficiency is considered related to the high concentration of the test material in the diet and not toxicologically relevant. Water consumption was significantly higher in the high-dose animals during weeks 1 and 3 (approximately 20–36%) and slightly higher (approximately 17%) in the 30 000 mg/kg diet group during week 1 compared with controls.

There was no relationship between treatment and urine analysis or ophthalmoscopic findings. Minor differences in some haematological parameters were noted in male rats only; for example, haematocrit and haemoglobin values were reduced by generally less than 5% in all dose groups compared with controls. A corroborative reduction in red blood cells was not observed in these animals. In addition, all treated female dose groups demonstrated slightly lower (20–25%) lymphocyte counts compared with controls. However, these inter-group differences were not considered to be toxicologically significant based on the lack of an apparent dose–response relationship, the observation that the changes occurred in one sex only and the finding that individual values were within normal background ranges of biological variation.

There were statistically significant ($P < 0.01$) lower absolute and relative thymus weights (approximately 22%) in high-dose males compared with controls.

In the absence of any macroscopic or histopathological changes correlated with this finding or a similar effect in females, it was not considered toxicologically relevant. There were no significant gross pathological findings, with the exception of the presence of pale-coloured material in the lower gastrointestinal tract of all high-dose females.

Histopathological findings included an increased incidence of corticomedullary mineralization in the kidneys of treated females in the high-dose group; the kidneys of other dose groups were examined, and the incidences of corticomedullary mineralization were 2/10, 3/10, 4/10, 7/10 and 8/10 in the control through 50 000 mg/kg diet groups, respectively. Statistical significance ($P < 0.05$) of this finding was achieved only at the high dose level. There were no other histopathological abnormalities. In the absence of similar findings in the males, as the mineralization was graded as minimal in the majority of rats and as there was no corroborative evidence of kidney dysfunction, this finding is not considered treatment related. It was noted that high incidences of these same lesions have been found in other strains of rat (mainly females) and are thought to be related to the calcium and phosphorus balance of purified diets (Rao, 2002).

The administration of advantame in the diet at concentrations up to 50 000 mg/kg was well tolerated, and 50 000 mg/kg diet was considered a suitable high concentration for the subsequent 13-week study in rats. The NOAEL following 4 weeks of dietary exposure to advantame was 50 000 mg/kg diet (equal to 6126 mg/kg bw per day), the highest dietary concentration tested (Chase, 2002b).

In a 13-week study, groups of 20 male and 20 female Wistar (Han) rats (36–44 days of age) were fed, ad libitum, diets containing advantame (purity 99.5%; batch no. 001227) at a concentration of 0, 1500, 5000, 15 000 or 50 000 mg/kg, equal to 0, 118, 415, 1231 and 4227 mg/kg bw per day for males and 0, 146, 481, 1487 and 5109 mg/kg bw per day for females, respectively. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Satellite groups of nine male and nine female rats were dosed similarly for toxicokinetic analysis. Additional groups of five male and five female rats were assigned to the control, 15 000 or 50 000 mg/kg diet groups and were treated for 13 weeks followed by a 4-week recovery period. Parameters studied included clinical signs, physical examinations, functional observational battery, body weights, feed and water consumption, test article intake, ophthalmoscopy, haematology, clinical chemistry, urine analysis and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues for each animal, as well as any tissues classified as abnormal. Additional blood samples were collected for immunotoxicity assays during weeks 4 and 13 and week 4 of the recovery period and for toxicokinetics analyses during weeks 1 and 13.

There were no treatment-related mortalities or clinical signs of toxicity. Pale-coloured faeces were observed in both male and female rats in the 15 000 (incidence: 10–35%) and 50 000 (incidence: 95%) mg/kg diet groups. The change in faecal colour was considered to be associated with the high concentration of advantame in the diet; the pale colour was considered to be due to unmetabolized advantame. In

addition, the occasional animal receiving 5000, 15 000 or 50 000 mg/kg diet also had green or pink staining of the cage lining; this discoloration appeared to be associated with contact of the faeces with the paper lining. The light-coloured faeces and tray paper staining were not apparent by the end of the recovery period for animals that had received diets containing advantame at 15 000 or 50 000 mg/kg. Although these effects are treatment related, they are not considered toxicologically relevant. Body weight, body weight gain, feed consumption and feed conversion efficiency were unaffected by treatment. There was a dose-related increase in water consumption that was statistically significant in high-dose males during week 5 (113% of the controls) and in animals in the 5000 mg/kg diet group and above during week 11 (110%, 115% and 128% of the controls at 5000, 15 000 and 50 000 mg/kg diet, respectively). In females, water consumption was significantly higher than the control in animals in the 15 000 mg/kg diet groups and above during weeks 5 and 11 (approximately 130% of the control).

There were no treatment-related ophthalmoscopic abnormalities, and there was no evidence of neurotoxicity observed during the functional observational battery. Changes in haematology parameters analysed at week 13 included significantly lower ($P < 0.05$ or 0.01) haematocrit and haemoglobin concentrations in both male and female high-dose groups compared with controls and in females at 15 000 mg/kg diet ($P < 0.01$). Mean red blood cell counts were lower ($P < 0.05$) than in controls in high-dose females. However, the magnitude of the change in these parameters was relatively small (2–4% lower than controls), and all values were within the reference ranges for these parameters. The changes observed in these parameters between treated and control animals were not observed at the end of the recovery phase. Furthermore, similar differences were not observed in the chronic rat study, which was conducted with the same dietary concentrations of advantame. Therefore, these differences are considered to be in the normal range of biological variation and are not considered toxicologically relevant.

There was a dose-related reduction in total white blood cell count in males, which was statistically significant ($P < 0.01$) in the 15 000 and 50 000 mg/kg diet groups (approximately 80% of the control value). This finding was considered to be related to a significant reduction in lymphocytes ($P < 0.01$) observed in these males (decreases up to 77% of the control value in the high-dose group). In females, only the high-dose group had significantly lower mean lymphocyte counts ($P < 0.01$; approximately 80% of control value). There was no significant difference between these groups and the controls during the recovery phase; however, it is noted that these end-points are typically highly variable. Given that all values for total white blood cell count and lymphocytes fell within the respective reference ranges (lymphocytes: 1.19–9.45 $\times 10^9$/l in males; 0.68–6.8 $\times 10^9$/l in females; CRL, 2008), these changes are not considered toxicologically relevant.

There were no adverse treatment-related changes in clinical chemistry or urine analysis parameters. Any statistically significant differences observed were not considered clinically relevant or did not follow a dose–response relationship. In females, the mean plasma urea concentration was significantly lower ($P < 0.05$) than that of the controls at 15 000 and 50 000 mg/kg diet (approximately 88% of the control value) during week 13, but not after 4 weeks of recovery (approximately 108%

of the control value). The lower plasma urea concentrations were considered to be associated with the reduced intake of dietary protein due to the high concentration of test material in the diet. Therefore, this finding is considered to be an adaptive response that is not adverse or toxicologically significant. This conclusion is further supported by the absence of corroborative evidence of liver dysfunction. All values for this parameter also fell within the normal range for female Han Wistar rats aged 17 weeks or older (4.18–8.93 mmol/l; CRL, 2008).

Terminal investigations revealed no remarkable changes in absolute or relative organ weights. The occurrence of pale-coloured contents of the caecum and colon of male and female rats at 50 000 mg/kg diet was the only macroscopic abnormality considered treatment related. This observation was attributed to the high concentration of test material in the diet.

Histopathology of sacrificed rats revealed an increased incidence of degeneration of optic nerve fibres in high-dose males (20% versus 10% in the controls) and in females at 15 000 mg/kg diet and above (15%, 20%, 25%, 37% and 55% at 0, 1500, 5000, 15 000 and 50 000 mg/kg diet, respectively), with the result at 50 000 mg/kg diet reaching statistical significance ($P < 0.01$) in females. However, these findings were stated to be unilateral and occasionally associated with degenerative changes in the Harderian gland. In rats sacrificed after the recovery period, there was no difference in the incidence of degenerate optic nerve fibres between treated and control rats. In the absence of corroborative evidence, such as abnormal ophthalmoscopy, this finding was considered by the study authors to be likely related to blood sampling via the retro-orbital sinus rather than a treatment-related effect. It is further noted that similar observations of degeneration of optic nerve fibres were not found in the chronic 2-year study in rats (see section 2.2.3 (b)). There was also a non-statistically significant increased incidence of mineralization of the medulla of the kidneys in high-dose females (13/20 versus 8/20 in the controls; graded as minimal), a finding that was not evident following the 4-week recovery period. The incidence of corticomedullary mineralization of the kidneys (graded as minimal) was elevated in females at 15 000 (4/5) and 50 000 (4/5) mg/kg diet relative to the controls (1/5) at the end of the 4-week recovery period. This finding was not noted during the treatment period and is not considered treatment related. The authors stated that corticomedullary mineralization of the kidneys is a common age-related finding in female rats of this strain.

There were no other treatment-related histopathological abnormalities.

The results of the immunotoxicity analysis are discussed in detail in section 2.2.6 (e).

The NOAEL following 13 weeks of dietary exposure to advantame was 50 000 mg/kg diet (equal to 4227 mg/kg bw per day), the highest dietary concentration tested (Chase, 2004).

(c) Dogs

In a 4-week study, groups of Beagle dogs (23–27 weeks of age; four of each sex per group) were given diets containing advantame (purity 99.2%; batch no. 000530) at a concentration of 0, 5000, 15 000 or 50 000 mg/kg, equal to doses of 0, 232, 737

and 2385 mg/kg bw per day for males and 0, 254, 743 and 2488 mg/kg bw per day for females, respectively. Dogs were offered 400 g of test or control diet each day, with water available ad libitum. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan) and test guidelines (Japan, USFDA and European Commission). Animals were examined daily throughout the study period, and body weight and feed consumption were monitored weekly. Ophthalmoscopic and electrocardiographic examinations were conducted prior to treatment and during week 4 and weeks 2 and 4, respectively. Blood and urine samples were collected for haematological, clinical chemistry, toxicokinetics and urine analyses prior to treatment and during week 4. Macroscopic and histopathological examination of all animals was undertaken, as well as measurement of organ weights at study termination.

There were no deaths during the study. The only clinical sign related to treatment was the observation of pale-coloured faeces, starting from week 1, passed by high-dose animals of both sexes. This finding was attributed to the high concentration of the test material in the diet rather than a toxicological effect. There were no toxicologically significant effects of treatment on body weight gain or on feed consumption. Males and females at the highest dose gained slightly less weight than respective controls; this finding was attributed to outlier data from one dog of each sex. It is noted that the feed conversion efficiency in the high-dose groups was reduced. Lower weight gain in this dose group is likely reflective of the high concentration of a non-caloric substance in the diet rather than a toxicological effect.

There was no relationship between treatment and the findings from the ophthalmic and electrocardiographic examinations. A few statistically significant inter-group findings from the haematological (lymphocytes) and serum chemistry analyses (mean plasma urea, glucose) were identified; however, these occurred in a non-dose-dependent manner, were limited to males only and were mild in nature. The study authors also stated that the level of these parameters fell within historical control background ranges. Therefore, none of these findings was considered to be related to treatment. There were no statistically significant differences in the urine analysis parameters between treated and control groups.

The absolute weights of the thymus of both sexes in the treated groups were lower than in the controls. Relative thymus weights were reduced by approximately 33% in males from the two highest dose groups, but not dose dependently, and by approximately 22% in the two highest female dose groups. Based on no correlating histopathological changes in this organ, corroborative haematology (lymphocyte differential counts) or similar observations in the 1-year dog study, the findings were not considered to be of toxicological significance. There were no macroscopic or microscopic pathology findings attributable to treatment.

The NOAEL following 4 weeks of dietary exposure to advantame was 50 000 mg/kg diet (equal to 2385 mg/kg bw per day), the highest dietary concentration tested (Barker, 2002).

In a 13-week study, groups of Beagle dogs (23–26 weeks of age; four of each sex per group) were given diets containing advantame (purity 99.5%; batch

no. 001227) at a concentration of 0, 5000, 15 000 or 50 000 mg/kg, equal to doses of 0, 205, 667 and 2230 mg/kg bw per day for males and 0, 229, 703 and 2416 mg/kg bw per day for females, respectively. Dogs were offered 400 g of test or control diet each day, with water available ad libitum. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan) and test guidelines (Japan, USFDA and European Commission). An additional two dogs of each sex per group in the control and the high-dose groups were then maintained untreated for 4 weeks. Parameters studied included clinical signs, physical examinations, body weights, feed consumption, ophthalmoscopy, electrocardiographic examinations, haematology, clinical chemistry, urine analysis, toxicokinetics and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues for each animal.

There were no deaths during the study. The only treatment-related clinical sign was the observation of pale-coloured faeces passed by high-dose animals, which continued to be observed through the 1st week of the recovery period. This observation is consistent with rodent studies and is attributed to the high concentration of test material in the gastrointestinal tract rather than to a direct toxicological effect. Loose/liquid stools occurred across all groups, including controls, with the incidence somewhat higher in treated groups. Although possibly treatment related, this condition was not considered to be of toxicological significance. In high-dose males, body weight gain was significantly reduced (0.5 ± 0.75 kg versus 1.7 ± 1.01 kg in the controls); body weight gains for other treated groups of male dogs, and all treated females, were comparable to those of the concurrent control animals over the study period. This result is considered to reflect the high concentration of a non-caloric substance in the diet of this group, and therefore the finding is not attributable to a toxicological effect. There was no treatment-related effect on feed consumption at any dose level.

There were no treatment-related ophthalmic or electrocardiographic findings. There were no treatment-related haematological effects in males. In females, a few statistically significant inter-group findings (compared with controls) were identified at week 13 (lower mean haematocrit, haemoglobin and red blood cells at 15 000 and 50 000 mg/kg diet and lower mean reticulocytes and white blood cells at 50 000 mg/kg diet). However, these differences were considered to reflect normal biological variation rather than being treatment related based on the following: haemoglobin, reticulocytes and white blood cells were lower in the high-dose group than in the controls prior to treatment; the magnitude of the difference in haematocrit and red blood cells compared with controls was relatively small (7–10% lower); a clear dose–response relationship was not evident for any of the parameters; and the direction of change observed in haematocrit and red blood cells during the recovery phase was inconsistent (~2% decrease in one dog and ~2% increase in the other). The study authors also stated that all of the values fell within the historical control range.

There was no treatment-related effect on any clinical chemistry parameters in males. In high-dose females, significant differences ($P < 0.05$) in a number of parameters were noted compared with controls (higher cholesterol and phospholipids, lower α2-globulin and higher β-globulin during week 6; lower potassium during weeks 6 and 13; and lower creatinine during week 13). However, these differences, although statistically significant, are not considered treatment

related on the basis of the following: changes in parameters were only slight (2–15%) when compared with pretreatment values rather than controls; cholesterol and phospholipids decreased during the study period when compared with pretreatment values rather than controls; and there was no remarkable change in parameters at the end of the recovery period when compared with values during the study period. There were no treatment-related changes in urine analysis parameters.

The mean relative pituitary gland weights of males treated at 15 000 and 50 000 mg/kg diet were decreased compared with controls by approximately 11%. In the absence of correlating histopathological changes in the pituitary gland, the differences were not considered toxicologically relevant. In high-dose males, a decrease of approximately 20% was noted for relative thymus weights compared with the control group. Although this decrease did not achieve statistical significance, observations of correlating macroscopic and microscopic findings were noted. Corroborative macroscopic observations included reduced thymus sizes in three of four dogs in the high-dose group compared with none of four dogs in the control group. In addition, histopathological evidence of an increased incidence and/or severity of thymic involution/atrophy was observed in the mid- and high-dose groups (1/4, 0/4, 1/4 and 2/4 dogs at 0, 5000, 15 000 and 50 000 mg/kg diet, respectively), graded as moderate at 15 000 and 50 000 mg/kg diet and only slight in the control group. The two dogs that were sacrificed after the 4-week recovery period did not display these findings. In an examination of the individual data, the occurrence of thymic involution/atrophy across all groups occurred in dogs that had the lowest absolute thymus weight and the lowest body weight gain over 13 weeks. In order to assess the significance of these observations, the authors examined the range of these findings in control Beagles of a similar age from the archived laboratory data. In six other studies conducted at the testing laboratory, the incidence of thymic involution/atrophy in male control dogs ranged up to 2/4, with the severity considered similar to that reported in the high-dose males treated with advantame. The size of the thymus in Beagles varies considerably in short-term toxicology studies, and thymic involution begun prior to sexual maturity may be enhanced by the direct or indirect effect (malnutrition) of the test compound (Haggerty, Thomassen & Chengelis, 1992). It is further noted in general background physiological data for Beagles that the thymus gradually decreases in weight as the dog ages. A 41% reduction in the mean thymus weight of 9-month-old male Beagles was observed when compared with 6-month-old Beagles (Choi et al., 2011). In this study, the high-dose males were approximately 9–10 months old at the termination of the study and exhibited significantly reduced overall body weight gains (approximately 30%) compared with the control group, an effect that is attributed to the high concentration (5%) of a non-caloric test material in the diet. Based on the inherent variability in thymic sizes and weights, the association between enhanced thymic involution and reduced nutritional status, the lack of corroborative haematology changes (lymphocyte differential counts) and the absence of similar thymus effects in the 1-year dog study, these findings are not considered to be of toxicological significance.

The NOAEL following 13 weeks of dietary exposure to advantame was 50 000 mg/kg diet (equal to 2230 mg/kg bw per day), the highest dietary concentration tested (Powell & Scott, 2005).

In a 1-year study, groups of Beagle dogs (22–26 weeks of age; four of each sex per group) were fed diets containing advantame (purity 99.1% or 99.2%; batch nos 010319 and 010626) at a concentration of 0, 2000, 10 000 or 50 000 mg/kg for 52 weeks. Dogs were offered 400 g of test or control diet each day for 15 weeks, after which they were offered 500 g/day for the remainder of the study. Samples of each diet formulation prepared for administration in weeks 1, 12, 26, 39 and 52 of treatment were analysed for achieved concentration of the test substance. The dose of test material received by each treated group was calculated from the recorded feed consumption, body weight and dietary concentration data. These concentrations are equal to average doses of 0, 82.5, 420.9 and 2058 mg/kg bw per day for males and 0, 81.9, 406.2 and 2139 mg/kg bw per day for females, respectively. Water was available ad libitum. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan) and test guidelines (Japan, USFDA and European Commission). An additional two dogs of each sex were assigned to the control, 10 000 and 50 000 mg/kg diet groups and maintained untreated for a further 6 weeks before necropsy. Parameter studies included clinical signs, body weight, feed consumption, ophthalmoscopy, electrocardiography examinations, haematology, clinical chemistry, urine analysis, toxicokinetics and organ weights. Complete gross pathology and microscopy were performed on a standard suite of tissues from all animals.

No treatment-related deaths occurred during the study; however, one high-dose male dog (#417) was sacrificed 1 day prior to the last day of the recovery phase due to polyarteritis ("Beagle pain syndrome"). This condition is not related to treatment. The only clinical sign related to treatment was the occurrence of pale-coloured faeces from dogs in the 10 000 and 50 000 mg/kg diet treatment groups. In the recovery phase, this finding was noted in only the 1st week and only in the high-dose treated animals. Loose stools were noted in all groups, including controls, and were not considered treatment related.

The mean pretreatment body weight of the male control group was higher than those of the three treatment groups, and this difference was maintained throughout the study. The mean body weight gain over 52 weeks was comparable between the controls and treatment groups. A few animals showed body weight losses during the first 14 weeks of the study; however, after the feed ration was increased from week 15, all animals showed satisfactory overall body weight gains, with the exception of one high-dose female. The body weight of this female stabilized following administration of an additional 100 g of plain pelleted diet from week 34. There was no adverse effect of treatment on feed consumption during the 52 weeks of treatment or during the recovery period. There were no treatment-related ophthalmic abnormalities.

The results of the electrocardiography investigations showed no treatment-related effects on intervals or waveforms. The mean heart rate of males in the 50 000 mg/kg diet group was increased compared with controls (15–33%) at all sampling intervals 1 hour after dosing, a finding that was not apparent at the 24-hour time point after dosing. However, this observation was attributed to a single dog that was tachycardic (heart rate above 180 beats per minute) throughout the sampling intervals, and the difference was not statistically significant. Another male in the 50 000 mg/kg diet group was tachycardic 1 hour after dosing only during week 39.

However, these findings were not considered treatment related based on the absence of similar findings in females and in the 13-week dietary study using the same doses. There were no treatment-related changes in haematological, clinical chemistry or urine analysis parameters. All observed differences from control values, including those that attained statistical significance, were considered to be minor, not dose related and not consistent across sexes, or they were essentially similar to pretreatment values.

No organ weight changes were reported that were attributed to treatment. No evidence of treatment-related pathological changes was found following macroscopic and histopathological evaluations. Findings were considered by the authors to be normal background lesions for the age of the animals. It is noted that there was no evidence of macroscopic change in the thymus compared with that reported in the 13-week study. Thymic involution/atrophy was present in all dogs, including controls, with no notable differences in the severity between groups (males: 4/4, 4/4, 4/4, 4/4; females: 3/4, 2/4, 3/4 and 3/4; at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively).

The NOAEL in the 1-year dietary dog study was 50 000 mg/kg diet (equal to 2058 mg/kg bw per day), the highest concentration tested (Powell, Lelasseux & Crome, 2005; Otabe, Fujieda & Masuyama, 2011a).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a study of carcinogenicity in mice, groups of 64 male and 64 female Crl:CD-1 (ICR) BR mice (40–44 days of age) were fed, ad libitum, diets containing advantame (purity 99.3%; batch no. 010228) at a concentration of 0, 2000, 10 000 or 50 000 mg/kg for 2 years. These concentrations were equal to doses of 0, 213, 1057 and 5693 mg/kg bw per day for males and 0, 272, 1343 and 7351 mg/kg bw per day for females, respectively. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). All animals were observed twice daily for mortality or signs of moribundity. Parameters measured included clinical signs, physical examinations including palpation for masses, body weight, feed consumption, limited haematology end-points (red blood cells, white blood cells, differential white blood cells, abnormal morphology) and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues from each animal. This study was specifically designed to assess carcinogenicity rather than chronic toxicity, and therefore only limited haematology parameters and no clinical chemistry, ophthalmoscopy or urine analysis parameters were analysed.

There were no treatment-related deaths or clinical signs of toxicity. Statistical analysis of the mortality indicated that there were no statistically significant differences between control and treated groups ($P > 0.05$). There was no difference in the incidence of palpable swellings between treated and control groups. There was no treatment-related effect on absolute body weight. Body weight gain of high-dose animals of both sexes was slightly reduced during the first 16 weeks of treatment (88% and 90% of control values for males and females, respectively). These differences continued through to week 78 of treatment (90% and 87% of

controls in males and females, respectively). In high-dose females, but not males, the overall body weight gain was significantly lower ($P < 0.05$; 76% of controls) than that of the controls. It is noted that, apart from when data for senile mice (more than 21 months old) were included, there were no statistically significant differences in body weight gains. There was no effect on feed consumption. A slight decrease in feed conversion efficiency was reported (weeks 1–16) in both sexes of the high-dose group and attributed to the slightly lower body weight gain.

In the analysis of peripheral blood samples from week 104, both sexes at 50 000 mg/kg diet had significantly lower mean red blood cell counts ($P < 0.01$ for males; $P < 0.05$ for females) than the respective control groups (86% and 89% of control values for males and females, respectively). In the absence of appropriate historical control data, the large inter-individual variation in the data and the small magnitude of the change were considered sufficient to support the conclusion that this finding is not treatment related. There were no treatment-related differences in blood smears evaluated at weeks 52 and 78. The study author concluded that these results show no evidence of elevated cell counts that could be indicative of leukaemia in treated groups.

There were no statistically significant differences in absolute or relative organ weights between treated and control mice. Given the large degree of inter-animal variation, the lack of a dose–response relationship and the absence of any corroborative evidence from other end-points, observed differences (thymus, uterus/cervix, left and right ovary) were not considered treatment related.

The results of the gross pathological examinations showed a higher incidence ($P < 0.05$) of an enlarged liver in high-dose males compared with controls (0/64 [0%], 5/64 [8%], 4/64 [6%] and 6/64 [9%] at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively). Although not statistically significant, the incidence of haemangiosarcoma of the liver was increased in all treated groups of males compared with the controls (Table 13). The incidence of haemangiosarcoma was within the historical control range for CD-1 mice (1.11–8.57%; CRL, 2005), and no other remarkable histopathological findings were noted in the liver.

In the absence of a dose–response relationship, a similar effect in females or an effect on absolute or relative liver weights, these findings were not considered treatment related.

Non-statistically significant increases in the incidence of exophthalmic eyes (1/64 [2%], 3/64 [5%], 4/64 [6%] and 5/64 [8%] at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively) and Harderian gland masses (1/64 [2%], 5/64 [8%], 5/64 [8%] and 6/64 [9%] at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively) were observed in male mice. These findings correspond with histopathological findings of an increased incidence of adenoma of the Harderian glands (Table 13). However, these findings are not considered treatment related based on the absence of a similar increase observed in females and the fact that the incidence of adenoma was within the historical control range for CD-1 mice (1.67–18.64%; CRL, 2005).

In treated female mice, the incidence of bronchioloalveolar carcinoma of the lung was increased relative to controls (Table 13), with statistical significance achieved in the pair-wise test in the low-dose group only. No other pair-wise or

Table 13. Neoplastic findings in mice administered advantame in the diet for 2 years

Organ (finding)	Incidence of finding[a]							
	0 mg/kg diet		2000 mg/kg diet		10 000 mg/kg diet		50 000 mg/kg diet	
	Males	Females	Males	Females	Males	Females	Males	Females
Harderian gland (adenoma)	1/64 (1.6%)	2/64 (3.1%)	2/64 (3.1%)	1/64 (1.6%)	3/64 (4.7%)	1/64 (1.6%)	6/64 (9.4%)	1/64 (1.6%)
Lung (bronchioloalveolar adenocarcinoma)	11/64 (17%)	1/64 (1.6%)	14/64 (2.2%)	7/64 (11%)*	6/64 (9.4%)	5/63 (7.9%)	6/64 (9.4%)	5/64 (7.8%)
Liver (haemangiosarcoma)	0/63 (0%)	0/63 (0%)	3/63 (4.8%)	1/63 (1.6%)	4/64 (6.3%)	1/63 (1.6%)	2/63 (3.2%)	0/64 (0%)

* $P < 0.05$

[a] Results expressed as the absolute number of mice showing the abnormality, with the % incidence contained in parentheses.

trend tests achieved statistical significance. Given the absence of a dose–response relationship, and noting that the incidence in control females was at the lower end of the historical control range while that in the treated groups was at the high end of the control range (0.77–18.37%; CRL, 2005) and the absence of any effects in males, the bronchioloalveolar carcinomas in treated females were considered to be unrelated to treatment with advantame.

There were no treatment-related non-neoplastic findings.

The NOAEL following 2 years of dietary exposure to advantame was 50 000 mg/kg diet (equal to 5693 mg/kg bw per day), the highest concentration tested (Horne, 2006).

(b) Rats

A 2-year study of toxicity and carcinogenicity was conducted in rats exposed in utero. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). In the in utero phase of the study, groups of F_0 HsdBrl Han:Wist (Han Wistar) rats (6–9 weeks of age; 65 of each sex per dose) were fed, ad libitum, diets containing advantame (purity ≥99.2%; lot no. 010212) at a concentration of 0, 2000, 10 000 or 50 000 mg/kg for 4 weeks prior to mating, throughout mating and until the offspring were transferred to the main study at approximately 4 weeks of age. Animals were randomly assigned for the toxicity and carcinogenicity phases when the litters reached 10–14 days of age. Animals selected to produce the F_1 generation continued treatment at the same dietary levels as their parents from the time of weaning until the nominal start of treatment for the main study.

In the carcinogenicity phase of the study, groups of 55 males and 55 females of the F_1 generation (28 days of age) were fed, ad libitum, diets containing advantame at a concentration of 0, 2000, 10 000 or 50 000 mg/kg for 104 weeks.

These concentrations are equal to doses of 0, 97, 488 and 2621 mg/kg bw per day for males and 0, 125, 630 and 3454 mg/kg bw per day for females, respectively. Twenty additional rats of each sex were allocated to each treatment group for interim sacrifice at week 52. Another 10 rats of each sex were assigned to the control, 10 000 and 50 000 mg/kg diet groups and allowed to recover for 6 weeks after week 52, prior to sacrifice. Satellite groups of 12 rats of each sex were treated for 104 weeks, and their blood was sampled at specific intervals for toxicokinetic analysis. Parameters studied included clinical signs, physical examinations including palpation for masses, body weight, feed and water consumption, feed conversion efficiency, test article intake, ophthalmoscopy, haematology, clinical chemistry, urine analysis and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues from each animal.

During the in utero phase, there were no treatment-related mortalities or clinical signs of toxicity in parental rats. There were no adverse treatment-related effects on mating or gestation length, litter sizes, sex ratio or growth and survival of offspring.

During the toxicity and carcinogenicity phases, there was no effect of treatment on mortality. Notable clinical signs consisted of a high incidence of pale-coloured and swollen anus from week 4 to week 32 in high-dose males and females. However, the findings were not considered toxicologically significant because there was no clear persistence over time. Discolorations of the faeces (light/dark green) were observed for animals at 10 000 and 50 000 mg/kg diet. Green, pink or purple staining of the bedding was also observed in animals dosed at 10 000 and 50 000 mg/kg diet. These findings resolved by the end of the recovery period. Although these findings are treatment related, they are not considered toxicologically significant.

The mean body weight gain of high-dose groups over the 104 weeks of the main study was lower than that of the controls (93% and 94% of control values for males and females, respectively), but was statistically significant ($P < 0.05$) only in males. In males receiving 50 000 mg/kg diet, the overall weight gain up to week 52 was 95% of the control value. Over the 6-week recovery phase, this same group had a significantly higher ($P < 0.01$) body weight gain compared with the controls (162% of control). Absolute body weight values were not significantly different among the treated and control groups at any point during the study. Feed consumption was slightly higher for females receiving 50 000 mg/kg diet (109% at 52 weeks; 108% at 104 weeks). Feed conversion efficiency was slightly reduced for males and females at 50 000 mg/kg, which is attributed to the lower body weight gain in males and slightly higher feed intake in males and females. These findings are considered to be associated with the addition of high concentrations of a non-caloric substance to the diet and are not of toxicological significance.

Water consumption was slightly increased compared with controls in all treated male and female groups during weeks 11, 24 and 50, with the differences sometimes achieving statistical significance (103–121% of controls). In the absence of a dose–response relationship or other evidence of a direct toxicological effect, these treatment-related effects are not considered toxicologically significant.

No treatment-related ophthalmic lesions were noted during any of the ophthalmologic examinations.

There were no treatment-related changes observed in the haematology, clinical chemistry or urine analysis parameters measured during the various phases of the study. All differences from controls, including those that attained statistical significance, were considered minor, fell within historical control ranges, were not dose related and/or were not consistent between the sexes or over time. Of note in the clinical chemistry evaluation were consistently lower plasma urea concentrations in high-dose animals (Table 14). Statistical significance was attained during weeks 26, 39 and 78 in both sexes and during weeks 52 and 104 in males. Individual animal data revealed that the lowest values for high-dose females at weeks 78 and 104 fell outside the historical control range (Han Wistar rats more than 17 weeks of age: 3.82–7.14 mmol/l in males and 4.18–8.92 mmol/l in females; CRL, 2008). During the 6-week recovery phase following 52 weeks of treatment, the plasma urea concentrations were similar to or slightly higher than those of the controls. Consistent with observations of lower plasma urea in high-dose females in the 13-week rat study, these findings are considered to be associated with the reduced intake of dietary protein due to the high concentration of test material in the diet. Therefore, this finding is considered to be an adaptive response that is not adverse or toxicologically significant. This conclusion is further supported by the absence of corroborative evidence of liver dysfunction.

No adverse treatment-related effects were observed in the results of the gross pathological and organ weight analyses. The results of organ weight analyses showed a number of inter-group differences (pituitary at week 52 only, seminal vesicles, liver, salivary gland at week 104 only) that attained statistical significance, but these differences either showed no dose–response relationship or were considered minor in nature, all occurred in one sex only and all either were not associated with any pathological findings or were known age-related findings in rats. Therefore, none of these findings is considered treatment related. The only treatment-related pathological finding noted was the presence of green stomach contents (five rats) and ileum contents (one rat) of high-dose females that died or were killed prior to termination. These findings are attributed to the high concentration of test material in the diet and are not considered adverse.

There were no histopathological findings on the incidence of non-neoplastic lesions related to treatment in rats sacrificed after 52 or 104 weeks of treatment or in rats sacrificed following the 6-week recovery phase.

There was no effect of treatment on the incidence of neoplastic lesions in rats sacrificed at week 52 or in rats that died during the 104 weeks of treatment.

In rats sacrificed after the 104-week treatment period, a slightly higher incidence of pancreatic islet cell carcinomas was observed in high-dose males (0/55 [0%], 1/55 [2%], 2/55 [4%] and 3/55 [5%] at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively). The combined incidence of pancreatic islet cell carcinoma and adenoma was 2/55 (4%), 5/55 (9%), 4/55 (7%) and 5/55 (9%) at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively. The combined incidence of the tumours was considered to be generally within historical control ranges provided by the testing

Table 14. Mean plasma urea concentrations in rats exposed to advantame for 2 years

Week	Mean plasma urea concentration (mmol/l)							
	0 mg/kg diet		2000 mg/kg diet		10 000 mg/kg diet		50 000 mg/kg diet	
	Males	Females	Males	Females	Males	Females	Males	Females
13	7.54	7.55	7.63	7.93	7.79	8.10	6.78	6.77
26	6.86	8.27	6.26	7.69	6.42	7.16	6.15*	6.26**
39	6.69	7.06	5.98*	6.89	6.00*	7.28	5.53**	6.11*
52	5.92	6.65	5.58	7.07	5.82	6.51	5.29**	6.29
Recovery 6	5.59	5.83	—	—	5.71	6.50	5.72	6.02
78	5.22	6.65	5.01	5.22*	5.56	5.69*	4.41*	5.54*
104	4.66	4.95	4.87	5.85	4.82	6.01	4.19*	5.40

* $P < 0.05$; ** $P < 0.01$

laboratory (adenomas: 0–10%; carcinomas: 0–3.3%; Horne, 2005). Furthermore, pair-wise and trend analyses conducted on the incidences of carcinoma or carcinoma and adenoma combined did not demonstrate a statistically significant effect compared with controls. This statistical method is recommended in the United States Environmental Protection Agency's (USEPA) proposed guidelines for carcinogen risk assessment and further referenced in OECD guidance (OECD, 2010) for differentiating whether an increased tumour incidence is treatment related or a chance occurrence. Therefore, this finding is not considered to be treatment related.

In high-dose females, a significantly higher incidence of mammary gland adenoma was observed ($P = 0.036$; 4/41 [10%] at 50 000 mg/kg diet compared with 0/41 in control, low-dose and mid-dose groups). The incidence of mammary gland adenocarcinomas was not statistically significant in a trend or pair-wise analysis. Combining the incidence of mammary gland adenoma, fibroadenoma and adenocarcinoma resulted in a positive trend test ($P = 0.015$); however, the pair-wise comparisons with controls did not achieve statistical significance at any dose. Combining the incidence of benign tumours, adenomas and fibroadenomas resulted in a positive trend test ($P = 0.026$) and a significant pair-wise comparison ($P = 0.042$) only at the high dose. The combined incidence of mammary gland adenomas and fibroadenomas was within the historical control range for female Han Wistar rats (12.7–37.5%; CRL, 2003); the control values are near the bottom of the range, and the high-dose group values are near the top of the range. Therefore, these findings were not considered a treatment-related effect.

The NOAEL following 2 years of dietary exposure of rats to advantame was 50 000 mg/kg diet (equal to 2621 mg/kg bw per day), the highest concentration tested (Horne, 2005; Otabe, Fujieda & Masuyama, 2011b).

2.2.4 Genotoxicity

The results of studies of genotoxicity with advantame are summarized in Table 15. Studies were conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan) and test guidelines (OECD Test Guidelines 471, 476 and 474, USEPA and European Commission). Positive and negative (vehicle) controls were tested in each study and gave expected results. Advantame showed no evidence of genotoxic effects in these assays. Results of studies of genotoxicity with the degradation products of advantame are described in section 2.2.6 (c).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

(i) Rats

In a two-generation study of reproductive toxicity, groups of rats (Sprague-Dawley (CRL : CD IGS BR)) were given, ad libitum, diets containing advantame (purity 98.6%) at a concentration of 0, 2000, 10 000 or 50 000 mg/kg. These concentrations were equal to the mean intakes of advantame illustrated in Table 16. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Groups of 30 male and 30 female rats (6 weeks of age) of the F_0 generation were provided test diets for 10 weeks before mating and then throughout mating, gestation and lactation, until the F_1 pups were weaned (28 days postpartum). At approximately 4 weeks of age, offspring (the F_1 generation) were allocated to groups of 25 males and 25 females, and they continued to receive the same dietary concentrations as their parental animals until the F_2 pups were weaned. The F_2 pups were sacrificed on postnatal day 30. All litter sizes were adjusted to 10 offspring 4 days after birth. Clinical signs, growth and reproductive parameters were assessed for the F_0 and F_1 generations. Growth, physical maturation and behaviour parameters were assessed for the offspring. All adults were subjected to a detailed necropsy and histopathological examination, and weights of specified organs were analysed. Offspring of the F_0 and F_1 generations were also subjected to macroscopic evaluation, including offspring culled on postpartum day 4, offspring that died prematurely and offspring sacrificed at 4 weeks of age, which were not allocated to treatment groups.

There were no treatment-related mortalities. Four treated F_0 females were killed in extremis 21–24 days after mating due to parturition difficulties (0, 1, 2 and 1 at 0, 2000, 10 000 and 50 000 mg/kg diet, respectively). One pregnant F_1 female from the 2000 mg/kg diet group was killed in extremis on day 23 after damaging its teeth on the cage. These deaths are not considered to be associated with treatment, based on the absence of a dose–response relationship and the inconsistency between the F_0 and F_1 generations. There were no clinical signs of overt toxicity. Treatment-related clinical signs included changes in the colour and/or form of the faeces and staining of the paper beneath the cages throughout the study. Many animals at 10 000 mg/kg diet and most of the animals at 50 000 mg/kg diet from both the F_0 and F_1 generations had white faeces and/or green and/or purple staining

Table 15. Results of assays for genotoxicity of advantame

Test system	Test object	Test article	Concentration/ dose	Results	References
In vitro					
Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA/pkM101 (CM891)	Advantame (purity 99.2%)	5–5000 µg/ plate	Negative	May, Watson & Burton (2001); Otabe, Fujieda & Masuyama (2011c)
Cell mutation[a,b]	L5178Y mouse lymphoma	Advantame (purity 100.1%)	Preliminary: 39–5000 µg/ well Main: 500–5000 µg/ well	Negative[c]	Clare, Kernahan & Clemson (2002); Otabe, Fujieda & Masuyama (2011c)
In vivo					
Micronucleus formation[d]	CD-1 mouse bone marrow	Advantame (purity 100.1%)	Preliminary: 2000 mg/kg bw (2 mice/sex) Main: 500, 1000 or 2000 mg/kg bw (7 males/group)	Negative	Mehmood et al. (2001); Otabe, Fujieda & Masuyama (2011c)

[a] In the presence and absence of Aroclor-induced rat liver microsomal fraction (S9).
[b] Short-term (3-hour incubation; ±S9) or continuous (24-hour incubation; –S9) treatment.
[c] Cytotoxicity observed at and above 2500 µg/well, 3-hour exposure, ±S9; and at and above 2000 µg/well, 24-hour exposure, –S9.
[d] 24- and 48-hour sampling times.

of cage paper. These observations are not considered to represent a toxicological effect but rather are attributed to the high concentration of advantame in the diet.

During the premating period and in dams during gestation and lactation, there was no treatment-related effect on absolute body weight or body weight gain in F_0/F_1 parental rats. Feed consumption was higher than in controls in the high-dose groups prior to mating, achieving statistical significance in the males ($P < 0.01$). In the high-dose F_1 males and females, feed consumption was significantly higher than in the controls ($P < 0.01$ or 0.05) during most weeks of the premating period (6–9% higher in males and 7–12% higher in females). The feed conversion efficiency was lower in the high-dose animals in both the F_0 and F_1 generations. These significant differences are not considered toxicologically relevant and are attributed to the high concentration of the non-caloric test substance in the diet.

Table 16. Mean intakes of advantame in a two-generation reproductive toxicity study in rats

Time period	Mean intake (mg/kg bw per day)[a]					
	2000 mg/kg diet		10 000 mg/kg diet		50 000 mg/kg diet	
	Males	Females	Males	Females	Males	Females
F_0 generation						
Prior to mating	164	184	833	907	4410	4776
Gestation[b]	—	163	—	795	—	4136
Lactation[c]	—	320	—	1575	—	8192
F_1 generation						
Prior to mating	204	229	1036	1139	5431	5920
Gestation[b]	—	167	—	865	—	4457
Lactation[c]	—	316	—	1592	—	8447

[a] Values represent group means for 30 animals of each sex.
[b] Mean calculated over total number of gestation days.
[c] Mean calculated over total number of lactation days.

There were no adverse effects on any reproductive parameters (mating, fertility, gestation length/index, F_0 sperm quality), litter observations (size, survival, sex ratio and pup body weight) or any measures of postnatal offspring development (reflex development, auditory, visual and neuromuscular function, motor activity, learning ability).

The mean absolute and relative uterine (with cervix and oviducts) weights of adult F_0 females from the high-dose group were significantly ($P < 0.05$; approximately 19%) lower than those of controls. In the absence of a similar finding in other F_1 adult females, an effect on fertility and correlating pathology, this finding is not considered treatment related. There were no treatment-related macroscopic or histopathological abnormalities detected in F_0/F_1 rats. There was no treatment-related effect on mean or absolute organ weights in F_1/F_2 offspring.

The NOAEL for parental, offspring and reproductive toxicity was 50 000 mg/kg diet (equal to advantame doses in the range of 4000–6000 mg/kg bw per day during the period prior to mating and gestation and over 8000 mg/kg bw per day during lactation), the highest concentration tested (Willoughby, 2004; Otabe, Fujieda & Masuyama, 2011d).

(b) Developmental toxicity

(i) Rats

Groups of 22 female Sprague-Dawley rats (CRL : CD IGS BR; 10–11 weeks of age) were fed, ad libitum, diets containing advantame (purity 100.1%) at a concentration of 0, 5000, 15 000 or 50 000 mg/kg diet from day 0 to day 20 of gestation. These concentrations were equal to doses of approximately 0, 465,

1418 and 4828 mg/kg bw per day. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). The females were mated 1:1 with stock males of the same strain and source and killed on day 20 of gestation, and their uterine contents were examined. Animals were examined twice daily for clinical signs, with more detailed examinations once a week during the study. Body weights and feed consumption were monitored every 3–4 days throughout gestation. Dams were macroscopically examined on gestation day 20, and reproductive parameters were assessed, including gravid uterine weight, number of corpora lutea, number of implantation sites, number of resorption sites and number and distribution of live and dead fetuses. Fetuses were weighed and examined externally at necropsy. Approximately half of the fetuses in each litter were examined for visceral abnormalities in the neck and the thoracic and abdominal cavities, and the other half of the fetuses were examined for skeletal development.

All dams survived to scheduled sacrifice. Nonspecific signs of coat staining and hair loss were seen in animals at all treatment levels. Treated animals also had changes in the colour of the faeces (mostly pale), especially at 50 000 mg/kg diet, with increasing incidence, so that all animals were affected 20 days after mating, along with green and/or purple/blue staining of the cage liners. These signs are considered attributable to the high concentration of advantame and/or its metabolites in the digestive tract and are not evidence of toxicity.

In the high-dose group, reduced body weight gain was observed during the first few days of treatment; subsequent weight gains paralleled those of the control group, but a deficit in body weight compared with controls was maintained throughout the study. Absolute final body weights were not significantly different between the controls and any dose group; however, when final body weights were adjusted for gravid uterine weight, mean body weight gain of high-dose dams from gestation days 0 to 20 was 84% of that of the controls. There was no difference in body weight gain at 5000 and 15 000 mg/kg diet compared with the controls. In the high-dose dams, mean feed consumption was initially significantly lower than that of the controls (days 0–2; 88% of controls; $P < 0.01$), but thereafter it was significantly higher ($P < 0.01$ or 0.05; 6–9%). The initially low feed consumption at 50 000 mg/kg diet was likely due to the decreased palatability of the diet. In the low- and mid-dose groups, feed consumption was similar to or marginally higher than that of the controls throughout gestation. Although these findings are considered to be treatment related, in the absence of an adverse effect on survival, growth or fetal development, the effect on maternal body weight likely reflects the high non-nutritive content of the diet rather than a direct toxicological effect of the test material. In comparison, in the rat multigeneration study conducted with the same dietary concentrations of advantame (see section 2.2.5 (a) (i)), no significant effects were noted on either body weight gain or final body weights of the dams.

All females were pregnant with live young at day 20 of gestation. There were no treatment-related effects on any of the litter parameters (implantations, resorptions, live young, preimplantation and post-implantation losses, sex ratio, placental, fetal or total litter weights). There were no treatment-related findings at necropsy of the dams on gestation day 20. There were no effects of treatment on the incidence of major abnormalities, minor visceral findings or minor skeletal findings.

The NOAEL for maternal and developmental toxicity was 50 000 mg/kg diet (equal to 4828 mg/kg bw per day), the highest concentration tested (Willoughby, 2002; Otabe, Fujieda & Masuyama, 2011e).

(ii) Rabbits

In a preliminary range-finding study in rabbits, groups of six mated and presumed pregnant New Zealand White rabbits (17–23 weeks of age) were given advantame (purity 99.8%; batch no. 001212) in 1% (w/v) aqueous methyl cellulose by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw per day on days 6–28 of gestation. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Rabbits were examined throughout the study for abnormal clinical signs, body weight and feed consumption were monitored daily, and water consumption was monitored weekly. Blood samples were collected on days 6 and 27 of gestation for toxicokinetics analysis. On day 29 of gestation, dams underwent detailed necropsy, and reproductive parameters were analysed, including gravid uterine weight, number of corpora lutea, number of implantation sites, number of resorption sites and number and distribution of live and dead fetuses. Fetuses were weighed and examined externally at necropsy. The neck and the thoracic and abdominal cavities of all fetuses were examined for visceral abnormalities. Approximately half of the fetuses in each litter were examined for skeletal development.

In the high-dose group, one dam showed marked reddening and swelling of the anogenital region and was sacrificed on day 21 of gestation. Necropsy revealed that this dam was pregnant with live young. One control and one low-dose dam were killed on day 20, as both showed evidence of abortion. Necropsy revealed a single implantation in the uterus of each animal.

Pale-coloured faeces were observed in two of six dams in the mid-dose group and in four of six dams in the high-dose group. Green and/or purple and/or pink staining of the cage lining was observed for the majority of treated dams (3/6, 5/6 and 6/6 at 500, 1000 and 2000 mg/kg bw per day, respectively). Green and/or purple urine was observed directly in four mid-dose dams. Green staining of paws and tail was observed in the majority of dams from the mid- and high-dose groups, with green staining of the perigenital region also occurring in some animals.

Group mean body weight and body weight gain (both adjusted for gravid uterine weight), feed consumption and water consumption were unaffected by treatment. The only treatment-related macroscopic finding noted was the presence of dark or green bladder contents in two dams in each of the low- and high-dose groups. The caecum of one, one and three animals at 500, 1000 and 2000 mg/kg bw per day contained gaseous and dark contents. These findings were not considered adverse.

There were no treatment-related effects on any litter parameters or fetal parameters.

On the basis of this study, 2000 mg/kg bw per day was selected as the high dose in the main developmental toxicity study (Fulcher, Renaut & Bottomly, 2002).

In the main embryo-fetal developmental toxicity study, groups of 24 mated and presumed pregnant New Zealand White rabbits (19–25 weeks of age) were given advantame (purity 99.8%) in 1% (w/v) aqueous methyl cellulose at a dose of 0, 500, 1000 or 2000 mg/kg bw per day by gavage (5 ml/kg bw) between days 6 and 28 (inclusive) of gestation. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Animals were examined daily for abnormal clinical signs, and body weight and feed consumption were assessed daily. All dams were subjected to a detailed necropsy on day 29 after mating, and reproductive parameters were assessed, including gravid uterine weight, number of corpora lutea, number of implantation sites, number of resorption sites and number and distribution of live and dead fetuses. Fetuses and placentas were weighed and examined externally at necropsy. The neck and the thoracic and abdominal cavities of all fetuses were examined for visceral abnormalities. Approximately half of the fetuses in each litter were examined for skeletal development.

During the course of treatment, seven animals (1, 0, 1 and 5 in the control, 500, 1000 and 2000 mg/kg bw per day groups, respectively) were sacrificed prior to necropsy. One out of the five dams in the high-dose group that were humanely sacrificed exhibited hunched and unsteady posture, hypoactivity, low muscle tone, weight loss and reduced feed consumption and was sacrificed on day 17. The four other high-dose dams were sacrificed later in the study (days 23–26). With the exception of one high-dose dam that had consumed normal amounts of feed prior to sacrifice on day 24, the three remaining high-dose dams lost body weight several days before sacrifice, had low feed and water consumption and had reduced faecal output and/or loose stools. Necropsy revealed evidence of gastrointestinal disturbance, such as the presence of dark gelatinous liquid in the gastrointestinal tract, especially in the caecum, along with caecal wall haemorrhage and poor faecal pellet formation in the rectum. The incidence of deaths at the high dose, the circumstances of these deaths and the similarity of necropsy findings were considered to be consistent with a treatment-related effect.

The one mid-dose dam that exhibited weight loss, collapsed posture, loss of coordination and locomotion, vocalization and respiratory difficulties was sacrificed on day 27. Necropsy revealed internal surfaces of the kidneys stained green (possibly due to the presence of test substance and/or metabolites), with punctate foci on both kidneys, green contents in the gastrointestinal tract, a gelatinous green material at the ileo-caecal junction and a slight haemorrhagic caecal wall. Based on the similarity of these findings to those at the high dose, this death was also considered likely to be a treatment-related effect. The study authors proposed that these findings are a result of inappetence and gastrointestinal tract distress associated with oral ingestion of large amounts of poorly absorbed material. However, there are insufficient data to discount a toxicological effect and attribute this maternal toxicity to a localized irritant effect on the gastrointestinal tract. Toxicokinetic data from the preliminary rabbit study demonstrated that there is systemic exposure to both advantame and advantame acid over the study duration, with some evidence of accumulation of advantame acid at the higher doses (1000 and 2000 mg/kg bw per day), but not at the lowest dose (based on AUC values). Discoloured urine observed in rabbits further suggests systemic

exposure to a metabolite or metabolites of advantame. Furthermore, it is noted that neotame, a physicochemically related sweetener that has similar absorption to advantame (approximately 10% of ingested amount), did not cause gastrointestinal disturbances in rabbits up to the highest dose tested (1000 mg/kg bw per day).

The control group dam was killed on day 17 shortly after dosing due to convulsive-like signs, vocalization and respiratory distress. No other abnormalities were observed in this dam, and the death was considered to arise from difficulties during the gavage dosing.

One dam in each of the control and high-dose groups aborted its entire litter. The high-dose dam was classified as having aborted after delivering a single dead fetus before necropsy on day 29 of gestation. Clinical observations for this dam included body weight loss and reduced feed intake from day 18, green/purple staining of the cage paper, loose faeces and little water consumption. Necropsy of this dam revealed that all but one of the remaining fetuses were dead, all placentas appeared pale-coloured with mottled areas and there was a large amount of serous red fluid in the uterus and cervix. The liver was pale-coloured, the caecal contents were dark and fluid and there was no faecal pellet formation in the rectum. Although reduced feed intake and body weight loss are frequently associated with abortion in rabbits (Matsuzawa et al., 1981; Petrere et al., 1993), the similarity of necropsy findings with those observed in the other dams sacrificed prior to scheduled necropsy indicates an association between an indirect treatment-related effect on appetite and subsequent effects on gastrointestinal function (Matsuzawa et al., 1981).

The control dam showed signs of abortion on day 21 of gestation. Necropsy findings revealed that this dam had only three implantations. No other necropsy observations were remarkable.

Out of the mated and presumed pregnant rabbits (24 per group), there were 17 non-pregnant animals (3, 6, 2 and 6 at 0, 500, 1000 and 2000 mg/kg bw per day, respectively). The assessment of litter responses and calculations of group mean values reflect animals with live young at termination on day 29 (19, 18, 21 and 12 at 0, 500, 1000 and 2000 mg/kg bw per day, respectively).

Green, purple or pink staining of the cage paper was observed for the majority of treated dams, with green/purple/blue or pink urine observed in 9, 12 and 9 dams at 500, 1000 and 2000 mg/kg bw per day, respectively. Green staining of external surfaces (muzzle, ventral body surface, urogenital region, paws and tails) was observed in all mid- and high-dose sacrificed dams and in 17 of the surviving dams (2, 7 and 8 at 500, 1000 and 2000 mg/kg bw per day, respectively). These findings are attributed to the presence of the test material and/or metabolites and are not considered adverse.

For high-dose animals surviving to necropsy, including rabbits that were not pregnant or aborted their litter, lower body weight gains were observed through treatment until gestation day 10, with the majority of animals showing body weight loss from day 6 to day 8 of gestation. Recovery of body weight gain was evident from day 10, with overall gain comparable to that of controls by termination (including after adjustment for the gravid uterine weight). In the low- and mid-dose groups,

there was no clear effect of treatment on body weight gain during gestation, and overall body weight gain (including adjustment for the gravid uterine weight) was essentially similar to that of controls. Transient periods of reduced feed intake were observed in all groups, including controls. There was no evidence of a consistent pattern of altered feed intake related to treatment.

Green bladder contents were observed in one, two and three dams at 500, 1000 and 2000 mg/kg bw per day, respectively, which includes one of the high-dose dams killed prior to the end of the treatment period. These findings are consistent with the cage observations of discoloured urine, cage paper and external body surfaces and are considered treatment related. There were no other consistent findings among the animals that survived to day 29 that were considered indicative of an adverse effect of treatment.

There was no clear effect of treatment on litter data (number of early, late and total resorptions, implantations and live young, and sex ratio). Although not statistically significant compared with the concurrent control, an increase was noted in the incidence of fetal deaths (mean late resorptions: 0.3, 0.4, 0.4 and 1.0; post-implantation loss: 5.6%, 8.0%, 10.5% and 12.3%; at 0, 500, 1000 and 2000 mg/kg bw per day, respectively). Historical control data for various strains of rabbit indicate the existence of a ceiling value for the number of implantations that can survive to full term. Post-implantation loss can vary from 8.4% to 60.2%, depending on the number of corpora lutea and implantations (Feussner et al., 1992). In the absence of a significant effect on the mean live litter size (8.2, 8.6, 6.9 and 7.7 at 0, 500, 1000 and 2000 mg/kg bw per day, respectively), this finding is not considered toxicologically relevant.

There was no clear adverse effect of treatment on group mean values of placental, litter or fetal weights at any of the doses. There were no treatment-related effects associated with the type, incidence or distribution of visceral and skeletal alterations or the incidence of skeletal variants.

The NOAEL for maternal toxicity was 500 mg/kg bw per day, based on the occurrence of morbidity and early humane sacrifice at and above 1000 mg/kg bw per day. The study authors allocated a developmental NOAEL of 1000 mg/kg bw per day based on a possible trend towards higher fetal deaths at the highest dose tested (Fulcher, Renaut & Bottomly, 2003; Otabe, Fujieda & Masuyama, 2011e). However, in the absence of a clear treatment-related effect on fetal survival, the NOAEL for developmental toxicity is considered to be 2000 mg/kg bw per day, the highest dose tested.

2.2.6 Special studies

(a) Behaviour and locomotor activity

Behavioural and physiological abnormalities were examined in male Wistar (Han) rats (approximately 7 weeks of age; six per group) administered advantame (purity 99.9%; batch no. 000825) at a dose of 0, 10, 100 or 1000 mg/kg bw via a single oral gavage. The vehicle (negative control) was 1% (w/v) aqueous methyl cellulose. The study was conducted in accordance with GLP requirements (OECD,

European Commission, United Kingdom, USFDA and Japan). Animals were monitored for 7 days post-dosing. Standard laboratory diet and water were provided ad libitum throughout the study period, with the exception of overnight fasting prior to dosing and until 180 minutes post-dosing. Detailed behavioural observations, such as restlessness, apathy, writhing, fighting, stereotyped behaviour, dispersion in cage and locomotor activity, were made at 30, 60, 180 and 300 minutes and 24 hours after dosing, with daily observations for mortalities and clinical signs. Rats were sacrificed at the end of the 7-day observation period.

No deaths or behavioural or physiological abnormalities were observed in rats administered an oral dose of advantame up to 1000 mg/kg bw, the highest dose tested (Williams & Murphy, 2001).

Locomotor activity was examined in male Wistar (Han) rats (approximately 6 weeks of age; 10 per group) administered advantame (purity 99.9%; batch no. 000825) at a dose of 0, 10, 100 or 1000 mg/kg bw via a single oral gavage. The vehicle (negative control) was 1% (w/v) aqueous methyl cellulose. A positive control group of 10 rats was given a single gavage dose of amphetamine sulfate in 10% (w/v) methyl cellulose at 3 mg/kg bw. The study was conducted according to GLP requirements (OECD, European Commission, United Kingdom, USFDA and Japan). Animals were monitored for 300 minutes after dosing. Standard laboratory diet and water were available ad libitum, with the exception of overnight fasting prior to dosing. Using an automated activity monitor, the locomotor activity of the rats was measured for 10 minutes at each interval beginning with the predosing period and followed by measurements at 30, 60, 180 and 300 minutes post-dosing. After the final measurements at the 300-minute interval, the rats were sacrificed.

The positive control rats responded as expected, with a statistically significant ($P < 0.05$) increase in spontaneous locomotor activity relative to the vehicle control at the 30-, 60- and 180-minute intervals. There were no statistical differences between the test and vehicle control groups. No effect on locomotor activity was observed in rats administered an oral dose of advantame up to 1000 mg/kg bw, the highest dose tested (Williams & Adams, 2001).

(b) Cardiovascular and respiratory effects

Cardiovascular and respiratory changes were examined in anaesthetized male Beagle dogs (7–12 months of age; three per group) administered advantame (purity 99.9%; batch no. 000825) at a dose of 0, 10, 100 or 1000 mg/kg bw as a single intraduodenal bolus dose. The vehicle (negative control) was 1% (w/v) aqueous methyl cellulose. The study was conducted according to GLP requirements (OECD, European Commission, United Kingdom, USFDA and Japan). Dogs were fasted for a minimum of 16 hours prior to dosing. The following cardiovascular and respiratory parameters were recorded at 5-minute intervals during a 30-minute stabilization period (prior to dosing), at 10-minute intervals for the 1st hour after dosing and thereafter every 15 minutes up to 240 minutes: arterial blood pressure, heart rate, left ventricular systolic pressure and dP/dt(max) (cardiac contractility), electrocardiograph, femoral blood flow, femoral resistance, respiration rate, respiratory minute volume and tidal

volume. A bolus dose of noradrenaline (1 µg/kg bw) was injected into each animal to test the validity and responsiveness of the system. Dogs were sacrificed at the end of the observation period.

The injection of noradrenaline elicited the expected pharmacological responses from the test animals. The mean arterial blood pressure of the 100 mg/kg bw advantame dose group was significantly lower than that of the control group at the 120-, 150- and 165-minute sampling times post-dosing. In addition, a non-significant, transient increase in mean tidal volume was also observed in the 100 mg/kg bw dose group. The increase was primarily attributed to a single dog, which demonstrated an increase of 50% in mean tidal volume. Based on the absence of any dose–response relationship, these findings were considered incidental and of no biological significance.

There were no treatment-related effects on cardiovascular or respiratory parameters in dogs administered an intraduodenal dose of advantame up to 1000 mg/kg bw, the highest dose tested (Jordan et al., 2001). This result is supported by the lack of treatment-related effects observed for the electrocardiographic examinations of dogs in the 4-week, 13-week and 1-year oral toxicity studies (see section 2.2.2).

(c) Degradation products of advantame

Advantame acid is a known impurity of the final advantame product, with its presence limited to 1.0% or less; it is also the major metabolite of advantame. Minor degradation products of advantame formed at levels of less than 1.0% were identified as β-advantame, β-advantame acid, advantame-imide, *N*-(3-(3-hydroxy-4-methoxyphenyl))-propyl-L-aspartic acid (HF-1) and L-phenylalanine methyl ester.

Advantame acid has been identified in the plasma, urine and faeces of humans, dogs and rats. Following consumption, advantame is hydrolysed to advantame acid in both the gut and the plasma. Because advantame acid is systemically available following the ingestion of advantame, the toxicological studies conducted with advantame are considered sufficient to support the safety of advantame acid.

Prior to excretion, advantame acid can be further hydrolysed to form HF-1. HF-1 has been identified in the faeces of humans and dogs and as a minor urinary metabolite in humans, dogs and rats following the ingestion of advantame. In contrast to advantame acid, HF-1 has not been identified in the plasma following the ingestion of advantame or as an impurity in the final product. Therefore, the safety of this degradation product was assessed individually in a series of in vitro genotoxicity assays. In vitro genotoxicity assays were also conducted with the minor degradation products β-advantame, β-advantame acid and advantame-imide. Results of assays are presented in Table 17 below.

L-Phenylalanine methyl ester was not detectable under the recognized conditions simulating carbonated soft drinks (pH of 3.2 stored at 20 °C for 8 weeks); however, L-phenylalanine methyl ester was identified as a minor (<1.0%) degradation product of advantame under storage conditions with higher

temperatures (30–35 °C) and over longer durations (14–26 weeks) (Kato, Uno & Nagami, 2009). L-Phenylalanine methyl ester is expected to hydrolyse to L-phenylalanine and methanol following the ingestion of advantame-sweetened products. Both methanol and phenylalanine are minor degradation products of the related sweeteners neotame and aspartame.

(i) Safety evaluation of the degradation products of advantame

The results of studies of genotoxicity with minor degradation products of advantame are summarized in Table 17. Studies were conducted according to GLP requirements (Japan). Positive and negative (vehicle) controls were tested in each study and gave expected results.

There was no evidence of mutagenic activity for any of the minor degradation products of advantame in bacterial reverse mutation assays and mammalian forward mutation assays, in the absence or in the presence of S9 mix, with the exception of advantame-imide monohydrochloride (HCl). Advantame-imide HCl induced a weak (approximately 2- to 3-fold), but significant and dose-dependent, increase in total mutation frequency in mouse lymphoma cells, in the presence and absence of S9 mix, compared with the negative control in all assays.

The genotoxic potential of advantame-imide HCl was further examined in vivo using a mouse micronucleus test. Animals treated with advantame-imide HCl did not exhibit statistically significant increases in the incidence of micronucleated immature (polychromatic) erythrocytes at any dose level, compared with the negative control group. Compared with the vehicle controls, treatment with advantame-imide HCl did not significantly decrease the proportion of immature erythrocytes. However, the ratio of immature erythrocytes to total erythrocytes was slightly reduced compared with controls at the highest dose (2000 mg/kg bw per day), which was taken as evidence that the test substance was able to reach the bone marrow. Therefore, under the conditions of the mouse micronucleus assay, advantame-imide HCl did not demonstrate any genotoxic potential.

(d) Gastrointestinal motility

Gastrointestinal motility was examined in male Wistar (Han) rats (approximately 7 weeks of age; 10 per group) administered advantame (purity 99.9%; batch no. 000825) at a dose of 0, 10, 100 or 1000 mg/kg bw via a single oral gavage. The vehicle (negative control) was 1% (w/v) aqueous methyl cellulose. A positive control group of 10 rats was given a single gavage dose of morphine sulfate at 10 mg/kg bw. The study was conducted according to GLP requirements (OECD, European Commission, United Kingdom, USFDA and Japan). Standard laboratory diet and water were available ad libitum, with the exception of overnight fasting prior to dosing. Thirty minutes after dosing, each rat was gavaged with 1.0 ml charcoal (5% [w/v]) in water and sacrificed 30 minutes later. The gastrointestinal tract was removed, and the distance migrated by the charcoal from the pyloric sphincter towards the caecum was measured. The results were expressed as a percentage of the total gut length.

The positive control gave the expected results; motility was significantly lower in the positive control group relative to the vehicle control group (30.4 ± 10.33%

Table 17. Results of assays for genotoxicity with the minor degradation products of advantame (β-advantame, β-advantame acid, advantame-imide and HF-1)

Test system	Test object	Test article	Concentrations	Results	Reference
In vitro					
Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	β-Advantame (purity 99.9%)	0, 156, 313, 625, 1250, 2500 or 5000 µg/plate	Negative	Kimura (2009a)
Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	Advantame-imide HCl (purity 93.5%)	0, 156, 313, 625, 1250, 2500 or 5000 µg/plate	Negative	Kimura (2009c)
Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	β-Advantame acid (purity 99.4%)	0, 156, 313, 625, 1250, 2500 or 5000 µg/plate	Negative	Kimura (2009e)
Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	HF-1 (purity 97.9%)	0, 156, 313, 625, 1250, 2500 or 5000 µg/plate	Negative	Kimura (2009g)
Cell mutation[a,b]	Mouse lymphoma L5179Y ($tk^{+/-}$ –3.7.2c) cells	β-Advantame (purity 99.9%)	0, 575, 1150, 2300 or 4600 µg/ml[c]	Negative	Kimura (2009b)
Cell mutation[a]	Mouse lymphoma L5179Y ($tk^{+/-}$ –3.7.2c) cells	Advantame-imide HCl (purity 93.5%)	0, 35.1, 52.7, 79.0, 119, 178, 267 or 400 µg/ml (–S9; short term)[d] 0, 15.6, 23.4, 35.1, 52.7, 79.0, 119 or 178 µg/ml (+S9, short term) 0, 79.7, 104, 135, 175, 228, 296, 385 or 500 µg/ml (–S9; continuous)[d]	Positive	Kimura (2009d)
Cell mutation[a,b]	Mouse lymphoma L5179Y ($tk^{+/-}$ –3.7.2c) cells	β-Advantame acid (purity 99.4%)	0, 563, 1125, 2250 or 4500 µg/ml[e]	Negative	Kimura (2009f)

Table 17 (continued)

Test system	Test object	Test article	Concentrations	Results	Reference
Cell mutation[a,b]	Mouse lymphoma L5179Y ($tk^{+/-}$ –3.7.2c) cells	HF-1 (purity 97.9%)	0, 125, 250, 500 or 1000 µg/ml[f]	Negative	Kimura (2009h)
In vivo					
Micronucleus formation	Crlj:CD1 (ICR) mouse bone marrow	Advantame-imide HCl (purity 93.5%)	0, 300, 1000 or 2000 mg/kg bw per day (oral; preliminary test) 0, 500, 1000 or 2000 mg/kg bw per day (oral; main test)	Negative	Kimura (2009i)

HCl, monohydrochloride

[a] In the presence and absence of Aroclor-induced rat liver microsomal fraction (S9).
[b] Short-term (3-hour incubation; ±S9) or continuous (24-hour incubation; –S9) treatment.
[c] Test article precipitation observed at the highest concentration (4600 µg/ml) at the start of treatment, but not at the end of treatment under either incubation conditions (short-term or continuous).
[d] Test article precipitation at concentrations ≥385 µg/ml at the start and end of short-term (–S9) and 24-hour treatments.
[e] Test article precipitation at concentrations of ≥2250 µg/ml in the short-term and continuous treatments; decreased relative suspension growth and total growth at 4500 µg/ml in the continuous treatment (–S9).
[f] Test article precipitation at 1000 µg/ml; dose-dependent decrease in the relative suspension growth and relative total growth in the short-term treatment (+S9).

versus 52.6 ± 11.08%, respectively). Gastrointestinal motility was unaffected by the administration of advantame at 10 and 100 mg/kg bw. The distance travelled by the charcoal in the rats was similar to the distance travelled in the vehicle-treated rats. There was a statistically significant reduction in gastrointestinal motility compared with the vehicle control group observed in the 1000 mg/kg bw dose group (37.6 ± 5.74% versus 52.6 ± 11.08%, respectively). The authors attributed this result to the bulking effect of the high-dose formulation rather than a specific pharmacological effect of advantame.

No significant effect on gastrointestinal motility was observed in rats administered an oral dose of advantame up to 100 mg/kg bw (Williams, Murphy & Smith, 2001).

(e) Immunotoxicity

Immunotoxicity was examined in Wistar (Han) rats (approximately 5 weeks of age; 20 of each sex per group) administered advantame (purity 99.9%; batch no. TA10X251) ad libitum in the diet at a concentration of 0, 1500, 5000 or 15 000 mg/kg for 28 days. These dietary concentrations are equal to doses of

0, 140, 450 and 1380 mg/kg bw per day, respectively. The study was conducted according to GLP requirements (OECD, European Commission, USFDA and Japan). Ten rats per group were allowed to recover for an additional 30 days after the treatment phase prior to sacrifice. Animals were monitored daily for clinical signs of toxicity and mortality. Body weights and feed consumption were recorded on a weekly basis. Blood samples were collected at termination for haematology (total white blood cell and differential white blood cell counts) and clinical chemistry (total protein, albumin and albumin to globulin ratio). Immunological parameters included analyses of lymphocyte subsets (T cells, T helper cells, T cytotoxic cells, splenic lymphocytes, B cells and natural killer cells) and analyses of lymphocyte proliferation and cytokine production following ex vivo stimulation with concanavalin A. At necropsy, the weights of the adrenals, spleen and thymus were recorded, and animals were subjected to gross examination. Histopathological examinations were performed on the control and high-dose animals of the treatment phase of the study for the following tissues: spleen, thymus, mesenteric lymph node, popliteal lymph nodes, gut-associated lymphoid tissue (Peyer's patches), sternum with bone marrow and all gross lesions. Histopathological examination was not performed on tissues from the recovery group because no compound-related effects were observed in the animals from the treatment phase of the study.

No mortality or clinical signs of toxicity were observed. Mean body weights and feed consumption were comparable between the test groups and controls. During the recovery phase, feed consumption was slightly elevated in mid- and high-dose males, which occasionally achieved statistical significance. The elevated feed consumption during the recovery period was sporadic in nature and therefore considered to be the result of chance occurrence.

There were no treatment-related differences observed in total or differential white blood cell counts at the end of treatment and the recovery period. A statistically significant reduction in the percentage of eosinophils, relative to the total white blood cell count, was observed in the two highest dose groups of females (46% and 33% lower than control value, respectively) at the end of treatment. However, there was no dose–response relationship, and the absolute eosinophil values reached statistical significance only in the mid-dose females. This finding was considered to be due to normal variation and therefore incidental. There were no treatment-related differences observed in the clinical chemistry parameters at the end of the test period. At the end of the recovery phase, a small (4%) but statistically significant increase in the level of total plasma protein was noted in low-dose males only. Based on the magnitude of the change and the fact that it was observed only in the low-dose group, this change was also considered to be an incidental observation. There were no significant differences observed in absolute or relative organ weights and no treatment-related effects observed in the gross and microscopic examinations of animals after the treatment and recovery phases of the study.

There were no treatment-related differences in lymphocyte subsets. A decrease of approximately 14% in the percentage of B cells compared with controls was statistically significant in mid-dose males; however, this change was not dose dependent and was not observed in female animals. The reduced percentage of B cells in mid-dose males was therefore considered to be a chance occurrence.

Splenocyte cultures were prepared from rats at necropsy and tested for proliferation response and cytokine production following treatment with concanavalin A at 5 or 20 µg/ml for 72 hours at 37 °C. Stimulation of cell suspensions of splenocytes from both the control and test group animals with concanavalin A at a concentration of 20 µg/ml resulted in no proliferation, which was attributed to cell death due to overstimulation. This concentration of concanavalin A was considered too high for purposes of the functional assessment of T cell proliferation and cytokine production. No treatment-related or statistically significant differences in the T cell proliferation or cytokine production responses to concanavalin A were observed after stimulation of splenocytes with concanavalin A at a concentration of 5 µg/ml.

Based on the results for the end-points analysed in this study, there are no significant effects on immune system function observed in rats following 28 days of dietary exposure to advantame up to 15 000 mg/kg diet (equal to 1380 mg/kg bw per day), the highest concentration tested (Lina & van Mierlo, 2013).

Immunotoxicity was also examined in a 13-week oral toxicity study. Groups of 20 male and 20 female Wistar (Han) rats (36–44 days of age) were fed, ad libitum, diets containing advantame (purity 99.5%; batch no. 001227) at a concentration of 0, 1500, 5000, 15 000 or 50 000 mg/kg, equal to doses of 0, 118, 415, 1231 and 4227 mg/kg bw per day for males and 0, 146, 481, 1487 and 5109 mg/kg bw per day for females, respectively. The study was conducted according to GLP requirements (OECD, United Kingdom, European Commission, USFDA and Japan). Additional groups of five male and five female rats were assigned to the control, 15 000 and 50 000 mg/kg diet groups and were treated for 13 weeks followed by a 4-week recovery period. Parameters studied included clinical signs, physical examinations, functional observational battery, body weights, feed and water consumption, test article intake, ophthalmoscopy, haematology, clinical chemistry, urine analysis and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues from each animal as well as any tissues classified as abnormal. Additional blood samples were collected for immunotoxicity assays during week 4, week 13 and week 4 of the recovery period.

Any differences observed in the total white blood cell count and lymphocytes were within historical control ranges and not considered toxicologically significant. There were no significant differences observed in absolute or relative organ weights, and no treatment-related effects were observed in the gross and microscopic examinations.

Immunotoxicity parameters examined during week 4 revealed that the percentage of total B cells was significantly reduced by less than 20%, but not dose dependently, compared with controls in the three highest dose groups of the females. There were no significant differences in the percentage of total B cells at week 13. In males during week 4, the percentage of total T cells was significantly reduced by up to 26% compared with controls in a non-dose-dependent manner; only males in the highest dose group had a significantly reduced (15%) percentage of total T cells during week 13. In addition, high-dose males had significantly reduced CD4+ and CD8+ T cells compared with controls at week 13. In contrast,

females had a significantly increased percentage of total T cells during week 13, up to 17% in the two highest dose groups, compared with controls, as well as significantly increased CD4+ and CD8+ T cells compared with controls. Based on the absence of a dose–response relationship for any of these findings, the small magnitude of the changes, the inconsistency of the changes between the sexes, the lack of persistence and the absence of any pathology of the thymus, these findings are not considered treatment related.

Mitogen responsiveness of the peripheral blood lymphocytes was examined at week 4, at week 13 and after 4 weeks of recovery. Peripheral blood samples were cultured with concanavalin A at concentrations of 1.25 and 5 µg/ml for 72 hours at 37 °C. During week 4, all treated males had significantly lower ($P < 0.05$ or 0.01) stimulation of peripheral T lymphocytes in response to both concentrations of concanavalin A compared with controls. However, the reduced stimulation index was not dose related at either concentration of concanavalin A, and the greatest reductions in the stimulation index at concanavalin A concentrations of 1.25 and 5 µg/ml were observed for the low-dose males (69% and 56% of the control value, respectively). The reduction in T cell responsiveness is consistent with the noted reduction in total T cells. This effect was not observed in females, during week 13 or after the recovery period in males. In the absence of a dose–response relationship and because of the transient nature of the response, these findings are not considered treatment related.

There were no significant effects on immune system function observed in rats following 13 weeks of dietary exposure to advantame up to 50 000 mg/kg diet (equal to 4227 mg/kg bw), the highest concentration tested (Chase, 2004).

2.3 Observations in humans

2.3.1 Studies of tolerance of single doses

Advantame (purity 100.1%; batch no. 010228) was administered as a single oral dose in 150 ml of water to groups of eight fasted healthy male volunteers at 0.1, 0.25 or 0.5 mg/kg bw followed by 2 × 75 ml (150 ml) of water. Written approval for this study was obtained from the Independent Ethics Committee and conformed to the appropriate regulations (EEC, 1991; ICH, 1996; EC, 2001; WMA, 2008; USFDA, 2012a,b) and in accordance with the standard operating procedures of Ajinomoto Pharmaceuticals Europe Ltd. The mean age, weight and body mass index of the eight male volunteers were 24.1 ± 5.2 years, 77.7 ± 9.2 kg and 23.8 ± 2.2 kg/m^2, respectively.

Blood samples were collected prior to dosing and at specified intervals throughout the observation period. From the blood samples, the following parameters were assessed: plasma concentrations of advantame and advantame acid, pharmacokinetics, haematology (haemoglobin, red blood cells, white blood cells, differential white blood cells, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count and packed cell volume), clinical chemistry (albumin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, chloride, cholesterol, creatinine, gamma-glutamyl transpeptidase, lactate dehydrogenase, phosphorus,

potassium, sodium, total protein, urea and uric acid) and blood glucose levels. Physical examinations, including monitoring of heart rate and blood pressure, and electrocardiographic analysis were performed prior to dosing and at specific intervals throughout the observation period. Urine was collected predosing and at specified intervals throughout the observation period. Urine was analysed for glucose, protein, bilirubin and ketones. Any adverse events were recorded.

All subjects completed the study. Five adverse events were recorded for 3 of the 24 subjects: 1 subject at the middle dose (0.25 mg/kg bw) and 2 subjects at the high dose (0.5 mg/kg bw). The study authors described all of the adverse events as mild according to clinically validated international medical terminology. In the high-dose subjects, one reported a headache and the other reported sore throat, light-headedness and an upper respiratory tract infection. The mid-dose group subject reported a headache 7 days after a single dose of advantame. The study authors concluded that these effects were unlikely to be or were not related to treatment. Taking into consideration the elimination half-life of advantame, it is very unlikely that advantame or advantame acid would be present in the body 7 days after dosing. In addition, headaches were not reported in any of the other human studies, including those involving larger groups of subjects, with repeated exposure over longer periods of time, at the same dose level. Based on the lack of consistency across studies, the adverse events reported in this study are not considered treatment related.

There were no treatment-related effects on vital signs, electrocardiography parameters, haematology, clinical chemistry or urine analysis parameters.

Based on the results of this study, a single dose of advantame up to 0.5 mg/kg bw was considered to be well tolerated by healthy male subjects (Warrington, 2004; Warrington et al., 2011).

[^{14}C]Advantame (purity ≥98%; batch no. AJO186/57) was administered as a single dose of 18.75 mg in 150 ml of water (approximately 0.25 mg/kg bw) to six fasted, healthy male volunteers. Dosing was followed by an additional 150 ml of water. Written approval for this study was obtained from the Brent Medical Ethics Committee. The study was carried out in accordance with Ajinomoto Pharmaceuticals Europe Ltd's standard operating procedures, which are designed to ensure adherence to good clinical practice. Additional water was ingested at 2 hours post-dosing, lunch was consumed at 4 hours post-dosing and an evening meal was given 10 hours post-dosing. Only water was consumed between meals. The mean age, weight and body mass index of the subjects were 49.5 ± 8.83 years, 80.37 ± 5.79 kg and 22.2–28.8 kg/m^2, respectively.

Blood samples were collected prior to dosing and at specified intervals throughout the observation period. From the blood samples, the following parameters were assessed: plasma concentrations of advantame and advantame acid, radioactivity, pharmacokinetics, haematology (haemoglobin, red blood cells, white blood cells, differential white blood cells, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count and packed cell volume), clinical chemistry (albumin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, chloride,

cholesterol, creatinine, gamma-glutamyl transpeptidase, lactate dehydrogenase, phosphorus, potassium, sodium, total protein, blood urea nitrogen and uric acid). Physical examinations, including monitoring of heart rate and blood pressure, and electrocardiographic analysis were performed prior to dosing and at specific intervals throughout the observation period. Urine was collected predosing and at specified intervals throughout the observation period. Urine was analysed for glucose, protein, bilirubin, ketones and metabolites. All faecal samples passed from 12 hours prior to dosing until 168 hours post-dosing were collected and analysed for metabolites. Any adverse events were recorded.

All subjects completed the study. Eight adverse events involving five subjects were reported. None of the reported adverse events is considered related to the ingestion of advantame. Seven of the eight events were classified as mild, with one classified as moderate. Four subjects reported dental injury (broken teeth, lost dental filling) attributed to the consumption of food during the observation period, and the fifth subject reported back pain. One of the subjects with a dental injury also reported insect bites (mild), pain at the cannula site (where blood was withdrawn for testing) and rectal haemorrhage (moderate), with the latter commencing 6 days after dosing.

There was no treatment-related effect on vital signs, electrocardiographic parameters, haematology, clinical chemistry or urine analysis parameters.

Based on the results of this study, a single dose of advantame, equivalent to 0.25 mg/kg bw, was considered to be well tolerated by healthy male subjects (Warrington, 2005; Ubukata, Nakayama & Mihara, 2011).

2.3.2 Studies of tolerance of repeated doses

In a double-blind, placebo-controlled study, six subjects of each sex per group were randomized to receive either 10 mg advantame (purity >99%; lot no. 0279A) or encapsulated cellulose (placebo) 3 times daily for 4 weeks. Written approval for this study was obtained from the Ethics Committee for Clinical Research of Medicine and Pharmaceutical Products at Pauls Stradins Clinical University Hospital Development Foundation in Latvia, and the study conformed to the appropriate regulations (ICH, 1996; WMA, 2008) and was conducted in accordance with Ajinomoto Pharmaceuticals Europe Ltd's standard operating procedures. Subjects were instructed to take a capsule with water at breakfast, lunch and dinner. Subjects randomized to the advantame group received advantame doses between 0.375 and 0.5 mg/kg bw, depending on the subjects' weight. The mean age and weight of the subjects were 34.5 ± 11.85 years and 71.2 ± 7.37 kg, respectively.

Physical examinations, including monitoring of heart rate and blood pressure, were performed prior to dosing and at specific intervals throughout the observation period and 1 week after the final dose. Electrocardiographic analysis was conducted prior to dosing and on day 29. Blood samples were collected prior to dosing and at specified intervals throughout the observation period and 1 week after the final dose. From the blood samples, the following parameters were assessed: plasma concentrations of advantame and advantame acid, haematology (haemoglobin, red blood cells, white blood cells, differential white blood cells,

haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and platelet count), clinical chemistry (albumin, total bilirubin, blood urea nitrogen, calcium, C-peptide, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, chloride, cholesterol, creatinine, lactate dehydrogenase, phosphorus, potassium, sodium, total protein, triglycerides and uric acid), fasting blood glucose, glycosylated haemoglobin and insulin. Urine was collected predosing and at specified intervals throughout the observation period and 1 week after the final dose. Urine was analysed for pH, specific gravity, glucose, protein, bilirubin, ketones, occult blood and urobilinogen. Any adverse events were recorded.

All subjects completed the study. Three subjects reported adverse events: two subjects in the advantame group developed pruritis, and one placebo subject contracted a viral respiratory infection. All three adverse events were categorized as mild. Among the two subjects reporting pruritis, both cases were considered to be of no clinical significance, as they did not appear with any skin lesions, did not generate significant scratching, were transient in nature and resolved spontaneously. One of the two cases of pruritis was deemed to be possibly related to treatment due to a reasonable temporal sequence from the administration of advantame. It is noted that other factors may have been responsible for the condition; however, a relationship to advantame could not be ruled out.

There were no treatment-related effects on vital signs, electrocardiography, haematology, clinical chemistry or urine analysis parameters. There was no difference between the treated and control groups with regard to the oral glucose tolerance test or glucose/insulin profiles.

Based on the results of this study, a repeated oral dose of advantame up to 0.5 mg/kg bw per day for 4 weeks was considered to be well tolerated in healthy males and females (Warrington et al., 2011).

In a double-blind, placebo-controlled study, 36 Type 2 diabetic subjects of each sex per group were randomized to receive either 10 mg advantame (purity >99%; lot no. 0279A) or encapsulated cellulose (placebo) 3 times daily for 12 weeks. A subgroup of 24 subjects (12 of each sex per group) was randomly selected to undergo oral glucose tolerance testing during the screening period. Written approval for this study was obtained from the Ethics Committee for Clinical Research of Medicine and Pharmaceutical Products at Paula Stradina Clinical University Hospital Development Foundation, and the study conformed to the appropriate regulations (ICH, 1996; WMA, 2008) and was conducted in accordance with Ajinomoto Pharmaceuticals Europe Ltd's standard operating procedures. Subjects were instructed to take a capsule with water at breakfast, lunch and dinner. Subjects randomized to the advantame group received advantame doses between 0.375 and 0.5 mg/kg bw, depending on the subjects' weight. The mean age and weight of the subjects were 60.1 ± 7.3 years and 81.8 ± 9.46 kg, respectively.

Physical examinations, including monitoring of heart rate and blood pressure, were performed prior to dosing and at specific intervals throughout the observation period and 1 week after the final dose. Electrocardiographic analysis was conducted

prior to dosing and on day 85. Blood samples were collected prior to dosing and at specified intervals throughout the observation period and 1 week after the final dose. From the blood samples, the following parameters were assessed: plasma concentrations of advantame and advantame acid, haematology (haemoglobin, red blood cells, white blood cells, differential white blood cells, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count), clinical chemistry (albumin, alkaline phosphatase, total bilirubin, blood urea nitrogen, calcium, chloride, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, creatinine, lactate dehydrogenase, phosphorus, potassium, alanine aminotransferase, aspartate aminotransferase, sodium, total protein, triglycerides and uric acid), fasting blood glucose, glycosylated haemoglobin and insulin. Urine was collected predosing and at specified intervals throughout the observation period and 1 week after the final dose. Urine was analysed for pH, specific gravity, glucose, protein, bilirubin, ketones, occult blood, urobilinogen and microscopy of spun deposits. Any adverse events were recorded.

All subjects completed the study. Five subjects in the treated group experienced a total of 9 adverse events, and nine subjects in the control group experienced a total of 10 adverse events. The majority of adverse events were classified as mild (11/19), with the remainder classified as moderate. The predominant adverse events reported were respiratory and/or urinary tract infections (six subjects in the control group and three in the treated group), followed by gastrointestinal disorders (one subject in each group), asthenia (one subject in each group), dyslipidaemia (one control subject) and neuralgia (one control subject), all classified as slight. Of the three subjects with moderate adverse events, two were in the treated group (one with pneumonia and one with dyspepsia, flatulence and nausea) and one was in the control group (urinary tract infection). With the exception of the urinary tract infection, all events resolved by the end of the study. The author considered the dyspepsia, flatulence and nausea of moderate severity in one treated subject to be possibly treatment related.

There were no treatment-related effects on vital signs, physical findings or electrocardiographic results. There was no treatment-related effect on any haematology, clinical chemistry or urine analysis parameters. There was no difference between the treated and control groups with regard to the glucose/insulin profiles or glycosylated haemoglobin concentration.

Based on the results of this study, a repeated oral dose of advantame up to 0.5 mg/kg bw per day for 12 weeks was considered to be well tolerated by Type 2 diabetic subjects, including the lack of any effect on blood glucose (Pirage, 2006; Otabe, Muraoka & Narita, 2009).

3. DIETARY EXPOSURE

Advantame is a high-intensity sweetener that is approximately 20 000–37 000 times sweeter than sucrose and approximately 100 times sweeter than the structurally related high-intensity sweetener aspartame (Amino et al., 2006; Ajinomoto, 2012).

Food Standards Australia New Zealand (FSANZ) approved the use of advantame in 2011 as a general-purpose high-intensity sweetener, with no restrictions other than good manufacturing practices (GMP). Approval for the use of advantame as a new sweetener is currently also being sought in several other global markets, including the USA and the European Union (EU). Food additive applications have been submitted in both jurisdictions and are currently under review.

An acceptable daily intake (ADI) of 0–5 mg/kg bw for advantame was established by FSANZ in 2011 (FSANZ, 2011).

3.1 Use of advantame

Advantame is intended for use as a tabletop sweetener and as a sweetener in all categories currently permitted to contain aspartame and other high-intensity sweeteners. It was assessed for a more limited number of uses as a tabletop sweetener, as a sweetener in powdered or liquid fruit- and milk-based drinks and as a flavouring agent by FSANZ in 2011. FSANZ determined that advantame was suitable as a high-intensity sweetener, being stable for 12 months in powdered beverage preparations and for 36 months in tabletop sweeteners. FSANZ noted that advantame also had a flavour-enhancing effect at low concentrations (FSANZ, 2011).

3.2 Advantame concentration data

The sponsor-proposed maximum use levels were derived from maximum use levels for aspartame from regulations in the USA or EU and adjusted for the sweetness of advantame relative to aspartame of 100:1, with further alterations based on taste perception made in some cases. The concentrations of advantame were then doubled by the sponsor for the EU proposal to allow manufacturers some flexibility in formulation and to account for different taste preferences in the two markets.

A summary of sponsor-proposed maximum use levels for the Codex General Standard for Food Additives (GSFA) food categories is given in Table 18. These were derived by the sponsor by taking the higher value for a given food category from the proposals for the regulations in the USA and EU (European Parliament, 1994, 2004).

3.3 Estimates of dietary exposure

3.3.1 Screening methods

(a) Budget method

The budget method can be used to estimate the theoretical maximum level of advantame in those foods and beverages that are likely to contain the food additive that would not result in the ADI being exceeded by the population (Hansen, 1979; FAO/WHO, 2009). Assuming an ADI of 0–5 mg/kg bw (as established by FSANZ), that 50% of advantame is used in solid food and 50% in beverages, and that only 25% of the total amount of solid food and 50% of the total amount of

Table 18. Summary of the sponsor-proposed food uses and maximum use levels for advantame in the GSFA[a,b,c]

Food category no. and food category		Maximum proposed use level for advantame (mg/kg)
01.0 Dairy products and analogues, excluding products of food category 02.0		
01.1.2	Dairy-based drinks, flavoured and/or fermented (e.g., chocolate milk, cocoa, eggnog, drinking yoghurt, whey-based drinks)	12
01.7	Dairy-based desserts (e.g., pudding, fruit or flavoured yoghurt)	20
02.0 Fats and oils, and fat emulsions		
02.3	Fat emulsions mainly of type oil-in-water, including mixed and/or flavoured products based on fat emulsions	20
02.4	Fat-based desserts, excluding dairy-based dessert products of food category 01.7	20
03.0 Edible ices, including sherbet and sorbet		20
04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds		
04.1.2.1	Frozen fruit	20
04.1.2.2	Dried fruit	20
04.1.2.3	Fruit in vinegar, oil, or brine	6
04.1.2.4	Canned or bottled (pasteurized) fruit	20
04.1.2.5	Jams, jellies, marmalades	20
04.1.2.6	Fruit-based spreads (e.g., chutney) excluding products of food category 04.1.2.5	20
04.1.2.7	Candied fruit	40
04.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	20
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	20
04.1.2.10	Fermented fruit products	20
04.1.2.11	Fruit fillings for pastries	20
04.1.2.12	Cooked fruit	20
04.2.2.1	Frozen vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	20
04.2.2.2	Dried vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	20

Table 18 (continued)

Food category no. and food category		Maximum proposed use level for advantame (mg/kg)
04.2.2.3	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds in vinegar, oil, brine, or soybean sauce	6
04.2.2.4	Canned or bottled (pasteurized) or retort pouch vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds	20
04.2.2.5	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g., peanut butter)	20
04.2.2.6	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed pulps and preparations (e.g., vegetable desserts and sauces, candied vegetables) other than food category 04.2.2.5	20
04.2.2.7	Fermented vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products, excluding fermented soybean products of food categories 06.8.6, 06.8.7, 12.9.1, 12.9.2.1 and 12.9.2.3	20
04.2.2.8	Cooked or fried vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds	20
05.0 Confectionery		
05.1.1	Cocoa mixes (powders) and cocoa mass/cake	40
05.1.2	Cocoa mixes (syrups)	40
05.1.3	Cocoa-based spreads, including fillings	20
05.1.4	Cocoa and chocolate products	40
05.1.5	Imitation chocolate, chocolate substitute products	40
05.2.1	Hard candy	120
05.2.2	Soft candy	40
05.2.3	Nougats and marzipans	40
05.3	Chewing gum	400
05.4	Decorations (e.g., for fine bakery wares), toppings (non-fruit) and sweet sauces	40
06.0 Cereals and cereal products, derived from cereal grains, from roots and tubers, pulses and legumes, excluding bakery wares of food category 07.0		

Table 18 (continued)

Food category no. and food category		Maximum proposed use level for advantame (mg/kg)
06.3	Breakfast cereals, including rolled oats	20
06.5	Cereal and starch based desserts (e.g., rice pudding, tapioca pudding)	20
07.0 Bakery wares		
07.1	Bread and ordinary bakery wares	80
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	34
09.0 Fish and fish products, including molluscs, crustaceans and echinoderms		
09.2	Processed fish and fish products, including molluscs, crustaceans, and echinoderms	15
09.3	Semi-preserved fish and fish products, including molluscs, crustaceans, and echinoderms	6
09.4	Fully preserved, including canned or fermented fish and fish products, including molluscs, crustaceans, and echinoderms	6
10.0 Eggs and egg products		
10.4	Egg-based desserts (e.g., custard)	20
11.0 Sweeteners, including honey		
11.4	Other sugars and syrups (e.g., xylose, maple syrup, sugar toppings)	60
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	GMP
12.0 Salts, spices, soups, sauces, salads, protein products (including soybean protein products) and fermented soybean products		
12.2.2	Seasonings and condiments	40
12.3	Vinegars	60
12.4	Mustards	7
12.5	Soups and broths	3
12.6	Sauces and like products	7
12.7	Salads (e.g., macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories 04.2.2.5 and 05.1.3	20
13.0 Foodstuffs intended for particular nutritional uses		
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	20
13.4	Dietetic formulae for slimming purposes and weight reduction	16

Table 18 (continued)

Food category no. and food category		Maximum proposed use level for advantame (mg/kg)
13.5	Dietetic foods (e.g., supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	20
13.6	Food supplements	110
14.0 Beverages, excluding dairy products		
14.1.3.1	Fruit nectar	12
14.1.3.3	Concentrates for fruit nectar	12
14.1.4	Water-based flavoured drinks, including “sport”, “energy” or “electrolyte” drinks and particulated drinks	12
14.1.5	Coffee, coffee substitutes, tea, herbal infusions, and other hot cereal and grain beverages, excluding cocoa	12
14.2.7	Aromatized alcoholic beverages (e.g., beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	12
15.0 Ready-to-eat savouries		10

GMP, good manufacturing practices

[a] Codex Alimentarius Commission (2012).
[b] FAO/WHO (2012b).
[c] Sponsor-proposed maximum use levels based on highest value for advantame in the proposals submitted for consideration by the USA or EU.

beverages in the food supply contain the food additive, the theoretical maximum level of advantame would be 400 mg/kg for food and 100 mg/kg for beverages. These theoretical maximum levels were higher than the sponsor-proposed GSFA maximum use levels (Table 18) for nearly all foods and beverages, with the exception of 400 mg/kg for chewing gum and the likely concentration for use in tabletop sweeteners (GMP in submission, 450 mg/kg assumed in the 2011 FSANZ advantame risk assessment). Hence, further investigations were made.

(b) Sugar replacement methods

An alternative screening method for intense sweeteners assumes that the sweetener replaces all sucrose in the diet (Renwick, 2008). For example, the population in the USA has one of the highest reported mean total sugar intakes of 44 kg/person aged 2+ years per year (120 g of sugar per day), with the highest reported total sugar intake of 160 g/day for 12- to 19-year-olds. In the unlikely event that total sugar intake was all replaced by advantame in the food supply in the USA, replacement would result in a mean population dietary exposure to advantame of 6 mg/day or 0.1 mg/kg bw per day (maximum of 8 mg/day or 0.13 mg/kg bw per day for 12- to 19-year-olds), assuming a sweetness relative to sucrose of 20 000:1 and a body weight of 60 kg for the relevant population (USDA, 2012). As for the

budget method, this is a very conservative estimate, because it is very unlikely that one sweetener will replace all sucrose; additionally, it ignores other technological functions of sucrose in food.

A similar calculation can be undertaken to cover all regions of the world, using food balance sheet data from the 2012 WHO Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) 17 Cluster Diets for the amount of total sugar, honey and candies available for consumption (WHO, 2012). Per capita sugar, honey and candy food amounts across the 17 groups of countries ranged from 30 to 134 g/day, equivalent to a dietary exposure to advantame of 0.02–0.11 mg/kg bw per day, assuming a sweetness relative to sucrose of 20 000:1, a body weight of 60 kg for the general population and full sugar replacement.

An alternative approach proposed by Renwick (2008) is to predict intakes of sucrose equivalents from reported dietary exposure estimates of other more widely used intense sweeteners and then back-calculate potential advantame dietary exposures. Renwick (2008) reported that sucrose equivalents for dietary exposures to sweeteners such as aspartame, saccharin and sucralose given in the literature for the general population averaged 255 mg sucrose equivalents per kilogram of body weight per day (range from 100 to 300 mg sucrose per kilogram of body weight per day or 6–18 g sucrose per day, assuming a body weight of 60 kg). For populations likely to have higher sweetener intakes, such as people with diabetes, predicted sucrose intakes for high consumers were up to 897 mg sucrose equivalents per kilogram of body weight per day (or 54 g sucrose per day, assuming a body weight of 60 kg). As expected, these predictions were much lower than the actual amount of total sugars consumed per day from the diet in countries similar to those where sweetener intakes were reported (Renwick, 2008). Predicted mean dietary exposures to advantame based on the sucrose equivalent intakes reported by Renwick (2008) were 0.01–0.03 mg advantame per kilogram of body weight per day for the general population and 0.03–0.05 mg advantame per kilogram of body weight per day for high consumers (Table 19).

3.3.2 Individual dietary records

To predict dietary exposure to advantame, the additive was assumed to be present in all foods in each of the food categories nominated in the submissions for consideration by the USA or EU at the sponsor-proposed maximum use level in each jurisdiction. The food categories used in the calculations for European countries were broader than those used in the USA predictions and included some foods for which advantame use is not intended. Predicted dietary exposures were given in the submission for the USA and European countries based on published summary food consumption data derived from individual food consumption data from relevant national nutrition surveys (Ajinomoto, 2012).

Dietary exposure estimates for advantame have been reported only for Australia and New Zealand based on individual food consumption data from their respective national nutrition surveys and proposed uses, which were limited compared with those proposed for the GSFA (FSANZ, 2011). Table 20 summarizes predicted dietary exposure estimates for advantame.

Table 19. Predicted dietary exposure to advantame based on sweetener surveys (submission, based on Renwick, 2008)

Population group	Intakes of sucrose equivalents, derived from reported intense sweetener dietary exposures (mg/kg bw per day)		Estimated dietary exposure to advantame[a]			
			mg/kg bw per day		mg/day[b]	
	Mean intake	Heavy consumer	Mean intake	Heavy consumer	Mean intake	Heavy consumer
Non-diabetic adults	255	675	0.01	0.03	0.77	2.03
Diabetic adults	280	897	0.01	0.04	0.84	2.69
Non-diabetic children	425	990	0.02	0.05	1.28	2.97
Diabetic children	672	908	0.03	0.05	2.02	2.72

[a] Based on an assumed sweetness for advantame relative to sucrose of 20 000:1.
[b] Assuming a body weight of 60 kg; as studies for diabetic children included children between the ages of 2 and 20 years, this assumption was maintained for all age groups.

3.3.3 Dietary exposure to degradation products

The potential exposures to methanol and phenylalanine from the consumption of advantame-sweetened products can be considered insignificant in comparison to the consumption of these substances from background dietary sources. In the previous JECFA assessment of the safety of neotame, the Committee noted that both methanol and phenylalanine are normal components of the diet and concluded that the amounts of methanol and phenylalanine resulting from the ingestion of neotame were insignificant in comparison to the amounts of these substances found in food (Annex 1, reference *167*). Taking into consideration that the sweetness potency of advantame relative to sucrose (=37 000) exceeds that of neotame (=7000) (Renwick, 2011), the exposures to methanol and phenylalanine from the use of advantame are expected to be less than that from neotame.

4. COMMENTS

4.1 Toxicological data

A comprehensive range of studies on pharmacokinetics and toxicokinetics, acute toxicity, short-term and long-term toxicity, carcinogenicity, genotoxicity, and developmental and reproductive toxicity undertaken with appropriate standards for study protocol and conduct were taken into consideration in the safety assessment of advantame.

In all species studied (mice, rats, rabbits, dogs and humans), advantame is rapidly converted to advantame acid (de-esterified advantame). Based on the data from studies with simulated gastric and intestinal fluids, the majority of this

Table 20. Predicted dietary exposures to advantame

Country	Survey type	Source of concentration data	Population group	Dietary exposure to advantame (mg/kg bw per day)
Australia	1995 NNS, 10 101 consumers aged 17+ years, 24-hour recall survey (1 record)	FSANZ (2011)[a]	17+ years mean consumers	0.04
			17+ years 90th percentile consumers	0.07
	2007 ANCNPAS, 4487 respondents, 24-hour recall survey (2-day average, non-consecutive records)	FSANZ (2011)[a]	2–6 years mean consumers	0.06
			2–6 years 90th percentile consumers	0.14
			7–16 years mean consumers	0.04
			7–16 years 90th percentile consumers	0.08
	2007 ANCNPAS, 4487 respondents, 24-hour recall survey (2-day average, non-consecutive records)	GSFA sponsor-proposed levels	2–6 years mean consumers	1.45
			2–6 years 90th percentile consumers	2.16
			7–16 years mean consumers	0.86
			7–16 years 90th percentile consumers	1.45
New Zealand	1997 NNS, 4636 respondents, 24-hour recall survey (1 record)	FSANZ (2011)[a]	15+ years mean consumers	0.03
			15+ years 90th percentile consumers	0.06
USA	2007–2008 and 2009–2010 NHANES combined data sets, 24-hour recall survey, 2 non-consecutive records	Sponsor-proposed MULs for advantame for regulations in USA, based on permitted uses for aspartame[b] in USA (CDC, 2011)	0+ years mean consumers	0.09
			0+ years 90th percentile consumers	0.19
			0–2 years mean consumers	0.25
			0–2 years 90th percentile consumers	0.52
			3–11 years mean consumers	0.18
			3–11 years 90th percentile consumers	0.32

Table 20 (continued)

Country	Survey type	Source of concentration data	Population group	Dietary exposure to advantame (mg/kg bw per day)
Europe	EFSA 2011 Food Consumption Database for 22 countries (32 different surveys, some covering different age groups, using different survey methods)	Sponsor-proposed MULs for advantame for regulations in EU, based on permitted uses of aspartame[c] in EU	18–64 years mean consumers	0.17–0.7
			18–64 years 95th percentile consumers[d]	0.41–1.26
			<18 years mean consumers	0.23–1.38
			<18 years 95th percentile consumers[d]	0.32[d]–2.27

ANCNPAS, Australian National Children's Nutrition and Physical Activity Survey; EFSA, European Food Safety Authority; GSFA, Codex General Standard for Food Additives; MUL, maximum use level; NHANES, National Health and Nutrition Examination Survey; NNS, National Nutrition Survey

[a] Proposed use levels in powdered and liquid milk-based and juice drinks, tabletop sweeteners.
[b] Based on MUL in USA regulations for aspartame, adjusted for sweetness for advantame to aspartame of 100:1, with alterations based on sensory data in some cases.
[c] Based on MULs in EU regulations for aspartame, adjusted for relative sweetness for advantame to aspartame of 100:1, then doubled to allow manufacturers flexibility in formulation, with alterations based on sensory data in some cases; broad food categories used.
[d] Two commodities at 95th percentile plus mean for all other commodities.

conversion occurs rapidly in the intestinal tract prior to absorption. Maximum plasma concentrations of advantame-associated radioactivity after oral dosing in rodents are reached within 15–45 minutes (T_{max}); this is similar to the T_{max} in fasted humans (1.25 hours) and contrasts with the T_{max} of 6–8 hours in fasted dogs. Overall bioavailability following oral doses is estimated to be less than 10% in rats, 8–15% in dogs and approximately 6% in humans.

Following oral dosing with radiolabelled advantame, the majority of advantame and/or its metabolites are found to be associated with the gastrointestinal tract, in particular the stomach and small and large intestine. The low levels of advantame-associated radioactivity found outside the gastrointestinal tract were consistent with the presence of these materials in plasma and provided no indication of tissue-specific distribution or accumulation. Based on autoradiography studies, advantame and/or its metabolites were not detected in the placentas or fetuses of pregnant rats after oral dosing. Human volunteers ingesting advantame daily over a 12-week period demonstrated no evidence of accumulation.

Advantame acid (de-esterified advantame) is generally the predominant metabolite found in plasma, urine and faeces of all species except dogs, where

advantame acid accounts for a low proportion (<1%) of the total absorbed dose in plasma. In this species, a sulfate conjugate was postulated to be the predominant metabolite in plasma based on HPLC and mass spectrometric analyses. This conjugate, together with possible enterohepatic circulation of other metabolites, likely explains the longer terminal half-life of plasma radioactivity in the dog (advantame-associated radioactivity half-life of 74–85 hours in dogs compared with 6.0–8.1 hours in rats and 3.9 hours in humans). Sulfate conjugates were not detected in the plasma of rats or humans.

L-Phenylalanine methyl ester was identified as a minor (<1.0%) degradation product of advantame in beverages under low-pH storage conditions. When ingested, L-phenylalanine methyl ester would hydrolyse to L-phenylalanine and methanol. However, it was considered that any potential exposure to either chemical would be insignificant compared with the usual dietary exposure to these chemicals.

The oral LD_{50} of advantame administered by gavage to rats was greater than 5000 mg/kg bw. The subchronic and chronic toxicity of dietary advantame was assessed in mice (13 and 104 weeks), rats (4, 13 and 104 weeks) and dogs (4, 13 and 52 weeks). NOAELs for each of these studies were established on the basis of an absence of adverse effects at the highest concentration tested (50 000 mg/kg in the diet, equal to doses ranging from approximately 2000 to 7400 mg/kg bw per day). A common observation in the subchronic and chronic rodent feeding studies was a significant decrease in body weight gain associated with the high-dose group (5% advantame in the diet) with an associated reduction in feed conversion efficiency, but not feed intake. A significant reduction in final body weight gain compared with controls, however, was noted in female mice in the high-dose group (50 000 mg/kg diet) in the chronic bioassay and occurred in the absence of a decrease in feed conversion efficiency. As this effect was mainly related to a decrease in body weight gain observed in senile mice (>78 weeks of age) and there was no significant difference in the final mean body weights, in the absence of any other adverse effects, the reduced body weight gain observed at the highest dose was attributed to the relatively high concentration of a non-caloric substance in the diet.

In the chronic bioassays designed to assess potential carcinogenicity in mice and rats, there was no treatment-related increase in tumour incidence at advantame doses of up to 5693 and 2621 mg/kg bw per day, respectively. There was no evidence of genotoxicity in any of the in vitro or in vivo tests conducted with advantame. A number of minor degradation products of advantame (formed at levels of less than 1%) were also tested for genotoxicity potential in vitro. Only advantame-imide gave a weak positive response in vitro, which was not confirmed on in vivo testing.

In a two-generation study of reproductive toxicity in rats, there were no treatment-related effects on reproductive parameters (mating performance, fertility, gestation length/index and sperm quality), litter observations (size, survival, sex ratio and pup body weight) or measures of postnatal offspring development at dietary concentrations of advantame up to 50 000 mg/kg (equal to advantame doses in the range of 4000–6000 mg/kg bw per day during the period prior to mating and gestation and over 8000 mg/kg bw per day during lactation). The developmental toxicity of advantame was examined in rats (diet) and rabbits (gavage) at

advantame doses of 0, 465, 1418 or 4828 mg/kg bw per day and 0, 500, 1000 or 2000 mg/kg bw per day, respectively. In neither species was there any evidence of embryotoxicity or teratogenicity up to the highest dose tested. In rats, there was a transitory decrease in feed consumption at the start of treatment, resulting in lower body weight gain and subsequent lower final body weights in high-dose dams; however, there was no adverse effect on survival, growth or fetal development. In the main rabbit developmental toxicity study, clinical signs of toxicity (lethargy, loss of coordination and locomotion, inappetence and body weight loss) leading to humane sacrifice were observed in the 1000 mg/kg bw per day (n = 1) and 2000 mg/kg bw per day (n = 5) dose groups. Necropsy findings of these animals included incidences of distended caecum with or without haemorrhagic walls, congestion of the gastrointestinal tract, kidneys reported with punctate cysts and/or foci on the surfaces and bladder filled with green-coloured urine. Based on the available data, it could not be concluded that the clinical symptoms necessitating humane sacrifice in maternal animals in the mid- and high-dose groups were not treatment related. In the high-dose group, there was an observation of an approximate 2-fold increase in post-implantation loss compared with controls. However, the Committee noted that this effect occurred without a significant reduction in the number of live offspring per litter and was within the historical control range for this strain of rabbits. Based on the similarity in clinical signs observed in gravid rabbits subject to humane sacrifice in the 1000 and 2000 mg/kg bw per day dose groups, a NOAEL for maternal toxicity of 500 mg/kg bw per day was assigned, whereas the NOAEL for developmental effects was 2000 mg/kg bw per day, the highest dose tested.

Studies of human tolerance of advantame included a single-dose pharmacokinetic study, a 4-week study in healthy males and a 12-week study in male and female diabetic subjects. There were no treatment-related adverse effects or withdrawals during the study periods. Advantame did not affect plasma levels of glucose or insulin in healthy subjects, exacerbate glucose tolerance or insulin resistance or affect levels of glycosylated haemoglobin in diabetic subjects with acceptable blood glucose control. The consumption of a single or repeated dose of advantame up to 0.5 mg/kg bw per day was considered to be well tolerated by both healthy and diabetic individuals.

4.2 Assessment of dietary exposure

Advantame is intended for use as a high-intensity sweetener in a number of food categories, including use in tabletop sweeteners, where there are existing provisions in regulations for aspartame. At low concentrations, advantame can also be used as a flavour enhancer. Application of a modified budget method as a screening method indicated a theoretical maximum use level of 400 mg/kg for advantame, assuming use in one quarter of the food supply and half the beverage supply and an ADI for advantame of 0–5 mg/kg bw, as established by FSANZ in 2011. The proposed use levels for advantame in chewing gum and tabletop sweeteners were at or above this maximum level, so further investigations were made.

In an additional screening method, mean dietary exposures to advantame were predicted, assuming total sugar replacement by advantame in the food supply and a sweetness relative to sucrose of 20 000:1. Using per capita data for the food

group "Sugar, honey and candies" for the 17 GEMS/Food Cluster Diets or reported total sugar intakes for the population of the USA, known to have one of the highest sugar intakes in the world, the predicted mean population dietary exposures to advantame were all less than or equal to 0.2 mg/kg bw per day, assuming a 60 kg body weight. Dietary exposures to advantame were also predicted assuming replacement of permitted high-intensity sweeteners, by calculating the sucrose equivalent intakes derived from their known use and converting back to advantame use. Predicted mean dietary exposures to advantame were, as expected, lower than those for total sugar replacement, at 0.01–0.03 mg/kg bw per day for the general population and 0.03–0.05 mg/kg bw per day for high consumers, including people with diabetes, assuming a 60 kg body weight.

Dietary exposures to advantame were predicted from individual food consumption records from national nutrition surveys for a number of populations with a known use of foods containing high-intensity sweeteners (the USA, 22 European countries, Australia and New Zealand) and sponsor-proposed maximum use levels for each jurisdiction, assuming that brand-loyal consumers always select foods proposed to contain advantame. The proposed use levels were derived from existing maximum permitted levels for aspartame in the relevant jurisdiction and adjusting for the relative sweetness of advantame to aspartame of 100:1, with further alterations based on taste perception, in some cases. The Committee noted that the maximum use levels proposed by the sponsor for the EU were double those proposed for the USA in some food categories, as an additional factor of 2 had been applied to the advantame levels derived from aspartame maximum permitted levels. Sponsor-proposed use levels for the GSFA were derived by taking the highest value from the regulations in the USA or EU. Predicted mean dietary exposures to advantame for consumers only across the different population groups evaluated ranged from 0.03 mg/kg bw per day (mean consumers aged 15 years and over in New Zealand, assuming restricted use in beverages and tabletop sweeteners) to 1.45 mg/kg bw per day (mean consumers aged 2–6 years in Australia, assuming sponsor-proposed GSFA use levels). Predicted dietary exposures for high consumers ranged from 0.06 mg/kg bw per day (90th percentile consumers aged 15 years and over in New Zealand, assuming restricted use in beverages and tabletop sweeteners) to 2.16 mg/kg bw per day (90th percentile consumers aged 2–6 years in Australia, assuming proposed GSFA use levels). The predicted dietary exposures for European populations and the estimate for Australian children based on applicant-proposed GSFA use levels tended to be higher than those for the population in the USA due to higher maximum levels of advantame proposed for similar food categories and the fact that broader food categories were used in these estimates, which included foods for which no use of advantame is intended.

5. EVALUATION

On the basis of the available studies, the Committee considered advantame to be a substance of low oral toxicity across a range of species, including humans. Appropriately conducted studies indicated that advantame is not carcinogenic, mutagenic or teratogenic or associated with any reproductive or developmental

toxicity. The main treatment-related effect that was considered adverse was the occurrence of morbidity that necessitated early humane sacrifice of dams in the main rabbit developmental toxicity study (where dosing was by gavage) at and above an advantame dose of 1000 mg/kg bw per day. While these clinical observations were not observed in any other species dosed with similar levels of advantame via the diet, in the absence of pharmacokinetic data for gravid rabbits, it could not be concluded that the effect was not toxicologically relevant. The rabbit is considered the most sensitive species in the database, with a NOAEL for maternal toxicity of 500 mg/kg bw per day.

Although a developmental toxicity study is not considered to be representative of a long-term toxicity study, an additional safety factor was not considered necessary, based on the lack of adverse effects observed in long-term dietary studies conducted with comparable levels of advantame in different species.

An ADI of 0–5 mg/kg bw is established for advantame on the basis of a NOAEL of 500 mg/kg bw per day for maternal toxicity in a developmental toxicity study in rabbits and use of a 100-fold safety factor for interspecies and intraspecies variability.

The Committee agreed that the ADI also applies to those individuals with phenylketonuria, as the formation of phenylalanine from the normal use of advantame would not be significant in relation to this condition.

Advantame is intended for use as a tabletop sweetener and in a large variety of solid and liquid foods. Conservative calculations based on its sweetness potency (20 000 times that of sucrose) suggest that a mean population dietary exposure to advantame of less than 0.2 mg/kg bw per day would result from total sugar replacement in the diet, even for the maximum reported mean intake of 160 g total sugars per day for the population of the USA, assuming a 60 kg body weight. Therefore, a total replacement of sugar with advantame would not lead to the ADI being exceeded. Using national dietary exposure estimates and making the “worst case” assumption that brand-loyal consumers always select foods intended to contain advantame at the sponsor-proposed maximum use levels for broad food categories suggest that the maximum mean dietary exposure to advantame would be 1.45 mg/kg bw per day (29% of the upper bound of the ADI), and the maximum high-percentile dietary exposure would be 2.16 mg/kg bw per day (43% of the upper bound of the ADI). The Committee considered these predicted dietary exposures to advantame to be overestimated due to the conservative assumptions made.

The proposed maximum use levels that the Committee considered for advantame for possible inclusion in the GSFA were not expected to lead to dietary exposures exceeding the upper bound of the ADI for any population group.

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GLUCOAMYLASE FROM TRICHODERMA REESEI *EXPRESSED IN* TRICHODERMA REESEI

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its Forty-fourth Session (FAO/WHO, 2012), the Committee evaluated the safety of the glucoamylase enzyme preparation (glucan 1,4-α-glucosidase; Enzyme Commission No. 3.2.1.3) from *Trichoderma reesei* expressed in *T. reesei*, which it had not evaluated previously. Glucoamylase catalyses the hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from the non-reducing end of the

chain with concomitant release of β-D-glucose in polysaccharide substrates. In this report, the expression "glucoamylase" refers to the glucoamylase enzyme and its amino acid sequence, and the expression "glucoamylase enzyme preparation" refers to the preparation formulated for commercial use. The glucoamylase enzyme preparation is used as a processing aid in the manufacture of sweeteners such as high-fructose corn syrup, in baking, in brewing and in the production of potable alcohol (spirits).

1.1 Genetic modification

Glucoamylase is produced from a genetically modified strain of *Trichoderma reesei* containing the glucoamylase gene from *T. reesei*. *Trichoderma reesei* is a mesophilic filamentous fungus that is ubiquitous in nature. It has a long history of use in the production of enzymes used in food processing, including enzymes from genetically engineered strains of the organism. Prior to the introduction of the glucoamylase gene, the *T. reesei* host strain was genetically modified through deletion of genes encoding cellobiohydrolase 1 and 2 and endoglucanase 1 and 2, resulting in a strain with a compromised ability to use cellulose as a carbon source. The modified host strain was then transformed using two expression cassettes containing the glucoamylase gene from *T. reesei*. The final recombinant production strain is genetically stable and free of any antibiotic resistance genes or vector deoxyribonucleic acid used during transformation.

1.2 Chemical and technical considerations

Glucoamylase is produced by submerged aerobic, straight-batch or fed-batch pure culture fermentation of a genetically modified strain of *T. reesei* containing the gene coding for glucoamylase from *T. reesei*. The enzyme is secreted into the fermentation broth and is subsequently purified and concentrated. The enzyme concentrate is formulated with glucose, sodium benzoate and potassium sorbate to achieve the desired activity and stability. The glucoamylase enzyme preparation contains commonly used food-grade materials and conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (Annex 1, reference *154*). Glucoamylase activity is measured in glucoamylase units (GAU). One GAU is defined as the amount of enzyme that releases 1 g (5.6 mmol) of glucose per hour from soluble starch substrate (*p*-nitrophenyl-α-D-glucopyranoside) at pH 4.3 and a temperature of 30 °C. The mean activity of glucoamylase enzyme from three formulated batches of the enzyme preparation was 523 GAU per gram of glucoamylase enzyme preparation.

A typical commercial formulation of the glucoamylase enzyme preparation will contain 10–15% of enzyme as total organic solids (TOS). TOS includes the enzyme of interest and residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism during the manufacturing process. The glucoamylase enzyme preparation is used at concentrations ranging from 0.05 to 0.32 g TOS per kilogram of food, depending on the proposed application. The glucoamylase enzyme is expected to be inactivated during processing and hence is not expected to remain in the final food.

2. BIOLOGICAL DATA

In this monograph, the expression "glucoamylase" is used when referring to the pure glucoamylase enzyme and its amino acid sequence, whereas the expression "glucoamylase enzyme preparation" is used when referring to the enzyme preparation used in toxicology studies.

2.1 Biochemical aspects

The glucoamylase from *T. reesei* has a molecular weight of 65 kDa based on the predicted amino acid sequence, as well as sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis.

The glucoamylase from *T. reesei* has been evaluated for potential allergenicity using the bioinformatics criteria recommended in the report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). According to the FAO/WHO allergenicity recommendation, the possibility of cross-reactivity between a query protein and a known allergen has to be considered if there is 1) more than 35% identity in the amino acid sequence of the expressed protein using a sliding window of 80 amino acids and a suitable gap penalty or 2) an identity of six contiguous amino acids. Using these two criteria, the glucoamylase from *T. reesei* was compared with the allergenic protein sequences in the Structural Database of Allergenic Proteins (SDAP; http://fermi.utmb.edu/SDAP/sdap_who.html) and the Allermatch database (http://www.allermatch.org/). These databases allow for the bioinformatics analysis of protein allergenicity using the FAO/WHO recommendation, specifically the six contiguous amino acids match.

A search for matches showing greater than 35% identity over a window of 80 amino acids using SDAP produced multiple matches with Sch c 1 protein of *Schizophyllum commune*. The matches show greater than 35% identity over several windows of 80 amino acids. A search using the Allermatch database also produced multiple matches with Sch c 1 protein and in addition produced one match with Pen ch 13 protein in *Penicillium chrysogenum*. The Sch c 1 and Pen ch 13 proteins are not identified as food allergens in the WHO–International Union of Immunological Societies (IUIS) list of allergens (http://www.allergen.org/).

Using SDAP, a search for six contiguous amino acid sequences of the glucoamylase that could be present in allergenic proteins produced multiple matches. The vast majority of these matches are with various sections of the Sch c 1 protein of *Schizophyllum commune*, which was expected. The Sch c 1 protein is not identified as a food allergen in the WHO-IUIS list of allergens. However, in addition to the Sch c 1 protein, a number of other allergenic proteins in SDAP were also found to share six contiguous amino acid sequence identity with glucoamylase. Therefore, the biological relevance of these matches was examined by investigating the distribution of these six contiguous amino acid sequences in proteins in general—that is, whether these sequence segments are present only in allergenic proteins or are present in both allergenic and non-allergenic proteins. This extended search was performed using the National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/protein), which contains the sequences of all known proteins. The search demonstrated that each of these

six contiguous amino acid sequences of the glucoamylase that was found in other allergenic proteins is widely distributed in many proteins, such as various prokaryotic and eukaryotic proteins, including proteins from non-allergenic sources, and even edible sources. This indicates that the six contiguous amino acid sequence matches found between the glucoamylase and various allergenic proteins occurred by chance and are not likely to be part of any allergenicity-associated epitopes.

The evidence from bioinformatics analysis suggests that these sequences in *T. reesei* glucoamylase will most likely be of no concern for cross-reactivity to allergic or sensitive individuals. Therefore, despite some similarity to some sequences in allergen databases, the glucoamylase from *T. reesei* does not appear to have the characteristics of a potential food allergen, and the Committee considered that oral intake of glucoamylase is not anticipated to pose a risk of allergenicity.

2.2 Toxicological studies

The glucoamylase from *T. reesei* expressed in *T. reesei* is used in food industries. *Trichoderma reesei* is a non-pathogenic and non-toxigenic fungus. The production strain was tested for the presence of total aflatoxin, zearalenone, sterigmatocystin, ochratoxin A and T-2 toxin, and none was found. It was also tested for any antimicrobial activity and antibiotic activity, and none was detected.

The glucoamylase enzyme preparation from the production strain, referred to as *T. reesei* 70H2-TrGA#32-9, was used to perform toxicology studies. The mean activity of this preparation (lot no. trg08050/52) was 735 GAU/g, with a TOS content of 20.73%, a total protein content of 199.63 mg/ml and a pH of 3.80. Therefore, in this preparation, 1 GAU corresponds to 0.282 mg TOS (= 3.55 GAU/mg TOS). TOS consists of the residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism and the manufacturing process.

The toxicological studies conducted using *T. reesei* glucoamylase enzyme preparation were a 90-day oral toxicity study and genotoxicity studies (Ames mutagenicity test and in vitro chromosomal aberration assay).

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

A subchronic oral toxicity study (Andreassen, 2009) was performed under good laboratory practice (GLP) and conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents; 1998). There were four groups of rats: one control group (Group 1) and three treatment groups (Groups 2, 3 and 4). The dose levels of the glucoamylase enzyme preparation were selected to be 11.1 mg TOS per kilogram body weight (bw) per day (Group 2; low dose), 55.5 mg TOS per kilogram body weight per day (Group 3; intermediate dose) and 166.4 mg TOS per kilogram body weight per day (Group 4; high dose). The glucoamylase enzyme preparation (lot no. trg08050/52) was administered in 0.9% saline daily to groups of Ntac:SD rats (10 of each sex per group) by oral gavage for 90

consecutive days. At the start of treatment, the rats were 5–6 weeks old, with body weights ranging between 180 and 202 g for males and between 161 and 180 g for females. The control group received an equivalent volume of vehicle (0.9% saline) by oral gavage. Animals were evaluated with respect to general clinical observations, functional observations, ophthalmoscopic examination, body weight, feed consumption, haematology, clinical chemistry, urine analysis, organ weights, macroscopic examination and histopathology of all organs. Mortality/viability, all visible signs of ill-health and any behavioural changes were recorded daily. Body weight and feed consumption were evaluated weekly; water consumption was recorded twice weekly. Detailed clinical observations were performed weekly, which included testing for skin/fur, eyes, mucous membranes, occurrence of secretions, excretions, autonomic activity (e.g. lacrimation, piloerection, pupil size and unusual respiratory pattern), changes in gait, posture and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards). Ophthalmoscopic observations were made on all animals before the start of treatment. Before termination of treatment, all animals in the control and high-dose groups were re-examined. Haematology, clinical chemistry and urine analysis were conducted on samples collected at study termination. A functional observational battery consisting of detailed clinical observation, reactivity to handling and stimuli (e.g. auditory, visual, tactile), grip strength and motor activity (open-field test) was conducted during week 13 for the control and high-dose rats. After a thorough macroscopic examination, selected organs were removed, weighed and processed for histopathological examination. Microscopic examination was initially conducted on selected organs from the control and high-dose animals. Because of questionable findings in males, the microscopic examination of the kidneys and testes was extended to the low- and mid-dose groups.

One control male had a fast-growing subcutaneous tumour and was sacrificed for animal welfare reasons. No other mortalities or toxicologically relevant, adverse treatment-related effects were observed with respect to feed consumption, water consumption, body weight, body weight gain, haematology, urine analysis and ophthalmological examinations. There were no differences in the functional observational battery and stimuli assays between treated and control animals.

Some statistically significant differences were noted in the treatment groups compared with the respective controls, such as 1) a lower (4%) mean cell haemoglobin in high-dose females, 2) a higher (2%) mean cell haemoglobin concentration in mid-dose females, 3) a higher (9%) mean glucose level in high-dose females, 4) a lower (9%) mean potassium level in low-dose females and 5) a lower (8%) mean magnesium level in low-dose females. These changes were not considered to be treatment related or toxicologically relevant because the effects were isolated, were not dose dependent and did not occur in both sexes. Statistically significant differences (12–19%) in the mean creatinine levels observed in all treated females were also not considered to be treatment related because the increase in the treatment groups was not dose dependent and the actual values were within the range of historical control data in the laboratory where the analysis was performed. It was concluded that the statistically significant difference was due to incidentally low creatinine values in the control group.

At necropsy, there were no toxicologically relevant, treatment-related findings of adverse effects on organ weights, macroscopic findings or histopathological examinations. In males, focal basophilic tubular dilatation, including hyaline casts, was seen in the kidneys of all groups, including the controls; the degree of severity was minimal (grade 1) to slight (grade 2). In females, the same findings were noted in the control and the high-dose groups, but not in the other groups; the degree of severity was minimal (grade 1). These findings were considered incidental and not treatment related because of their occurrence in the control group and the absence of a dose–response relationship in the treatment groups, because there were no indications of treatment-related renal insufficiency in the clinical pathology data and because there were no statistically significant differences among groups. In the testes, minimal to moderate tubular atrophy was found in the high-dose group, whereas it was found to a minimal degree in all other groups, including the controls. The finding was not considered to be toxicologically relevant for a number of reasons: first, the difference in severity between groups was not statistically significant; second, the historical control data from the testing laboratory showed the occurrence of spontaneous testicular atrophy in control rats; and third, spontaneous testicular atrophy has been reported to be one of the most common non-neoplastic lesions in various strains of laboratory rats (Lee et al., 1993; Pettersen et al., 1996). All other microscopic findings were considered to be within the background incidence of findings reported in this age and strain of rats.

Overall, it can be concluded that no toxicologically relevant effects were seen in this study of general toxicity in Sprague-Dawley rats when glucoamylase enzyme preparation from *T. reesei* was administered daily by oral gavage for 90 consecutive days at doses up to 166.4 mg TOS per kilogram body weight per day. The no-observed-adverse-effect level (NOAEL) was identified as 166.4 mg TOS per kilogram body weight per day, the highest dose tested (Andreassen, 2009).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two genotoxicity studies with glucoamylase enzyme preparation from the production strain, referred to as *T. reesei* 70H2-TrGA#32-9 (lot no. trg08050/52), are summarized in Table 1. The bacterial reverse mutation assay was conducted in accordance with OECD Test Guideline 471 (Bacterial Reverse Mutation Test; 1997). The in vitro chromosomal aberration assay was conducted in accordance with OECD Test Guideline 473 (In Vitro Mammalian Chromosome Aberration Test; 1997). Both studies were certified for compliance with GLP and quality assurance. The results of the studies showed that glucoamylase enzyme preparation was not genotoxic.

2.2.5 Reproductive and developmental toxicity

No information was available.

Table 1. Genotoxicity of glucoamylase enzyme preparation in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5000 µg test substance per plate using treat and plate method[b] ±S9[c]	Negative	Edwards (2009a)
Chromosomal aberrations[d]	Human lymphocytes	1st experiment: 78.1, 156, 313, 625, 1250, 2500 and 5000 µg/ml ±S9 2nd experiment: 313, 625, 1250, 2500 and 5000 µg/ml ±S9	Negative	Edwards (2009b)

[a] The positive control substances used without S9 mix were cumene hydroperoxide (100 µg/plate) for TA102, sodium azide (1 µg/plate) for TA100 and TA1535, 2-nitrofluorene (1 µg/plate) for TA98 and 9-aminoacridine (10 µg/plate) for TA1537. The positive control agent used with S9 mix was 2-aminoanthracene (2-AA, 2 µg/plate) for TA98, TA100, TA1535 and TA1537 and 2-AA (4 µg/plate) for TA102. The positive control substances produced marked increases over the concurrent negative control values, and the data were consistent with the historical control data. Toxicity was assessed in all tests by examining the plates for thinning of the background lawn of non-revertant bacteria, the appearance of micro-colonies or a reduction in the number of revertant colonies on the test plates in comparison with the negative control plates. Based on these criteria, the test substance was not toxic to the test bacteria. The test substance was considered positive (mutagenic) if all of the following criteria were met: 1) increases in the number of revertant colonies were observed at one or more test points, 2) the mean number of revertant colonies at the test point showing the largest increase was more than twice the corresponding negative control value, 3) there was a credible scientific explanation for the observed dose–response relationship that involved a mutagenic effect of the test item, 4) the increases were reproducible between replicate plates and were observed in both main tests (when treatment conditions were the same), 5) the increases were statistically significant and 6) the increases were not directly related to increased growth of the non-revertant bacteria.

[b] The tests were performed using the “treat and plate” treatment method. In the treat and plate method, the bacteria were first incubated in liquid culture, that is, with the test substance in solution, nutrient broth and buffer or S9 mix, for a period of 3.5 hours. The bacteria were then washed to remove the test substance and any histidine before mixing with top agar and plating on minimal glucose agar plates.

[c] The S9 is the 9000 × *g* supernatant fraction of liver homogenate. It contains microsomes and cytosol and is devoid of mitochondria. The livers used to obtain the S9 fraction were obtained from rats treated with Aroclor 1254, which is a broad-spectrum inducer of cytochrome P450 enzymes. Hence, the S9 fraction acts as an exogenous metabolic activation system.

[d] The positive control cultures were treated with daunomycin (0.015 µg/ml) in the absence of S9 mix and cyclophosphamide (6 µg/ml) in the presence of S9 mix. The positive control chemicals daunomycin and cyclophosphamide both produced a statistically significant increase in the incidence of cells with chromosomal aberrations. The test substance was considered positive (clastogenic) if all of the following criteria were met: 1) the increases in the frequency of metaphases with aberrant chromosomes were observed at one or more test concentrations, 2) the increases were reproducible between replicate cultures and between tests (when treatment conditions were the same), 3) the increases were statistically significant and 4) the increases were not associated with large changes in pH or osmolarity of the treated cultures.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

3.1 Introduction

Glucoamylase is used as a processing aid in the manufacture of corn sweeteners, such as high-fructose corn syrup, and other sweeteners (glucose, dextrose), in baking, in brewing and in the production of potable alcohol (spirits). The Committee evaluated one submission received from DuPont Industrial Biosciences, a manufacturer of the preparation.

The enzyme is used internationally and is generally recognized as safe in the USA. Although the enzyme is not expected to remain in sugar, beer or spirit products following purification processes used in their manufacture and is expected to be inactivated in bread and bakery products, the enzyme preparation is used across a number of food products, and therefore an upper-bounding dietary exposure assessment approach was taken. A theoretical "worst case" dietary exposure estimate was made, assuming that 100% of food products in which glucoamylase could be used would be manufactured using the product and that 100% of the preparation would remain in the final food products. Additionally, it was assumed that the glucoamylase enzyme preparation contained as much total protein and TOS as were used in the toxicity study in rats (see section 2.2). The Committee concluded that a tiered approach to assessing potential dietary exposure to glucoamylase was not necessary, as the upper-bounding exposure estimate was evaluated.

3.2 Dietary exposure assessment

Four broad uses of glucoamylase were nominated in the submission (manufacture of sweeteners, baking, brewing and potable alcohol products). These were considered individually to arrive at a total dietary exposure estimate.

3.2.1 Sweetener production

Assuming that all sweeteners in the USA are produced from grain using glucoamylase as a processing aid, potential dietary exposures can be calculated from the estimate of per capita annual consumption of all sweeteners. The Committee considered that the consumption of high-fructose corn syrup, glucose and dextrose was likely to be higher for the population of the USA than elsewhere; therefore, it was assumed that sugar consumption figures for the USA were sufficient to cover all possible use scenarios for sweeteners. For the USA, per capita consumption of total sugars was 44 kg/person aged 2 years and over per year from the 2009–2010 surveys (120 g sugar per day or 2 g sugar per kilogram body weight per day assuming a body weight of 60 kg) (USDA, 2012). Reference to the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) 17 Cluster Diets (WHO, 2012) confirms that cluster G10,

which includes the USA, has the highest apparent consumption of total sugars, honey, candies (excluding chocolate), sweeteners and molasses, at 130 g/day, of all the diets; however, as this also included other products, the USDA (2012) figure for total sugar consumption was used in the dietary exposure estimate.

If glucoamylase is applied at a rate of 174 000 GAU/tonne, the maximum concentration would be 0.174 GAU/g or, assuming that 1 GAU equals 0.282 mg TOS, 0.049 mg TOS per gram sweetener. Hence, the maximum dietary exposure to glucoamylase from all sweeteners would be 0.098 mg TOS per kilogram body weight per day (2 g sweeteners per kilogram body weight per day × 0.049 mg TOS per gram sweetener).

3.2.2 Baking products

The glucoamylase preparation is used in the baking industry at the rate of 1–2 g/kg flour. If the enzyme preparation is applied to flour at the maximum level of 2 g/kg flour or 1140 GAU/kg flour (assuming 570 GAU/g enzyme in the food matrix as per sponsor submissions), this equates to 321 mg TOS per kilogram flour (assuming that 1 GAU equals 0.282 mg TOS). To estimate the contribution from flour, the maximum consumption of bread was taken for the population of the USA rather than the mean consumption amount, to account for consumption of other bakery products. Assuming a maximum consumption of bread of 250 g/day (about eight slices per day) and a flour content of bread of 65%, the maximum consumption of flour from bread would be 162.5 g/day or 2.71 g/kg bw per day (assuming a body weight of 60 kg). Applying the maximum concentration level of 321 mg TOS per kilogram to the maximum bread consumption amount results in a maximum dietary exposure to glucoamylase of 0.870 mg TOS per kilogram body weight per day (2.71 g flour per kilogram body weight per day × 0.321 mg TOS per gram).

3.2.3 Brewing

The glucoamylase enzyme preparation is used for mash brewing of beer at the rate of 1.5–7 kg/tonne of grist (e.g. malted barley). Taking the maximum concentration of 7 g/kg and assuming that 17 g grist is required to produce 100 litres of beer, the concentration of the enzyme product in the beer would be 1.19 g/l beer or 678 GAU/l beer (assuming 570 GAU/g enzyme in the food matrix). This equates to 191 mg TOS per litre (assuming that 1 GAU equals 0.282 mg TOS).

The United States Department of Agriculture's (USDA) Continuing Survey of Food Intakes (USDA, 2009) indicates that the per capita intake of beer for adults was 82 litres/year, or 0.225 litre/day, which equates to 3.75 ml/kg bw per day, assuming a 60 kg body weight. However, from the GEMS/Food 17 Cluster Diets, it appears that the population of the USA may not be the highest beer-consuming population, as cluster G08 (Austria, Germany, Poland and Spain) has a higher apparent beer consumption of 260 g/day (approximately 0.260 litre/day, as density is close to 1, or 4.33 ml/kg bw per day). Applying the maximum concentration of 191 mg TOS per litre to this value for the cluster G08 diet, the maximum dietary exposure to glucoamylase would be 0.827 mg TOS per kilogram body weight per day (4.33 ml beer per kilogram body weight per day × 0.191 mg TOS per millilitre).

3.2.4 Potable alcohol (spirits)

The glucoamylase enzyme preparation is used for the saccharification of liquefied starch to glucose in the manufacture of potable alcohols at 0.5 kg enzyme product per tonne of equivalent starch dry. It is estimated that the ethanol yield from 1 tonne of equivalent starch dry is 650 litres of potable alcohol. The concentration of the enzyme product is 0.5 kg/650 litres potable alcohol or 438 GAU/l potable alcohol (assuming 570 GAU/g enzyme in the food matrix). This equates to 124 mg TOS per litre (assuming that 1 GAU equals 0.282 mg TOS).

The USDA Continuing Survey of Food Intakes (USDA, 2009) indicates that the per capita intake of spirits for adults was 5.3 litres/year, or 0.0145 litre/day, which equates to 0.24 ml/kg bw per day, assuming a 60 kg body weight. Applying the maximum concentration of 124 mg TOS per litre to the consumption of spirits, the maximum dietary exposure to glucoamylase would be 0.03 mg TOS per kilogram body weight per day.

3.2.5 Combined dietary exposure estimate

Combining the maximum dietary exposure from each of the four uses of glucoamylase (0.098 + 0.870 + 0.827 + 0.03 mg TOS per kilogram body weight per day) results in a potential total exposure of 1.73 mg TOS per kilogram body weight per day, if maximum use concentrations and 100% market penetration for the production of sugars, bread, beer and spirits are assumed, in addition to assuming that the enzyme is present and not inactivated at all during each production process. The Committee noted that under these conditions, the major contribution to total dietary exposure to glucoamylase would be from the consumption of bakery products, followed by beer.

4. COMMENTS

4.1 Assessment of potential allergenicity

The glucoamylase from *T. reesei* was evaluated for potential allergenicity according to the bioinformatics criteria recommended by FAO/WHO. The amino acid sequence of glucoamylase was compared with the amino acid sequences of known allergens. A similarity search in SDAP for matches showing greater than 35% identity over a window of 80 amino acids produced multiple matches with Sch c 1 protein of *Schizophyllum commune*. A similar search using the Allermatch database also produced multiple matches with Sch c 1 protein and in addition produced one match with Pen ch 13 protein in *Penicillium chrysogenum*. The Sch c 1 and Pen ch 13 proteins are not identified as food allergens in the WHO-IUIS list of allergens. Using SDAP, a search for six contiguous amino acid sequences of the glucoamylase that could be present in allergenic proteins produced multiple matches as well. The vast majority of these matches are with various sections of the Sch c 1 protein of *Schizophyllum commune*, which was expected. The Sch c 1 protein is not identified as a food allergen in the WHO-IUIS list of allergens. However, in addition to the Sch c 1 protein, a number of other allergenic proteins in SDAP were also found to share six contiguous amino acid sequence identity with

glucoamylase. Therefore, the biological relevance of these matches was examined by investigating the distribution of these six contiguous amino acid sequences in proteins in general—that is, whether these sequence segments are present only in allergenic proteins or are present in both allergenic and non-allergenic proteins. This extended search was performed using the National Center for Biotechnology Information protein database, which contains the sequences of all known proteins. The search demonstrated that each of these six contiguous amino acid sequences of the glucoamylase that was found in other allergenic proteins is widely distributed in various prokaryotic and eukaryotic proteins, including proteins from non-allergenic sources, and even edible sources. This indicates that the six contiguous amino acid sequence matches found between the glucoamylase and various allergenic proteins occurred by chance and are not likely to be part of any allergenicity-associated epitopes. Therefore, the Committee considered that oral intake of glucoamylase is not anticipated to pose a risk of allergenicity.

4.2 Toxicological data

In a 13-week study of general toxicity in rats, no treatment-related adverse effects were seen when glucoamylase enzyme preparation was administered daily by gavage at doses up to 166 mg TOS per kilogram body weight per day. The glucoamylase enzyme preparation was not mutagenic in a bacterial reverse mutation assay in vitro and was not clastogenic in an assay for chromosomal aberrations in human lymphocytes in vitro.

4.3 Assessment of dietary exposure

A theoretical "worst case" dietary exposure estimate was made assuming that the enzyme remains in the food at its maximum concentration following its use as a processing aid in the production of sugars, bread/bakery items, beer and spirits and that it is present in 100% of each range of products. Based on these very conservative assumptions, the estimate of total dietary exposure was 1.73 mg TOS per kilogram body weight per day. However, the enzyme is not expected to remain in sugar, beer or spirit products following purification processes used in their manufacture, and it will be inactivated in bread and bakery products.

5. EVALUATION

Based on its low toxicity and because it is reasonably anticipated that dietary exposure would be very low, the Committee established an acceptable daily intake (ADI) "not specified" for the glucoamylase enzyme preparation from *T. reesei* expressed in *T. reesei* used in the applications specified and in accordance with good manufacturing practice.

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NISIN

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1. EXPLANATION

Nisin is a mixture of antimicrobial polypeptides produced by *Lactococcus lactis* subsp. *lactis*. Nisin preparations are currently used in commerce as an antimicrobial preservative in processed cheeses, pasteurized dairy products and processed meats. The Committee evaluated the safety of nisin at the twelfth meeting of JECFA (Annex 1, reference *17*) and established an acceptable daily intake (ADI) of 0–33 000 units of nisin per kilogram body weight.

At its Forty-fourth Session (FAO/WHO, 2012), the Codex Committee on Food Additives requested JECFA to verify the calculation of the ADI for nisin; clarify the basis of the ADI for nisin; and provide the calculation to convert units of nisin to milligrams of nisin.

In the original JECFA evaluation of nisin, the ADI of 0–33 000 units of nisin per kilogram body weight was derived from the highest dose in a 2-year repeated-dose toxicity study (Frazer, Sharratt & Hickman, 1962), which the Committee interpreted as being 3 330 000 units of nisin per kilogram of body weight, but which was actually 3 330 000 units of nisin per kilogram of feed. As the ADI in the original JECFA evaluation was derived incorrectly, the present Committee undertook a re-evaluation of nisin, taking previously evaluated as well as new studies into account.

1.1 Chemical and technical considerations

Nisin is a mixture of closely related antimicrobial polypeptides that are 34 amino acids in length. Nisin is produced by strains of *Lactococcus lactis* subsp. *lactis* under appropriate fermentation conditions. The major polypeptide from the fermentation is nisin A. Nisin is produced in a sterilized medium of non-fat milk solids or non-milk-based fermentation sources, such as yeast extract and carbohydrate solids. The fermentation process is controlled for time and pH, until optimum nisin production has been achieved. Nisin is recovered, concentrated and purified from the fermentation medium by various methods, such as sterile injection, membrane filtration, acidification, salting out or spray-drying. The purified nisin is then standardized to make nisin preparation at the desired activity level. The remaining components of the preparation are milk solids and products of fermentation, which include proteins and carbohydrates. Nisin is commercially available as nisin preparation and contains commonly used food-grade materials. A typical batch of a commercial nisin preparation contains 2.5% (weight per weight [w/w]) nisin and approximately 75% (w/w) sodium chloride.

The activity of nisin in commercial nisin preparation has been described in the literature in Reading Units (RU) and International Units (IU). In 1970, the WHO Expert Committee on Biological Standardization (WHO, 1970) established the International Unit as the international reference for units of nisin activity. One IU was defined as the amount of nisin required to inhibit the growth of one bacterial cell in 1 ml of broth. This amount is 0.025 µg of nisin (= 1 IU). Therefore, 1 µg of nisin is equivalent to 40 IU. The Committee at the current meeting reviewed the scientific literature in order to harmonize the Reading Unit and International Unit and concluded that they are equivalent (i.e. 1 IU is the same as 1 RU). The Committee at the current meeting clarified that the specification for the activity of nisin is reported as International Units and provided a conversion factor to obtain the quantity of active nisin in a given sample. The revised JECFA specifications for nisin include this conversion. The revised assay for nisin activity in the JECFA specifications is not less than 900 IU of nisin per milligram or not less than 22.5 µg of nisin per milligram. The revised specifications also removed the synonym “nisin preparation”.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

Nisin is ribosomally synthesized as a precursor protein (pre-nisin) that is 57 amino acids long and that contains a 23-residue leader region (Buchman, Banerjee & Hansen, 1988). There are two natural variants of nisin, designated nisin A and

nisin Z. Single *Lactococcus* strains make only one nisin variant; both nisin A and nisin Z producers are widely distributed. Nisin A and nisin Z differ at residue 27 (histidine in nisin A and asparagine in nisin Z) (de Vos et al., 1995). The pre-nisin is post-translationally modified to produce nisin, which is a peptide that is 34 amino acids long with a molecular weight of 3354.07 daltons (EFSA, 2006). Nisin is a class I bacteriocin[1] and a lantibiotic[2]. Like other bacteriocins, nisin is mostly active against Gram-positive bacteria; however, purified nisin has been found to kill some Gram-negative bacteria as well (Cotter, Ross & Hill, 2013). Nisin contains three uncommon amino acids: α-aminobutyric acid, dehydroalanine and dehydrobutyrine, produced during post-translational modification.

2.1.1 Absorption

In a study in dogs aimed at determining whether biologically active nisin is systemically absorbed, 24 dogs were orally dosed with nisin twice daily, with half of the daily dose given at each of the two treatment times, separated by at least 4 hours. The dogs were divided into four groups of six animals each (three males and three females). The dose levels used were 0 (control), 150 (low dose), 500 (middle dose) and 2000 (high dose) mg/kg body weight (bw) per day, and the animals were dosed for 28 days. Blood samples were collected on day 28, 2 hours after the first daily treatment. The presence of biologically active nisin in the serum was detected using agar diffusion assay.

Of the 18 dogs receiving nisin, only one from the high-dose group was found to have a detectable concentration of nisin in its blood serum (0.54 µg/ml nisin); the detection limit of nisin in the agar diffusion assay used was 0.45 µg/ml (Daly, 1997). This study report did not specify whether the test material was purified nisin or nisin preparation.

2.1.2 Inactivation/degradation

An early study by Heinemann & Williams (1966) showed that nisin was inactivated in vitro by pancreatin. Subsequently, the nisin-inactivating component of pancreatin was shown to be α-chymotrypsin (Jarvis & Mahoney, 1969).

A study by Bernbom et al. (2006) provided evidence that nisin is degraded or inactivated in the intestine. Bernbom et al. (2006) investigated the effect of nisin on the composition of the intestinal microbiota of human flora–associated (HFA) rats. HFA rats were created by gavaging human faecal suspension to germ-free rats. Twenty days after the introduction of human microbiota, the rats were randomly allocated to five groups, the number of rats per group varying between 3 and 5.

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[1] Bacteriocins are small, bacterially produced, ribosomally synthesized peptides that are active against other bacteria and against which the producer has a specific immunity mechanism. Bacteriocins are usually classified into peptides that undergo significant post-translational modifications (class I) and unmodified peptides (class II). Nisin is a class I bacteriocin and a lantibiotic (Cotter, Ross & Hill, 2013).

[2] The lantibiotics are a group of ribosomally synthesized, post-translationally modified peptides containing unusual amino acids, such as lanthionine, L-methyllanthionine and a number of dehydrated amino acids (McAuliffe, Ross & Hill, 2001; Willey & van der Donk, 2007).

Rats were dosed orally once on each of 2 days with nisin-producing *Lactococcus lactis* (6×10^9 colony-forming units [CFU] on day 1; 4×10^9 CFU on day 2), non-nisin-producing *L. lactis* (3×10^9 CFU), high-dose nisin A (60 mg/rat), low-dose nisin A (0.6 mg/rat) or phosphate-buffered saline. Faecal and intestinal (both small and large) samples were obtained directly from the rectums of rats during a 7-week period. The authors employed a competitive enzyme-linked immunosorbent assay (ELISA) to detect intact nisin or immunoreactive fragments of nisin that could still be detected by the anti-nisin antibody used. The presence of biologically active nisin in faecal samples was detected by agar diffusion assay. The limit of detection was 50 ng/g for the biological assay and 5 ng/g for the competitive ELISA.

Nisin could be detected in the duodenum and ileum of HFA rats 3 hours after dosing with 60 mg pure nisin by the biological assay (1.8 ± 0.1 µg/g in duodenum and 6.9 ± 1.5 µg/g in ileum) as well as by the competitive ELISA (18.9 ± 5.6 µg/g in duodenum and 62.4 ± 1.5 µg/g in ileum). Therefore, the concentrations estimated by ELISA were approximately 10-fold higher than the concentrations estimated by the biological assay, a difference that reflects the differential detection limits of the two assays. Similarly, nisin was also detectable by both assays in faecal samples taken 24 hours after dosing. However, the average concentration estimated by the biological assay was 0.4 µg/g faeces, whereas the concentration estimated by ELISA was 76.8 µg/g faeces. In other words, 24 hours after dosing, 200-fold higher concentrations of nisin were detected in the faeces by ELISA compared with the biological assay, indicating a sharp decline in the amount of biologically active nisin in the faeces over a 24-hour period under the conditions of this study. Additionally, in rats receiving high-dose nisin A (60 mg/rat), no nisin could be detected by biological assay or competitive immunoassay in caecum or colon 3 hours after dosing, indicating that nisin had been hydrolysed and inactivated in the intestinal tract.

The results of this study demonstrate that nisin is degraded and inactivated in the intestine over time. Nisin was also not detected by biological assay or competitive ELISA in faecal or intestinal samples taken either 3 hours or 25 days after the first dosage from rats inoculated with either of the *L. lactis* strains (Bernbom et al., 2006).

2.1.3 Development of resistance

In a study that aimed to determine whether nisin consumption could lead to the development of nisin-resistant bacteria and alter the nature of the bacterial flora in the oral cavity, 11 human subjects were fed chocolate milk containing 200 units of nisin preparation per millilitre (equivalent to 5 µg/ml). No nisin was detected in the saliva of the subjects 10 minutes after the consumption of chocolate milk containing nisin preparation (Claypool et al., 1966).

In another study, chocolate milk containing nisin preparation (25 000 IU of nisin per person per day, equivalent to approximately 10 µg of nisin per kilogram body weight per day, assuming a body weight of 60 kg) and control milk without nisin were given to two groups of volunteers (10 per group) for 14 days. Saliva specimens collected before and after the treatment showed no difference in standard blood agar and Rogosa agar plate count or in the number of nisin-resistant bacteria in saliva between the nisin and control groups. It was concluded that the residence

time of nisin in the mouth is too short to permit the development of resistance (Cowell, Allen & Jarvis, 1971).

Another study investigated whether sublethal concentrations of nisin present in food could induce the development of 1) nisin resistance in foodborne microbes or 2) cross-resistance to commonly used therapeutic antibiotics. This study investigated the potential for nisin to alter the sensitivity of 19 strains of 11 common pathogenic bacteria to 17 commonly used therapeutic antibiotics. The study showed that nisin was inactive against Gram-negative bacteria; however, exposure of nisin-sensitive bacteria (staphylococcal strains used) to sub-inhibitory concentrations of nisin induced increased resistance to nisin, which is indicated by an increase in the minimum inhibitory concentration. However, there was no evidence of the development of cross-resistance to the antibiotics used in the study (Hossack, Bird & Fowler, 1983).

Harris, Fleming & Klaenhammer (1992) summarized the literature on the development of acquired nisin resistance in various bacteria and stated that the mechanism of nisin resistance has not been investigated and may differ from strain to strain.

2.1.4 Cytotoxicity

As an antimicrobial peptide, nisin binds to the lipid II molecule and forms pores in the cytoplasmic membrane, causing leakage of the cell contents (Arnusch, Pieters & Breukink, 2012). A number of studies have been performed to address the cytotoxic potential of nisin in microbes as well as in eukaryotic cells.

In an in vitro study, the cytotoxic potential of a number of antimicrobial peptides, such as gallidermin, nisin A, natural magainin peptides and melittin, was investigated and compared in two gastrointestinal cell models (HT29 and Caco-2). The end-points studied were cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, haemolytic activities in sheep erythrocytes and epithelial integrity and concomitant paracellular permeability, as revealed by transepithelial electrical resistance. These effects were compared with those of vancomycin. The study revealed that gallidermin was the least cytotoxic, followed by nisin A, magainin I, magainin II and melittin. Melittin and nisin also caused significant haemolysis. However, whereas nisin caused haemolysis at concentrations that were 1000-fold higher than those required for antimicrobial activity, melittin was haemolytic at concentrations in the same order of magnitude as its antimicrobial activity. In addition, melittin, but not nisin, also affected paracellular permeability (Maher & McClean, 2006).

Purified nisin caused no haemolysis in 22 hours at a concentration of 20 000 units/ml (equivalent to 500 µg/ml) (Hirsch & Mattick, 1949). Nisin tested in vitro did not cause haemolysis in freshly isolated rabbit erythrocytes up to a concentration of 600 µg/ml, but haemolysis increased thereafter with increasing concentration of nisin (Reddy et al., 2004). Nisin demonstrated cytotoxicity after 48 hours of incubation with SV40-HC cells and Vero cell lines (3.4 mmol/l); more pronounced toxicity was noticed for SV40-HC cells (0.85 mmol/l) using a trypan blue exclusion test (Murinda, Rashid & Roberts, 2003). At a concentration of

400 µg/ml, nisin was found to be spermicidal in vitro (Reddy et al., 2004). Similarly, nisin tested at 5–640 µg/ml demonstrated differential cytotoxicity in the MTT assay when incubated in vitro with rabbit red blood cells, vaginal epithelial HeLa-S3 cells and spermatozoa, the cytotoxicity being the highest for spermatozoa and the lowest for the vaginal cells. Nisin at a dose up to 50 mg/day administered intravaginally to rabbits for 14 consecutive days did not cause gross abnormalities in the vaginal tissue (Reddy et al., 2004).

In summary, purified nisin is cytotoxic to a number of eukaryotic cell types in vitro. From the reported studies, the order of nisin's cytotoxicity is sperm > red blood cells > SV40-HC cells > Vero cell lines.

2.2 Toxicological studies

The Committee evaluated many toxicity studies submitted by the sponsor, some of which are unpublished. The studies include acute toxicity studies, short-term and long-term studies of toxicity, genotoxicity studies and reproductive and developmental toxicity studies. Some studies discussed at the twelfth meeting of the Committee in 1968 (Annex 1, reference *17*) are cited briefly.

In addition, a separate literature search was performed in PubMed (last search done on 26 April 2013) using the following search terms, with the corresponding number of retrieved records indicated in parentheses: "nisin" (1488), "nisin toxicity" (32), "nisin safety" (146), "nisin and human consumption" (4), "nisin and safety of consumption" (4), "nisin and oral" (41) and "nisin and oral gavage" (0). The majority of these records are not relevant for the current evaluation, as they discuss nisin in the context of its efficacy in killing various microbes, the use of nisin as a potential therapeutic molecule, genetic modification of nisin to create a more potent nisin molecule, use of nisin as a safe food preservative, etc. Many of these studies are also in vitro studies comparing the cytotoxicity of different bacteriocins, including nisin. Therefore, in the context of this document, the records retrieved with the search terms "nisin and safety of consumption", "nisin and human consumption" and "nisin toxicity" were focused upon. Some relevant publications obtained from this literature search have been incorporated in this narrative. In brief, the present literature search did not reveal information that would contradict the extensive body of literature that was taken into consideration to assess the safety of the use of nisin in food under the conditions of proposed use.

2.2.1 Acute toxicity

The median lethal dose (LD_{50}) of purified nisin administered to rats by the oral route was more than 2000 mg/kg bw (Dreher, 1995), and the LD_{50} for nisin preparation in mice was 6950 mg/kg bw (Hara et al., 1962). Table 1 lists the acute toxicity studies provided by the sponsor.

2.2.2 Short-term studies of toxicity

There were no consistent adverse effects that could be ascribed to nisin treatment in short-term studies ranging in duration from 7 days to less than 28 days. Table 2 lists these studies.

Table 1. Acute toxicity studies with nisin

Test species	Treatment period	Route of administration	No. of animals of each sex per group	Test material; dose	LD_{50}	Reference
Mouse (DD strain)	Single dose	Gavage, intraperitoneal and subcutaneous	10 males	Nisin preparation (10^6 U/g preparation, along with sodium chloride and milk proteins; no other details on the test material were available)	6950 mg/kg bw (gavage) 4450 mg/kg bw (intraperitoneal) 4750 mg/kg bw (subcutaneous)	Hara et al. (1962)[a]
Rat (Wistar)	Single dose (7-day observation)	Gavage and intraperitoneal	Not specified	Nisin preparation (up to 10^6 U/kg bw; no other details on the test material were available)	>10^6 U/kg bw (= >25 mg/kg bw)[b]	Frazer, Sharratt & Hickman (1962)[c]
Rat (SD)	Single dose (14-day observation)	Gavage	5 males + 5 females	Purified nisin (>80% of total peptide); 2000 mg/kg bw	>2000 mg/kg bw	Dreher (1995)
Rat (strain not specified)	Single dose (7-day observation)	Gavage	3 (sex not specified)	Nisin suspension; 0.5×10^6, 1.0×10^6, 1.5×10^6 RU/kg bw (suspension of crystallized nisin in distilled water added to milk; no other details on the test material were available)	>1.5×10^6 RU/kg bw (= >37.5 mg/kg bw)[d]	Pesquera (1966)

IU, International Unit; LD_{50}, median lethal dose; RU, Reading Unit; U, Unit

[a] The publication is in Japanese. The information was recorded as provided by the sponsor.
[b] FSANZ (2007), p. 22.
[c] Evaluated previously (see Annex 1, reference *17*).
[d] The conversion was made based on the determination that 1 RU = 1 IU and 1 IU = 25 ng of nisin.

Table 2. Short-term studies of toxicity of nisin of less than 28 days' duration

Test species	Treatment period; test material	No. of animals of each sex per group	Dose; route	Results	Reference
Mouse (BALB/c)	21 days; nisin preparation (no other details on the test material were available)	8 males	0.825 mg/kg bw per day; gavage	Significant increase in serum ALT level after 21 days, enlargement of spleen, histopathological changes in spleen and liver Remarks: The authors concluded that the observed effects could be due to the presence of 12% whey protein and 77% NaCl in the nisin preparation used. Because of the lack of a concurrent NaCl treatment group in this study, the observed adverse effects cannot be ascribed to nisin treatment, particularly due to other longer-term studies that included a NaCl treatment group, which are discussed below.	Vaucher et al. (2011)
Rat (SD, Crl:CDBR strain)	7 days; purified nisin (>80% purity; no other details on the test material were available)	5 males + 5 females	0, 500, 1000 and 2000 mg/kg bw per day; gavage	An apparent dose-related reduction in haemoglobin, red blood cell count and packed cell volume in males in the treated groups (~7–9% lower at the highest dose compared with controls); in females, values in all treated groups were also lower than in controls, but there was no dose–response relationship; no treatment-related histopathological findings	Hardie (1995a)
Dog (Beagle)	12 days (MTD phase); purified nisin (>80% purity)	2 males + 2 females	0, 500 (days 1–4), 1000 (days 5–8) and 2000 (days 9–12) mg/kg bw per day (animals were dosed twice daily, 4 h apart, to achieve the dose); gavage	No adverse effects; no treatment-related histopathological findings were reported	Hardie (1995b)
	7 days (fixed-dose phase); purified nisin (>80% purity)	2 males + 2 females	2000 mg/kg bw per day (animals were dosed twice daily, 4 h apart, to achieve the dose); gavage		

ALT, alanine aminotransferase; MTD, maximum tolerated dose; NaCl, sodium chloride

Several short-term studies have investigated the toxicity of nisin over durations of 28 days and longer. These are described below.

(a) Rats

In a study conducted following Organisation for Economic Co-operation and Development (OECD) and United Kingdom Department of Health guidelines as well as good laboratory practice (GLP), groups of 10 male and 10 female Crl:CDBR strain rats were administered purified nisin by oral gavage at a dose level of 0, 150 (low dose), 500 (middle dose) or 2000 (high dose) mg/kg bw per day for 28 consecutive days. The two batches of the test material had nisin contents of 85.21% and 83.66%. The body weight ranges of the rats at the start of treatment were 154.2–193.5 g for males and 132.5–177.6 g for females. Animals were observed daily for clinical signs and were given a detailed physical examination at weekly intervals. Body weights and feed consumption were recorded weekly. Ophthalmoscopy was performed on all animals pretreatment and on control and high-dose animals in week 4. Blood samples were taken from all animals at weeks 2 and 4 and from female animals before necropsy for haematology and clinical chemistry evaluation. Urine samples were collected from all animals in week 4. At the end of the dosing period, all animals were necropsied, and various internal organs were weighed, processed and examined histologically.

Statistical analysis was carried out on body weights, body weight gains, clinical chemistry, haematology, urine parameters and organ weights. Some statistically significant ($P < 0.05$) changes were observed in week 4, compared with control values. For example, packed cell volumes of mid-dose and high-dose males were lower (~5%); mean corpuscular volume of high-dose males was lower (~4%); individual haemoglobin values were slightly lower (~0.6–4%), producing a statistically significant dose–response relationship; total white blood cell count was lower (29%) in mid-dose females; the percentage of lymphocytes was higher in mid-dose and high-dose females (~7–8.5%) ($P < 0.01$); serum urea level was lower (~22%) in low-dose males; and serum creatinine level was lower (~5%) in low-dose and high-dose males. These observed effects were random, did not occur in both sexes and did not show a dose–response relationship, except for the haemoglobin values. Therefore, these effects were not considered to be treatment-related adverse effects. The authors did not specify the no-observed-adverse-effect level (NOAEL), but concluded that purified nisin administered at doses up to 2000 mg/kg bw per day for 28 days was well tolerated and produced little or no evidence of toxicity (Kelly, 1996a). The effects of nisin on the red blood cell parameters seen in the high-dose group in this study were also reported in the high-dose group in the 7-day study (Hardie, 1995a; Table 2).

In a published study in which groups of 10 weanling male Wistar rats were fed diets containing 20%, 30% or 40% cheese without nisin (control group) or with nisin (treatment group) for 12 weeks, no treatment-related, toxicologically relevant adverse effects were observed. The nisin intakes were 1.2×10^6, 1.8×10^6 and 2.4×10^6 U/kg bw, equivalent to nisin doses of 30, 45 and 60 mg/kg bw. This study was discussed in the previous JECFA evaluation (Annex 1, reference *17*); hence, it is not elaborated here. The nisin used was a commercial preparation of nisin

containing 1 million units of nisin per gram of preparation, equivalent to 25 mg nisin per gram of preparation. No other details on the test material characterization were described (Frazer, Sharratt & Hickman, 1962).

In another published study, rats (five males and five females) were given diets mixed with nisin suspension at a concentration of 10^4 RU/g of feed for 12 weeks. Because 1 RU = 1 IU, 10^4 RU of nisin per gram of feed is equivalent to 250 mg nisin per kilogram of feed. The control group (three females and two males) received diet without nisin. The nisin preparation was a suspension of nisin in distilled sterile water, and the concentration of nisin was expressed in Reading Units per millilitre. No other details on the test material were available. The initial group average body weight of rats ranged between 65 and 72 g. Body weight was measured weekly. Males from the treatment group were mated with females from both the treatment and control groups. Males from the control group were mated with females from both the treatment and control groups. The reproductive ability of rats in the parental generation (F_0) was investigated. All animals were necropsied.

The author reported that there were no differences in body weight gains between the control and treatment groups, and no abnormalities were found in the treatment groups. Fertilities of males in the treatment and control groups were nearly equal, and all the neonates were normal (Pesquera, 1966).

In a 90-day GLP study conducted following the Japanese Ministry of Health and Welfare guidelines (Hagiwara, 2007) and later published (Hagiwara et al., 2010), groups of 10 male and 10 female F344/DuCrlCrlj rats were fed a preparation of nisin A at a dietary level of 0% (control), 0.2% (low dose), 1.0% (mid dose) or 5.0% (high dose) for 90 consecutive days. Average intakes of nisin A were 0, 117, 586 and 2996 mg/kg bw per day for males and 0, 129, 638 and 3187 mg/kg bw per day for females, respectively. The selection of the dietary levels of nisin A was based on a 2-week range-finding study. The authors described the composition of two different batches of nisin A preparations; the nisin A preparation used in the range-finding study had an activity of 3300 IU/mg (= 82.5 µg/mg) of the preparation and a 79.95% sodium chloride content, whereas the other nisin A preparation had an activity of 3000 IU/mg (= 75 µg/mg) of the preparation and a 74.23% sodium chloride content. Rats in a reference group were given sodium chloride at a dietary level of 3.712%, which was the same as that in the 5.0% nisin A diet. Average intakes of sodium chloride were 2196 and 2423 mg/kg bw per day for males and females, respectively, at the 3.712% sodium chloride dietary level. The body weight ranges of rats at the start of treatment were 109–135 g for males and 86–99 g for females. The animals were observed daily for clinical signs; individual body weights as well as 2-day feed and water consumption per cage were measured weekly. Ophthalmological examination was performed for six animals of each sex per group at week 13. Samples for urine analysis were collected from six animals of each sex per group at weeks 4, 8 and 13. Haematology and clinical chemistry were performed on all animals at the termination of the study. Gross observations were made at necropsy; organs were collected, weighed and used for histopathology.

There were no signs of any treatment-related abnormalities in either sex. Some statistically significant differences between control and treated groups

were observed sporadically, such as higher (~3–4%) body weights in males in the mid-dose group in weeks 3 and 13. These differences were not considered to be toxicologically relevant, because the changes occurred sporadically, did not show any dose–response relationship and were not found in both sexes. The statistical significance of differences between control and treated groups for each parameter was calculated, excluding general condition, feed consumption and water consumption. A marked elevation in water consumption was observed in both sexes, particularly in males, in the high-dose group and the reference control group; the increases were similar. A corresponding elevation in urine volume was also observed in the high-dose males. These changes were considered to be due to the high (and similar) sodium chloride intake in both the high-dose group and the reference control group. There was a significant increase in urinary sodium and chloride levels and a decrease in urinary potassium levels in both sexes in the high-dose group and in the reference control group at weeks 4, 8 and 13. These changes were considered to be primarily due to the high sodium chloride intake. A significant increase in urinary sodium and chloride levels at week 4 in low- and mid-dose males and a significant decrease in urinary potassium level in mid-dose males at week 8 were observed. A lowering of urinary potassium level at week 8 in mid-dose males was noted. An increase in urinary chloride level at weeks 8 and 13 was also noted in mid-dose females. The specific gravity of urine was lowered in the high-dose group and in the reference control group at weeks 4, 8 and 13, and also in the mid-dose group in week 8; however, the difference was statistically significant in males, but not in females. Some other parameters that showed statistically significant increases between controls and the high-dose group were the red blood cell parameters (red blood cell count, haemoglobin, mean corpuscular haemoglobin, mean corpuscular volume, haematocrit value); the differences in all these parameters were less than 4% and within the range of historical data and were not considered to be toxicologically relevant. Some statistically significant differences between control and various treatment groups were also observed in clinical chemistry results, such as slight (<1%) but statistically significant lowering of sodium level in both sexes and increases in blood urea nitrogen in both sexes (~19–25%), alanine aminotransferase activity in females (~11%), albumin level in females (~2.5%) and albumin to globulin ratio in females (~3%) in the high-dose group. A significant increase in glucose level (~7%) in the mid-dose males and a lower level of total protein (~2%) in the low-dose males were also observed. Although most of the statistically significant changes observed did not show any dose–response relationship, those in the high-dose group occurred at most time points analysed (4, 8 and 13 weeks). Statistically significant changes observed in the low-dose and the mid-dose groups were not considered to be toxicologically relevant because they were few and sporadic and did not occur in both sexes.

There were statistically significant increases in absolute and relative kidney weights (~8–9%) in both males and females of the high-dose group. Similar changes were also observed in the sodium chloride reference group. It was considered that these alterations were related to sodium chloride intake, as reported previously (Shirai et al., 1982, 1984; Takahashi et al., 1983). Histopathological examination revealed squamous cell hyperplasia of the limiting ridge in the forestomach in all animals of the high-dose group, as well as in the sodium chloride reference group.

These alterations were also considered to be related to high sodium chloride intake (Shirai et al., 1982, 1984). The authors proposed the NOAEL to be the 5.0% dietary level of nisin preparation, which was equal to 2996 and 3187 mg/kg bw per day of nisin preparation for males and females, respectively. These nisin preparation doses correspond to nisin doses of 224.7 mg/kg bw per day for males and 239.0 mg/kg bw per day for females, as the nisin preparation used contained 7.5% nisin (Hagiwara, 2007; Hagiwara et al., 2010).

Groups of six male Holtzman strain rats were administered nisin by oral gavage at 0 (control) or 10 mg/kg bw per day[3] for 13 weeks. The authors themselves purified nisin from the preparation. Nisin preparation was dissolved in distilled water and column-purified to separate nisin from denatured milk solids and salts. The absorbance of each fraction was spectrophotometrically measured at 280 nm. The presence of nisin was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The fractions positive for nisin were pooled, dialysed and lyophilized. However, the purity of nisin was not specified in the publication. The average body weight of male rats at the start of treatment was 100 g. On day 92, three animals from each group were sacrificed, and blood was collected for haematological and serum biochemical analysis. The vital non-reproductive tissues (liver, kidney and brain) were also collected.

No differences were observed in body weight or weight of the non-reproductive tissues (liver, kidney and brain). There were no treatment-related changes in the haematological and serum biochemical profiles (data not shown by the authors) (Reddy et al., 2004). The authors did not specify a NOAEL, but, based on the lack of any adverse effects, 10 mg nisin per kilogram body weight per day can be regarded as the NOAEL from this study.

(b) Dogs

In a 28-day GLP study conducted following OECD and United Kingdom Department of Health guidelines, groups of three male and three female Beagle dogs were administered purified nisin by oral gavage at dose levels of 0, 150 (low dose), 500 (middle dose) and 2000 (high dose) mg/kg bw per day for 28 consecutive days. There were six different batches of the test material, with a purified nisin content ranging between 84.44% and 87.75%. The body weight ranges of the dogs at the start of treatment were 7.00–10.25 kg for males and 7.65–9.15 kg for females. Animals were observed daily for signs of ill-health or overt toxicity and given a detailed physical examination at weekly intervals. Body weights and feed consumption were recorded weekly. Ophthalmoscopy and electrocardiography were performed on all animals pretreatment and in week 4. Blood samples were taken from all animals in weeks 2 and 4 for haematological examination and evaluation of clinical chemistry. Urine samples were collected for analysis in weeks 2 and 4. At the end of the dosing period, all animals were necropsied, and various internal organs were weighed, processed and examined histologically.

[3] Dose is as given in the Materials & Methods section of the published paper. However, in the Results section, the dose is given as 5 mg/kg bw per day.

At week 4, there was a slight reduction (<1%) in body weight gain in high-dose males, as well as in treated females, compared with the respective controls. In the study report, this was attributed to decreased feed consumption, likely due to large dosing volumes and viscosity changes in test formulations. No treatment-related histopathological or other findings were reported. The author did not specify the NOAEL but concluded that purified nisin administered at up to 2000 mg/kg bw per day for 28 days was well tolerated and produced little or no evidence of toxicity (Kelly, 1996b).

2.2.3 Long-term studies of toxicity and carcinogenicity

In a 2-year repeated-dose study of general and reproductive toxicity, groups of 15 male and 30 female rats were fed diets containing 0 (control), 33 300 units (low dose) or 3 330 000 units (high dose) of nisin per kilogram of diet for their lifespan (2 years). Nisin was added to the diet as Meganisin, which is a commercial preparation of nisin containing 1 million units of nisin per gram of preparation.

A significant increase in relative kidney weight was observed in the animals in the high-dose nisin group when they were caged individually, but not when the animals were caged in fives. Detailed studies showed no differences in hepatic, renal or gastrointestinal function in the control and experimental groups. This study was discussed in the previous JECFA evaluation (Annex 1, reference *17*); hence, it is not elaborated here. Based on the absence of adverse effects, 3 330 000 units of nisin per kilogram of diet (not per kilogram of body weight, as interpreted incorrectly in the previous JECFA evaluation) was identified as the NOAEL. This NOAEL of 3 330 000 units of nisin per kilogram of diet is equivalent to 83.3 mg/kg diet. Based on the authors' statement that the average weight of the rats was 250 g and the average daily feed consumption was 15 g, this NOAEL is equivalent to a nisin dose of 5.0 mg/kg bw per day (Frazer, Sharratt & Hickman, 1962). This study provided the basis for the (incorrectly calculated) ADI established by the Committee at its twelfth meeting.

In a long-term study, groups of 10 male and 10 female Wistar rats (initial body weight 100–120 g) were fed nisin preparation at a dose of 2 mg/kg bw per day (equivalent to 50 µg nisin per kilogram body weight per day) in a paste form prior to the main feed, for 18 months. The nisin preparation had an activity of 1×10^6 IU/g (equivalent to 25 mg nisin per gram); it was of largely unknown composition but contained a stabilizer.

No signs of adverse effects were reported in the nisin treatment group compared with the control group, including on feed consumption, water intake, body weight gain, blood alkalinity, C-reactive protein and blood morphology (Shtenberg & Ignat'ev, 1970).

2.2.4 Genotoxicity

The results of genotoxicity studies with nisin are summarized in Table 3. They were performed in compliance with OECD guidelines (OECD, 1982) as well as those of other international regulatory bodies and with GLP. Nisin and nisin

Table 3. Genotoxicity studies with nisin

End-point	Test system	Test substance/concentrations	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* (strains CM 881 and CM 891)	Purified nisin 0.15–1500 µg/plate ±S9[a]	Negative	Kitching (1995)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Nisin (75% purity) 10–10 000 µg/plate ±S9	Negative	Richold (1980)
Mouse bone marrow micronucleus	5 male and 5 female CD-1 mice per group	Purified nisin 500–2000 mg/kg bw	Negative	Marshall (1996)
Mouse bone marrow micronucleus[b]	5 male and 5 female CD-1 mice per group	Purified nisin 2000–8000 mg/kg bw	Negative	Richold & Richardson (1980)
Chromosomal aberrations in vitro	Human lymphocytes	Purified nisin 1st experiment: 62.5, 125, 250 µg/ml ±S9 2nd experiment: 62.5, 125, 250 µg/ml –S9; 125, 250, 500 µg/ml +S9	Negative	Adams (1996)
Mouse lymphoma assay	Mouse lymphoma L5178Y cells (tk^+/tk^-)	Purified nisin Two independent assays were performed, both in the absence and in the presence of S9 mix. The combined ranges were: 50–400 µg/ml –S9 100–800 µg/ml +S9	Negative	Riach (1995)

[a] The S9 is the 9000 × *g* supernatant fraction of liver homogenate. It contains microsomes and cytosol and is devoid of mitochondria. The livers used to obtain the S9 fraction were obtained from rats treated with Aroclor 1254, which is a broad-spectrum inducer of cytochrome P450 enzymes. Hence, the S9 fraction acts as an exogenous metabolic activation system.

[b] Purified nisin was given as two oral gavage administrations, 24 hours apart, and animals were sampled 6 hours after the final administration.

preparation were not genotoxic. The highest dose levels in the actual experiments were determined based on the results of the toxicity studies. The Committee recognized that nisin's bacteriocidal activity, including on Gram-negative bacteria under certain circumstances (Stevens et al., 1991), raises the potential of false negatives in the bacterial reverse mutation assay. However, a polypeptide such as nisin is not expected to be genotoxic.

2.2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

Groups of six male Holtzman strain rats were administered nisin by oral gavage at a dose of 0 (control) or 10 mg/kg bw per day[4] for 13 weeks. The authors purified nisin from the nisin preparation, as described in section 2.2.2. However, the purity of nisin was not specified in the publication. The goal of the study was to investigate the effects of nisin treatment on male reproductive performance; hence, only male rats were dosed with nisin for 13 weeks before they were cohabited with untreated females. The average body weights of the rats at the start of treatment were 100 g for males and 80 g for females. On day 92, three animals from each group were sacrificed, and reproductive tissues (testes, epididymis, prostate and seminal vesicles) were collected. The remaining three animals from each group were cohabited with proven fertile females (1:2) in the proestrus–estrus transition phase during the 1st week after treatment. Duration of gestation, number of pups and weight of pups were recorded. After mating, the male rats were necropsied, and the epididymis was removed. Sperm were collected in physiological saline; total sperm count and motility were recorded. The experiment was repeated twice with a similar number of rats.

No differences were observed in body weight or weight of the reproductive tissues, such as testes, epididymis, prostate and seminal vesicle. There was no treatment-related change in the total sperm count in the cauda epididymis. The reproductive performance of the nisin-treated rats remained unaffected. Nisin treatment also did not reduce the number of pups born (6–10 in both groups) or pup weight (3–4 g) and general health of the pups. There were no perinatal or postnatal effects. Growth of the pups was observed until the end of lactation and was found to be normal and similar to that of the control group (Reddy et al., 2004).

In a 2-year repeated-dose study of general and reproductive toxicity (Frazer, Sharratt & Hickman, 1962, discussed above), no differences were found in the survival and reproductive performance between the control and the experimental groups. This study was discussed in the previous JECFA evaluation (Annex 1, reference *17*); hence, it is not elaborated here.

[4] Dose is as given in the Materials & Methods section of the published paper. However, in the Results section, the dose is given as 5 mg/kg bw per day.

In a study performed under GLP and investigating the effect of nisin preparation on the reproductive function of multiple generations in rat (CD CrL:COBS CS(SD) BR strain), groups of 12 male and 24 female rats were given diet containing 0% (control), 0.2% (low dose), 1.0% (middle dose) or 5.0% (high dose) nisin preparation for 26 weeks. Test diets were prepared each week, and treatment continued throughout the study for all generations. The nisin preparation contained 2.5% nisin, 76% sodium chloride and 20% non-fat milk solids. The study author stated that commercial nisin preparation has a standard antibacterial activity of 1000 IU/mg preparation. A reference control group received a diet containing 3.8% sodium chloride, which was equivalent to the sodium chloride content of the nisin preparation in the 5.0% diet group. The study author reported the nominal dosages of nisin preparation in units of milligrams per kilogram body weight per day. On average, 0.2%, 1.0% and 5.0% dietary levels of nisin preparation were equal to approximately 165, 851 and 4477 mg nisin preparation per kilogram body weight per day. As the commercial nisin preparation contained 2.5% nisin, these nominal dosages are equal to approximately 4, 21 and 112 mg nisin per kilogram body weight per day. The Committee noted that feed consumption values were provided in the study report for the premating period only. The body weight ranges of the rats at the beginning of treatment of the F_0 generation were 189–196 g for males and 152–155 g for females. Animals of the F_0 generation were maintained on their respective diets for at least 60 days prior to mating. Animals were then mated (one male to two females) for a period of 20 days to obtain F_{1A} litters. F_{1A} litters were sacrificed 21 days after birth and subjected to postmortem examination for macroscopic changes. After a short rest period (10 days following weaning of the F_{1A} litters), the F_0 females were remated using alternative one male to two female pairing for a period of 20 days. Offspring were again reared to 21 days postpartum, and then 12 males and 24 females from each group were selected to form the second generation (F_{1B}). The remaining F_0 parents and F_{1B} pups were sacrificed and examined macroscopically. The rats of the F_{1B} generation were reared on their respective diets until they were 90 days old, when they were mated for 20 days (one male to two females, pairing of animals from similar parents was avoided). The resulting F_{2A} litters were reared for 21 days after birth, then sacrificed and examined for macroscopic changes. F_{1B} animals were rested (10 days following weaning of the F_{2A} litters) before remating using alternative one male to two female pairing for a period of 20 days to obtain the second round of F_{2B} litters. The rearing of subsequent F_{2B} litters and selection of the third generation were continued as outlined for the previous generation. From the F_{3B} generation, 10 males and 10 females were selected approximately 21 days postpartum for detailed macroscopic examination, which included organ weight analysis. Animals in the control and high-dose groups were given histopathological examinations. All F_{2B} parents and remaining F_{3B} pups were sacrificed and examined macroscopically.

Some isolated mortality occurred, such as one male from the F_{1B} generation high-dose group, one female from the F_0 generation high-dose group and one female from each of the F_2 generation mid-dose and control groups. The cause of death could not be confirmed. Animals of the F_0 and F_{1B} generations from the high-dose nisin group showed an apparent increase in urination and a corresponding increase in water consumption; similar findings were observed among animals receiving the comparable concentration (3.8%) of sodium chloride. Weekly body weights of F_0

and F_{2B} males in the high-dose nisin group showed retardation in body weight gain compared with both the control and the sodium chloride reference control groups. Females in the high-dose nisin group did not show such retardation in body weight gain. No treatment-related changes were observed in reproductive performance as assessed by pregnancy rate. No treatment-related changes were observed in gestation period, postpartum litter loss, litter size, mortality or necropsy findings. There were no treatment-related changes in organ weights or histopathology at the end of the study. The mean pup weights for the high-dose nisin group were statistically significantly lower than those of the controls and the sodium chloride group in the F_{1B} generation, but not in the F_0 and F_{2B} generations. Occasional kidney changes were observed at macroscopic examination of pups, such as mild to severe increase in renal pelvic cavitation; however, the effects were seen more in the high-dose group, and the effect was not dose related. No other treatment-related differences were reported in any of the parameters measured (survival, growth, reproductive performance and gross/microscopic pathology) (Cozens, 1981).

(b) Developmental toxicity

In a study of the developmental toxicity of nisin, groups of 20 mated female Holtzman strain rats (average body weight 200 g) were administered purified nisin by oral gavage at a dose of 0 (control), 10 (low dose), 25 (middle dose) or 50 (high dose) mg/kg bw per day during the period of organogenesis (days 6 through 15). The authors purified nisin from the nisin preparation, as described above (Reddy et al., 2004). Changes in body weight, feed consumption and water intake were recorded daily throughout the study. Clinical signs of genital swelling, genital redness and vaginal bleeding were also recorded daily during the treatment period. On day 20 of pregnancy, the animals in the control and treated groups were further divided into subgroups consisting of 10 animals per group. Ten animals in each subgroup were allowed to continue to term. The remaining 10 animals in each subgroup were sacrificed. The weights of maternal gravid and empty uteri, kidneys, liver, thymus, ovaries and adrenal were recorded. The numbers of implantation sites, corpora lutea, embryo resorptions, and live and dead fetuses were also recorded. Fetuses were subjected to morphometric measurements and examined for body weight and any external signs of malformations. Half of the fetuses were processed and double stained with alcian blue/alizarin red-S to facilitate detailed skeletal examination in order to identify any signs of skeletal abnormalities. The other half of the fetuses of each litter were fixed in neutral buffered formalin for histopathological analysis to discern any visceral malformations that were not apparent with external examination. The postnatal effect of nisin on the F_1 progeny of female rats was also evaluated. Six male and six female pups from each treated group were kept with their mothers until weaning. The pups were observed for survival, weight gain, hair growth, eye opening, pinna opening, testes descent and vaginal opening, as well as behaviour. Reproductive performance of the male pups was assessed around 70–80 days of age by cohabiting them with normal fertile females. The males and females kept for mating were not from the same mother.

The authors reported that there were no statistically significant differences between nisin-treated and control groups of rats in maternal body weight, feed consumption, number of corpora lutea per ovary, number of implantations, number

of resorptions and gravid uterine weights. Fetuses from the nisin treatment group did not show any signs of gross external morphological alterations, skeletal malformations or visceral defects. The growth and reproductive performance of the F_1 progeny were also unaffected. Therefore, exposure to nisin during embryological development in rats did not cause toxicity to maternal growth or to fetal development, suggesting that purified nisin did not show developmental effects at doses up to 50 mg/kg bw per day (Gupta, Aranha & Reddy, 2008).

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

Nisin has been used commercially for over 25 years in a number of food types, primarily processed cheese and meat products. The Committee received information concerning dietary exposure patterns from one sponsor and independently obtained additional published information. The sponsor data had been submitted as part of a premarket evaluation of nisin for expanded uses in Japan in the mid-2000s. The additional information was taken from regulatory publications in Australia/New Zealand, Europe and the USA. Four national/regional estimates of dietary exposure to nisin were reviewed by the Committee: from the European Union (EU), Australia/ New Zealand, Japan and the USA (EFSA, 2006; FSANZ, 2007).

The estimate of mean consumers-only dietary exposure for the EU from consumption of cheeses, cream, desserts and egg products was 0.008 mg/kg bw per day, with exposure at the 97.5th percentile of 0.026 mg/kg bw per day. From Food Standards Australia New Zealand (FSANZ), the estimated consumers-only mean dietary exposures to nisin from consumption of cheese, cream, meat products, sauces, toppings and mayonnaise were 0.009 mg/kg bw per day (all ages) and 0.02 mg/kg bw per day (2- to 6-year-olds). Estimated consumers-only 95th percentile dietary exposures to nisin were lowest for New Zealanders aged 15 years and above, at 0.03 mg/kg bw per day, and highest for Australian children aged 2–6 years, at 0.07 mg/kg bw per day. The consumers-only dietary exposure estimate from the USA from consumption of cheese spreads, dressings, egg products and processed meat products was 0.04 mg/kg bw per day at the mean. The Committee also noted that a letter responding to a Generally Recognized as Safe notification in the USA for nisin use only in frankfurters included an estimate of consumer-only mean dietary exposure to nisin of 0.6 mg/day, or 0.01 mg/kg bw per day, with exposure at the 90th percentile of 1.1 mg/day, or 0.02 mg/kg bw per day (USFDA, 1988). The Japanese per capita estimate was from consumption of cheeses, buns, meat and egg products, tofu and miso and was reported as 2.06 mg/person per day or approximately 0.04 mg/kg bw per day for a 50 kg individual. The FSANZ, Japanese and USA estimates were consistent and higher than the EU estimate due to broader food categories in which nisin could be applied.

The Committee concluded that the use of a dietary exposure of 0.07 mg/kg bw per day (95th percentile, Australian children, 2–6 years old) was appropriate for the safety evaluation of nisin.

4. COMMENTS

4.1 Toxicological data

In vitro studies demonstrated that nisin is inactivated by α-chymotrypsin. Nisin administered by gavage to rats was also hydrolysed and inactivated in the intestinal tract, with no biologically active nisin being detected in the colon or caecum. In a gavage study in dogs dosed with nisin (test material description not available) at 1000 mg/kg bw twice per day for 28 days, biologically active nisin was detected in blood serum in one of six dogs at a level of 0.54 µg/ml (limit of detection: 0.45 µg/ml). As the experimental design did not involve multiple time points to confirm the presence of biologically active nisin in plasma, the Committee did not find the results of this gavage dosing study to be conclusive evidence that nisin in food would result in systemic exposure to biologically active nisin.

A range of studies on the acute toxicity, short- and long-term toxicity, genotoxicity, and reproductive and developmental toxicity of nisin was taken into consideration for the safety assessment.

The oral LD_{50} of nisin preparation in mice was 6950 mg/kg bw, and the oral LD_{50} of purified nisin in rats was greater than 2000 mg/kg bw. The toxicity of orally administered (diet or gavage) nisin or nisin preparation was assessed in short-term studies in mice, short- and long-term studies in rats and short-term studies in dogs. A common observation in animals treated with nisin preparations (with ~75% [w/w] sodium chloride) was a significant increase in the absolute and relative kidney weights, coupled with high water consumption and increased urination. The increased kidney weight and minimal squamous cell hyperplasia of the limiting ridge in the forestomach that were seen in these studies in the nisin preparation–treated groups were also seen in the sodium chloride control groups, and these effects are known to be typical of high sodium chloride treatment. Slight but statistically significant changes in haematological parameters at high doses of nisin (2000 mg of purified nisin per kilogram body weight per day in two studies and a 5% dietary level of nisin preparation in another study) were inconsistent in direction and magnitude.

In a 90-day toxicity study, rats were fed nisin preparation (with a nisin A potency of 3000 IU/mg, corresponding to a 7.5% nisin A content) in the diet at 0%, 0.2%, 1.0% or 5.0% (equal to 0, 117, 586 and 2996 mg of nisin preparation per kilogram body weight per day for males and 0, 129, 638 and 3187 mg/kg bw per day for females, respectively). A sodium chloride reference group was given sodium chloride at a dietary level of 3.712%, which was the same as that in the 5.0% nisin A diet. Significant increases in the absolute and relative kidney weights, coupled with high water consumption, increased urination and minimal squamous cell hyperplasia of the limiting ridge in the forestomach, were observed. These changes were also noted in the sodium chloride reference group and were considered to be related to sodium chloride intake. Other parameters that showed a statistically significant increase in the high-dose group were the red blood cell parameters (red blood cell count, haemoglobin, mean corpuscular haemoglobin, mean corpuscular volume, haematocrit value). The increase in these parameters was less than 4% and within the range of historical data and was not considered to be toxicologically

relevant. Based on the lack of treatment-related adverse effects in the highest dose group, the NOAEL was identified as 5.0% of the nisin preparation in the diet, which was equal to 2996 mg/kg bw per day of the nisin preparation, or 224.7 mg/kg bw per day of nisin.

In three one-generation reproductive toxicity studies, the reproductive performance of the nisin-treated rats was unaffected. There were no perinatal or postnatal effects. Growth of the pups was normal and similar to that of the control group. In a three-generation reproductive toxicity study, a nisin preparation containing 2.5% nisin A was fed to rats in a standard diet containing 0%, 0.2%, 1% or 5% nisin preparation for 26 weeks. A further group of animals received a diet containing 3.8% sodium chloride, which was equivalent to the sodium chloride content of the 5% diet group. No treatment-related changes were observed in reproductive performance as assessed by pregnancy rate, gestation length, postpartum litter loss, litter size, mortality or necropsy findings. There were no treatment-related changes in organ weights or histopathology at the end of the study. A decrease in body weight gain was observed in males in the F_0 and F_2 generations, but not in the F_1 generation. Decreased body weight gain was not observed in females. Furthermore, a decrease in body weight gain in rats of both sexes was not observed in other studies. Therefore, the Committee considered this finding to be unrelated to nisin treatment.

In a developmental toxicity study in rats administered purified nisin by gavage at doses up to 50 mg/kg bw per day, no effects on any developmental end-points were observed.

Purified nisin was not genotoxic in reverse mutation, chromosomal aberration, mouse lymphoma or mouse bone marrow micronucleus assays. Nisin was not carcinogenic in the 2-year rat study at dietary concentrations up to 3.33 million units of nisin per kilogram diet. Based on the authors' statement that 1 g of nisin had an activity of approximately 40 million units and their assumptions that the average weight of the rats was 250 g and the average feed consumption of the rats was 15 g, this is equivalent to 83.3 mg of nisin per kilogram diet, or 5.0 mg/kg bw per day. This was the study from which the Committee derived the ADI at the twelfth meeting.

4.2 Assessment of dietary exposure

Nisin has been used commercially for over 25 years in a number of food types, primarily processed cheese and meat products. The Committee received information concerning dietary exposure patterns from one sponsor and independently obtained additional published information. The sponsor data had been submitted as part of a premarket evaluation of nisin for expanded uses in Japan in the mid-2000s. The additional information was taken from regulatory publications in Australia/New Zealand, Europe and the USA. Four national/regional estimates of dietary exposure to nisin were reviewed by the Committee: from the EU, Australia/New Zealand, Japan and the USA. The estimate of mean consumers-only dietary exposure for the EU from consumption of cheese, cream, desserts and egg products was 0.008 mg/kg bw per day, with exposure at the 97.5th percentile of 0.026 mg/kg bw per day. From FSANZ, the estimated consumers-only mean dietary exposures to nisin from consumption of cheese, cream, meat products, sauces,

toppings and mayonnaise were 0.009 mg/kg bw per day (all ages) and 0.02 mg/kg bw per day (2- to 6-year-olds). Estimated consumers-only 95th percentile dietary exposures to nisin were lowest for New Zealanders aged 15 years and above, at 0.03 mg/kg bw per day, and highest for Australian children aged 2–6 years, at 0.07 mg/kg bw per day. The consumers-only dietary exposure estimate from the USA from consumption of cheese spreads, dressings, egg products and processed meat products was 0.04 mg/kg bw per day at the mean. The Committee also noted that a letter responding to a Generally Recognized as Safe notification in the USA for nisin use only in frankfurters included an estimate of consumer-only mean dietary exposure to nisin of 0.6 mg/day, or 0.01 mg/kg bw per day, with exposure at the 90th percentile of 1.1 mg/day, or 0.02 mg/kg bw per day. The Japanese per capita estimate was from consumption of cheeses, buns, meat and egg products, tofu and miso and was reported as 2.06 mg/person per day or approximately 0.04 mg/kg bw per day for a 50 kg individual. The FSANZ, Japanese and USA estimates were consistent and higher than the EU estimate due to broader food categories in which nisin could be applied. The Committee concluded that the use of a dietary exposure of 0.07 mg/kg bw per day (95th percentile, Australian children, 2–6 years old) was appropriate for the safety evaluation of nisin.

5. EVALUATION

At the meeting, the Committee provided clarifications on the identity of nisin and on the units of activity of nisin and a calculation to convert from International Units of nisin to micrograms of nisin based on the available data on different forms of nisin in commerce.

On the basis of the available studies, the Committee considered nisin to be a substance of low oral toxicity. Ingested nisin is inactivated in the upper part of the intestinal tract. Nisin is not carcinogenic or mutagenic and is not associated with any reproductive or developmental toxicity. The 2-year toxicity study was not considered to be an appropriate basis for establishing an ADI because it was not conducted to current standards and did not include an appropriate saline control group and because a number of later studies investigated higher doses of nisin.

After evaluating the new studies as well as the previously reviewed studies, the Committee concluded the 13-week subchronic toxicity study to be the pivotal study in the current safety evaluation of nisin because it was a higher-dose study and it took into consideration more parameters compared with other studies, such as the three-generation reproductive toxicity study. Based on the observation that there were no treatment-related adverse effects at the highest concentration tested, the 5.0% dietary level of nisin preparation (containing 7.5% nisin), a NOAEL of 224.7 mg of nisin per kilogram body weight per day was identified. Applying a safety factor of 100 to the NOAEL to account for interspecies and intraspecies variability, the Committee established an ADI for nisin of 0–2 mg/kg bw. The Committee did not consider it necessary to use an additional safety factor to account for the short duration of the study because no compound-related effects were observed at any dose in any of the other studies, including the reproductive toxicity study, and because ingested nisin is degraded in the upper part of the intestinal tract, such that systemic exposure to nisin is not likely to occur.

The highest estimated dietary exposure of 0.07 mg of nisin per kilogram body weight per day determined at the current meeting did not exceed the upper bound of the ADI.

The Committee withdrew the previous ADI of 0–33 000 units of nisin per kilogram body weight established at the twelfth meeting.

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ANNEXES

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases*. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.
13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents*. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents*. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. *Specifications for the identity and purity of some antibiotics*. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
19. *Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances* (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances*. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives*. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents* (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.
23. *Toxicological evaluation of some extraction solvents and certain other substances*. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. *Specifications for the identity and purity of some extraction solvents and certain other substances*. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.
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26. *Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.
27. *Toxicological evaluation of some enzymes, modified starches, and certain other substances*. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. *Specifications for the identity and purity of some enzymes and certain other substances*. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. *A review of the technological efficacy of some antioxidants and synergists*. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. *Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate*. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents*. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers*. FAO Food and Nutrition Paper, No. 4, 1978.
35. *Evaluation of certain food additives* (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. *Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives*. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.
37. *Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives*. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. *Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances*. (Nineteenth report of the

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40. *Specifications for the identity and purity of certain food additives*. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.
41. *Evaluation of certain food additives* (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 10, 1976.
43. *Specifications for the identity and purity of some food additives*. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.
44. *Evaluation of certain food additives* (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. *Summary of toxicological data of certain food additives*. WHO Food Additives Series, No. 12, 1977.
46. *Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others*. FAO Nutrition Meetings Report Series, No. 57, 1977.
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48. *Summary of toxicological data of certain food additives and contaminants*. WHO Food Additives Series, No. 13, 1978.
49. *Specifications for the identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 7, 1978.
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53. *Evaluation of certain food additives* (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 15, 1980.
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56. *Evaluation of certain food additives* (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

57. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 16, 1981.
58. *Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives)*. FAO Food and Nutrition Paper, No. 19, 1981.
59. *Evaluation of certain food additives and contaminants* (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.
60. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 17, 1982.
61. *Specifications for the identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 25, 1982.
62. *Evaluation of certain food additives and contaminants* (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.
63. *Toxicological evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 18, 1983.
64. *Specifications for the identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 28, 1983.
65. *Guide to specifications—General notices, general methods, identification tests, test solutions, and other reference materials*. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
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67. *Toxicological evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 19, 1984.
68. *Specifications for the identity and purity of food colours*. FAO Food and Nutrition Paper, No. 31/1, 1984.
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84. *Toxicological evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 24. Cambridge University Press, 1989.
85. *Evaluation of certain veterinary drug residues in food* (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.
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90. *Specifications for identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 49, 1990.
91. *Evaluation of certain veterinary drug residues in food* (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.
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95. *Toxicological evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 28, 1991.
96. *Compendium of food additive specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Combined specifications from 1st through the*

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ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

ADI	acceptable daily intake
ALT	alanine aminotransferase
ANCNPAS	Australian National Children's Nutrition and Physical Activity Survey
AUC	area under the 24-hour mean plasma concentration–time curve
AUC_{24}	area under the plasma concentration–time curve after 24 hours
AUC_t	area under the 24-hour mean plasma concentration–time curve at the final quantifiable time point
bw	body weight
CFU	colony-forming unit
C_{max}	maximum plasma concentration
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
eq	equivalent
EU	European Union
F	female; filial generation
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GAU	glucoamylase unit
GEMS/Food	Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme
GLP	good laboratory practice
GMP	good manufacturing practices
GSFA	Codex General Standard for Food Additives
HFA	human flora–associated
HPLC	high-performance liquid chromatography
IU	International Unit
IUIS	International Union of Immunological Societies
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-effect level
LSC	liquid scintillation counting
M	male
MTD	maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUL	maximum use level
N/A	not applicable
nd	not detected
NHANES	National Health and Nutrition Examination Survey
NNS	National Nutrition Survey
NOAEL	no-observed-adverse-effect level

OECD	Organisation for Economic Co-operation and Development
RU	Reading Unit
SDAP	Structural Database of Allergenic Proteins
$t_{½}$	half-life
T_{max}	time to reach maximum plasma concentration
TOS	total organic solids
USA	United States of America
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Rome, 4–13 June 2013

MEMBERS

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ANNEX 4

TOXICOLOGICAL AND DIETARY EXPOSURE INFORMATION AND INFORMATION ON SPECIFICATIONS

Food additives considered for specifications only

Food additive	Specifications[a]
Aluminium silicate	R, T
Annatto extracts (solvent-extracted bixin)	M
Annatto extracts (solvent-extracted norbixin)	M
Benzoe tonkinensis	M, T
Calcium aluminium silicate	R, T
Calcium silicate	R, T
Food additives containing phosphates	R[b]
Mineral oil (medium viscosity)	R
Modified starches	R[c]
Paprika extract	R[d]
Phytase from *Aspergillus niger* expressed in *Aspergillus niger*	R
Potassium aluminium silicate	R[d]
Potassium aluminium silicate–based pearlescent pigments, Type I	N[e]
Potassium aluminium silicate–based pearlescent pigments, Type II	N[e]
Potassium aluminium silicate–based pearlescent pigments, Type III	N[e]
Silicon dioxide, amorphous	R, T
Sodium aluminosilicate	R, T

[a] M, existing specifications maintained; N, new specifications; R, existing specifications revised; T, tentative specifications.

[b] The inductively coupled plasma – atomic emission spectrophotometric (ICP-AES) method for the assay of phosphate additives was added to the *Combined Compendium of Food Additive Specifications*.

[c] The method for determination of percentage of octenyl succinate groups in starch sodium octenyl succinate was revised.

[d] The tentative status of the specifications was removed.

[e] The existing combined specifications for potassium aluminium silicate–based pearlescent pigments were split into three separate specifications (Type I: coated with titanium oxide only, Type II: coated with iron oxide only and Type III: coated with both titanium dioxide and iron oxide). The tentative status of the specifications was removed.

Food additives evaluated toxicologically, assessed for dietary exposure and considered for specifications

Food additive	Specifications[a]	Acceptable daily intakes, other toxicological recommendations and dietary exposure assessment
Advantame	N, T	**The Committee established an acceptable daily intake (ADI) of 0–5 mg/kg body weight (bw) for advantame** on the basis of a no-observed-adverse-effect level (NOAEL) of 500 mg/kg bw per day for maternal toxicity in a developmental toxicity study in rabbits and application of a 100-fold safety factor to account for interspecies and intraspecies variability. The Committee agreed that the ADI also applies to those individuals with phenylketonuria, as the formation of phenylalanine from the normal use of advantame would not be significant in relation to this condition. Using the proposed maximum use levels and conservative assumptions, the maximum mean dietary exposure to advantame would be 1.45 mg/kg bw per day (29% of the upper bound of the ADI), and the maximum high-percentile dietary exposure would be 2.16 mg/kg bw per day (43% of the upper bound of the ADI).
Glucoamylase from *Trichoderma reesei* expressed in *Trichoderma reesei*	N	Based on its low toxicity and because it is reasonably anticipated that dietary exposure would be very low, **the Committee established an ADI "not specified"[b] for the glucoamylase enzyme preparation from *T. reesei* expressed in *T. reesei*** used in the applications specified and in accordance with good manufacturing practice.
Glycerol ester of gum rosin (GEGR)	R, T	As the requested two unpublished 90-day oral toxicity studies on GEGR in rats and complete information on the composition of GEGR were not submitted, **the Committee withdrew the temporary group ADI of 0–12.5 mg/kg bw for GEGR and glycerol ester of wood rosin (GEWR)** (see below).
Glycerol ester of tall oil rosin (GETOR)	W	No data on GETOR were submitted, and the Secretariat was informed that this compound is no longer supported by the previous data sponsor. Therefore, **the Committee did not evaluate GETOR**.
Glycerol ester of wood rosin (GEWR)	R[c]	As the requested data on GEGR were not submitted, **the Committee withdrew the temporary group ADI of 0–12.5 mg/kg bw for GEGR and GEWR and re-established the ADI of 0–25 mg/kg bw for GEWR**.

Food additive	Specifications[a]	Acceptable daily intakes, other toxicological recommendations and dietary exposure assessment
Nisin	R	**The Committee established an ADI for nisin of 0–2 mg/kg bw** on the basis of a NOAEL of 224.7 mg of nisin per kilogram body weight per day from a 13-week study in rats and application of a safety factor of 100 to account for interspecies and intraspecies variability. The Committee did not consider it necessary to use an additional safety factor to account for the short duration of the study because no compound-related effects were observed at any dose in any of the toxicity studies, including a reproductive toxicity study in rats, and because ingested nisin is degraded in the upper part of the intestinal tract, such that systemic exposure to nisin is not likely to occur. The highest estimated dietary exposure of 0.07 mg of nisin per kilogram body weight per day determined at the current meeting did not exceed the upper bound of the ADI. **The Committee withdrew the previous ADI of 0–33 000 units of nisin per kilogram body weight established at the twelfth meeting**.
Octenyl succinic acid (OSA) modified gum arabic	R, T	**The Committee decided to retain the temporary ADI "not specified"**[b] pending submission of additional data on the stability of OSA modified gum arabic in food by the end of 2013, which may help to explain contradictory hydrolysis data.

[a] M, existing specifications maintained; N, new specifications; R, existing specifications revised; T, tentative specifications; W, existing specifications withdrawn.

[b] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice—i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

[c] The tentative status of the specifications was removed.

Contaminants

Cadmium: Assessment of exposure from cocoa and cocoa products

The Codex Committee on Contaminants in Foods, at its Sixth Session, requested that the Committee conduct an assessment of dietary exposure to cadmium from cocoa and cocoa products.

The estimates of mean population dietary exposure to cadmium from products containing cocoa and its derivatives for the 17 new Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) Cluster Diets[1] ranged from 0.005 to 0.39 µg/kg bw per month, which equated to 0.02–1.6% of the provisional tolerable monthly intake (PTMI) of 25 µg/kg bw. Similar mean population cadmium dietary exposures for individual cocoa products were estimated from national data, ranging from 0.001 to 0.46 µg/kg bw per month (0.004–1.8% of the PTMI).

The potential dietary exposures to cadmium for high consumers of products containing cocoa and its derivatives in addition to cadmium derived from other foods were estimated to be 30–69% of the PTMI for adults and 96% of the PTMI for children 0.5–12 years of age. The Committee noted that this total cadmium dietary exposure for high consumers of cocoa and cocoa products was likely to be overestimated and did not consider it to be of concern.

Detailed information on cadmium occurrence data and national food consumption data used in the evaluation will be available on the JECFA web site[2].

[1] http://www.who.int/foodsafety/chem/cluster_diets_2012.pdf
[2] http://www.who.int/foodsafety/chem/jecfa/publications/reports/en/index.html

This volume contains monographs prepared at the seventy-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 4 to 13 June 2013.

The toxicological monographs in this volume summarize the safety data on three food additives: advantame, glucoamylase from *Trichoderma reesei* expressed in *Trichoderma reesei* and nisin. Toxicological and dietary exposure information and information on specifications for all of the food additives and contaminants considered by the Committee are annexed to the volume.

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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